

Light-induced structural changes in pigment-protein complexes studied with optical spectroscopy

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ABSTRACT

Light-induced structural changes in pigment-protein complexes studied with optical spectroscopy

High-resolution frequency-domain optical spectroscopy methods such as single-molecule spectroscopy (SMS) and non-photochemical spectral hole burning (NPHB) have been extensively employed to study various amorphous systems including glasses, polymers and proteins, and in particular pigment-protein complexes involved in photosynthesis. In all these systems one can observe, even at cryogenic temperatures, the shifts of the zero-phonon lines. These shifts are attributed to small structural changes in the pigment's environment that can be represented as switching between the minima of the energy landscape. They are observed directly in single-molecule spectroscopy and are the basis of spectral hole formation in NPHB. Understanding these shifts is important for proper interpretation of NPHB and SMS experiments in the context of photosynthesis research. NPHB also can be used to explore the protein energy landscapes. Joint analysis of the hole growth and recovery data for the same pigment-protein system may allow for determination of the molecular origins of the respective structural changes.

In this thesis I address several topics arising in NPHB experiments on pigment-protein complexes:

- The mismatch between the widths of the distributions of the barrier heights and of the tunneling parameter was explained with the presence of at least two distinct NPHB mechanisms featuring overlapping tunneling parameter distributions that arise due to quite different combinations of barrier heights, widths and the masses of the moving/tunneling entities.

- The slowdown of NPHB with increased light intensity can be explained with the presence of triplet state(s), though strong solvent deuteration effect likely indicates local heating.
- Differences in NPHB and hole recovery behavior of different pigments in the same protein pocket indicate that pigment molecules themselves likely contribute to the small structural changes behind NPHB. Alternatively, pigment molecules affect the relevant barriers by constraining the movements in their immediate protein environment.
- I also describe successfully building and testing a closed-cycle optical cryosystem that will be used in future experiments exploring protein dynamics as well as energy and charge transfer in pigment protein complexes.

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Contribution of Authors

This research is a result of collaboration between our group that performs optical spectroscopy experiments and the biochemists that provide the highly purified samples (R. Picorel, M. Lujan).

Within Zazubovits group, I have been the most senior PhD student (other student co-authors were MSc students) and have been responsible for proper functioning of all the relevant equipment. I contributed significantly to the experiments, data analysis and preparation of the published manuscripts on which several subsections of chapter 5 are based.

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List of Abbreviations

BChl	Bacteriochlorophyll
BRC	Bacterial Reaction Centre
Car	Carotenoid
CCD	Charge-Coupled Device
Chl	Chlorophyll
CT	Charge Transfer
Cyt	Cytochrome
EET	Excitation Energy Transfer
ETC	Electron Transfer Chain
FLN	Fluorescence Line Narrowing
FRET	Förster Resonance Energy Transfer
FWHM	Full Width at Half Maximum
GM	Gifford–McMahon (refrigeration cycle)
HB	Hole Burning

HGK	Hole Growth Kinetics
HR	Hole Recovery
LH1 / LH2	Light-Harvesting Complex 1 / 2
NPHB	Non-Photochemical Hole Burning
OD	Optical Density
PEL	Protein Energy Landscape
PHB	Photochemical Hole Burning
PMT	Photomultiplier Tube
PSB	Phonon Side Band
PSBH	Phonon Side Band Hole
PSI / PSII	Photosystem I / Photosystem II
RC	Reaction Center
SD	Spectral Diffusion

1. Introduction

For the past several years I have been studying various processes involved in photosynthesis. For all of these years I am still delighted and surprised by the flawless operation of this mechanism, brought to perfection by nature. Even though we as humanity have made significant progress in science and technology over the past centuries, we are still far from being able to compete with nature in terms of efficiency, sustainability and elegance. The present situation in the world is a challenge to mankind which tests the ability of redesigning energy production systems and solving associated problems such as greenhouse emission, fossil fuels exhaustion and even humanitarian crises bound to energy supply of fuels from particular countries. Fossil fuels that we have relied on for a century are not just finite but also destructive for our environment. That is, the extraction of fossil fuels and production of energy from them results in accumulation of waste which is extremely aggressive both for our health and the equilibrium of the surrounding nature. This pollution includes gaseous pollutants like carbon dioxide (CO_2) that contribute to global warming, and sulfur dioxide (SO_2) and nitrogen oxides (NO_x) which cause acid rain.

Many of these problems can be addressed by studying photosynthesis. For example, we can learn how to sequester the greenhouse gas carbon dioxide very efficiently from air and develop ways to clean the atmosphere efficiently. In fact, hydrocarbons in addition to serving as fuel, are used to produce a number of chemicals, plastics, and other commodities. At some point we should be able to replace fossil fuel as an energy source or raw material for chemical synthesis with the products of artificial photosynthesis. The research in this field is crucial to ensure the

wellbeing of our planet. In fact we could be able to stop using fossil fuels if we would know how to trap and store solar energy more efficiently. Every 2.5 hours more solar energy hits the surface of the Earth than the population of the Earth consumes in one year. The global annual energy consumption is estimated to be around 178,000 TWh per year (592 EJ), while solar energy reaching Earth's surface is approximately 85,000 TWh per hour. [1] [2] [3]

However, currently conversion from light energy to electric energy has a relatively low efficiency of up to 20% for common cells (although up to 50% has been reported in proof-of-principle research) and modern solar cells are expensive because of production costs.[4] Solar light-harvesting devices borrowing some design principles from photosynthesis may become a cheaper option; however, in order to produce them one should understand how plants, bacteria and algae exploit physics, reaching nearly 100 % quantum efficiency of the primary steps of photosynthesis (defined as number of electrons moved per photon absorbed). At the heart of Photosynthesis are special protein complexes which have naturally embedded chromophores. The most abundant and important of these chromophores is Chlorophyll (*Chl*) which is an intrinsic or natural fluorophore. Importantly, spectroscopic methods such as fluorescence line-narrowing (FLN) and spectral hole burning (SHB), along with time domain methods, have long been employed to probe excitation energy transfer (EET) and charge transfer processes in these complexes. These techniques are traditional in the photosynthesis research, as they directly connect underlying physics to biological function.

In particular, my thesis focuses on spectral dynamics observed in photosynthetic complexes at cryogenic temperatures. This spectral dynamics is a fairly universal phenomenon, observed in all pigment-protein complexes studied so far, and originates from small structural

changes in the protein surrounding the pigments. Small structural changes are part of the mechanism protecting pigment-protein complexes from excessive illumination. They may be responsible for modifying energy transfer pathways and details of electron transfer within these complexes. In the context of non-photochemical spectral hole burning and single-molecule spectroscopy, understanding mechanisms behind light-induced spectral shifts allows one to disentangle the effects of distributions of energy landscape barrier parameters from those of the energy transfer time distributions, and determine the latter more reliably. Last but not least, similar methods could be used to explore dissipation of vibrational energy in the proteins.

1.1 Thesis structure

Besides the first, introductory section, the thesis is structured as follows:

In Section 2, I introduce the basics of optical spectroscopy needed to establish the fundamental theoretical and technical background. This includes concepts, theories, and models such as Absorption/Fluorescence, Excitation Energy Transfer (EET), and the behavior of impurity centers in amorphous solids. This section also covers the foundational experimental techniques used in this research, specifically Non-Photochemical Spectral Hole Burning (NPHB).

Section 3 focuses on the necessary biological context, covering both oxygenic and anoxygenic photosynthesis and their main photosynthetic complexes, detailing the pigments, pigment-protein complex structures, and the associated electron and energy transfer pathways.

In Section 4, I discuss the motivation, principles, and specific modifications made to our cryosystem. In particular, we explore replacing conventional regenerator materials or combining them with magnetic intermetallic compounds. These compounds undergo magnetic phase

transition at cryogenic temperatures, enhancing the overall cooling power of the cryosystem. This work was done in an attempt to address the limitations of bath cryostats and to modify and build a closed-cycle cryosystem suitable for long-run and low-vibration optical experiments.

Section 5 is the core experimental/research section, where I present three distinct research papers/studies along with a brief overview of previous articles that lay a foundation for the current research.

Finally, Section 6 discusses future work and outlines several potential directions for follow-up research.

2. Basics of optical spectroscopy

Optical spectroscopy is a great tool employed in different fields of science from astronomy to nanoscience, in chemistry, material science, physics and more. This technique uses the interaction of light and matter to analyze the composition, structure and function of various substances. Historically one can date optical spectroscopy back to Isaac Newton with his work “Opticks: or, A Treatise of the Reflexions, Refractions, Inflexions and Colours of Light”, however there were many others whose work predated his optics experiments. Nowadays, different types of spectroscopic techniques have been developed, covering nearly all of the electromagnetic spectrum and often combining a variety of modalities. In the upcoming chapter we will discuss particular techniques and approaches employed in our research, which are tuned to suit our samples and research interests as well as possible, but the basics of optical spectra presented below are the foundation pillars.

Energy levels are the most fundamental concept, as conservation of energy dictates which light would interact with given type of matter. In sufficiently simple systems, such as atoms and molecules, the discrete nature of energy levels is explained by quantum mechanics. Unlike in classical physics, where an electron could orbit a nucleus at any distance and possess any amount of energy, the quantum mechanical model dictates that an electron is confined to specific, quantized energy levels and it cannot exist in a state between these levels. This means that for a molecule or an atom to absorb or emit energy, it must do so in discrete packets, or quanta. This fundamental principle explains why optical spectra of atoms appear as distinct lines rather than a

quasi-continuous broadband spectrum. As molecule or atom undergoes excitation or relaxation process, an electron makes a transition between bound states, that is an absorption or emission occurs. Normally the electronic transitions involving lower excited states occur in near-IR, visible and ultraviolet parts of the electromagnetic spectrum. Upon transition from lower to higher discrete energy state the photon of particular resonant energy is absorbed, and while the transition occurs from higher to lower bound state the photon of a particular energy is emitted. These processes form absorption and emission (line) spectrum, respectively. (Figure 2-1)

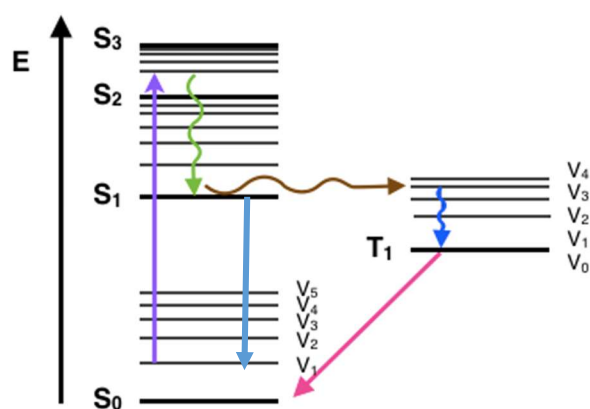


Figure 2-1: Jablonski diagram Illustrate the excited states of a molecule and the radiative and non-radiative transitions that can occur between them. The singlet ground state (S₀), the first three singlet excited states (S₁, S₂, S₃), and the first triplet excited state (T₁). The vibrational energy levels (v₀, v₁, v₂, v₃, v₄) are denoted by the thinner lines. [Jablonski diagram is shared under a CC BY 4.0 license and was authored, remixed, and/or curated by Jordan McEwen.]

However, in solids and liquids electronic spectra contain many more lines than there are possible purely electronic transitions because electronic transitions are accompanied by changes in vibrational and rotational states. Furthermore, chemically identical pigment molecules often experience slightly different local environments, leading to distributions of electronic transition frequencies (“Inhomogeneous broadening”, more details will be presented in 2.3). When the narrow lines can be resolved despite inhomogeneous broadening, electronic spectra become a

rich source of information about molecular properties. There is the set of interrelated parameters that characterise optical transitions: Oscillator strength, Einstein coefficients, transition dipole moment, extinction coefficient and the single site line shape function.

2.1 Absorption and Fluorescence

A molecule can be promoted to an excited state upon absorption of a photon, with the excited state energy being higher than the ground state energy of the molecule. Eventually, the molecule undergoes nonradiative decay back to the ground state or returns there via a radiative transition, emitting a photon in the process. (Figure 2-1) This transition can be stimulated by the presence of other resonant photons, yet even with no presence of other photons the molecule will decay back to the ground state with excitation-independent rate. The energy radiated as a result of the transition from higher to lower state is termed fluorescence.

2.1.1 Extinction coefficient

The extinction coefficient is a fundamental measure of how strongly a chemical specie absorbs light at a specific wavelength. It is a proportionality constant that links the concentration of a substance to its absorbance, as described by the Beer-Lambert law. The relation between absorbance and concentration is provided by Beer's Law, which is used to empirically determine the extinction coefficient of a substance of interest if its concentration is known.

$$A = OD = \epsilon Cz \tag{1}$$

The absorbance (A), or Optical Density (OD), is a unitless quantity that can also be expressed in relation to light intensities as

$$I = I_0 10^{-OD} = I_0 10^{-C\epsilon z} \quad (2)$$

where C is the concentration of the attenuating species, ϵ is the molar extinction coefficient, and z is the length of the optical path, I_0 is light intensity before interacting with the sample.

2.1.2 Absorption cross-section

Another important parameter related to extinction coefficient (ϵ) is absorption cross-section (σ) which affects the probability of an absorption process. (Eq. 3) Essentially, the extinction coefficient describes absorption for a molar concentration, while the absorption cross-section describes the effective area that a single molecule presents to an incoming photon.

$$\sigma = \frac{\ln(10)}{N_A} \epsilon \quad (3)$$

The absorption cross section is wavelength dependent via the line-shape function,

$$\sigma(\nu) = \frac{8\pi^3 \nu g(\nu) \mu^2}{3ch} \quad (4)$$

where μ is the transition dipole moment vector (see next subsection, c is the speed of light, h is Planck's constant, and $g(\nu)$ is the line-shape function. This function describes the intensity distribution of an absorption or emission line as a function of frequency and usually is considered to be a Lorentzian or a Gaussian function (or their convolution; depending on underlying broadening mechanisms). The absorption cross section has the units of area, but it should not be interpreted as the real geometrical cross section area of a molecule. Rather, it is the area of the “target” representing the molecule for the purpose of absorbing a photon. The Integrated cross

section takes into account all probabilities over the spectral range, thus indicating the total oscillator strength of the transition. (Section 2.1.4)

$$\sigma_{int} = \int \sigma(\nu) d\nu = \int \frac{8\pi^3 \nu f(\nu) \mu^2}{3ch} d\nu \quad (5)$$

2.1.3 The transition dipole moment

The dipole moment, μ is a vector quantity that describes a molecule's charge distribution. It quantifies the separation of positive and negative charge within the molecule. The dipole moment for a state described by the wavefunction ψ_i is calculated as the expectation value of the dipole moment operator $\hat{\mu}$ in this state. Quantum mechanically it is defined as:

$$\mu_i = \langle \psi_i | \hat{\mu} | \psi_i \rangle \quad (6)$$

where $\hat{\mu}$ is the dipole moment operator for a specific molecule.

$$\hat{\mu} = \sum_i q_i r_i \quad (7)$$

which sums over all the electrons and nuclei with charge q_i and position r_i . A non-zero *dipole* moment in a molecule's ground state indicates that the entity is polar.

In contrast to the permanent dipole moment which describes the static charge distribution of a molecule in a defined quantum state, transition dipole moment describes the probability of specific atom or molecule to change its state by absorbing electromagnetic wave. It is a vector quantity so its direction determines how the system interacts with an electromagnetic wave of a given polarization. Quantum mechanics apparatus defines transition dipole moment as

$$\mu_{nm} = \langle \psi_n | \hat{\mu} | \psi_m \rangle \quad (8)$$

Note that the wavefunctions belong to two different states. There could be a situation, mostly because of the symmetry, where transition dipole moment is zero - that is no absorption occurs.

The direction of the transition dipole is a consequence of the chemical structure of the molecule, therefore it is fixed with respect to the molecule's structure. For instance, the transition dipoles for the Q_y and Q_x bands of a chlorophyll (or related) molecules are oriented along specific, orthogonal axes within the planar porphyrin ring. (Figure 2-2) The Q_y -band's transition dipole is largely oriented along one axis, while the Q_x -band's orientation is along a nearly perpendicular axis. Consequently, light absorption is sensitive to the molecule's orientation relative to the polarization of the incoming light.

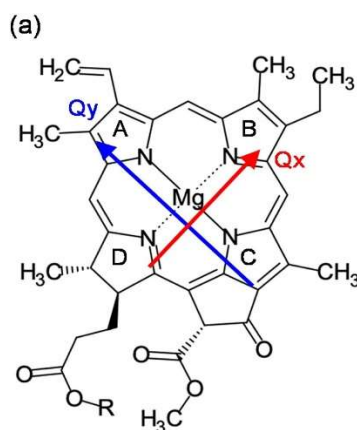


Figure 2-2: Chemical structure of Chl *a*. The red and blue arrows indicate the orientations of the Q_y and Q_x transition dipole moments, respectively. *R* represents the phytol group. [5]

Transition dipole moment is related to experimentally measured absorption via extinction coefficient ϵ which determines how absorbing the molecule is. Extinction coefficient is directly proportional to the square of the magnitude of the transition dipole moment. That means that a larger transition dipole moment leads to a higher probability of light absorption and thus a larger extinction coefficient.

In order to account for frequency distribution in measured (experimental) absorption spectra this relationship is expressed in terms of integrated extinction coefficient (absorbance) which is proportional to the square of the transition dipole moment.

$$\int \varepsilon(\nu) d\nu = \frac{8\pi^3 N_A}{3hc} |\mu|^2 \quad (9)$$

2.1.4 Oscillator strength

Oscillator strength (f) is another quantity that determines the probability of transitions between energy levels and serves as a theoretical reference for the intensity of a spectroscopic transition.

The oscillator strength for a specific transition can be obtained from the measured integral absorption coefficient which is defined as the integral of the molar extinction coefficient over the frequency range of the absorption band. The oscillator strength also can be expressed as

$$f = \frac{|\mu_{12}|^2}{|\mu_{12}|_{osc}^2} \quad (10)$$

Here f is the ratio of strength of a given transition to that of an electron oscillating harmonically in three dimensions. For allowed electrical dipole transitions f is close to unity, while for forbidden transitions it can be orders of magnitude smaller. For instance, singlet-triplet transitions typically have an oscillator strength of about 10^{-5} . This is due to quantum mechanical spin selection rules, which state that the total spin of the system ΔS must remain unchanged during a spectroscopic transition. A singlet-singlet transition ($\Delta S=0$) is allowed and has a high probability, resulting in a large oscillator strength. In contrast, for a singlet-triplet transition ($\Delta S \neq 0$), the total spin changes, making the transition highly improbable. Forbidden transitions are only possible due to weak spin-orbit coupling (electron's intrinsic spin and its orbital angular momentum), which acts as a perturbation that mix the pure spin states.

Combining an Integrated cross section and the square of the transition moment for the three-dimensional harmonic oscillator for the transition from the ground state to the first excited state results in:

$$f = \frac{2303m_e c^2}{\pi N_A e^2} \int \epsilon(\tilde{\nu}) d(\tilde{\nu}) \quad (11)$$

where m_e is the mass of the electron, c is the speed of light, e is the elementary charge, N_A is Avogadro's number, $\epsilon(\tilde{\nu})$ is the molar extinction coefficient as a function of wavenumber $\tilde{\nu}$. Thus this equation represents the experimentally obtained oscillator strength.

2.1.5 Einstein coefficients

Another way to represent the probabilities of electronic transitions is via Einstein coefficients.

The spectrum of a molecule is spectrally decomposed as a series of possible transitions, represented by lines at the frequencies of these transitions. The intensity of the lines depends on the number of molecules per unit volume (N_i) (population density) and the probabilities of their transitions.

Einstein postulated that the absorption rate of photons of a given frequency is proportional to the density of electromagnetic radiation ρ (radiant energy density) at the same frequency.

Spectral radiant energy is a measure of radiant energy of a particular frequency ρ_ν and is defined as $\rho_\nu = d\rho/d\nu$. To excite a molecule/atom from state 1 with population density of N_1 and energy E_1 to state 2 with population density of N_2 and energy E_2 the required energy is expressed as $\rho(\nu_{12})$.

$$Rate_{absorption} = \left(\frac{dN_1}{dt} \right)_{abs} = B_{12} \rho_\nu(\nu) N_1 \quad (12)$$

B_{12} is the Einstein coefficient for absorption.

The excited state of a molecule has a finite lifetime (τ), which is directly linked to the probability of spontaneous emission, where the molecule relaxes back to a lower energy state by emitting a photon without any external stimulation. The rate of spontaneous emission is independent of the radiation density and spontaneous emission is an incoherent process with regard to the phase, frequency, and direction of the emitted photon (note that emission direction distribution is not isotropic but corresponds to the dipole antenna emission distribution).

The rate of spontaneous emission N is given by:

$$\left(\frac{dN_2}{dt}\right)_{spont} = -A_{21}N_2 \quad (13)$$

where A_{21} is Einstein coefficient for spontaneous emission and N_2 the population density of the excited state. Index 1 refers to the ground state and 2 refers to the excited state. The coefficient of spontaneous emission is a measure of the lifetime of the excited state. The total radiative lifetime (τ_{rad}) for all possible transitions from the excited state is:

$$\frac{1}{\tau_{rad}} = A \quad (14)$$

While no stimulating radiation is present, dependence of excited state population on time is:

$$N_2(t) = N_2(0)e^{-A_{21}t} = N_2(0)e^{-t/\tau} \quad (15)$$

In the presence of an electromagnetic field, the third possible process is stimulated emission characterized by coefficient B_{21} . The rate of this process is proportional to the energy density and this process produces radiation coherent to the stimulating one.

$$Rate_{stimulated} = \left(\frac{dN_2}{dt} \right)_{stim} = -B_{21}\rho_v(v_{12})N_2 \quad (16)$$

All three Einstein coefficients are related to each other and all three processes can occur in the same system over time, but a single molecule is only undergoing one of these three processes at any given instant. The rates of changes of the populations are then

$$\left(\frac{dN_1}{dt} \right) = - \left(\frac{dN_2}{dt} \right) = -B_{12}\rho_v(v_{12})N_1 + A_{21}N_2 + B_{21}\rho_v(v_{12})N_2 \quad (17)$$

For the system in equilibrium the relation between the coefficients is

$$B_{12} = B_{21} \quad (18)$$

And

$$A_{21} = \frac{8h\pi v_{12}^3}{c^3} B_{21} \quad (19)$$

The coefficients are related to the transition dipole moment as its square is proportional to the coefficients.

$$A = \frac{16\pi^3 v^3 g_1}{3h^3 \epsilon_0 g_2} |\mu_{12}|^2 \quad (20)$$

$$B = \frac{2\pi^2 g_1}{3h^2 \epsilon_0 g_2} |\mu_{12}|^2 \quad (21)$$

Here g_1 and g_2 are the degeneracies of the ground state and the excited state, respectively.

2.2 Excitation Energy Transfer

The primary process in photosynthesis starts with the absorption of the light by the pigments. These pigments have strong overlap of their fluorescence bands with absorption bands of the same or other type pigments. Under these conditions, the electronic excitation can move from one pigment (donor) to the next (acceptor) without radiation being emitted and reabsorbed. The simplest version of this mechanism is referred to as Fluorescence resonance Energy Transfer, or FRET. Alternatively, “F” in FRET refers to Förster, the creator of the respective theory.[6] [7] [8] FRET is a nonradiative mechanism of energy transfer from a donor fluorophore in the electronically excited state to an acceptor fluorophore in the electronic ground state via dipole–dipole coupling (*Figure 2-3*) creating favorable conditions for excitation energy transfer between them. In general, all aforementioned resonance energy transfer processes can be expressed by the following photophysical equation:



where D^* and A^* are excited states of donor and acceptor respectively, and D and A are their ground states.

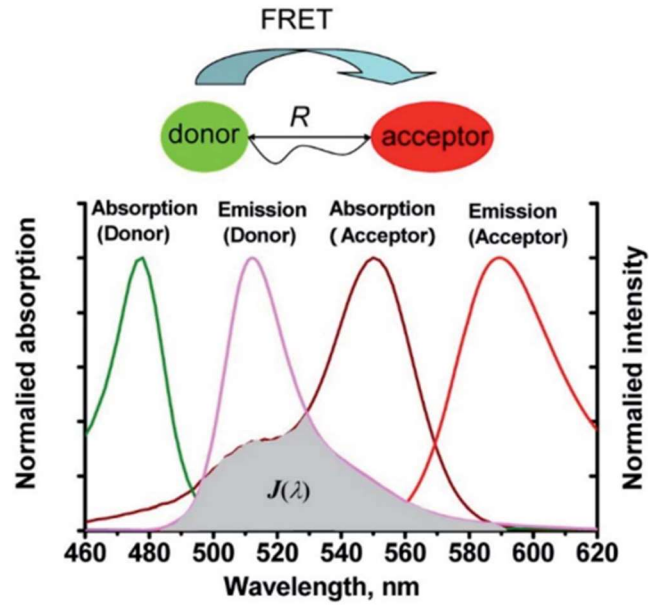


Figure 2-3: The mechanism of FRET. R is the distance between the energy donor and acceptor, $J(\lambda)$ represents the degree of spectroscopic overlap between the donor emission and the acceptor absorption. [9]

Förster resonance energy transfer (FRET) theory states that excitation energy transfer probability is inversely proportional to R^{-6} due to the induced dipole – induced dipole coupling mechanism, where R is the distance between the donor and acceptor.

That probability E (transfer efficiency) is given by:

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (23)$$

where R_0 is the Förster radius. Figure 2-4 depicts the typical transfer efficiency dependence on distance.

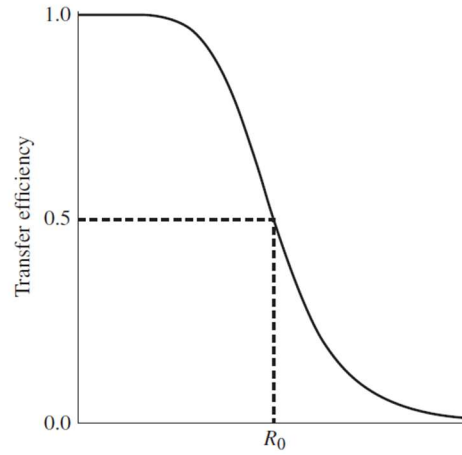


Figure 2-4: Distance dependence of the transfer efficiency between a pair of chromophores.
[10]

Förster radius is defined as a distance at which the energy transfer efficiency reaches 50% or the distance at which the rates of donor deactivation by FRET and by spontaneous fluorescence become equal. FRET efficiency depends through R_0 on many physical parameters. The Förster radius can be expressed by the following equation:

$$R_0 = 0.2108[k^2\phi_D^0n^{-4}J]^{\frac{1}{6}} \quad (24)$$

where ϕ_D^0 is the fluorescence quantum yield of the donor in the absence of the acceptor, κ is the dipole orientation factor, n is the refractive index of the medium, and J is the spectral overlap integral calculated as

$$J = \int f_D(\lambda)\epsilon_A(\lambda)\lambda^4d\lambda \quad (25)$$

Here f_D is the donor emission spectrum normalized to an area of 1 and $\epsilon_A(\lambda)$ is the acceptor molar extinction coefficient. Some spectral overlap of the donor emission spectrum and the acceptor absorption spectrum is necessary (it is a manifestation of the conservation of energy), while better overlap enhances the efficiency of FRET. The rate of energy transfer k_{et} can be expressed as:

$$k_{et} = \frac{1}{\tau_D^0} \left[\frac{R_0}{R} \right]^6 \quad (26)$$

here τ_D^0 is the radiative decay lifetime of the donor, R the distance between chromophores and R_0 is Förster radius. The rate of energy transfer is proportional to the square of the electronic coupling V and the spectral overlap J (in SI units).

$$k_{et} = \frac{2\pi}{\hbar} |V|^2 J \quad (27)$$

The inter-pigment coupling V is proportional to the transition dipole moments of the donor and acceptor. For the point-dipole approximation used in FRET, the coupling is

$$V = \frac{5.03}{n^2} \frac{\mu_D \mu_A \kappa}{R^3} \quad (28)$$

where V is in $[\text{cm}^{-1}]$, μ_A , μ_D are the transition dipole moments of the donor and acceptor (in Debye), κ is orientation factor and R is the distance between molecules (in nm).

The Debye unit is used to express molecular dipole moments because it has a more convenient size for molecular dipole moments. It is defined based on the SI units of charge and distance. 1 Debye = $3.33564 \cdot 10^{-30}$ Coulomb-meters (C·m).

The orientation factor κ (Figure 2-5) is determined by the mutual orientation of the two dipoles:

$$\kappa = \hat{\mu}_A \cdot \hat{\mu}_D - 3(\hat{\mu}_D \cdot \hat{R})(\hat{\mu}_A \cdot \hat{R}), \quad (29)$$

where $\hat{R}$ stands for normalized inter-fluorophore displacement and the $\hat{\mu}$ are normalized transition dipole moments of the donor and the acceptor.

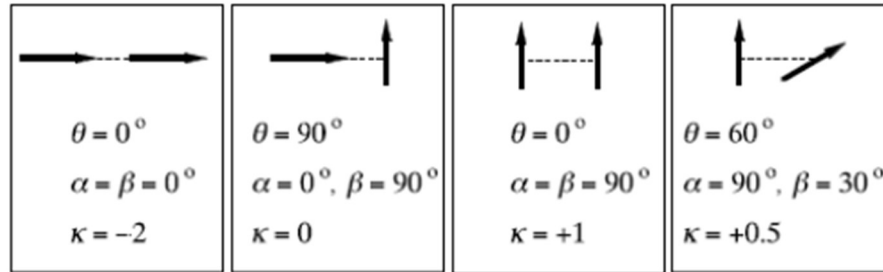


Figure 2-5: The dependence of the orientation factor on mutual orientation of the pigments. α is the angle between the donor and the displacement vector, β angle between the acceptor and the displacement vector and θ the angle between the two transition dipole moment [67].

The above discussion is applicable for cases of relatively weak inter-pigment coupling where no wave function overlap between the chromophores exists. In some circumstances (e.g., small inter-pigment distances, large interactions and/or presence of delocalized excitonic states) conventional Förster's theory cannot adequately describe energy transfer. Another possible mechanism was suggested by Dexter (1953) where the simultaneous exchange of electrons between the excited and ground states takes place. For example, green plants have a system to efficiently dissipate excess excitation energy absorbed by chlorophylls. This is attributed to the Dexter-type excitation energy transfer between chlorophylls and carotenoids in their vicinity.[11] Furthermore, in systems with short inter-pigment distances such as LH2 light harvesting complex,

significant wavefunction overlap results in delocalized excitonic states where point-dipole approximation of Förster's theory becomes inapplicable.

2.2.1 Lifetimes and spectral line widths

At the beginning of 20 century the classical physics has been challenged by the many phenomena's it could not explain. The absence of ultraviolet catastrophe predicted by classical electrodynamics, an explanation where Planck assumed that radiation is quantized, Born postulates of existence of stable orbits in atoms, Einstein explanation of photoelectric effect, the invention of wave mechanics by Schrödinger and contributions of many others led to the rise of Quantum Mechanics. There, Heisenberg uncertainty principle elaborates the limitation of our ability to measure the energy level of an electron, yet provides us with the tool to measure the lifetime of a state.

$$\Delta t \Delta \frac{E}{h} \geq \frac{1}{4\pi} \quad (30)$$

$$\Delta t \Delta E \geq \frac{\hbar}{2} \quad (31)$$

Long excited state lifetime will result in nearly monochromatic radiation while short lifetime will result in a broad range of frequencies, which will be manifested by sharp or wide spectral line, respectively. In other words, spectral line width is determined by the lifetime of the excited state and, conversely, the lifetimes can be determined from the line widths.

2.3 Theory of impurity centers in amorphous solids

Proteins can be treated as amorphous solids in the sense that some structural fluctuations may occur, but overall structure is well defined. Thus, one needs to consider the theories of spectral lines specific to pigments inside solids.

The interaction of electronic properties of the molecule with its surrounding is reflected by their chemical and physical behavior. In order to be able to decipher a multimolecular guest / pigment spectrum in a host solid, one should first consider the spectrum of a single molecule in vacuum, which is a set of narrow lines belonging to purely electronic transitions (in the first approximation) whose width is determined by the excited state lifetimes. Single molecule emission or absorption line has Lorentzian shape with Full Width at Half Maximum (FWHM)

$$\gamma_{nat}(cm^{-1}) = \frac{1}{2c\pi\tau_1} \quad (32)$$

where τ_1 is an excited state lifetime in *ps* and c is speed of light in *cm/ps* (i.e. $\sim 3 \cdot 10^{-2}$ *cm/ps*). Here the cm^{-1} (wavenumbers) are used as units of frequency. The speed of light serves as the conversion factor; thus, 1 cm^{-1} roughly equals to 30 GHz. Lorentzian line shapes can be derived from quasi-classical treatment of emission of electromagnetic radiation as the Lorentzian is the square of the Fourier transform of a sinusoid modulated by exponential decay. It is important to note that a purely electronic transition may be accompanied by additional sharp lines corresponding to internal vibrational modes of the molecule. These vibronic transitions are a characteristic feature of molecular spectra but are absent in atomic spectra due to the lack of internal vibrations. The Franck-Condon principle is the key concept that explains the relative intensities of these vibrational modes as they are coupled to an electronic transition. (*Figure 2-5*) Namely, according to Born-Oppenheimer approximation, the movements of the light electrons

occur fast enough that the nuclei do not have a chance to move. This corresponds to electronic transitions represented by vertical arrows in *Figure 2-6*. The probability of a transition between a pair of vibronic levels is determined by the overlap of the respective vibrational wavefunctions.

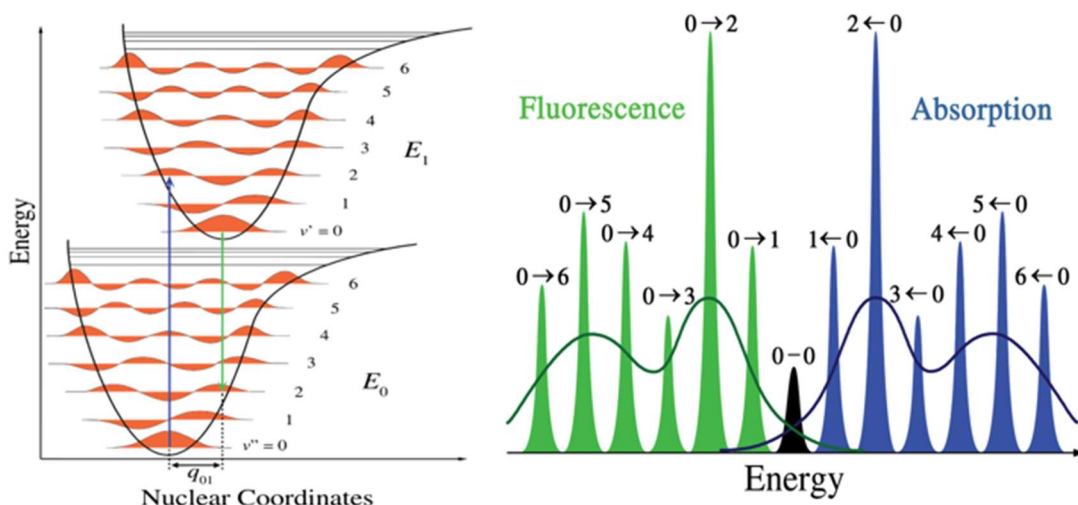


Figure 2-6: Franck–Condon principle energy diagram. Since electronic transitions are very fast compared with nuclear motions, vibrational levels are favored when they correspond to a minimal change in the nuclear coordinates. The shown favoring transitions between $v = 0$ and $v = 2$. [12]

If the chromophore is located in a solid, the spectroscopic measurement will also be affected by the presence of phonons i.e. delocalized lattice vibration of the solid. This interaction between the electronic states of the guest and the vibrational states of the host lattice is known as electron-phonon coupling. It obeys similar principles as the intra-molecular vibrations described above, except the “nuclear coordinate” is a linear combination of the coordinates of all atoms in the solid. As a result, the single spectral line of a purely electronic transition splits into two distinct components: the Zero-Phonon Line (ZPL) and the Phonon Sideband (PSB). (*Figure 2-7*) ZPL is a sharp, intense spectral line that represents the purely electronic transition without any energy being exchanged with the lattice. The PSB is a much broader band of frequencies alongside the ZPL. PSB originates from electronic transitions where a photon's energy is coupled

with the creation or annihilation of one or more phonons due to energy transfer from or to the surrounding environment via linear electron-phonon coupling.

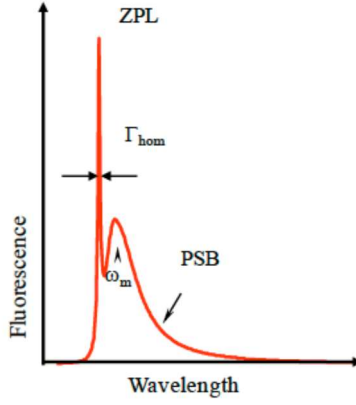


Figure 2-7: Absorption spectrum of a single molecule in solid, including ZPL and PSB.[8]

Vibrational relaxation may take place between the vibrational levels in both excited and ground state. The relative intensity of ZPL (I_{ZPL}) is related to the Debye-Waller factor α :

$$\alpha = \frac{I_{ZPL}}{I_{ZPL} + I_{PSB}} \quad (33)$$

where I_{PSB} is the integral intensity of the Phonon Sideband (PSB) and I_{ZPL} is the integral intensity of the ZPL. On the other hand Debye-Waller factor is related to linear electron-phonon coupling (Huang-Rhys factor S) as:

$$\alpha = \exp(-S \times \coth(\frac{\Omega_M}{2k_B T})), \quad (34)$$

where Ω_M is the difference between maximum energies of ZPL and PSB, T is absolute temperature and k_B is the Boltzmann constant. At cryogenic temperatures the absorption

spectrum is dominated by ZPL, and the overall tendency of the temperature dependency of a single molecule spectrum is shown in *Figure 2-8*.

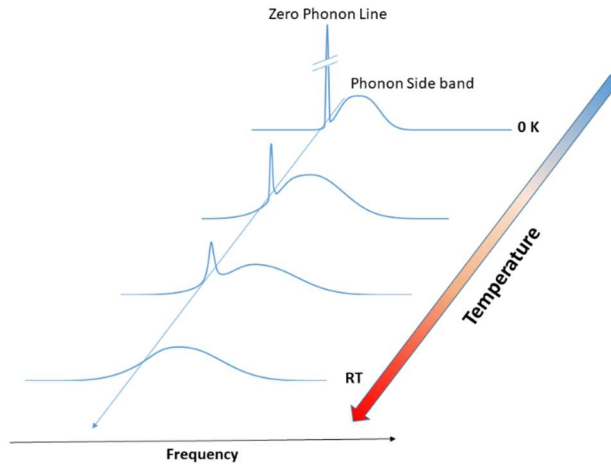


Figure 2-8: Dependence of the shape of ZPL and PSB on temperature. [13]

One may notice that not only the Debye-Valler factor but also the ZPL width are temperature-dependent. The width of the ZPL corresponds to the minimum absorption linewidth of the guest molecule in the host environment. This width is called Homogenous Linewidth, Γ_{hom} . The Homogeneous Linewidth is determined by the total dephasing time (τ_2) and is given by the equation:

$$\Gamma_{\text{hom}} = \frac{1}{\pi\tau_2} = \frac{1}{2\pi\tau_1} + \frac{1}{\pi\tau_2^*(T)} \quad (35)$$

Total dephasing time is also sometimes called the excited coherence lifetime and it is affected by the excited state radiative lifetime τ_1 and the pure dephasing time $\tau_2^*(T)$. The excited state lifetime τ_1 is the time that system spends in the excited state before relaxation occurs, i.e. it is

$1/A$ where A is the Einstein coefficient for spontaneous emission. The pure dephasing time (τ_2^*) is the time for which the phase of the excited-state wavefunction is preserved. Interactions with phonons (lattice vibrations) may cause a phase shift of the excited-state wavefunction without a loss of energy, thus causing pure dephasing. This phenomenon is related to quadratic electron-phonon coupling (unlike the PSB above). It should be noted that the pure dephasing time (τ_2^*) is a function of temperature. While it is infinitely long at absolute zero (no phonons are available to be scattered; no effect on the line width), its effect cannot be neglected at liquid helium temperatures.

Now the absorption of light by many identical molecules embedded in an optically transparent perfect crystal can be considered. (Figure 2-9) The relevant quantum-mechanical system consists of the electronic and vibrational degrees of freedom of the molecule (impurity center) and of the vibrational motion of the surrounding solid. Since the local environment of all the molecules is the same and in this approximation, and they are far away from each other to ensure there are no interaction between them, all these molecules will have identical spectra with ZPL peaked at the same frequency.

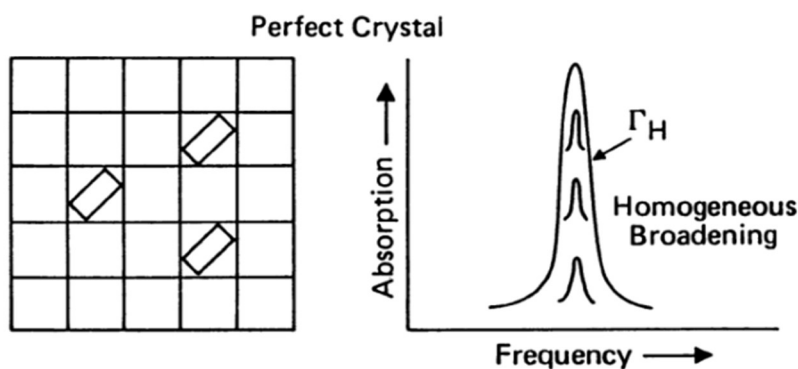


Figure 2-9: The illustration of a perfect crystal and resulting absorption line. All molecules absorb at exactly the same frequency/wavelength and the absorption band width is equal to the homogeneous width. [13]

In real crystals (and more so in amorphous solids, including proteins), (Figure 2-10), the immediate surrounding will be slightly different for each impurity center, resulting in so called inhomogeneous broadening of the absorption band of a collection of multiple pigment molecules.

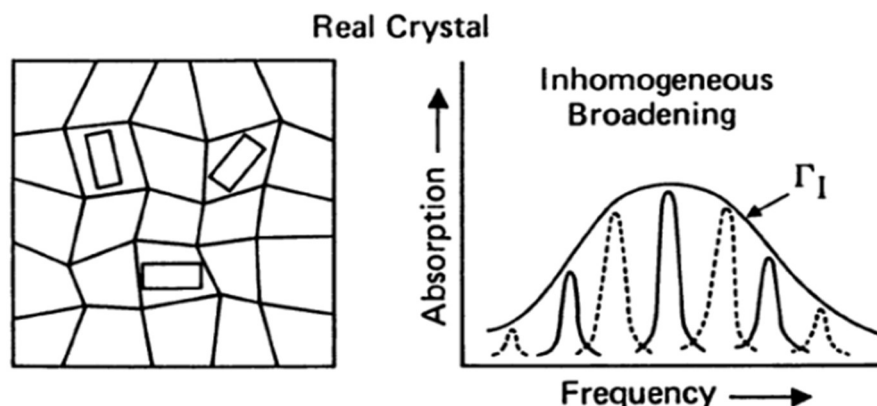


Figure 2-10: The illustration of real crystal or amorphous solid and the resulting inhomogeneously broadened absorption band.

From the mathematical standpoint, the observable absorption spectrum of the multiple pigments in an amorphous solid is a convolution of the Site Distribution Function (SDF) which is the distribution of probabilities of ZPL transition frequencies of pigment molecules (usually a Gaussian distribution), with the Single Site Absorption (SSA) spectrum which is an absorption of the single guest molecule including ZPL and PSB (as well as any replicas of this structure due to intramolecular vibrations), Figure 2-11.[13]

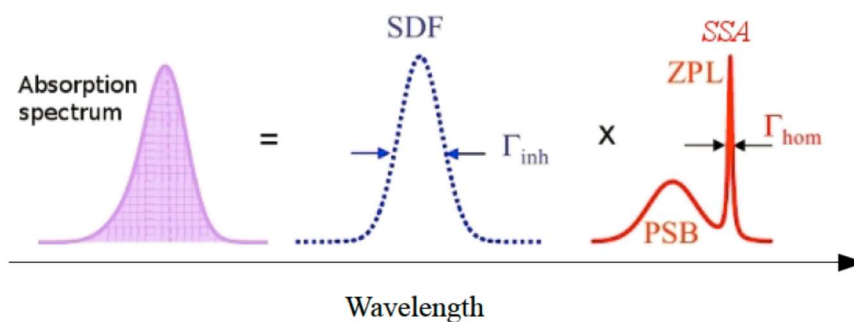


Figure 2-11: The slightly asymmetrical absorption spectrum as a result of convolution of a Single Site Absorption and Site Distribution Function. [8] [9]

As stated above, homogeneous line width contains information on characteristic times of various processes (pure dephasing, energy transfer, charge transfer) and the frequencies / shifts of the spectral lines contain information on the local environment. However, in the spectra of macroscopic samples homogeneously broadened lines are obstructed by inhomogeneous broadening. To extract relevant information from the SSA spectrum one has to somehow overcome the inhomogeneous broadening. One obvious solution is to employ single-molecule spectroscopy. While this is definitely feasible, single-molecule spectroscopy has certain disadvantages, namely triplets-related saturation, local heating and light-induced spectral shifts. These drawbacks are a direct result of the need to use high excitation intensities in order to collect sufficient signals from single molecules. [14] Spectral hole burning (SHB) is another technique suitable for uncovering SSA.

2.4 Spectral Hole Burning

In this section, I briefly review the theories behind Spectral Hole Burning (SHB). The first observations of persistent hole burning were made by Kharlamov et al. (1974) in organic glasses

and by Gorokhovskii et al. (1974) in organic crystals. These foundational experiments immediately established SHB as a powerful technique for probing the intricate interactions between a guest molecule and its surrounding solid matrix. While the method became more sophisticated and the spectral resolution increased, a deeper understanding of the details of these interactions was necessary to fully interpret the measured spectra. This led to the discovery and confirmation of phenomena like spectral diffusion in numerous subsequent investigations. Since then, the technique of spectral hole burning has developed into a highly efficient method, particularly well-suited for investigating the low-temperature tunneling dynamics in organic amorphous solids.

[15] [16]

In general, SHB is the frequency-selective bleaching of the absorption spectrum of a material, which leads to an increased transmission (a "spectral hole") at the selected frequency. (*Figure 2-12*) Spectral Hole Burning techniques may employ two different pathways that are distinguishable by the nature of the change caused by the light absorption.

The Photochemical Spectral Hole Burning (PSHB) is a process in which the absorption leads to a chemical change in the molecule of interest causing irreversible change (in most cases; one example of a reversible photochemical process would be a flip of a proton pair in free-base chlorin [17]). On the contrary, in Non-Photochemical Spectral Hole Burning (NPHB) no chemical change occurs, but physical and structural changes in the host environment are considered to be a reason for hole formation. In other words, small conformational change induced by the light of burning frequency is registered by the means of spectroscopic measurements. This is a universal phenomenon for amorphous solids and it is particularly suitable for photosynthesis research as it preserves the networks of interacting pigments in a protein.

In SHB normally smooth inhomogeneously broadened absorption bands in solids at low temperatures can be spectrally modified for time periods much longer than the lifetime of any excited state or a typical time resolution of frequency-domain spectroscopy experiments. There are a number of fundamental requirements to perform SHB.[7] [18]

- The optical absorption spectrum of the guest (impurity) molecule under consideration must be inhomogeneously broadened,
- There must exist more than one ground state configuration of the total system (host + guest/impurity/probe),
- The optical absorption frequencies for transitions from the various ground states must differ by more than the homogeneous line width and the linewidth of the tunable light source in use.
- There must exist an optical pumping pathway that connects the ground state configurations.
- The relaxation among the ground state configurations must be much slower than the decay rate of any excited state or the relaxation between the excited states of different configurations.

During the SHB experiments we excite the molecules of interest by a narrow-band laser ensuring this way that only a small fraction of pigment-protein systems, mostly those possessing zero-phonon transition frequencies resonant with the laser, is excited. It is worth noting that excitation can also occur through the phonon sidebands (PSB). Under carefully chosen experimental

conditions and with proper data analysis, the contribution from the PSB excitation can be minimized and/or accounted for. The excitation of a pigment may trigger a small structural change in the surrounding protein leading to the system being stuck in the new conformation (non-photochemical hole burning, NPHB; the pigment molecule is not chemically transformed). SHB provides information about different processes relevant for photosynthesis (see next chapter) such as energy transfer, electron transfer, and charge separation. SHB method can also yield information on lifetimes, Γ_{inh} values, Franck–Condon or Huang-Rhys factors S of phonons and intramolecular vibration modes (Electron–phonon and electron-vibration coupling parameters), PSB shape, pure dephasing time and parameters of the barriers on the energy landscape (Section 2.9).

The Hole (Burning) Spectrum is defined as

$$HBS = A(\Omega, t) - A(\Omega, 0) \quad (36)$$

where $A(\Omega, 0)$ is the pre-burn spectrum and $A(\Omega, t)$ is the post burn spectrum after burning for time t . The simplified sketch of post-burn and pre-burn spectra is shown in *Figure 2-12*. It is easy to see that the main feature of the hole in the absorption spectrum is an image of the ZPL. The Antihole represents the ZPLs shifted as a result of Non-Photochemical Burning, while Real PSHB stands for Phonon Side Band of the burnt resonant molecules (molecules that were burnt through ZPL), and Pseudo PSHB is made of the ZPL of the molecules that were burnt through their PSB. As a result of these processes, the overall post burn spectrum shows a central dip flanked by two side-wings (Real and pseudo PSBH wings).

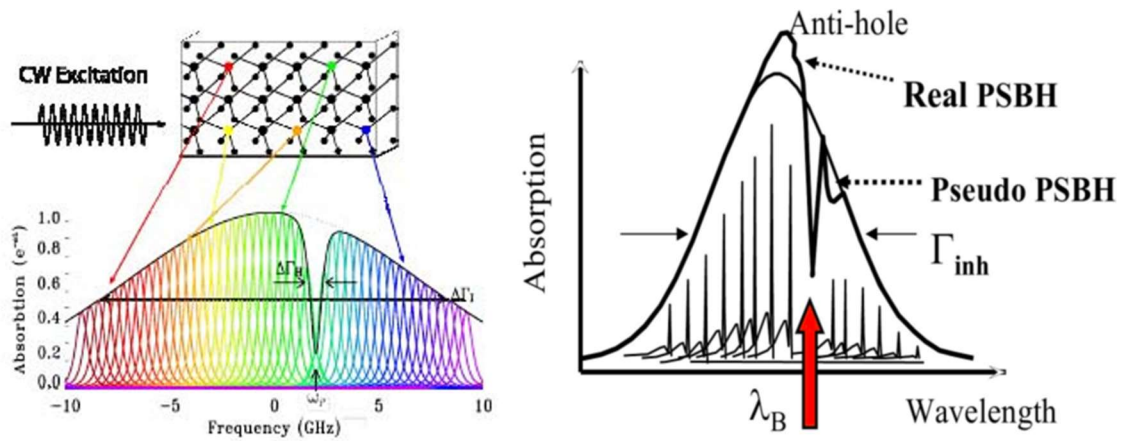


Figure 2-12: Left frame: Absorption profile of an amorphous solid with a spectral hole burned at ω_b . $\Delta\Gamma_i$: inhomogeneous bandwidth. $\Delta\Gamma_h$: homogeneous hole width.[11] Right frame: The different features of NPHB including ZPH, real PSBH, and pseudo PSBH [35].

SHB is an ensemble technique and any single molecule in the fraction that is in resonance with the burning frequency will be excited and burned out in a probabilistic fashion, therefore we can observe gradual growth of the spectral hole. In order to describe this process and make meaningful comparisons between different samples and different experimental conditions, Fractional Hole Depth (FHD) is introduced:

$$FHD = 1 - \frac{A(\Omega, t)}{A(\Omega, 0)} \quad (37)$$

2.5 The Mechanism of NPHB

NPHB mechanism for amorphous solids is based mainly on the Two-Level System (TLS, or double-well) model that was originally employed to explain anomalous specific heat and heat conductivity in glasses at low temperatures. [53] Two Level System (TLS) is a simplified model that may be applied to the conformational changes in the proteins and that features energy landscape with only one generalized coordinate. Figure 2-13 shows the schematic representation of a TLS.

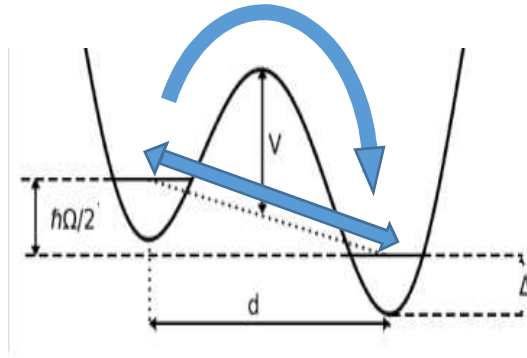


Figure 2-13: Two Level System model. There are two local energy minima, separated by the distance d , and potential barrier of the height V . The asymmetry Δ represents the difference between two zero-levels of the phonon/vibrational mode in two local minima. Zero-levels are $\hbar\Omega/2$ above the well bottoms and represent the minimal quantum-mechanical phonon/vibrational energy in each well.

The system must surmount the potential barrier in order to switch from one configurational state to another. At all but the lowest temperatures, the transition between two states may occur either by the classical thermally-activated process (“barrier hoping”) or by tunneling process through the potential barrier. It is important to emphasize that this is true quantum tunneling; the system does not lower the potential barrier through a catalytic mechanism, but rather tunnels through the barrier itself. At low temperatures (below 10K-12K) the tunneling is the dominant process for transition between these two states. In the simplest tunneling model, for particle energies far below barrier top energies, the tunneling probability can be represented as $\exp(-2\lambda)$. The tunneling parameters λ then can be expressed in terms of the potential barrier height V and other system parameters:

$$\lambda = \frac{d}{\hbar} \sqrt{2mV} \quad (38)$$

Here m is the effective mass of the tunneling entity and d is the thickness of the barrier. One should note that this model is simplified in several ways. First, the above equations are based on

rectangular barriers and free-particle wavefunctions, and actual number of the minima of the energy landscape is most likely greater than two shown in *Figure 2-14*, especially in proteins. Note also that in our case there are two TLS – for the excited electronic state and for the ground state of the guest molecule. For the purpose of hole burning process the relevant V and λ are the excited state ones.

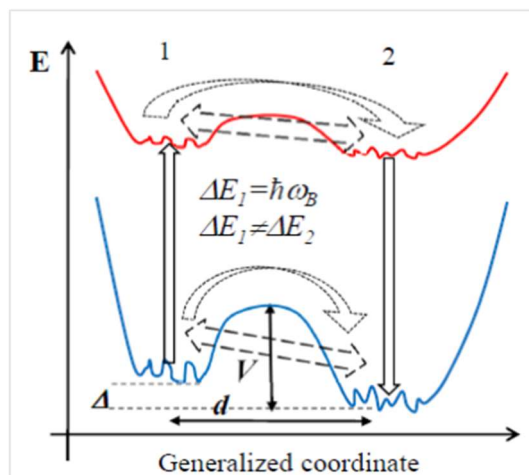


Figure 2-14: Schematic representation of TLS for NPHB. The structure at the bottoms of the two wells represents lower tier of the protein energy landscape. The lower tier is responsible for spectral diffusion - shifts smaller than homogeneous line width, affecting hole width but not hole area. [19]

After the absorption of a photon, the system starting from minimum 1 in the ground state may be excited to the minimum 1 of the excited state, and then if the barrier height in the excited state is significantly lower than in the ground state there is a non-negligible probability of transition from the minimum 1 to the minimum 2 in the excited state. The subsequent relaxation will take the system into minimum 2 in the ground state and it will stay there for some significant time period which will be defined by the ground state barrier parameters. In the figure $\Delta E_1 = \hbar\omega_1$ corresponds to the burning frequency matching the energy difference between the corresponding wells of

excited/ ground state landscapes; $\Delta E_2 = \hbar\omega_2$ is the new, post-burn transition frequency. The overall process will result in a light-induced transition from the minimum 1 of the ground state to minimum 2 of the ground state. As a result of the transition one can observe the shift in the absorption spectrum of the chromophore. This tunneling process is phonon-assisted process since there is an energy gap between the relevant energy levels in two wells; the chromophore-protein system may not just lose some energy but also acquire some, depending on the energy of the new state. Thus, we can introduce such concepts as downhill transition and uphill transition (or uphill tunneling and downhill tunneling). As a result of the uphill transition the scattered lattice phonon would lose some energy, while during the downhill transition the phonon gains some energy. The rate of the uphill tunneling is always lower than the rate of downhill tunneling, as the phonons must have some energy to lose it but do not need to have any to gain it. Using the tunneling prefactor (attempt frequency) Ω_0 we can write the rates for uphill and downhill processes in a simple manner:

$$R_{downhill} = \Omega_0 \exp(-2\lambda)(n(\Delta, T) + 1) \quad (39)$$

and

$$R_{uphill} = \Omega_0 \exp(-2\lambda)(n(\Delta, T)) \quad (40)$$

Here $n(\Delta, T)$ is the so called phonon population number.

Under the assumption that the linewidth of the laser is much narrower than the homogeneous line width, the post burn spectrum is given by the NPHB Master Equation

$$A(\Omega, t) = 1.5 \int d\omega L(\Omega - \omega) G(\omega) \int d\lambda f(\lambda) \int d\alpha \sin(\alpha) \cos^2(\alpha) e^{-Pt\sigma\phi(\lambda)L(\omega_B - \omega)\cos^2(\alpha)} \quad (41)$$

This is the post-burn absorption spectrum after burning at ω_B , with photon flux P for time t . $G(\omega)$ is pre-burn Site Distribution Function (SDF) which is the distribution of probabilities of ZPL transition of pigment/guest molecules (usually Gaussian), $L(\Omega - \omega)$ is Single Site Absorption (SSA) spectrum which is an absorption of the single guest molecule including ZPL and PSB and replicas due to intra-molecular vibrations with total area normalized to one on the frequency ω scale. α is the angle between the polarization of the laser and the transition dipole vector of the chromophore, σ in the exponential is the integrated absorption cross-section of the chromophore.[35] The distribution of α explicitly encoded into the above master equation corresponds to the case of random orientation of molecules in 3D and linear polarization of light. λ is the tunneling parameter (Eq. 38) while $f(\lambda)$ is the distribution of λ in the excited state of the pigment-protein system (See section 2.7). This distribution is usually considered Gaussian with mean value of λ_0 and standard deviation of σ_λ ; in some systems this distribution may be flat within a range (corresponding to the $1/\sqrt{V}$ barrier distribution from [20]). $\phi(\lambda)$ is the quantum yield (probability) of non-photochemical hole burning which is given by

$$\phi(\lambda) = \frac{\Omega_0 \exp(-2\lambda)}{\Omega_0 \exp(-2\lambda) + \tau_1^{-1}} \quad (42)$$

where Ω_0 is the attempt frequency, which defines how often system tries to pass the potential energy barrier between two wells in TLS model by tunneling and τ_1 is the observable fluorescence lifetime, that may or may not be affected by energy transfer or other form of quenching, depending on the sample. The attempt frequency is considered to be on the order of 10^{12} Hz [20],

a value that has recently been confirmed through simulations of tunneling in a realistic landscape.

[21] Equation 42 does not explicitly contain the phonon population number terms.

In the previous sections it was mentioned that the shape of the homogeneous spectral line (ZPL) is Lorentzian and for small fractional hole depth hole width $\Gamma_H = 2\Gamma_{\text{hom}}$. The factor of 2 results from the fact that a convolution of two Lorentzians is a Lorentzian that has the width that is the sum of the widths of the original Lorentzians. In the case of SHB, the modified SDF with the hole with the width Γ_{hom} is convoluted with the single-molecule spectrum whose ZPL has the same width Γ_{hom} , yielding $\Gamma_H = 2\Gamma_{\text{hom}}$.

For small fractional hole depths and with the absence of spectral diffusion (see below) the hole width may be represented as

$$\Gamma_H = 2\Gamma_{\text{hom}} = \frac{2}{\pi\tau_2} = 2\left(\frac{1}{2\pi\tau_1} + \frac{1}{\pi\tau_2^*(T)}\right) \quad (43)$$

However, in practice the real measured hole width (even for shallow holes) is affected by an additional factor Γ_{SD} that stands for Spectral Diffusion (SD), which is time and temperature dependent. Thus, the realistic line width becomes:

$$\Gamma_H = 2\Gamma_{\text{hom}} + \Gamma_{\text{SD}} \quad (44)$$

Spectral diffusion occurs because of the conformational changes on the smallest barrier tier of the energy landscape (see the bottom of the wells in *Figure 2-14*). For this tier spectral shifts are smaller than the homogeneous line width and therefore contribute to the apparent width of the hole and not to larger shifts that appear as NPHB. The hole broadening due to SD and

burning/recovery represent similar process on two distinct hierarchical tiers on the Protein Energy Landscape (PEL), (see section 2.9).

2.6 Hole Growth Kinetics

Hole Growth Kinetics (HGK) technique is one of the most useful tools for TLS or energy landscape barrier parameters determination. Amorphous solids including proteins do not have a single, rigid structure but rather a collection of nearly isoenergetic conformational substates which can be modeled as TLSs. A TLS is characterized by two nearly degenerate energy minima, representing distinct conformations, which are separated by a potential barrier. The barrier's height is determined by the collective, cooperative motion of many atoms or groups within the protein matrix. Their distribution reflects the complex energy landscape of the protein, and provides quantitative insight into a state of “rigidity” or “glassiness” of the protein. Using HGK or hole recovery (HR) techniques one can observe the rate at which molecules overcome these barriers (either to convert to an antihole configuration, undergo spectral diffusion or to recover) to model a Protein Energy Landscape (PEL) across the entire protein ensemble and to use barrier distribution as a fingerprint of the protein's glass-like heterogeneity.

Despite hole burning experiments being performed at cryogenic temperatures in order to suppress thermal fluctuations and isolate quantum phenomena (like tunneling), the barrier distributions reveal mechanisms and parameters that can be used to model and predict molecular dynamics at physiological conditions. Therefore, understanding the distribution of these barriers may help one to understand how and why a protein might fold, unfold, or undergo a functional conformational changes crucial for their biological activity. However it is worthy to mention that there is no consensus about what actually determined the observable protein

dynamics at physiological temperatures. The "slaving" hypothesis is a dominant framework used to explain how biological molecules function. At physiological temperatures the hydration shell is highly mobile, with water molecules exchanging on a picosecond to nanosecond timescale. The solvent provides the thermal energy and the "fluctuation space" necessary for the protein to transition between different substates. [22], [23], [24]

In HGK experiments one investigates the growth of a spectral hole, or in other words the decrease of absorption or fluorescence (in fluorescence excitation mode) while the sample is illuminated by a narrow-band laser at fixed frequency (and temperature). The fluorescence or transmission is monitored continuously while the spectral hole is being burnt. The burning is done with constant (stabilized) photon flux P in Eq. 41. The decrease of the fluorescence (or absorbance) signal usually is presented versus the irradiation dose (the product of time and intensity). The signal is collected by the photomultiplier tube; however other photodetector options are possible. As the ZPLs of the burnt molecules are no longer excited effectively and therefore do not fluoresce any more, the fluorescence signal at the burn frequency should decrease in an exponential fashion. In practice, however, it cannot be fitted to a single-exponential curve because of the dispersion of the barrier parameters. This dispersion can be traced back to the distribution of local environments, i.e. qualitatively the same phenomenon as the one responsible for inhomogeneous broadening. The fact that barrier parameters are not dependent on frequency within the inhomogeneously broadened band proves that both the frequency and the barriers are determined by a combination of many factors. To finalize the experiment and gain additional insights one must compare the fractional depth of the scanned hole spectrum measured after the HGK measurement is finished to the fractional depth of the hole spectrum obtained directly from the HGK. The Hole Spectrum scanning is usually done with

the intensities several orders of magnitude lower than the burning intensity and in the spectral region that is at least ten times wider than the spectral hole width. This comparison allows to determine if the hole and/or HGK had any transient component that must be taken into account in the subsequent analysis. The mismatch of the hole depth and HGK fractional depths may be due to fast recovery, fast spectral diffusion or triplets (See chapter 5.3).

HGK technique probes the parameters of the barriers (or, more precisely, of the distribution of the tunneling parameter) in the excited state, since the key step of burning is the transition between the wells in the excited state (recall *Figure 2-14*). The parameters of the tunneling parameter λ include; for example, in the case this distribution is Gaussian - the tunneling parameter distribution mean (λ_0) and its standard deviation (σ_λ). More details on the λ - and barrier distributions will be presented in Section 2.7.

Other parameters that can be obtained using HGK include electron-phonon coupling (S). Correct interpretation of the HGK requires the knowledge of S . To find the electron-phonon coupling parameter S one should burn out all the resonant molecules (through their ZPL) or in other words saturate the ZPH. Hole saturation (no more ZPLs are available at this burning frequency) is manifested by significant decrease in burning rate that is visible even on the logarithmic dose scale. The relation of the depth of the saturated hole (ZPH) to the electron-phonon coupling parameter S is given by Equation (45):

$$[\text{saturated fractional depth}] \approx \exp(-S) \quad (45)$$

After that the burning continues more slowly, due to burning through the PSB. To find the parameters of the distribution of the tunneling parameter (mean λ_0 and σ_λ in case of Gaussian distribution) one has to fix all the other parameters in the Eq. 46 to the values obtained

independently from other experiments (e.g. PSB shape (See section 2.8), S , fluorescence lifetime, etc.) and then to determine the best possible values for λ_0 and σ_λ of this HGK curve. *Figure 2-15* shows the theoretical dependence of HGK curve on σ_λ for the same λ_0 . Here changing σ_λ leads to different curvature in HGK curve.

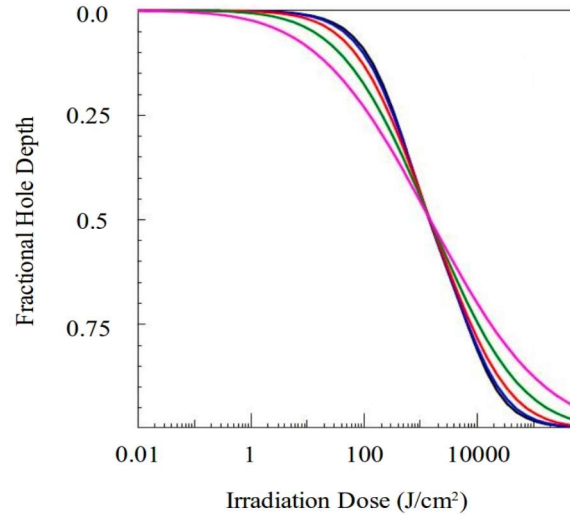


Figure 2-15: Modeled HGK curves for different parameters $\lambda_0 = 10$ and $\sigma_\lambda = 0.0$ (no dispersion; black), 0.3, 0.6, 1.0 and 1.5 (magenta).

The change in the mean of the λ_0 with all other parameters staying the same leads to shifting HGK curve towards higher or lower illumination doses without changing its shape. (*Figure 2-16*)

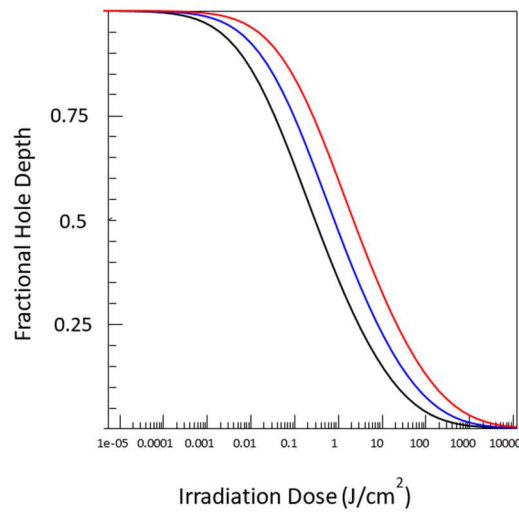


Figure 2-16: Simulated HGK curves at 5K with burn wavelength coinciding with the SDF peak. The Gaussian distribution of tunneling parameter was peaked at various $\lambda_0 = 11, 10.5$ and 10 for red, blue and black curves respectively. $\sigma_\lambda = 1$.

2.7 Hole Recovery

Complementary to HGK technique which allowed for investigating the parameters of the excited state landscape barriers, the Hole Recovery techniques give the insights into the ground state barrier distributions. There are two types of hole recovery experiments that may be conducted. The first one explores hole recovery that occurs spontaneously at the same temperature as burning while the second one involves exploring recovery that is induced by the temporary temperature increases. Note that to properly account for spectral diffusion, one is ultimately analyzing the remaining area of the spectral hole rather than depth.

At low (e.g. 5K) burn temperatures the recovery of the hole may take days, or even months, since this process occurs only through the tunnelling in the ground state (from substate two to substate one in Figure 2-14). It was shown by modeling that full recovery of the hole does

occur, but requires very long time.[12] Full recovery does occur since the populations altered by holeburning represent a departure from thermodynamic equilibrium, and eventually the system will always reach equilibrium, it just may take very long time. In the pigment-protein systems that are the focus of this thesis, the recovery observed within the first day is on the order of several tens of percent.

Another type of hole recovery experiment involves Thermocycling. When using this technique one should raise the temperature to the certain level (after the hole was burnt) and then temperature must be decreased to the temperature of burning in order to measure the hole spectrum again. This reduces the hole width (almost) back to burn T values and makes it easier to measure any changes reliably. (Spectral diffusion is irreversible, but the increase of the homogeneous line width with temperature is reversible). These steps are repeated with increasing the thermocycling peak temperature. During this process the recovery speeds up as heating of the sample causes both increasing (phonon-assisted) tunneling rate and opening up the barrier hopping mechanism (the tunnelling continues contributing to the recovery, but its temperature dependence is weaker than the exponential one for barrier-hopping and at some point barrier-hopping becomes dominant).

To analyze the hole recovery data we should determine the hole area as a function of time and temperature. That is necessary for explanation of both spontaneous fixed-temperature and thermocycling-induced recovery. To carry out this analysis a number of new variables and additional quantities have to be introduced.

- The number of pigments in the burned and unburned states are N_0 and N respectively

- λ distribution in the ground state is $g(\lambda)$ (the distribution $f(\lambda)$ from the master equation (Eq. 41) belongs to the excited state)
- The relaxation rate from burn to unburned state is $R(\lambda)$ at specific temperature T .

The number of recovered molecules that have relaxation rate between $R(\lambda)$ and $R(\lambda+d\lambda)$ is $N_0 g(\lambda)d\lambda$ [23]. We can present the change in the number of the burned states as:

$$dN(t, T) = N_0 g(\lambda) d\lambda \exp(-R(\lambda)t) \quad (46)$$

where t is the recovery time. By integrating Eq. 46 over all possible λ the total number of unrecovered (that are still in the burned state) molecules is:

$$N(t, T) = N_0 \int_0^{\infty} g(\lambda) d\lambda \exp(-R(\lambda, T)t) \quad (47)$$

According to the previous studies [19, 23] the dominant process at low temperatures around 5K is tunneling. It follows from the near independence of the burning rates on temperature.

Considering the tunneling rate until recently we relied on (Eq. 39) for downhill tunneling.[19] We used to assume downhill tunneling, because results (dependence of HGK on temperature) were more consistent with that than with other possibilities. By 2019 we adopted the viewpoint that the hole is a departure from thermodynamic equilibrium and return to equilibrium occurs with the rate that is the sum of the uphill and downhill rates. So in a two-state system one gets the rate $\sim 2n+1$ instead of $n+1$ for downhill tunneling and twice the rate for barrier hopping. $2n+1$ has similar temperature dependence to $n+1$, or at least more similar than n . For thermocycling the rate of transition due to barrier hopping must be taken into consideration. The barrier hopping rate is determined by Equation 48:

$$R(\lambda, T) = 2\Omega_0 \exp\left(\frac{-\hbar^2 \lambda^2}{2md^2 k_B T}\right) \quad (48)$$

where Ω_0 is the attempt frequency and where the potential barrier height is expressed with the help of Equation (36). The total relaxation rate may be written as a sum of (55) and (48).

$$R(\lambda, T) = \Omega_0 (2n(E, T) + 1) \exp(-2\lambda) + 2\Omega_0 \exp\left(\frac{-\hbar^2 \lambda^2}{2md^2 k_B T}\right) \quad (49)$$

Inclusion of both rates for the thermocycling experiments yields:

$$N(t, T) = N_0 \int_0^\infty g(\lambda) q(\lambda, T, t) d\lambda \quad (50)$$

where q is defined as:

$$q(\lambda, T, t) = \exp\left(-\Omega_0 t \left[(2n(E, T) + 1) \exp(-2\lambda) + 2 \exp\left(\frac{-\hbar^2 \lambda^2}{2md^2 k_B T}\right) \right]\right) \quad (51)$$

Function q shows how large is the fraction of unrecovered systems with specific λ after time t at temperature T . Since $q(\lambda, t, T)$ is a step-like function, equation (50) can be interpreted as follows:

by multiplying $q(\lambda, t, T)$ by the ground-state λ distribution $g(\lambda)$; or, more precisely, the partial distribution encoded into the hole (see *Figure 2-18 and 2-18*), the smaller- λ part of this distribution is chopped off, and the area remaining under the (partial) lambda-distribution curve, which is proportional to the hole area, is reduced (meaning the hole is partially recovered).

Figure 2-17 shows the simplified visualisation of modifying the hole area by the q function during the recovery.

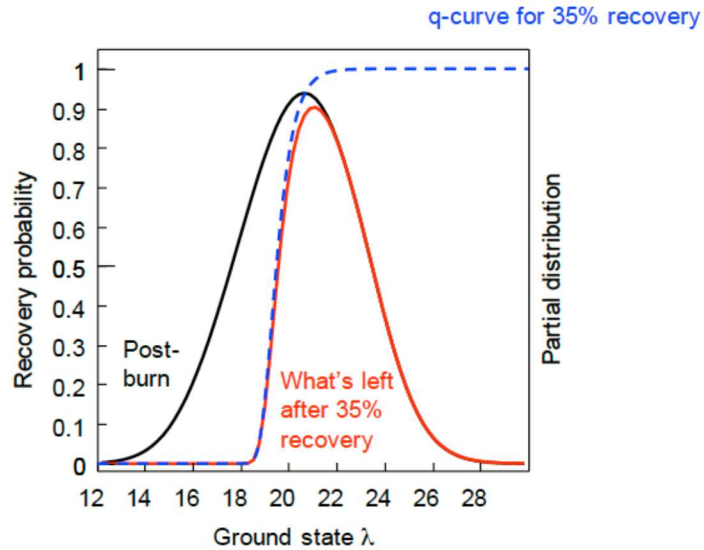


Figure 2-17: Post-burn partial distribution (black); q-curve resulting in 35% recovery of the hole (blue) and the partial distribution of λ encoded into the remaining hole (red)

Figure 2-18 shows the examples of partial distributions encoded into the holes in the cases of Gaussian and Boxlike (uniform) full λ -distribution (dashed lines). The boxlike λ -distribution follows from $1/\sqrt{V}$ barrier distributions proposed and observed for glasses. [25], [26] The distributions actually encoded in the holes (solid lines) are just (the lower-barrier) fractions of the full original distributions. The apparent area ratios do not match the percentages shown since the lowest- λ fraction of the distribution has all resonant molecules burned out very quickly and burning continues on account of slightly non-resonant molecules. In other words, the lowest-lambda fraction of systems exhibits saturation broadening of spectral holes.

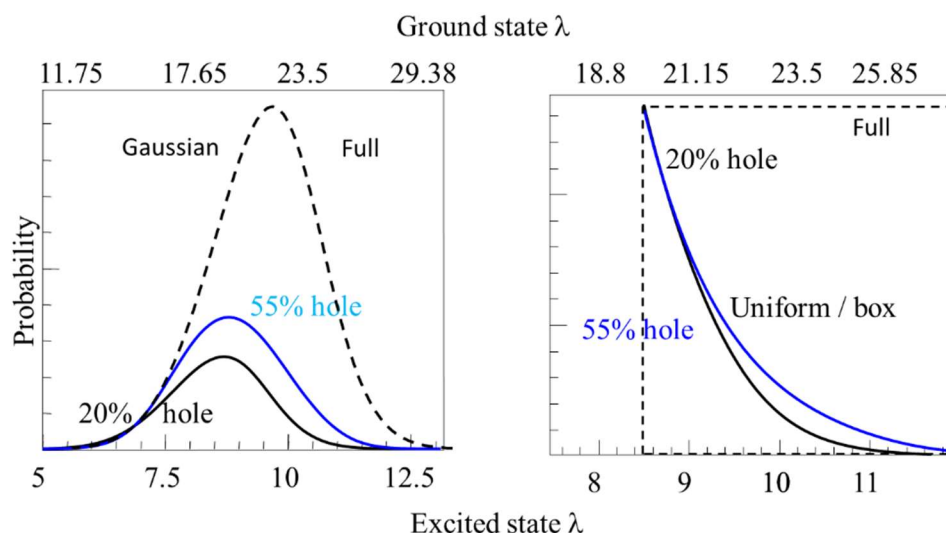


Figure 2-18: Partial distribution encoded in hole and manifesting in hole recovery in the case of a perfect spectral memory.

In order to find out the distribution of the tunneling parameter in the ground state one should measure the hole area evolution with time, calculate $q(\lambda, t, T)$ and fit the experimental data to the Eq. 50. In the case of thermocycling, a similar logic is followed; however, this technique requires applying a series of q -curves corresponding to various times and temperatures to fully map the barrier distribution.

2.8 Fluorescence Line Narrowing

Fluorescence Line Narrowing is another line narrowing technique that is used to overcome the problem of inhomogeneously broadened absorption spectrum of a disordered solid. When using FLN the resonant fraction of the inhomogeneously broadened absorption spectrum is preferentially excited (via ZPL), even though some molecules can be excited via PSB. [27] Thus the collected fluorescence emission mostly belongs to the portion of the molecules excited via ZPL. Therefore, compared to conventional fluorescence spectrum (obtained with broadband

and/or higher-energy excitation) the FLN spectrum is much narrower and more structured [28]. Additional advantage of FLN is applicability to systems without NPHB mechanism. Under ideal experimental conditions the main feature of FLN spectrum is the ZPL, but in practice it is often smeared by scattered excitation light. FLN spectrum contain also pseudo PSB and real PSB (see *Figure 2-12*), however both of them are located to the red with respect to the ZPL / excitation frequency in contrast to SHB where the real PSB are to the blue of the ZPH.

Δ FLN is a technique combining FLN with NPHB, with two FLN spectra measured before and after burning a spectral hole.[29] [30] Subtraction allows for elimination of the contribution from scattered excitation light. This technique is especially useful for defining the electron-phonon coupling parameters. [28] The shape of the Δ -FLN is equal to the shape of the Single Site Emission spectrum since the double spectral selection effect of SHB and FLN eliminates the pseudo PSB contribution.

Figure 2-19 is an example of Cytochrome b_{6f} (Cyt b_{6f}) Δ FLN spectra. The figure depicts the FLN spectra for two different detergents (n-dodecyl β -D-maltoside (DM) and n-octyl β -D-glucopiranoside (OGP)). The blue spectrum is different from red and black ones due to energy transfer between two Chls in Cyt b_{6f} . For long enough excitation wavelengths the Δ FLN spectra for DM- based samples (red, black) do not depend on wavelength, proving that pseudo-PSB is suppressed.

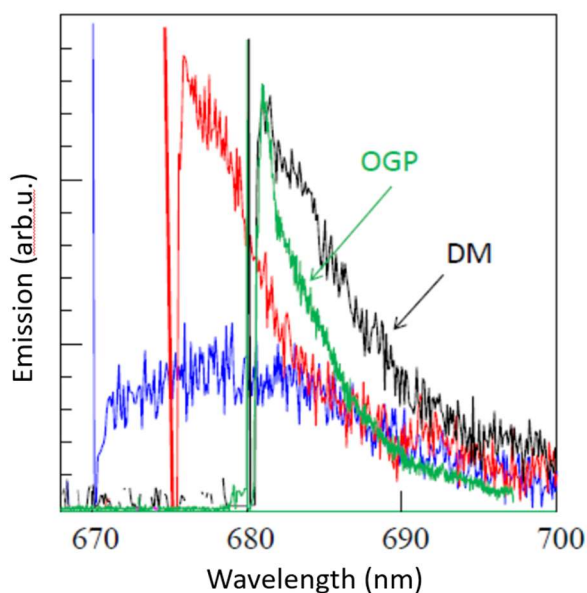


Figure 2-19: Δ FLN spectra of sample of Cytochrome b_6f solubilized with dodecylmaltocyclodextrin detergent. Green curve belongs to Cyt b_6f sample where Cytochrome was solubilized with OGP detergent. The latter sample features smaller electron-phonon coupling.

Both SHB and FLN techniques provide independent information on the shape of the single-site spectrum. That shape is an important part of the model which is used in the study of energy landscapes of proteins.

2.9 Protein Energy Landscape

Finally, we provide more details on the Protein Energy Landscapes that were modeled with TLS in the previous subsections. The interactions responsible for this energy are electrostatic and entropic in nature.

A protein is a highly complex biological molecule, a long chain of amino acids that folds into intricate secondary, tertiary, and quaternary structures. Unlike a rigid crystal lattice, a protein is a somewhat amorphous substance, meaning it lacks the long-range order of a crystal. This structural complexity makes it nearly impossible to map its absolute, exact energy landscape.

Moreover, high resolution X-ray diffraction structures appear to be misleading because they create an impression that the structure is very well defined. In reality, they represent an average of several similar configurations.

The process of protein folding is currently best described by the folding funnel hypothesis which states that the native state of a protein corresponds to its global free energy minimum under its given environmental conditions. [31] [32] As a protein folds, it progressively reduces its conformational entropy and energy. The energy landscape has many local minima which can correspond to partly folded or misfolded protein states where the protein can become kinetically trapped during the folding process.

The Protein Energy Landscape (PEL) or multidimensional potential energy surface of a protein is a theoretical model used to describe the complex interactions between structure and free energy of the protein. This concept provides a statistical description of a protein's potential energy, creating a framework needed to understand protein folding and conformational dynamics of the protein's native states. The PEL near the energy minimum is also rugged, and the protein constantly fluctuates between these substates, enabling different biological function. This behavior resembles those of amorphous material like glasses, where the potential energy surface has multiple local minima. The *Figure 2-20* visualizes the PEL in two dimensions for simplicity, however, in reality the PEL is a multidimensional space representing all possible degrees of freedom of the protein. [33]

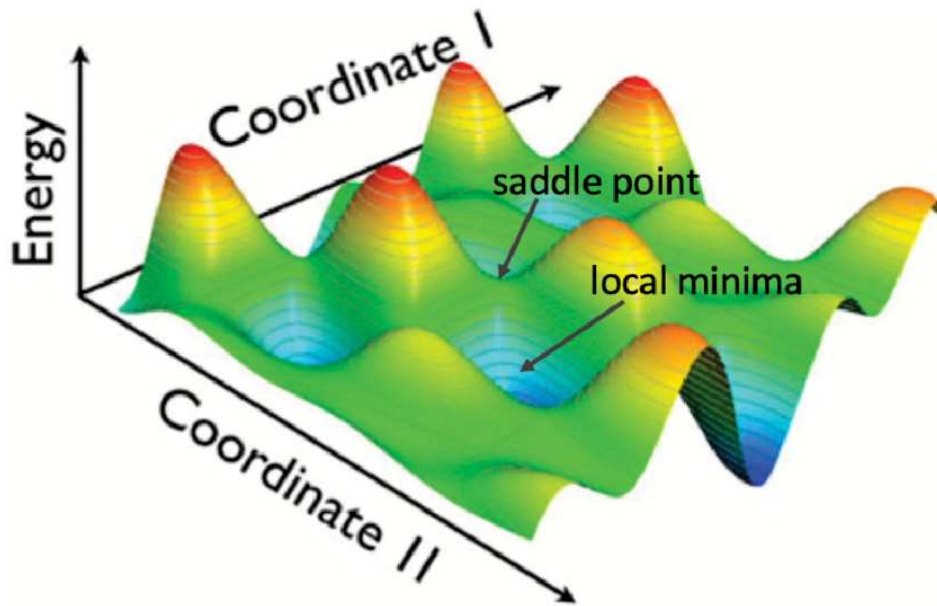


Figure 2-20: Protein energy landscape with two generalized coordinates. [34]

According to protein folding theory, a protein's stable, native state is well-defined and corresponds to a relatively narrow range of low-energy configurations. The native structure of a protein is not a single, static state. Instead, it is an ensemble of substates that correspond to specific molecular configurations, each a local energy minimum. These substates represent local energy minima separated by potential barriers that must be overcome for the protein to switch from one conformation to another. The PEL, therefore, can be thought of as a collection of these local minima and potential barriers along a generalized coordinate that represents the protein's conformational space.

3. Photosynthetic Pigment-Protein Complexes

Photosynthesis is one of fundamental processes of the ecosystems on Earth. Nearly every oxygen atom in the atmosphere was at some point in time released from a water molecule by photosynthesis. For the past 3.5 billion years the Photosynthesis has been the main mechanism to convert solar energy into chemical energy needed to support life. The critical evolutionary step to oxygenic photosynthesis likely occurred in a lineage ancestral to cyanobacteria between 3.3 and 2.9 billion years ago, long before the oxygen it produced began to significantly alter the planet's atmosphere. Nowadays, if photosynthesis ceased, there would soon be little food or other new organic matter on Earth, most organisms would disappear, and the oxygen in the atmosphere would be depleted.

In addition, photosynthesis is biologically important in a number of more indirect ways. For instance, the release of molecular oxygen is a prerequisite for the formation of stratospheric ozone. Photosynthetic assimilation of CO₂ in aquatic environments also plays a critical role in the global carbon cycle, as it facilitates the formation of carbonate shells by aquatic organisms. [35] By consuming dissolved CO₂ photosynthesis shifts the carbonate equilibrium in the water, which raises the pH and makes it easier for organisms to precipitate calcium carbonate for their shells. In this chapter, I will give an overview of the process and its main components and pathways.

3.1 Photosynthesis

Photosynthesis occurs in leaves of higher plants and in many kinds of bacteria and algae and to some extent in the stems of green plants. Plant cells contain organelles called chloroplasts that are actually in charge of the photosynthesis. In the more primitive organisms, the apparatus of

photosynthesis may instead be incorporated into the cell membrane. Photosynthesis can be divided into two types: oxygenic photosynthesis and anoxygenic photosynthesis. Oxygenic photosynthesis is the most common and is seen in plants, algae and cyanobacteria. Anoxygenic photosynthetic and oxygenic photosynthetic differ based on the electron donors used for the primary steps of photosynthesis. In both oxygenic and anoxygenic photosynthesis, the reactions begin with the absorption of light by pigments in the light-harvesting complexes embedded in the thylakoid membrane (or in case of cyanobacteria, also in phycobilisomes). Anoxygenic photosynthesis involves only one type of reaction center (RC), in contrast to oxygenic photosynthesis that involves two, each of which absorbs at slightly different wavelengths. [3]

3.1.1 Photosynthesis Efficiency and Proteins

The efficiency of the first steps of excitation energy conversion in the photosynthetic reaction centers is close to ideal - the internal quantum yield is close to unity. This means that the absorption of one photon anywhere in the antenna usually leads to charge separation in the RC. Currently, there are no artificial devices that can achieve such results, despite significant advances in photovoltaic cells in recent decades. The thoughtful scaffolding and structural composition of proteins organize the processes of energy and charge transfer with maximum efficiency (*Figure 3-1*). Therefore, a deep understanding of the actors involved in EET and CS is necessary for better design of the artificial solar energy harvesting devices.

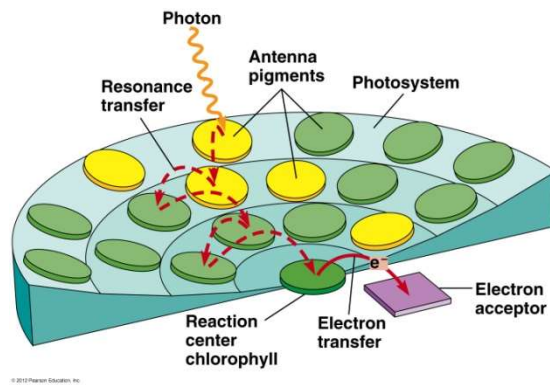


Figure 3-1: Primary steps of energy and electron transfer in photosynthesis. [36] Yellow and green – antenna pigments. The protein arranges pigments in such way that they form a funnel for energy transfer purposes, directing the energy to the RC featuring Chl electron donor (dark green) and electron acceptor (purple).

3.2 Pigments

Pigments are the means by which the energy of sunlight is captured for photosynthesis. However, since each pigment absorbs only a narrow range of the spectrum, there is usually a need to employ several kinds of pigments, each of a different color, to capture more of the Sun's energy. In higher plants there are two main classes of pigments that play an essential role in the photosynthesis process: the chlorophylls (Chls) and the carotenoids (Cars), which could be further divided to subgroups associated with their spectral properties. In anoxygenic photosynthesis, phototrophic bacteria use bacteriochlorophylls (BChls) instead of Chls. They are similar to Chls, but their absorption extends into the infrared region. Each of them has slightly different absorption spectrum that helps to cover and maximize the sunlight absorption. *Figure 3-2* illustrates the absorption spectra of most common pigments involved in photosynthesis:

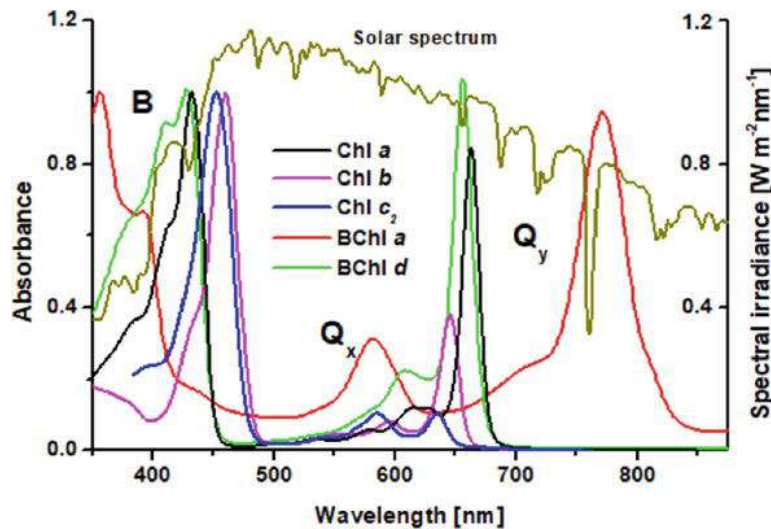


Figure 3-2: Absorption spectra of selected Chls in methanol solution, Chls, BChls of whole cells, and carotenoids in hexane. Pigments dissolved in solvents have absorption peaks slightly shifted from those in cells.[37] .

3.2.1 Chlorophyll

Chlorophyll is the most common pigment that is involved in harvesting light energy and is found in all oxygenic photosynthetic organisms. The chlorophylls are a group of tetrapyrrolic pigments with common structural elements and functions. In chemical terms they are cyclic tetrapyrrole derivatives of porphyrin, chlorin (17,18 – dihydroporphyrin) or bacteriochlorin (7,8,17, 18 – tetrahydroporphyrin). Most of Chls molecules are chemically classified as chlorin because of the reduction of its D ring (*Figure 3-3*).

The most common types of chlorophyll are Chl *a* and Chl *b* which differ with respect to both role and relative abundance in higher plants. Chl *a*/Chl *b* ratios commonly range from 3.3 to 4.2 in well-nourished sun-adapted species but can be as low as 2.2 in shade-adapted species grown at low light. Such variation is easily reconciled with contrasting functional roles for both Chl *a* and Chl *b*. [38]

Chlorophyll *a*

Chl *a* is found in most organisms that are capable of oxygenic photosynthesis, where it occurs in both reaction centers (RC) and in all light-harvesting complexes (LHC) with very few exceptions [5]. It functions as the primary donor in the RC of photosystem II (PS II), and Chl *a* or a closely related pigment is also the primary donor of photosystem I (PS I)[5]. Chl *a* absorbs strongly in the visible range, but its intense bands are narrow and therefore it must be supplemented by Chls *b* and Carotenoids in its role of an antenna. Chemical structure of Chl *a* is shown in *Figure 3-3* with two different types of atoms numbering system commonly used in literature.

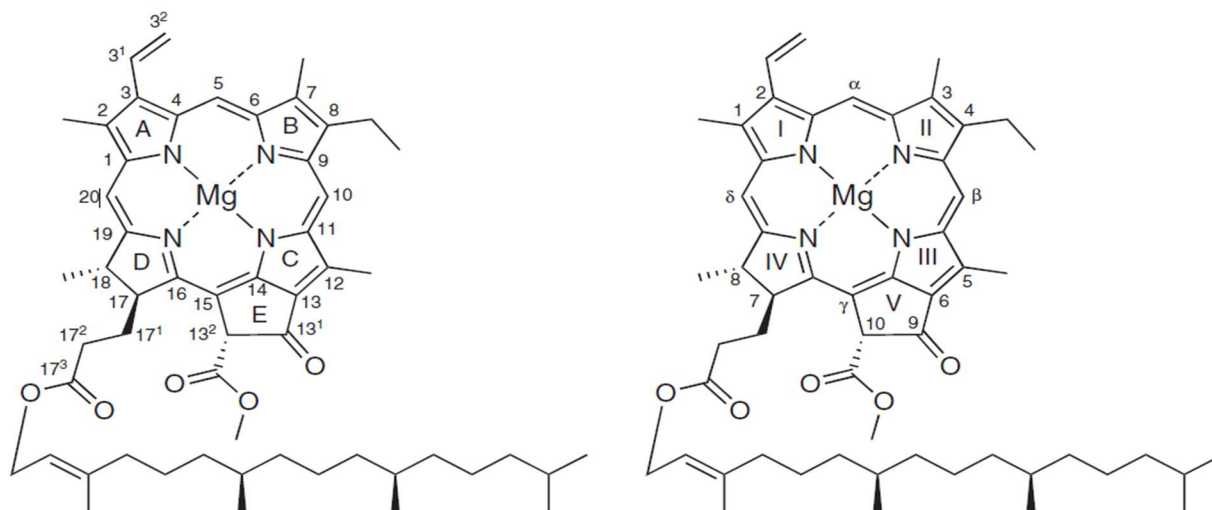


Figure 3-3: Chlorophyll a chemical structure with IUPAC and Fischer numbering system. Chlorophyll a is shown, although the same basic numbering scheme applies to all chlorophyll-type pigments. Left: the current IUPAC standard system. Right: the older Fischer numbering system. Hydrogen atoms are not shown. [39]

Chlorophyll *b*

Chl *b* is considered as accessory pigment for Chl *a*. It slightly differs in chemical structure (See Fig. 1.10) by substitution of methyl group to aldehyde group, resulting in shifted absorbance peaks. Namely, this results in the shift of the Q_y band from 670 to about 640-650 nm. In fact, it helps

better coverage of sunlight emission spectrum, in the same manner as Carotenoids help to absorb from violet to green light. (Figure 3-4)

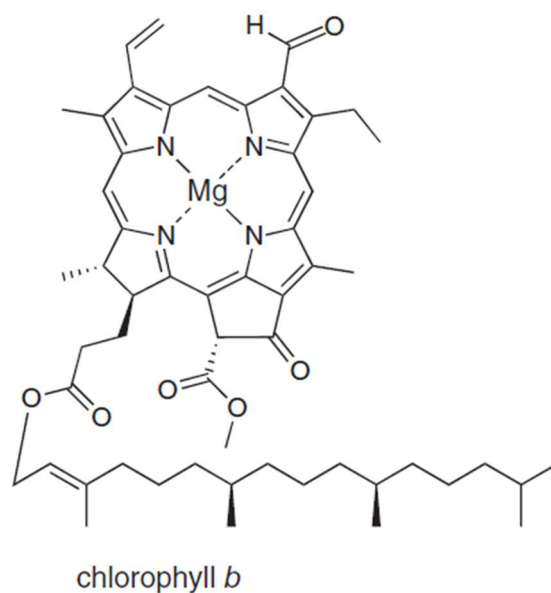


Figure 3-4: Chlorophyll *b* chemical structure. [39]

3.2.2 Bacteriochlorophyll

In anoxygenic photosynthetic complexes, Bacteriochlorophylls (BChls) are the main pigments, closely related to Chl pigments in both their structure and functions. Most of BChls are chemically classified as bacteriochlorins due to reduction of both pyrrole rings *II* and *IV*. (Figure 3-5)

However, some BChls feature a chlorophyll- chlorin nature. For example, BChls *c*, *d*, *e*, and *f* have a chlorin macrocycle ring with only one reduced pyrrole ring(*IV*). In contrast, BChls *a*, *b*, and *g* have a “standard” bacteriochlorin macrocycle ring with two reduced pyrrole rings (*II* and *IV*). [40]

The difference between a chlorin and a bacteriochlorin lies in their hydrogenation state. A chlorin has a single reduced double bond (of porphyrin), which shifts its absorption peak to the red part of the spectrum (around 670-700 nm). In contrast, a bacteriochlorin has two reduced double bonds, which results in a further redshift, shifting its absorption peak into the near-infrared region (around 800–1000 nm).

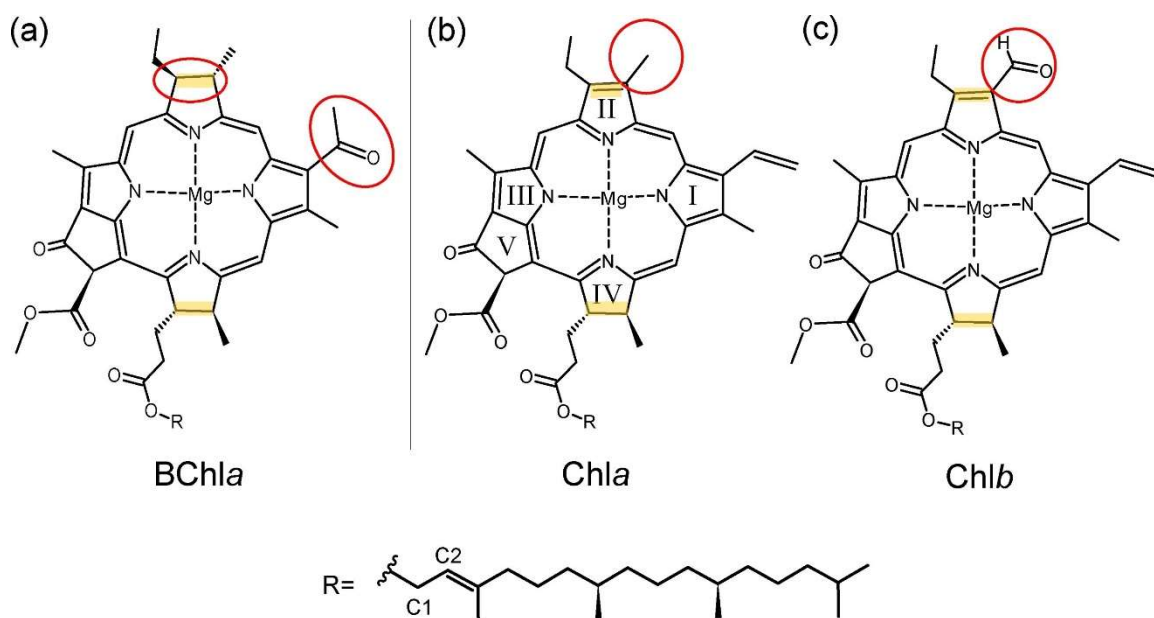


Figure 3-5: Molecular structures of (a) BChl, (b) Chl a and (c) Chl b, where R represents the phytol tail. Red circles highlight the specific differences between various chlorophylls. The hydrogenation states differences highlighted in yellow. [41]

Bacteriochlorophyll *a* is the most abundant pigment found in Purple bacteria. The coordination number for the central Mg atom is usually 5 in protein environment (In Figure 3-5 the axial ligands are not shown). However, it may exist in other states with 4 (no axial ligands) or 6 ligands. The latter may occur when the Mg atom binds to two axial ligands, although it is considered to

be a rare state in native protein pigment complex (It holds true also for other Chls pigments). [42]

The immediate surrounding inside the protein complex, coordination number of pigments and inter-pigment interactions modify spectral properties resulting in different absorption maxima in different organisms of the same pigment.

3.2.3 Oxygenic photosynthesis.

During oxygenic photosynthesis, light energy is used to transfer electrons from water to carbon dioxide, producing carbohydrates. In this transfer, the carbon dioxide is reduced and the water becomes oxidized. Ultimately, oxygen is produced along with carbohydrates. The photosynthesis process consists of two main classes of reactions. The first requires the presence of light, and is called “Light reactions”, the second that does not require presence of light, is called the “Dark reactions”.

The very first event in photosynthesis is the light absorption by pigments: chlorophylls (Chl), carotenoids, and phycobilins, contained in antenna protein complexes. (*Figure 3-1*) The absorbed energy is transferred in the form of (electronic) excitation energy until it reaches reaction centers (RCs), located in two large membrane-bound pigment– protein complexes named photosystem I (PSI) and photosystem II (PSII). The light-induced excitation (indirect, via EET, or direct) of a pigment molecule in the RC of PSII initiates the transfer of an electron to the primary electron acceptor, and this electron is eventually replaced by an electron taken from water. *Figure 3-6* shows the overall scheme for transfer of 4 electrons from water to NADP⁺, release of one oxygen molecule (O₂) and the operation of the Q-cycle. Thus, each Photosystem (I and II) will need to receive at least 4 photons to power these steps. [43]

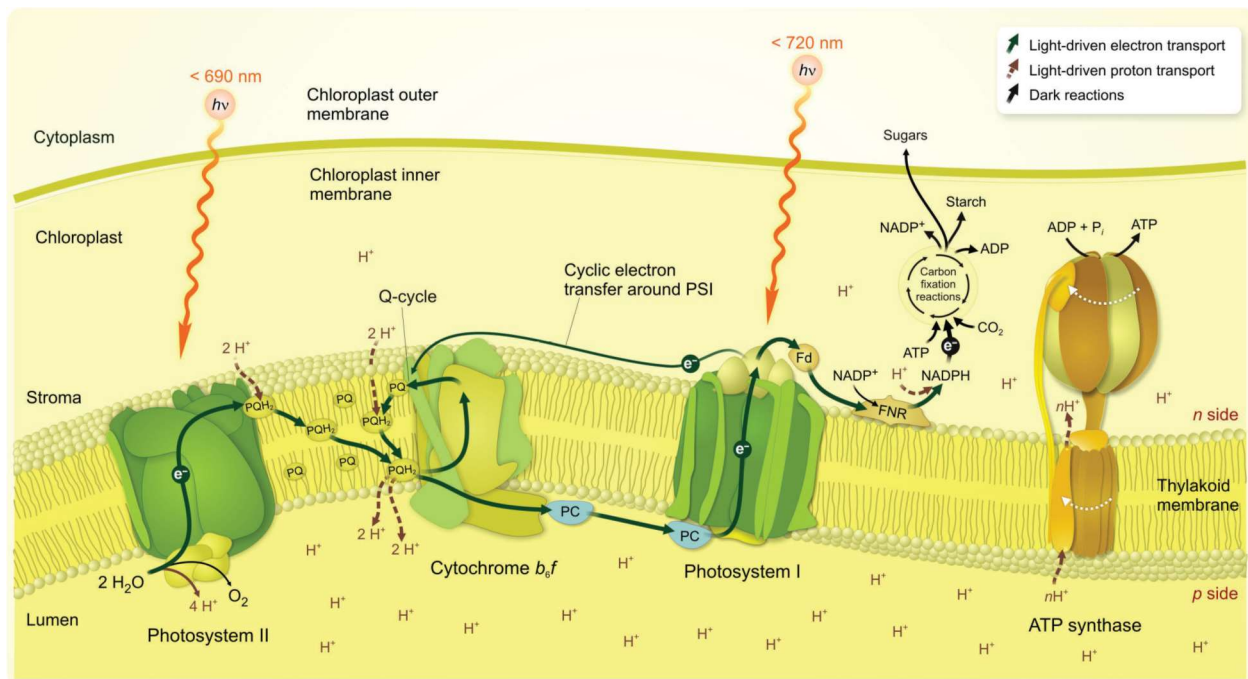


Figure 3-6: A simplified schematic representation of a thylakoid membrane, photosynthetic protein complexes and some intermediates (metabolites) involved in the electron and the proton transport reactions of photosynthesis in higher plants and green algae. The dark green arrows indicate the light-driven electron transport, brown arrows indicate the proton flow, and some dark reactions are colored in black. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; FNR, ferredoxin-NADP⁺ oxidoreductase; NADP⁺ and NADH, oxidized and reduced forms of nicotinamide-adenosine dinucleotide phosphate; Pi, orthophosphate; PQ, plastoquinone. [4]

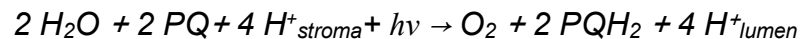
At each step, the resulting "electron hole" in PSII is filled by a multi-step process ultimately taking an electron from a water molecule. This also contributes to proton concentration gradient across the membrane. The electron then passes through an electron transport chain located in the thylakoid membrane. The flow of these electrons through Cyt *b₆f* generates further contributions to the proton gradient across the membrane, which is then used by an enzyme called ATP synthase to produce ATP (adenosine triphosphate), through a process known as chemiosmosis. At the end of the electron transport chain, the electron is used to reduce NADP⁺ to NADH via PSI. These energy-rich molecules, ATP and NADH, then serve as the primary power sources for the

light-independent reactions. During this process, carbon dioxide and water combine to form carbohydrates like glucose, a process known as carbon fixation.

3.3 Protein Complexes of Oxygenic Photosynthesis

3.3.1 Photosystem II (PSII)

In any organism capable of carrying out oxygenic photosynthesis, PSII functions as a water-plastoquinone oxidoreductase, using light energy to drive the transfer of electrons from water to plastoquinone, resulting in a splitting of water molecule into molecular oxygen and protons. The overall reaction may be shown as:



Plastoquinone (PQ) is a molecule that functions as a mobile electron and proton carrier between photosystem II (PSII) and the cytochrome *b₆f* complex. By accepting two electrons from PSII at the binding site (Q_b) and simultaneously two protons from the stroma, PQ is reduced to plastoquinol (PQH₂). Chemically, PQ consists of a quinone head group and an isoprenoid tail. The quinone has a benzoquinone ring that undergoes reversible oxidation and reduction, allowing it to transfer electrons. The isoprenoid tail has a hydrophobic polyisoprenoid side chain, which anchors the molecule in the lipid bilayer.

PSII is a large membrane-integral pigment–protein complex, which exists as a dimer with a total mass of ~700 kDa. [4] Each monomer of PSII contains 20 proteins and about 90 cofactors (mostly pigments). 17 of the proteins are transmembrane subunits and 3 are peripheral subunits on the luminal side of the complex. Some of these proteins and major redox cofactors are shown

in Figure 3-2. All electron transfer cofactors of PSII are bound to two central transmembrane proteins D1 and D2. These proteins exist as a D1/D2 heterodimer, which, together with a few small proteins, forms the core of the PSII RC. The D1/D2 heterodimer binds two branches of redox cofactors related by pseudo- C_2 symmetry. However, the electron transfer within PSII occurs via cofactors located mainly on the D1 side of the D1/D2 heterodimer. By analogy with the Bacterial Reaction Center of purple bacteria (Section 3.5.2), it was believed for a long time that the primary donor in PSII is a special pair PD1-PD2. (P680, labeled according to its peak absorption). However, many inter-pigment couplings energies in PSII RC are comparable to PD1-PD2 coupling, so alternative models have been proposed where primary charge separation starts from PD1-Chl D1. [4] Figure 3-7 shows schematic arrangement of central redox cofactors of cyanobacterial PSII monomer. This figure includes the D1 and D2 subunits, several minor RC subunits as well as CP43 and CP47 core antenna complexes.

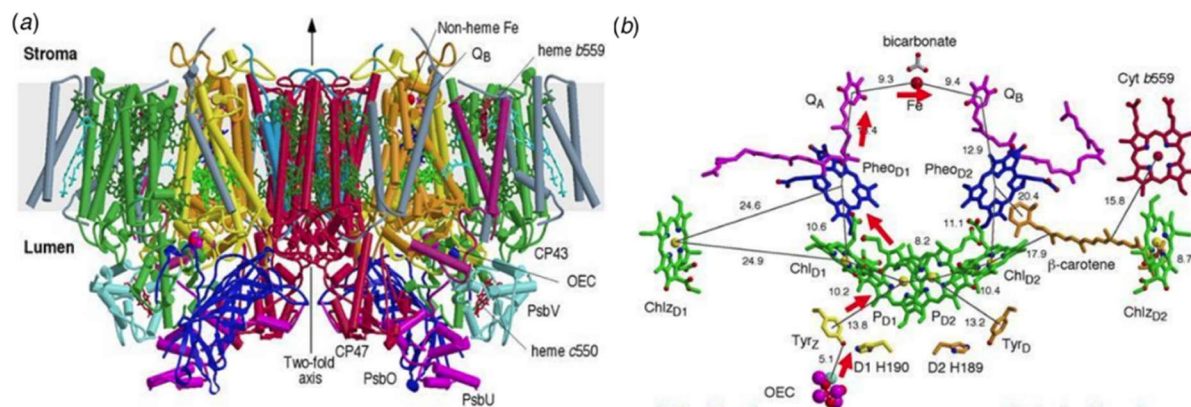


Figure 3-7: (a) Side view of the structure of Photosystem II, the water splitting enzyme of photosynthesis. The D1- and D2-proteins that compose the RC are shown in yellow and orange respectively. (b) Organisation of the electron transfer cofactors that make up the RC of Photosystem II. Excitation of the RC via the chlorophylls (Chl) shown in green leads to the reduction of pheophytin (Pheo) resulting in the formation of the radical pair $P680^{++}Pheo^{\bullet-}$. The radical cation of P680 is localised on P_{D1} , while the radical anion is located on $Pheo_{D1}$. The electron on $Pheo_{D1}^{\bullet-}$ is rapidly donated to a firmly bound plastoquinone Q_A (shown in purple) and then transferred to a second, mobile, plastoquinone Q_B (also shown in purple). [44]

3.3.2 Photosystem I

PSI is a membrane-integral protein complex that is present in all oxygenic photosynthesizers. PSI, just like PSII, is also a main player in converting light energy for driving the redox reactions of the ETC. Thus, PSI uses the energy of light to power the electron transfer from one mobile electron carrier PC (Plastocyanin; or alternatively Cyt c_6) to another carrier, Fd, and thereby provides the electrons for the reduction of NADP⁺. It therefore acts as a light-driven plastocyanin-ferredoxin oxidoreductase. [45] In plants, PSI always exists as a monomeric complex, however in cyanobacteria PSI exists mainly as a trimer having a molecular weight of ~1100 kDa. PSI monomer contains 12 protein subunits and about 130 noncovalently bound cofactors. [89, 90] The core of PSI complex is formed by two proteins, PsaA and PsaB. These two proteins coordinate the majority of the cofactors of the ETC within PSI, as well as most of the antenna Chls and the Cars of PSI. Moreover, PsaA/PsaB heterodimer is known to be involved in the docking of the mobile electron carrier (PC or Cyt c_6) near P700 on the electron donor (luminal) side of PSI. *Figure 3-8* shows schematic arrangement of the central redox cofactors of PSI. [4]

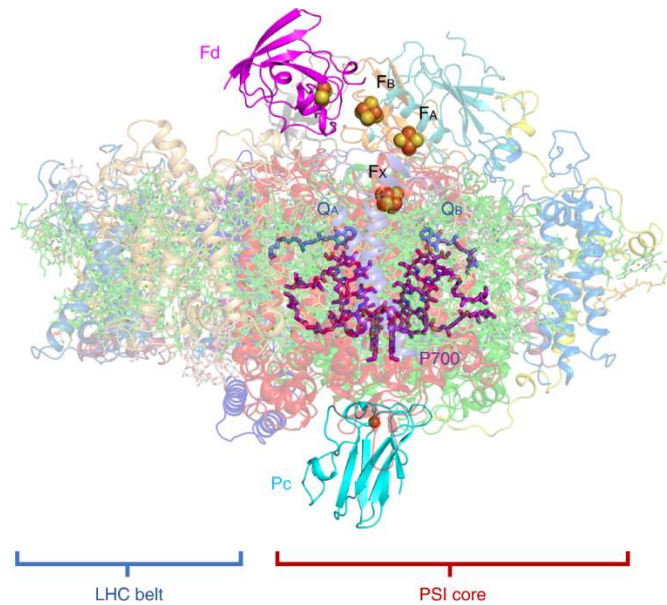


Figure 3-8: The structure of a complex of plant photosystem I with ferredoxin and plastocyanin. PC is coloured in cyan, Fd in magenta, ETC chlorophylls in purple and the quinones in slate. Cu^+ , [4Fe-4S] and [2Fe-2S] clusters are shown as spheres. PsaA is coloured in red, PsaB in green, PSI chlorophylls are coloured in green, carotenoids in orange and lipids in white. Q_A and Q_B are the quinones of subunits PsaA and PsaB, respectively.[46]

3.3.3 Cytochrome b_6f

Light-induced electron transfer between the two photosystems is mediated by the membrane-integrated Cyt b_6f complex. This complex catalyzes the transfer of electrons from lipophilic PQH_2 to the soluble PC. Moreover, the intersystem electron transport performed by the Cyt b_6f complex is coupled with the transport of protons from the stroma to the lumen, thus contributing to the generation of a proton gradient and an electrochemical potential across the thylakoid membrane that is further utilized by the ATP synthase for ATP synthesis. In both cyanobacteria and plants, the Cyt b_6f complex exists as a dimer and contains several prosthetic groups. Each monomer is composed of eight subunits.[84] These include four large subunits: a 32 kDa cytochrome f with a c-type cytochrome, a 25 kDa cytochrome b_6 with a low- and high-potential

heme group, a 19 kDa Rieske iron-sulfur protein (ISP) containing a [2Fe-2S] cluster, and a 17 kDa subunit IV; along with four small subunits (3-4 kDa): PetG, PetL, PetM, and PetN. The total molecular weight of the dimer is 217 kDa.

When PQH₂ is oxidized by the FeS protein, protons are released into the lumen. One of the two available electrons delivered by PQH₂ passes along a linear Electron Transfer Chain (ETC) to the Cyt *f* subunit and further to a small mobile PC (or Cyt *c*₆), which then carries the electron toward PSI. The other electron is transferred through the two Cyt *b*₆ hemes, which then reduces a PQ to PQ⁻ (semiquinone). Upon receiving one of the two electrons from a new PQH₂ molecule (oxidized by the FeS), PQ⁻ is reduced to PQ²⁻ at the same time picking up two protons (and forming PQH₂), participating thereby in a cyclic process called the Q-cycle. Thus the Q-cycle increases the number of protons pumped across the membrane. Overall, for every two electrons that reach PSI, four protons are translocated across the thylakoid membrane. Figure 3-9 depicts the Cyt *b*₆*f* structure. [4] One Chl per monomer are present in Cyt *b*₆*f*. The role of these Chls is unclear as they do not participate in light harvesting or transfer energy to other pigments in adjacent complexes. They might be an evolutionary relic. The Chls of two monomers are far apart (around 6 nm). This makes Cyt *b*₆*f* a good model system to explore slow EET and protein dynamics by means of optical spectroscopy. Note that in the subsequent chapters we focus not on the biochemical functions described above, but utilize Cytochrome *b*₆*f* as a model system to study light-induced spectral dynamics. Due to relatively large distance between two Chlorophylls a of the Cyt *b*₆*f* dimer, excitonic effects in this system are negligible.

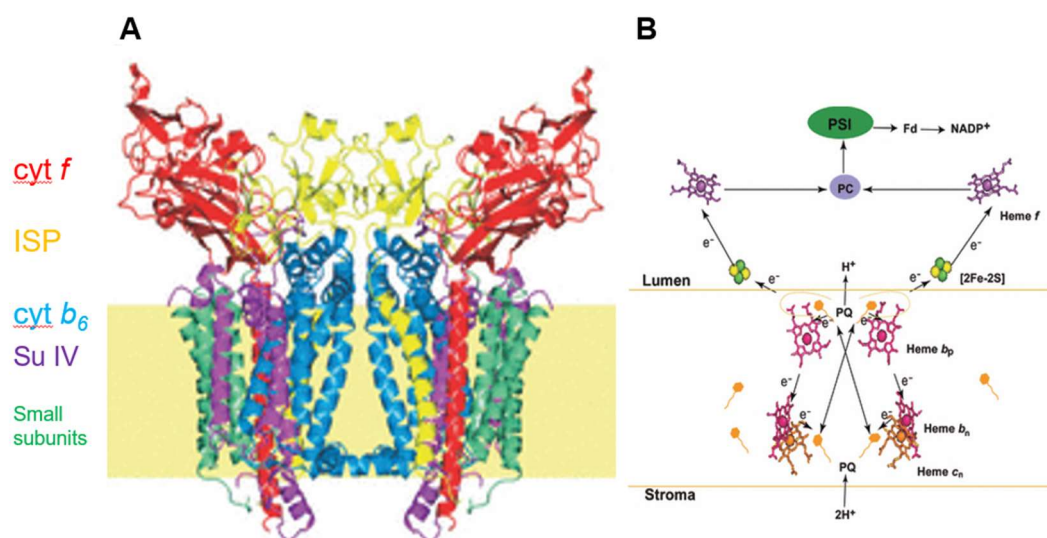


Figure 3-9: Structure and transfer pathways in the dimeric cytochrome b_6f . Frame A: Structure of the cytochrome b_6f . Ribbon diagram shows a three-dimensional structure of the eight subunit cyanobacterial b_6f complex (PDB id 2E74). [47] Frame B: Schematic of the transmembrane electron, proton, and plastoquinone(ol) transfer pathways in the dimeric cytochrome b_6f complex. After entry from a pool (~ 10 PQ per cyt f) in the lipid bilayer membrane into the inter monomer quinone exchange cavity, PQ/PQH₂ are transferred across the quinone exchange cavity between the reducing site near heme b_n -heme c_n on the stromal (n) side of the complex to the oxidation site near the [2Fe-2S] cluster of the Rieske ISP on the p (lumen) side.[47]

3.4 Electron transfer

Electron transfer is the central process in the light-dependent reactions of photosynthesis. The details of the process are common to different plants and bacteria. [48] [49] In photosynthesis, there are two primary types of electron transfer pathways: Linear Electron Flow and Cyclic Electron Flow. These pathways occur within the thylakoid membranes of chloroplasts (in plants and algae) or the plasma/internal membranes of cyanobacteria, and both contribute to the production of chemical energy. The electron transfer pathway from water to NADP⁺ is driven by two membrane-bound photosystems. The excitation energy harvested by the antenna is captured

by the assembly of unique photoactive Chls molecules, denoted as the Reaction Center of photosystems (*Figure 3-1*).

The electron transfer process is summarized by the Z-scheme depicted in *Figure 3-10*.

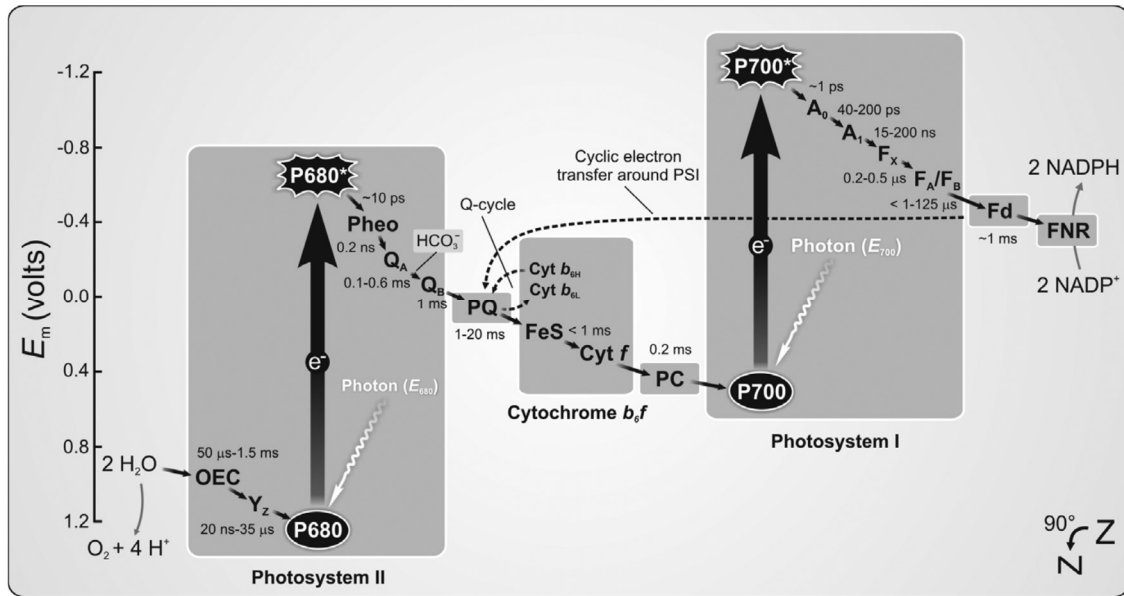


Figure 3-10: The zig-zag or Z-scheme of oxygenic photosynthesis representing the energetics of linear electron transfer from H₂O to NADP⁺ plotted on the redox midpoint potential (E_m , at pH 7) scale. The diagram also shows a cyclic electron transfer around PSI, Q-cycle, and half-times of several linear electron transfer steps. The two black vertical arrows symbolize the excitation of RC Chl a molecules (P680 and P700 in PSII and PSI, respectively); these lead to electrons in the ground state to be raised into a higher (singlet) excited state in response to the absorption of excitation energy from the light-harvesting antenna or by direct absorption of photons (wiggly white arrows). [43]

Under specific conditions, in particular when the cell needs more ATP than NADPH to balance energy requirements for different metabolic processes, electrons can take a cyclic pathway.

Electrons from ferredoxin are transferred back to the plastoquinone or directly to the cytochrome b₆f complex instead of going to NADP⁺ reductase. These electrons then cycle back through the

Cyt *b₆f* complex and plastocyanin to PSI. In this pathway no NADPH is produced, but it contributes to the proton gradient to facilitate production of ATP molecules.

3.5 Protein Complexes of Anoxygenic Photosynthesis

3.5.1 Anoxygenic Photosynthesis

Anoxygenic photosynthesis is carried out by a large number of photosynthetic bacteria where most of them use inorganic carbon as their carbon source, while others use organic compounds. In oxygenic type of photosynthesis water is used as an ultimate electron donor, whereas an anoxygenic photosynthesis employs a variety of electron donors such as sulfur compounds, ferrous ions, or molecular hydrogen. Instead of chlorophyll pigments of oxygenic photosynthesis, anoxygenic bacteria use bacteriochlorophylls BChls, whose absorption is shifted to a red side of the spectrum relatively to Chls, therefore covering the part of the spectrum not exploited by oxygenic organisms. [50] For example, in Purple Bacteria photosynthesis relies on the pigments that harvest light in blue–green (Carotenoids and BChl Soret band)) and far–red and IR (BChls *Q_y* bands) spectral regions. These lower-energy photons are sufficient for the anoxygenic process but lack the energy needed to split water. The evolution of oxygenic photosynthesis later allowed organisms to use higher-energy photons absorbed by chlorophylls (Chls), enabling them to use water as an electron donor. This shift to oxygenic photosynthesis, while more energetically demanding, was a major evolutionary leap.

3.5.2 Light-Harvesting Complex I (LH1) and Bacterial Reaction Centre (BRC)

At the heart of the purple bacterial photosynthetic unit is the Reaction Center (RC), which is surrounded by the Light-harvesting 1 (LH1) complex. All species of purple bacteria have LH1 complexes, while many of them such as *Rbl. acidophilus*, also have additional peripheral antenna complexes such as LH2. The LH2 complex (see next section) has two near-IR absorption bands at ~ 800 nm and at ~ 850 nm. These two absorption bands reflect the presence of two distinct groups of BChl *a* molecules in LH2. This difference in absorption maxima in LH1 and LH2 are of great importance since they set up an energy gradient in the complex. (Figure 3-11) This allows to funnel and direct energy from the periphery into the LH1 complexes and after into the RCs.[51]

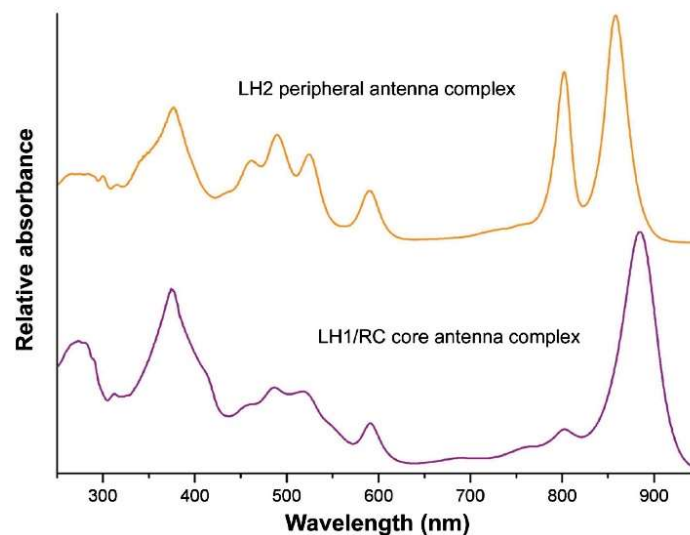


Figure 3-11: The absorption spectra of the isolated LH2 (top) and LH1–RC (bottom) core complexes from *Rps. acidophila*. The absorption bands ~ 380 nm, 590 nm and 800–900 nm are the BChl *a* Soret, Q_x and Q_y transitions, respectively. The peaks between 450 and 550 nm come from the carotenoids. The peaks at 800 and 760 nm in the LH1–RC ‘core’ complex are due to the RCs, not the LH1 component. [52]

Light harvesting complex 1 (LH1) is a membrane integral protein (Figure 3-12). It has an elliptical open ring structure surrounding the reaction center RC (Figure 3-13). The inner ring mean radius varies depending on the species between 50Å to 65Å [53] [54] [55] [56]. LH1 is composed of 16

α, β - dimers, it has 32 molecules of strongly interacting BChl a that are responsible for the Q_y absorption band at 880 nm. This complex has a lot of similarities with LH2 complex that will be discussed more thoroughly in the next section.

The structure of LH 1 – RC complex is shown in *Figure 3-13*. The alpha chains are depicted in yellow and form the inner ring. The outer ring is formed by the α subunits, depicted in deep purple. The BChls form a ring structure close to the luminal side of the membrane and are depicted as B880 and are responsible for the absorption band at 880 nm.

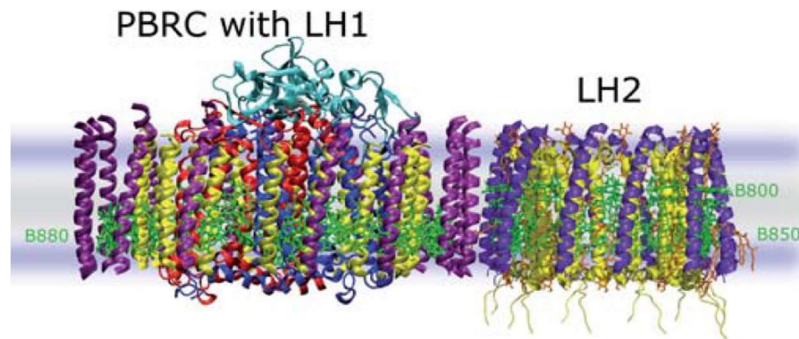


Figure 3-12: Structure of the purple bacterial reaction center with the peripheral Lh1 and Lh2 antenna rings. [2]

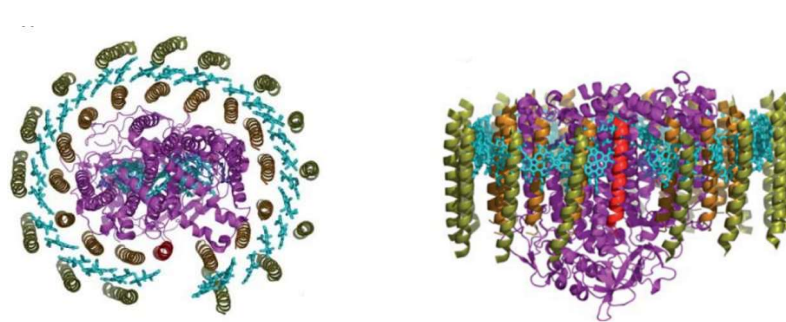


*Figure 3-13: Left: A section through the crystal structure. Right: A side view of the complete RC - LH1 complex from *Rps. palustris*. The α - apoproteins are brown, the β - apoproteins green, the RC purple, and Protein W red. [52]*

The spectral distribution of the pigments is highly optimized to allow a very efficient excitation energy transfer from LH2 to LH1 to RC.

Reaction centers from purple bacteria have three subunits, L, M, and H (some types of bacteria have only two subunits) surrounding the cofactors.[57] The overall protein is organized around a two-fold symmetry axis. Each of the L and M subunits consists of five transmembrane α -helices. These helices, which are characterized by a pronounced hydrophobic nature, are composed of 24 to 31 amino acid residues. Four of these helices—two from the L subunit and two from the M subunit—form a central helical bundle that spans the cell membrane. H subunit consist of single α -helix and globular domain on the cytoplasmic side of the bacterial membrane. In contrast to L and M subunits it does not get involved in the primary electron transfer pathway and rather plays a structural, stabilizing role in the reaction center complex. (*Figure 3-14*) All the cofactors which participate in electron transfer are located in that core. The cofactors of the electron transport chain consist of the special pair of BChls, accessory BChls, Bacteriopeophytins (BPheo) and two quinones, Q_a and Q_b . The others helices are considered to be peripheral (non transmembrane) helices. Three helices in each subunit are aligned on the periplasmic surface of the protein along the edge of the cell membrane while additional one forms a binding site for quinone cofactors.

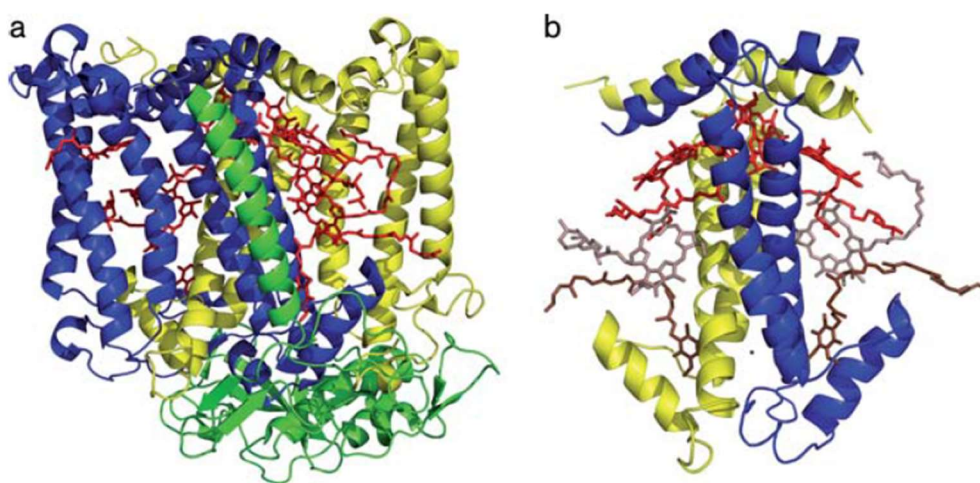


Figure 3-14: (a) Structure of the reaction center from R. sphaeroides showing the three protein subunits: L (yellow), M (blue), and H (green) and the cofactors (red). (b) Structure of the core motif found in photosynthetic complexes formed by the L (yellow) and M (blue) subunits: the four central transmembrane helices and six smaller helices surrounding the A (right hand side) and B (left hand side) branches of cofactors consisting of the bacteriochlorophyll dimer (red), two bacteriochlorophyll monomers (purple), two bacteriopheophytins (orange), quinones and non-heme iron (brown).[52]

3.5.3 LH2

Light-harvesting complex 2 (LH2) is of special interest for our research. LH 2 is an integral membrane protein which has a nine- or eight-unit ring structure, depending on the species. Each monomeric unit has α -apoprotein and β -apoprotein, together binding three BChl molecules and one carotenoid molecule. The outer wall of the complex is formed by the nine (or eight) α -helices of the β -apoproteins. The α -helices from the nine (or eight) α -apoproteins form the inner wall of the complex. All of the pigments are arranged between these rings of α -helices. The structure is capped on either side by the N- and C-termini folding over and interacting with each other. That is a set of helices (α and β) forms its own ring or scaffold, separated by space filled by pigments. Therefore the stability and connection between α - and β -apoproteins come mainly

from the pigments coordinated between them, interactions at the protein termini (N- and C-ends) outside the membrane and hydrophobic interactions with the surrounding membrane lipids. The N - termini of both apoproteins are found at the cytoplasmic surface of the membrane and the C - termini are located at the periplasmic surface. Beginning at the cytoplasmic side, and proceeding downward into the structure, the first group of BChl a molecules are the eight or nine molecules of the B800 ring. [52] Sixteen or eighteen BChls denoted as B850 are arranged in a double-ring structure. These two concentric rings are very close to each other, forming a single, strongly coupled excitonic band with absorption at Q_y maximum around 850 nm. [58] This B850 ring is located between the α -apoproteins and β -apoproteins. Carotenoids in the complex are believed to play a photoprotective role by dissipating the excess energy and preventing singlet state oxygen which can lead to oxidative damage of pigments, lipids and proteins. This belief exists despite some recent publications claiming that presence of carotenoids amplifies singlet oxygen damage on BChls. [59] Carotenoids also have other important functions, such as structure stabilization and light harvesting in blue –green region with consecutive ET to BChls. [60]

Figure 3-15 shows an overall structure of LH2 complex where α - apoproteins are shown in brown, β -apoproteins in green, BChls are purple and carotenoids are turquoise.

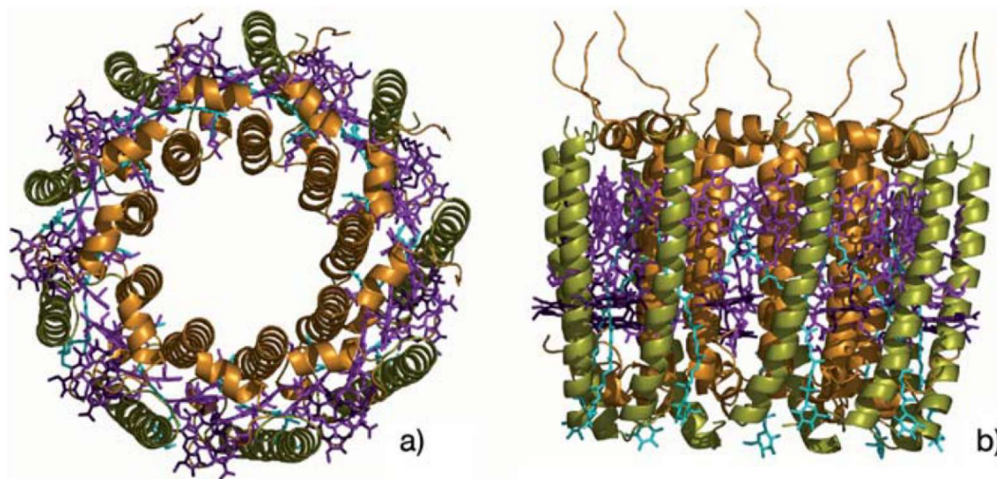


Figure 3-15: A top (a) and side view (b) of LH2 complex from *Rps. Acidophila* strain 10050. [51]

Figure 3-16 shows the arrangement of all the pigments in LH2 with the apoproteins removed for clarity.

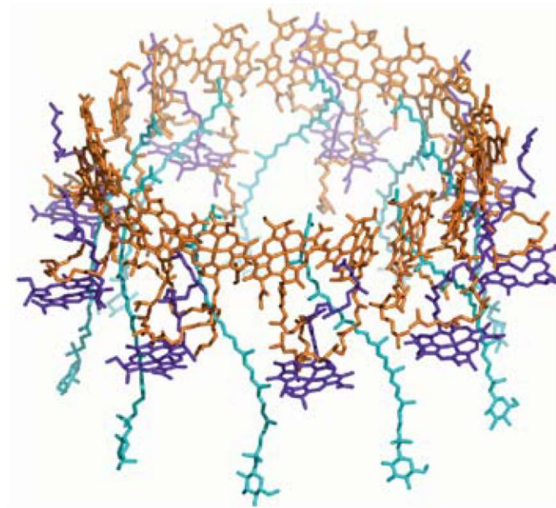


Figure 3-16: The arrangement of all the pigments in LH2 based on Crystal Structure of the LH2 Complex from *Rps. acidophila* strain 10050 The B850 BChls are brown, the B800 BChls purple, and the carotenoids turquoise. [52]

The phytol chains of B800 and 850 are very important structural elements of the protein complex, even though they are a passive part from the viewpoint of energy and charge transfer. The phytol chains from the B800 BChls and the β -bound B850 BChls fold around each other. The interactions with the phytol chains appear to be the way in which the proteins are able to orientate the bacteriochlorin rings, controlling the positioning of bacteriochlorin rings in the complex. [61] The correct orientation of the head groups and consequently the Q_x and Q_y dipoles is crucial for the efficiency of energy transfer.

3.5.4 Electron transfer Pathways in anoxygenic photosynthesis

In purple bacteria anoxygenic photosynthesis utilizes various Light Harvesting Complexes (LH1 and LH2) and two additional protein complexes (the RC and cytochrome bc_1). Light is absorbed by LH2, transferred to LH1, and then transferred to the “special pair” of two BChl molecules found in the Reaction Centre (RC) with absorption around 870 nm. Within RC an electron is transferred from the special pair to bacteriopheophytin (BPh) and then to a quinone which varies depending on the species (Q_A , either menaquinone or ubiquinone). Next, the electron is transferred to ubiquinone (Q_B) which accepts two such electrons along with two protons from the cytoplasm before moving into the quinone pool (Q_P). [62] The ubiquinol (reduced ubiquinone), is then oxidized back to ubiquinone in cytochrome bc_1 complex and its protons are released outside the cell. (*Figure 3-17*)

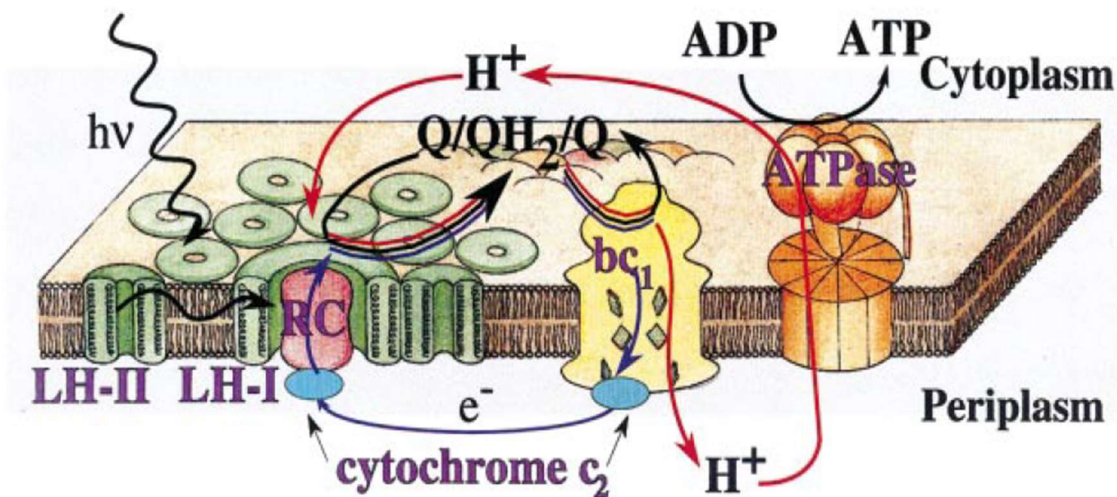


Figure 3-17: Schematic representation of the photosynthetic apparatus in the intracytoplasmic membrane of purple bacteria. The reaction center (RC, red) is surrounded by the light-harvesting complex I (LH-I) green, which is surrounded by multiple light-harvesting complexes (LH-IIs) (green), forming altogether the photosynthetic unit. Photons are absorbed by LHs and excitation is transferred to the RC initiating a charge (electron-hole) separation. [63]

One of the electrons from ubiquinol is passed to cytochrome c_2 which diffuses to the RC and the electron is transferred to the special pair to complete the cycle. Once ubiquinol gives up its first electron, it becomes a ubisemiquinone (an unstable radical). The ubisemiquinone moves back into the quinone pool. After the RC absorbs two photons, the net result is the transfer of four protons from the cytoplasm to the exterior of the cell.[64] Figure 3-18 below shows electron transfer pathways for two categories of photosynthetic bacteria, green sulfur bacteria, which has a similar reaction pathway, except its special pair absorbs at 840 nm (P840) and it uses Fe-S proteins and ferredoxin (Fd) instead of quinones for transferring electrons. [62]

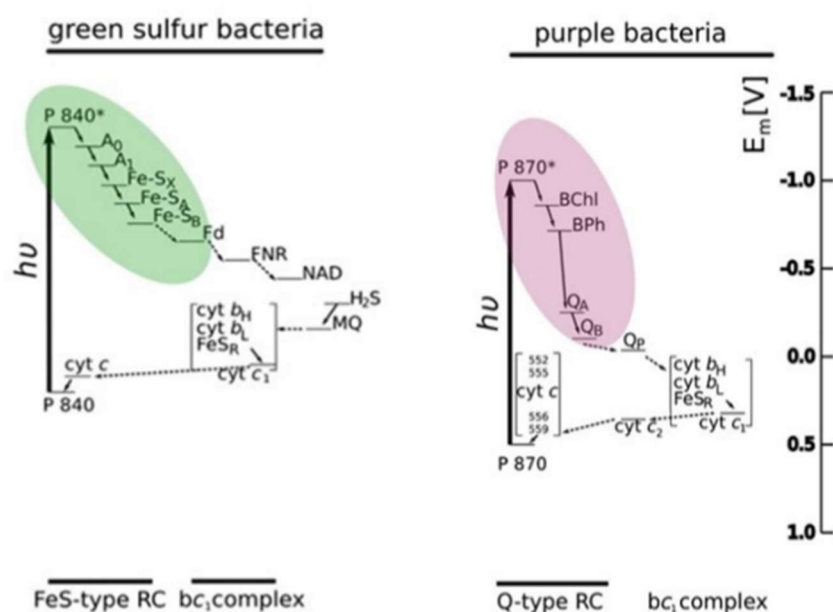


Figure 3-18: Electron transport diagram indicating the types of RCs and electron transport pathways found in different groups of photosynthetic organisms. Green highlighting indicating a type I RC, while purple highlighting indicates a type II RC. [65]

3.6 Excitation Energy Transfer – considerations specific to pigment-protein complexes and excitonic effects.

As it was mentioned before, the primary process in photosynthesis starts with the absorption of light by the pigments. These pigments have strong overlap of their fluorescence bands with absorption band of the same or other type pigments (*Figure 2-2*) creating favorable conditions for excitation energy transfer between them in accordance with the Förster theory (see section 2.2).

[66] Although FRET is a well-established approach for modeling energy transfer between chromophores, it relies on the assumption of weak electronic inter-pigment coupling and relatively large spatial separation between donor and acceptor molecules. In natural photosynthetic pigment-protein complexes, however, pigments are densely packed, with intermolecular distances often in the range of one-two nanometers. Under these conditions, the

point dipole approximation central to the FRET theory becomes invalid. Also, strong short-range interactions between chemically identical pigments lead to delocalization of excited states across multiple chromophores. This excitonic coupling alters the nature of energy transfer, making it more coherent and collective in character rather than purely incoherent hopping. To accurately describe energy dynamics in such systems, more sophisticated theoretical frameworks are required—such as Redfield theory, which accounts for coherent exciton dynamics and environmental interactions, or Extended Förster theory, which generalizes traditional FRET by incorporating exciton delocalization and intermediate coupling regimes. In some cases, quantum mechanical approaches may be taken in order to describe the time evolution of a quantum system interacting with its environment. They are particularly useful for modeling open quantum systems, such as an exciton in a pigment-protein complex, where the system is not isolated and loses energy and coherence to the surrounding environment (the protein matrix). [67] [68] [69]

3.6.1 Interaction between molecules

Photosynthetic protein complexes consist of many pigment molecules bound to the protein. The pigment molecules are close enough to each other so that excited states of the pigments are delocalized, forming so called excitonic states. Strictly speaking, the excited state becomes a superposition of the excited states of all the molecules involved, which is the defining characteristic of an exciton. In general, an exciton is defined as a quasiparticle, an excited electronic state, which is an electronic excitation in a dielectric, semiconductor or metal, propagating throughout the crystal and not associated with the transfer of electrical charge and mass. In the context of protein complexes, we consider a more specific group called Frenkel

excitons. Conceptually, these exciton states determine the spectroscopic properties of molecular complexes and aggregates. In delocalized states, this excitation energy spreads simultaneously over all involved molecules, which allows for energy transfer between different delocalized excitonic states.

For a case of two fixed identical molecules close to each other, the wavefunction of the dimer is a superposition of two wavefunctions. The Hamiltonian (H) of the isolated molecules determined by:

$$H_n \varphi_n^i = E_n^i \varphi_n^i \quad (52)$$

where i =g or e for ground or excited state respectively and n is molecule's index. Then for wavefunctions of the excitonically coupled dimer:

$$\psi_{\pm} = 1/\sqrt{2} (\psi_1^e \psi_2^g \pm \psi_2^e \psi_1^g) \quad (53)$$

where $\psi_{\pm}$ stands for the excitation shared with a positive(+) and a negative (-) phase resulting in a low and a high energy optically active state. $1/\sqrt{2}$ is a normalization factor ensuring that the total probability of the system being in the excited state is equal to 1. The excited state(s) can formally be written as

$$\psi = c_1 \psi_1^e \psi_2^g + c_2 \psi_1^g \psi_2^e \quad (54)$$

Or for case with many chromophores:

$$\varphi_k = \sum_{n=1}^N c_{nk} \psi_n \quad (55)$$

Detailed mathematical treatment is well elaborated in [40]. *Figure 3-19* illustrates the consequences of delocalization of the excited state, namely the energy level splitting and the absorption frequencies of the coupled dimer. Delocalization results in an increase in the spatial extent of the excited state, changing the nature of the chromophore. [10]

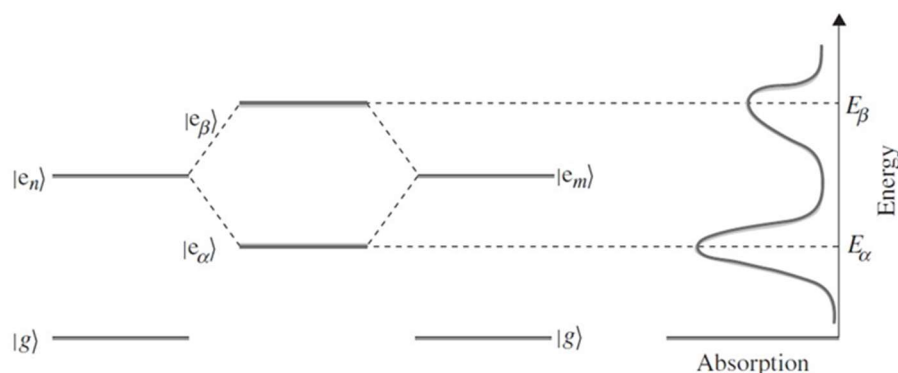


Figure 3-19: Illustration of the electronic energy spacing of a homodimer, which consists of two identical chromophores, each modeled as a two-level system. Molecular excited states e_n and e_m are coupled by an electronic potential, V_{nm} , resulting in delocalized excited states e_α and e_β . Absorption frequencies of the dimer occur at the corresponding energies E_α and E_β . [10]

The degree of exciton delocalization is a measure of how many molecules are involved in this shared excited state. It is directly related to the exciton splitting, which is the energy difference between the new, delocalized excited states. When molecules couple, their individual excited-state energy levels split into a series of new states—a lower-energy state and one or more higher-energy states. The energy separation between these states is a direct measure of the strength of the coupling. A larger splitting indicates stronger coupling and, therefore, a greater degree of exciton delocalization.

If the distances between pigments are greater than about 1 nm, dipole-dipole approximation is applicable which is leading to:

$$V_{1,2} = \kappa \frac{\mu_1 \mu_2}{4\pi\epsilon R^3} \quad (56)$$

where μ are transition dipoles and ϵ is the effective dielectric constant of the protein environment. The sign and magnitude of $V_{1,2}$ depend on the same orientation factor κ that we first introduced when discussing FRET in Section 2.2.

The wavefunctions of the delocalized excitonic states can be determined by diagonalizing the matrix containing V_{ij} as off-diagonal elements and transition energies of the pigments in the absence of interaction as the diagonal elements. The eigenvectors of the matrix determine the relative oscillator strengths of the excitonic states, while eigenvalues are the energies of the excitonic states. Collectively, they determine the shape of the optical spectrum of the excitonically coupled pigment system. It is particularly important that excitonic states may “steal” oscillator strength from each other. For instance, in the B850 ring of Bchl *a* in LH2, the doubly-degenerate second lowest state steals almost all the oscillator strength, while splitting pushes the band further to the red.

Calculating excitonic couplings is generally considered more straightforward than determining absolute transition energies in the absence of inter-pigment interactions. However, even when the dipole-dipole approximation is applicable, significant uncertainty remains. This is because the local protein dielectric constant can vary, leading to screening effects and local field corrections at the boundary between the pigment molecules and their dielectric protein environment. Ultimately, the value of V_{12} is a critical factor that determines both the rate of energy transfer and the exciton splitting of the dimer. The diagonal elements (determined by pigment-protein interactions) are often treated as fit parameters when experimental spectra are fitted to the results of matrix diagonalization mentioned above.

Summarizing, the optical spectra and energy and charge transfer processes are determined by the interactions between pigments and between pigments and the protein. Protein provides scaffolding determining transition energies of the pigments and interaction energies between them. Therefore it is important to study pigment-protein interactions in photosynthetic complexes.

4. Construction of a closed-cycle optical cryosystem

4.1 Motivation

As follows from the above discussion on optical spectra, information on energy and charge transfer times and many aspects of protein dynamics are obtained from low-temperature measurements. There is ample evidence that with proper handling/ use of cryoprotectants, the pigment protein complexes do not denature upon cooling since the spectra still match the structures as well as the cryo-EM structures match the X-ray diffraction structures. Our samples in general do not experience degradation between the experiments; their absorption and emission spectra do not change. Partly because we work most of the time at cryogenic temperatures and store the samples at -80°C , and partly because we employ NPHB (no photo-transformations of the pigment molecules). However, we are unable to conduct as many experiments as we would like, especially extended-time experiments, because we are currently using He-bath cryostat, which consumes several liters of liquid helium per a day of experiment. Even if one neglects the scarcity of liquid helium and its high price, the longest uninterrupted experiment could last 16 hours, before a refill of helium is required. During the refill process the sample experiences significant temperature fluctuations and mechanical shifts and the sample volume could be exposed to higher humidity resulting in water condensation and ice formation on the sample or the inner windows of the cryostat. In short, the purity of the experiment is compromised significantly. Although the helium bath cryostat is relatively simple, its drawbacks include both the inability to conduct long-run experiments and the prohibitive cost of liquid helium. Over the past 20 years the prices of liquid helium have grown by about an order of magnitude. While in 2005 we were able to purchase liquid helium at 7.5 CAD per liter, by now the

prices have reached 60 CAD per liter before taxes. Helium shortages occur because it is a finite, non-renewable resource that is a byproduct of natural gas extraction, with supply concentrated in a few countries like the US, Qatar, Russia and Algeria. (see *Figure 4-1*),

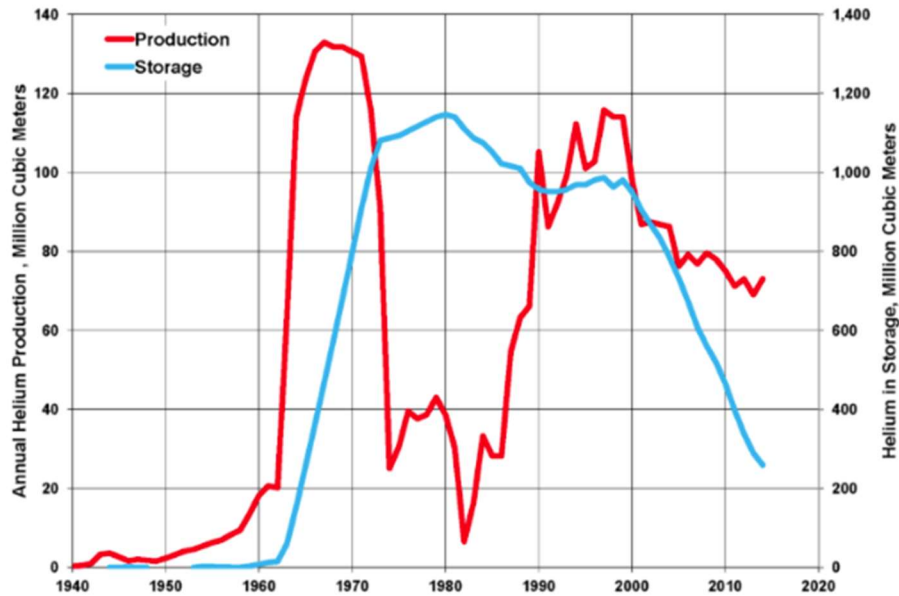


Figure 4-1: Helium production and Storage. [70]

Disruptions from political conflicts, plant maintenance, and increasing demand have caused price spikes and prices are expected to rise further as demand grows, mostly due to expanding use of liquid helium for cooling medical MRI equipment, as well as semiconductor production, aerospace applications, quantum computers and many others. Closure of the US Bureau of Land Management's helium reserve in 2021 removed a significant portion of global capacity. As our research funding has not increased proportionally, we have found ourselves increasingly unable to afford our liquid helium experiments. Closed cycle cryosystems offer a reliable alternative to cooling with liquid helium. Unfortunately, we were not able to secure funding for a new

cryosystem capable of satisfying our needs (~100,000 USD) and had to resort to building such a cryosystem by ourselves. We have already been in possession of a Cryomech GB15 cryosystem based on Gifford-McMahon cycle that was built in the 90-ies and was left behind by a retired faculty member. [71] That system was restored to specified performance in 2016 as a part of a joint project between Zazubovich and Champagne groups which involved Raman measurements on graphene nanodevices. It was demonstrated that while specified minimal temperature of 10 K could indeed be reached, other aspects of system's performance were unsatisfactory. In particular, high levels of vibration did not permit measurements on nanodevices as they simply did not stay in the focus of the optical system. Furthermore, 10 K minimal temperature achievable in GB15 was somewhat too high for our experiments on photosynthetic complexes (see previous Chapters.) The 10 K minimal temperature was due to the use of lead as the regenerator (heat exchanger) material in the second stage of the GB15. Most regular materials, including lead, feature very low specific heat below 10 K which makes heat exchange between helium gas and the coldfinger of the cryostat extremely ineffective. That is, below this temperature the volumetric heat capacity decrease while the volumetric heat capacity of compressed He increases. It results in thermal-mass imbalance between the regenerator and the pressurized helium, which in its turn makes the regenerator matrix not able to absorb enough heat to cool the gas sufficiently before the expansion step of the cycle. In the middle of 70's Buschow et al. suggested that lanthanide materials with low magnetic ordering (phase transition) temperatures (< 10 K) could be used as cryogenic regenerators, see *Figure 4-2*

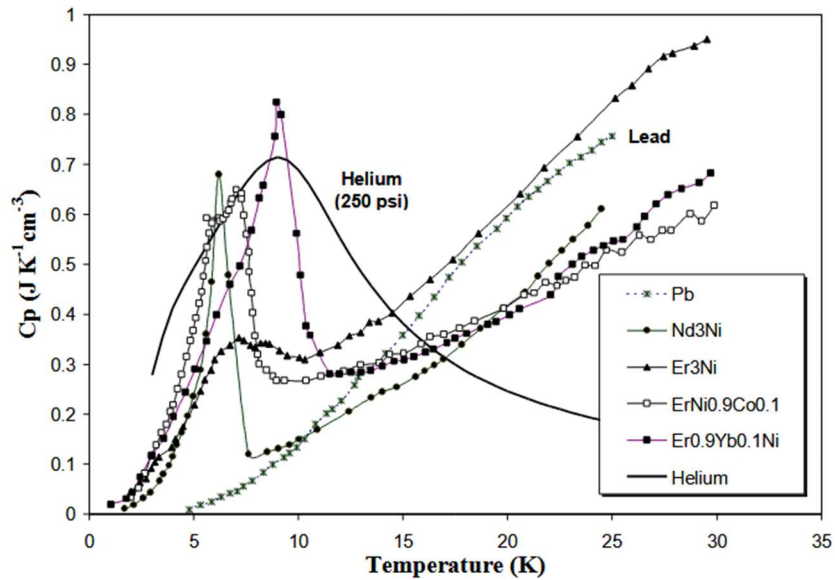


Figure 4-2: Volumetric heat capacities of some RE/TM alloys compared to lead and pressurized helium. [72]

While at these times those materials were not available commercially and, therefore, were extremely expensive, later the concept was proven to be viable.

To address the above deficiencies, we attempted to:

- a) Modify the GB15 cryocooler by exchanging the regenerator material to HoCu_2 alloy which, along with ErNi, is the standard regenerator material in modern 4 K closed cycle cryosystems.
- b) Rebuild the optical cryostat setup in a manner that promises to reduce sample vibrations without compromising the thermal performance, by emulating engineering solutions featured in Montana Instruments (MI) cryostats (Figure 4-3). [73] MI cryostats feature sample plate that is vibrationally decoupled from the vibrating cryocooler by using a flexible thermal link, as well as various measures for vibrationally isolating the cryocooler from the optical table.

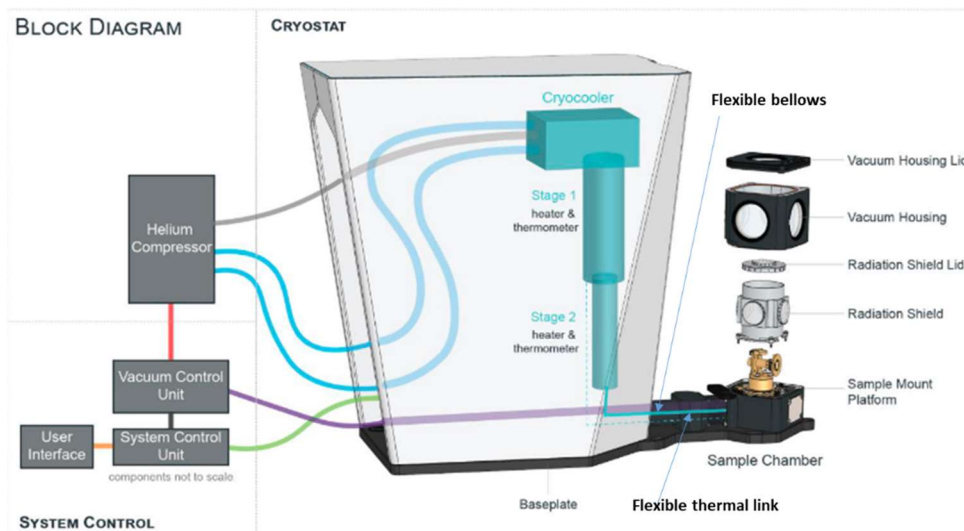


Figure 4-3: Design of a Montana Instruments cryostat. CryoCore | CryoAdvance-50 | CryoAdvance-100. [73]

The cryocooler (left) is placed in vacuum and is connected to the sample (located in a separate vacuum chamber with optical windows, right) by a thermal link which contains rigid and flexible parts. The cryocooler assembly is placed on vibration isolators and its outer wall is connected to the sample chamber by a flexible bellow.

4.2 Principles of operation of GM cryosystem

The closed-cycle cryosystems consist of the following essential components (Figure 4-4):

- A compressor to compress the helium gas,
- A displacer to move the gas from the cold to the warm end and vice-versa
- A regenerator, to enable the gas to exchange heat with other parts.

The last two components make up the cold head part. Cold head in the GM refrigerator cycle is connected to the compressor by two lines supplying high and low pressure helium at the constant rate. In the classical GM cycle an internal pressure differential moves the displacers, although they can also be moved mechanically using a crankshaft. The synchronization of the motion of the displacers is achieved by a rotary valve. The cycle starts with the rotation of the valve disk by electric motor that opens the high pressure path. The helium expands and cools. Then the valve switches to a low pressure path, which lets cooled gas pass through regenerators and cool them down. Displacers move back to the starting position and the cycle starts over again.

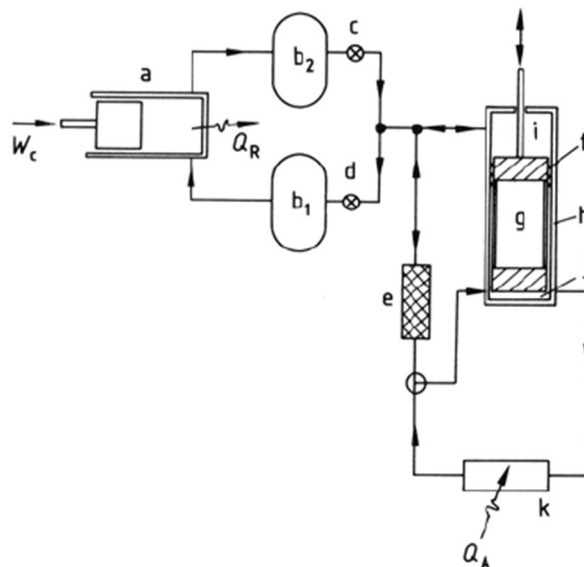


Figure 4-4: Gifford–McMahon refrigerator a) Compressor; b) Surge vessels (b_1 is at p_1 , b_2 is at p_2); c) Inlet valve; d) Outlet valve; e) Regenerator; f) Seal; g) Displacer piston; h) Cylinder; i) Upper expansion space; j) Lower expansion space; k) Load heat exchanger. [74]

The operations for the GM cycle consist of 4 main steps (see Figure 4-4).

Step 1 (Pressure buildup) the pressure builds up while the displacer (g) is at the bottom of the cylinder (h) and the outlet valve (d) closed, the inlet valve (c) is opened and the pressure

within the cylinder is increased from the low pressure P_1 to the high pressure P_2 . The volume of the lower expansion space (j) is practically zero during this process.

Step 2 (intake stroke). With the inlet valve open and the outlet valve closed, the displacer piston is moved from the bottom position to the top of the cylinder. This piston motion moves the gas that was originally in the upper (warm) expansion space (i) through the regenerator to the lower (cold) expansion space. Because the gas is cooled as it passes through the regenerator (e), its volume tends to decrease and additional gas is drawn in through the inlet valve to maintain a constant pressure within the system.

Step 3 (Pressure release and expansion). With the displacer at the top of the cylinder, the inlet valve is quickly closed and the outlet valve is slowly opened, thus allowing the gas within the lower expansion space to expand from pressure P_1 to the low pressure P_2 . The gas that remains in the lower expansion space does work in forcing out the gas that leaves during this process; therefore, the temperature of the remaining gas drops. Ideally, the expansion takes place reversibly and adiabatically (isentropic expansion).

Step 4 (exhaust stroke). With the outlet valve open, the low-temperature gas is forced from the lower expansion space by moving the displacer down to the bottom of the cylinder. The cold gas flows through a heat exchanger (k) to absorb energy from the low temperature source.

Step 5 (Return to step 1). The gas flows back through the regenerator, where it is warmed to higher temperature, and the cycle is ready to be repeated. [74]

To increase cooling capacities of GM type cryocooler or in systems where refrigeration is required at intermediate temperatures, an additional stage is introduced. In this case the system has a

higher thermodynamic performance than a single-stage unit. Multi-staged systems with a lead-based second stage regenerator were able to reach temperatures as low as 6.5 K but without much cooling capacity left. A typical two-stage GM refrigerator is shown in *Figure 4-5*.

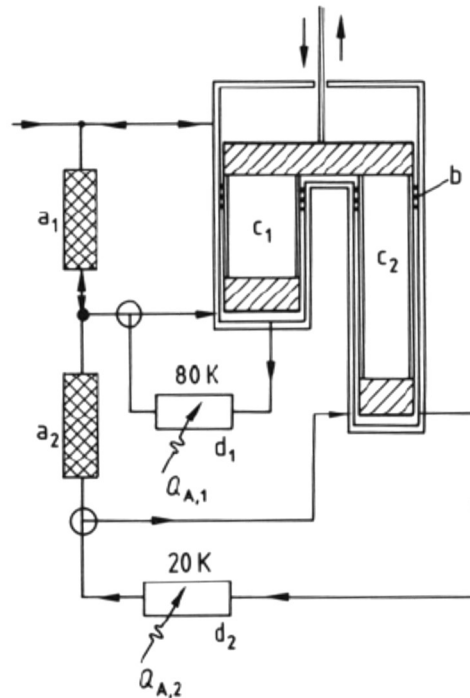


Figure 4-5: Two-stage Gifford–McMahon refrigerator a) Regenerators; b) Seals; c) Displacer pistons; d) Load heat exchanger. [74]

4.3 Modification of the Regenerator in Cryomech GB15

As stated above, achieving 4K requires different regenerator material. In order to modify the regenerator one should consider the following: The regenerator should be designed to allow relatively free gas passage – this is a necessary condition for the desired thermodynamic processes. The heat capacity of the regenerator material must be high in the temperature range of interest to provide efficient heat storage. A thermal conductivity in the direction of fluid/gas

flow must be low to keep sufficient temperature difference between warm end and cold end of the regenerator. It is also desirable to have good thermal conductivity in the direction perpendicular to fluid/gas flow. In the original two-stage setup of GB15 the regenerator material of the second stage (lowest temperature stage) is lead in the form of spheres of 0.2-0.3 mm diameter. This shape prevents tight packing and allows a relatively free passage of the gas and thus ensures low pressure drop in the regenerator. Lead is suitable for ~ 10 K since it has lower Debye Temperature than copper (the material of the first stage) and higher heat capacity at cryogenic temperatures above 10 K. However, below 10K the thermal capacity of such conventional materials is quite low, compared to the thermal capacity of helium. This is reflected in decreasing efficiency of lead regenerators toward 10K and below,

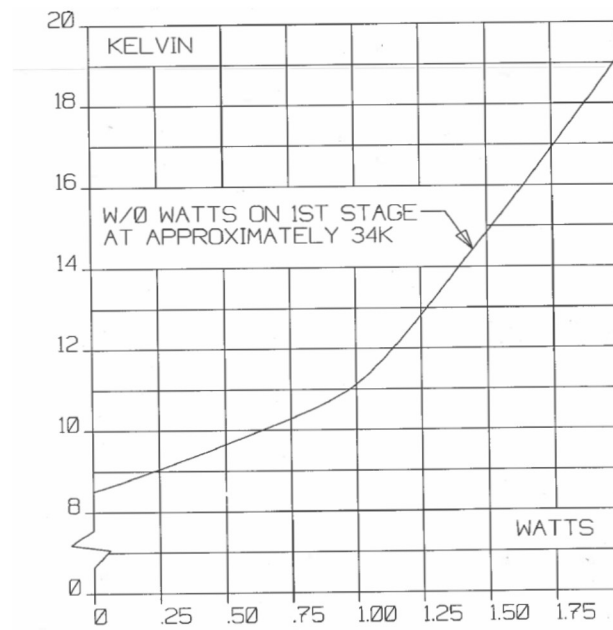


Figure 4-6: Non-modified GB-15 capacity graph. Horizontal axis is Watts at the second stage. (Figure from manual of the Cryostat – not available online)

The cooling capacity of such system decreases with temperature and approaches zero at around 9K (*Figure 4-6*). In the last decades magnetic materials, which have a large magnetic specific heat at very low temperatures, have been developed for use as regenerator material as well as some techniques were developed to improve a system's ability to remove heat and move threshold towards lower temperatures. These materials order magnetically in the temperature range 4 to 15 K. Such an ordering is associated with a magnetic phase transition resulting in high specific heat in that temperature regime.[72] In the last two decades, it was reported that several teams have successfully modified GM cryocoolers to reach temperatures < 4 K with second-stage regenerators upgraded with those materials, and Sumitomo commercialized such coolers. For example, authors of [75] have modified a G-M cryocooler to reduce and maintain its minimum, no-load temperature from 6.1 ± 0.1 K to 3.42 ± 0.05 K at a nominal operating frequency of 1.2 Hz. The cooling power at 4.2 ± 0.1 K was measured to be 0.43 W with zero thermal load at the first stage. To achieve the result the authors composed a hybrid monolithic regenerator consisting of three layers of particles made of different materials.

The specific heat of various regenerator materials, including lead used in the original GB15, is shown in *Figure 4-7*. It is clear that, for example, HoCu_2 possesses higher heat capacity down to at least 3 K than lead possesses at 10 K. We were able to purchase sufficient amount of HoCu_2 spheres with the diameter in the range of 200 to 300 microns (*Figure 4-8*), from Toshiba Inc, which is the preferred supplier of the major cryocooler manufacturers such as Sumitomo, Cryomech/BlueForce, ARS and others. These materials are somewhat controlled substances, as portable cryocoolers are used also in the missile guidance systems.

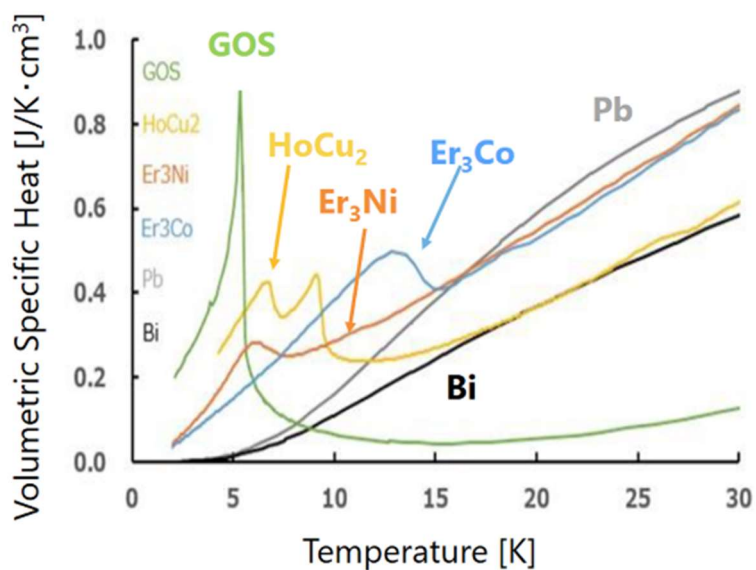


Figure 4-7: Volumetric heat capacities of some RE/TM alloys compared to lead and pressurized helium. [76]

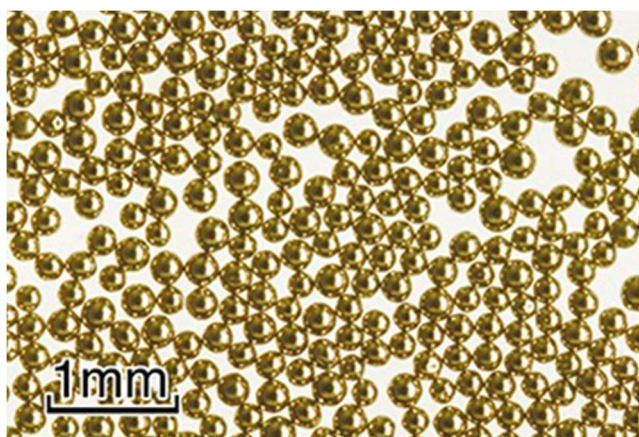


Figure 4-8: HoCu_2 spheres of 200-300 μm used in modified Cryomech cryostat. [76]

Unfortunately, despite our best efforts, we were unable to reach stable 4 K performance after the regenerator exchange. We have experimented with different combination of materials of the second stage regenerator including substages composed of varies combinations of HoCu_2 and

lead spheres, variety of heat conductive copper, aluminum and felt meshes in an attempt to achieve best helium passage and lateral heat conductivity of the second stage. (*Figure 4-9*)

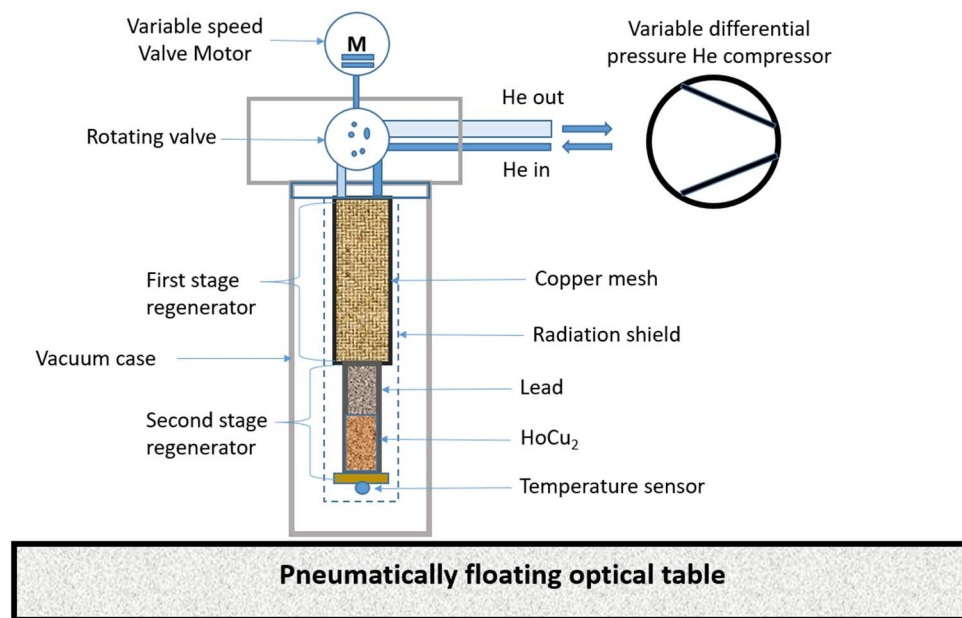


Figure 4-9: GM Cryomech modified 2 Stage regenerator.

These efforts also included varying the frequency of the motor driving the pistons, as suggested in [77]. In order to cope with increased heating capacities at lower temperatures the differential pressure of the original Cryomech compressor was pushed to the very edge of its abilities as well. While the minimal temperature we were able to reach was below 5.5 K (at the coldfinger), this temperature was not stable, and the system tended to oscillate between 5.5 and 8.5 K. Furthermore, there was obviously no cooling capacity left at 5.5 K, which would mean that we would not be able to cool any realistic sample arrangement involving thermal links to 5.5 K. Apparently, merely replacing the regenerator material was not enough, and some changes to the volumes of various internal parts would be required as well as modifications to the helium

compressor. Although we did receive some valuable support from Cryomech throughout this process, this free charity support obviously would not include the extensive engineering advice and assistance that would be required for such a major redesign. Thus, we had to resort to purchasing another second-hand cryocooler that was designed to reach 4 K to begin with, and to have sufficient cooling capacity at that temperature. Finding a second-hand cryocooler with reasonable characteristics, price and documented history (ensuring high probability of actually being operational) was not an easy task and required some time. Eventually we were able to purchase a Sumitomo RDK-415D coldhead with specified 1.5 W cooling capacity at 4.16 K. See

Figure 4-10.

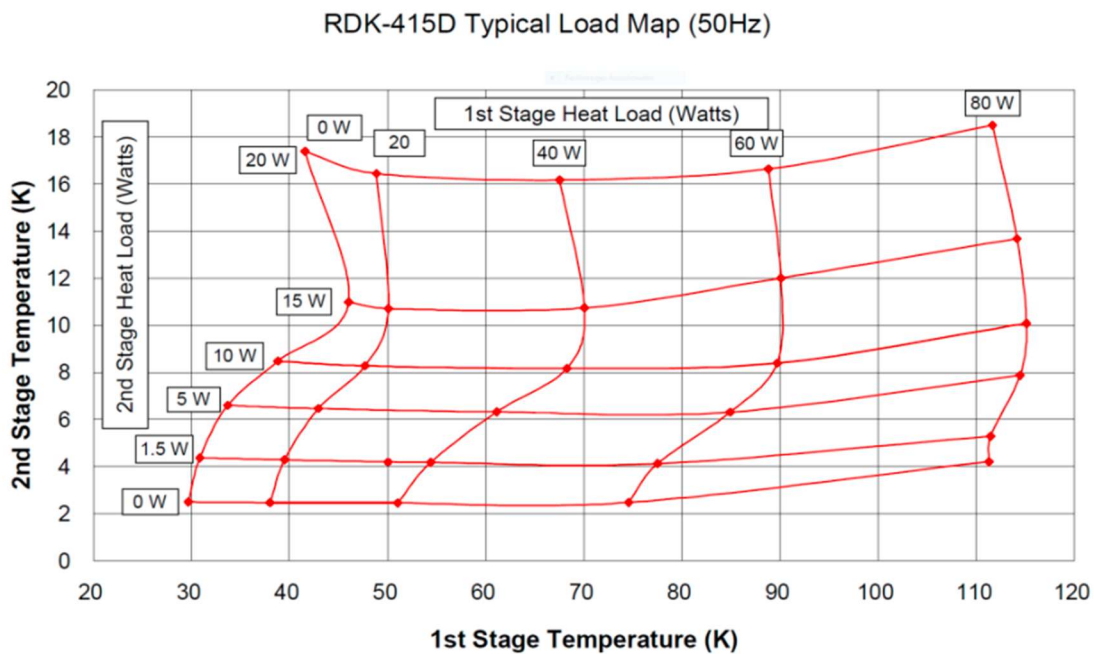


Figure 4-10: Typical load map of an RDK-415D cryocooler operated with a 50 Hz compressor at high thermal loads on the second stage. [78]

The cryocooler was part of a medical MRI equipment, preventing evaporation of liquid helium that was in turn cooling the superconducting magnets. Despite being almost 20 years old, the cryocooler showed great performance early on, reaching 14 K when powered by the 1.5 kW Cryomech helium compressor of the GB15. However, reaching specified performance required more powerful helium compressor and we had to eventually purchase it as well. Again, finding a second-hand compressor with reasonable characteristics, price and documented history / high likelihood of functioning took some time. The Sumitomo CSW-71C compressor is the compressor officially recommended for Sumitomo RDK-415D coldheads. Given that it requires 7 kW of electrical power, a dedicated power line had to be introduced in our lab. With the complete Sumitomo system, we were able to reliably reach coldfinger temperatures below 4 K within one hour. We are currently investigating if this means that our temperature sensor and controller are malfunctioning / miscalibrated or better-than-specifications performance has indeed been reached. Our system has been tested with properly designed radiation shield (see more details below) while the original use of the cryocooler implied that its coldfinger was used to condense the boil-off of liquid helium cooling the MRI magnet. Thus, it is conceivable that 4.16 K in the specifications was achieved with a partial radiation shield. Furthermore, there are reports of deliberately modified Sumitomo cryocoolers similar to ours reaching temperatures as low as 2.2 K, [79] while non-modified RDK-415D is reported to achieve 2.9K. [78] Montana Instruments also uses Sumitomo cryocoolers with some of their systems specified for 3.2 K.

4.4 Overall cryostat design

Overall design closely follows that of the Montana Instruments cryostats (*Figure 4-3*). All 5 K parts, including the coldfinger, rigid and flexible thermal links and the sample cell, were surrounded by the radiation shield made of AL-6063 aluminum alloy. This alloy has four times higher heat conductivity at 50 K (approximate first stage / radiation shield temperature) than the most widely available AL-6061. (*Figure 4-11*)

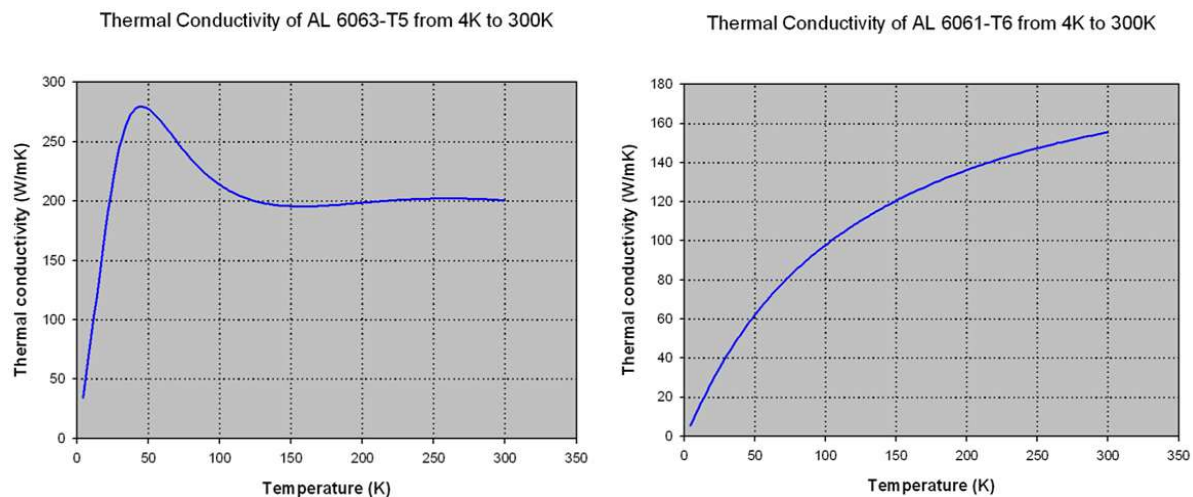


Figure 4-11: Thermal conductivities of AL 6063-T5 and AL 6061-T6 alloys.

The 4 K rigid and flexible thermal links were made of 101 (deoxygenated) copper. The rigid part of the link was machined locally from material provided by McMaster-Carr while the flexible part was space-grade custom-made item by Thermal Space Inc, Boulder CO. This flexible link is specified for 0.22 W/K at 20 K. All copper parts were gold-coated by us using Caldwell 24-karat (100% pure gold) goldplating solution to prevent oxidation of copper that would negatively affect thermal contact between different parts. Indium foil was pressed between the parts as they were bolted together (currently we exploit electroplating to coat a layer of indium onto matching surfaces). Silicone diode thermal sensor by Scientific Instruments (provided with a “standard

curve” and three points specific to the sensor) and resistive heater were attached to the sample cell. The heater is necessary to achieve equilibrium at temperatures higher than the minimal achievable temperature. The sample cell was machined locally from McMaster-Carr 101 copper and features 2 mm thick and 20 mm diameter optical windows by Edmunds Optics. The sample volume is a cylinder 12 mm in diameter and 4 mm in thickness. Liquid solution is introduced via small hole that is blocked by a screw with a polished conical end pushed into a matching receptacle. The two-window design of the optical sample cell requires that sample is illuminated from one side and transmitted beam or fluorescence is measured from the other side. The sample cell is shifted from the axis of the sample chamber by about 1” towards the optical output, in order to maximize the amount of fluorescence passing through the windows on the radiation shield and the outside windows of the sample chamber.

The radiation shield optical windows are 1” in diameter and were previously used as the radiation shield windows in the liquid helium cryostat. They are easily replaceable by windows with the same shape but different antireflection coatings. The outer (RT) windows are 1.5” in diameter (clear aperture 1” to allow for gaskets) and we have several versions of them with different anti-reflection coatings as well as fused-silica non-coated windows for UV spectroscopy that we may need for non-photosynthesis projects.

The steel flexible bellows were designed and custom-made by BellowsTech LLC, Ormond Beach, FL. The sample chamber with internal diameter of 4 inches is bolted to the optical table. The 4” diameter allows for comfortably working with the contents of the sample chamber, avoiding unwanted thermal contacts, and potentially introducing various new sample configurations (e.g. microscopy objective for single-molecule or single-nanoparticle imaging or spectroscopy). A horizontal aluminum plate connected to the radiation shield and therefore kept at about 50 K is

separated from the (RT) bottom of the sample chamber with four G10 glass-epoxy rods $\frac{1}{4}$ " in diameter and 2.5" in length. Two sets of three G10 glass-epoxy rods $\frac{1}{4}$ " in diameter and about 3" in length connected in series separate the above plate from the sample cell assembly. G10 features low thermal expansion ("warp" direction is along the rod) and the above nested structure allows for partial cancellation of thermal expansion of different parts. (*Figure 4-12*)

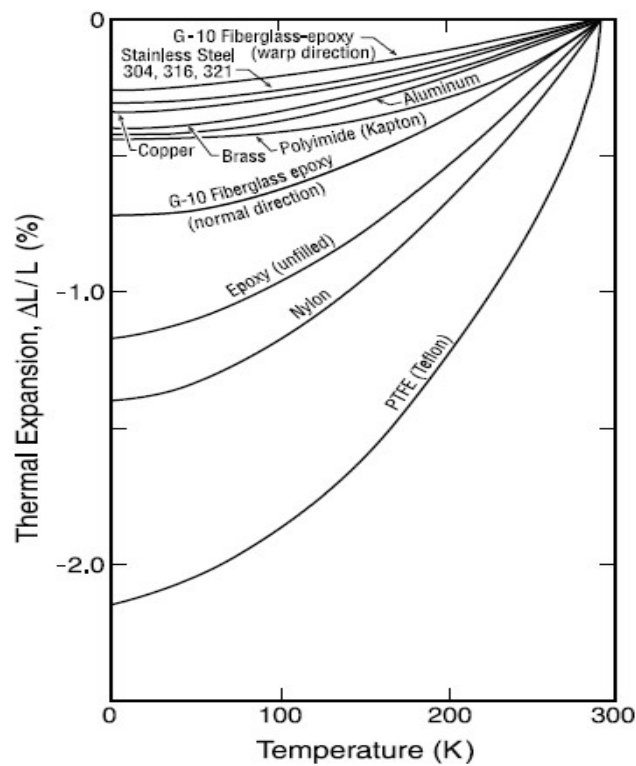


Figure 4-12: Thermal Expansion of selected materials .

The thermal conductivity of G10 is also quite low, see *Figure 4-13* below.

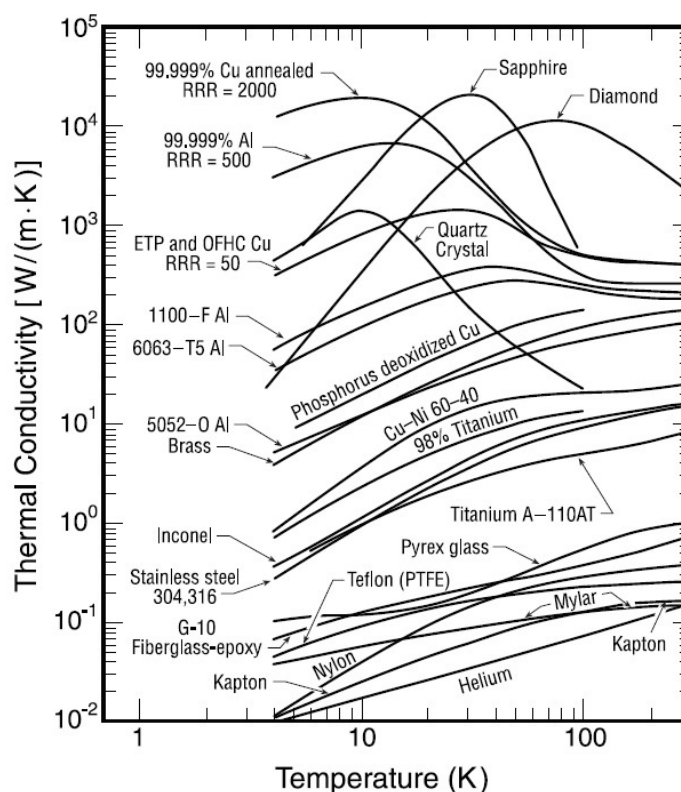


Figure 4-13: Thermal conductivity of various materials including copper, aluminum, steel and G10. The purity of McMaster-Carr OFHC (101) copper is estimated as RRR~200 .

Below we present the estimates of the amounts of energy introduced to different parts within the cryostat via the G10 rod structure, electrical wires and black-body radiation. No convection is possible as under normal operating conditions the coldfinger and the sample are in vacuum. Let's assume that the first stage of the cryocooler and the radiation shields are at 50 K and the second stage is at 5 K. The total length of the rods separating the 50 K plate from the 5 K plate is about 6". The cross-section of one rod is 0.0000307 m². Thus, for three rods $Q = kA\Delta T/d = 0.2 \text{ W/(m K)} \cdot 0.00009 \text{ m}^2 \cdot 45 \text{ K} / 0.15 \text{ m} = 0.005 \text{ W}$. The wires include 4 36AWG phosphor bronze sensor wires and 2 copper wires for the heater, yielding further 10 mW of energy transmitted from the 50 K stage to the 5 K stage. The largest contribution to energy introduced to the 5 K stage is thermal

radiation, $Q = \epsilon \sigma A T^4$ where emissivity $\epsilon = 0.1$, Stefan-Boltzmann constant $\sigma = 5.6703 \times 10^{-8} \text{ (W/m}^2\text{K}^4\text{)}$ with $50^4 = 6,250,000 \text{ K}^4$ and $5^4 = 625 \text{ K}^4$. The total area of the radiation shield surrounding the sample is about 350 cm^2 or 0.035 m^2 . Thus, $Q = 0.014 \text{ W}$. The total estimated flux of energy to the sample (not including optical excitation during the experiment) is therefore about 30 mW . Given the heat conductivity of the flexible thermal link, 0.22 W/K , and that the heat conductivity of the rigid part of the link is much larger than 0.22 W/K , one could expect the sample to be only 0.2 K above the coldfinger. Of course, the coldfinger itself and the rigid link also absorb some thermal radiation, but it does not have to be removed by the flexible link that is the bottleneck in terms of sample cooling. Even several hundred mW could be easily removed at 5 K given the 1.5 W cooling power at the second stage. One can also estimate the radiation flux from the outside (RT) walls to the radiation shield. $300^4 = 8100000000$. The estimate of the combined surface area of all parts of the radiation shield (including the part surrounding the parts of the cryocooler below the first stage) is about 0.12 m^2 , yielding thermal energy flux of about 50 W that is within the capacity of the first stage of the cryocooler.

As RDK-415D is one of the largest Sumitomo cryocoolers, and is much larger than the Cryomech GB15, the outer walls of the cryostat and the external support system with vibration isolators had to be redesigned and rebuilt upon switching to RDK-415D. Care has been taken to estimate the thermal contraction of all parts to ensure that the rigid part of the thermal link, the horizontal tube part of the radiation shield enclosing the link and the bellow connecting the cryocooler chamber with the sample chamber do not end up touching each other over the whole operational temperature range. Note that the RDK-415D cryocooler is 392.5 mm long and

contracts about 1.5 mm upon reaching minimal temperature (from the upper vacuum flange to the coldfinger).

A cryocooler operating at 4 K represents a powerful “vacuum pump” as most substances would condense on it and thereby leave the gas phase. On the other hand, some of our experiments take many hours to complete, and new information may be gained from even longer experiments (e.g. fixed-temperature spectral hole recovery over many days, covering the whole ground-state λ -distribution). Thus, one may be required to operate the system while vacuum is being maintained by the outside pump. In light of the above, oil rotary pumps are not a good choice for this application (as the coldfinger would suck the oil vapor into the cryostat) and molecular turbopump has to be used instead. We have acquired a second-hand Pfeiffer HiPace 80 turbopump, which required some service both by us and the manufacturer. The Pfeiffer HiPace 80 is designed to operate in the High Vacuum (HV) and Ultra-High Vacuum (UHV) ranges and requires fore-vacuum (backing pump) oil free pump to operate. We installed second-hand MD Vario diaphragm pump refurbished to the working condition in our lab. The overall cryogenic system is presented by *Figure 4-14*. Currently our system maintains steady vacuum of at least 5×10^{-3} Torr or below as reported by a calibrated Agilent Varian 531 Measurement Gauge. Note that this is the lower end of the sensor's range and in fact vacuum is better.

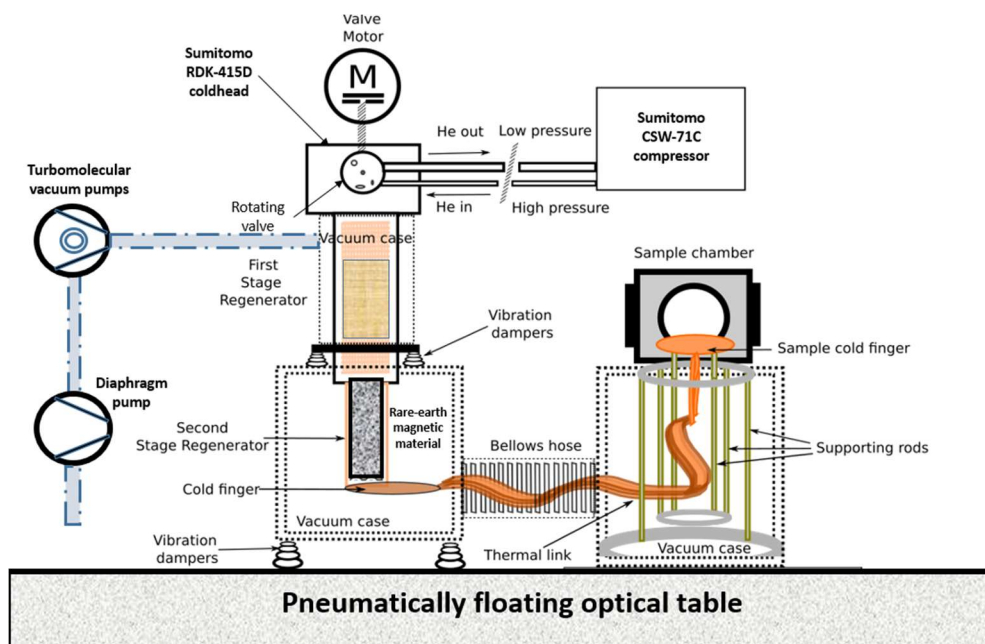


Figure 4-14: The schematics of overall design of the cryosystem. The radiation shields are not shown for clarity.

An image of current state of the cryosystem is depicted in Figure 4-15:

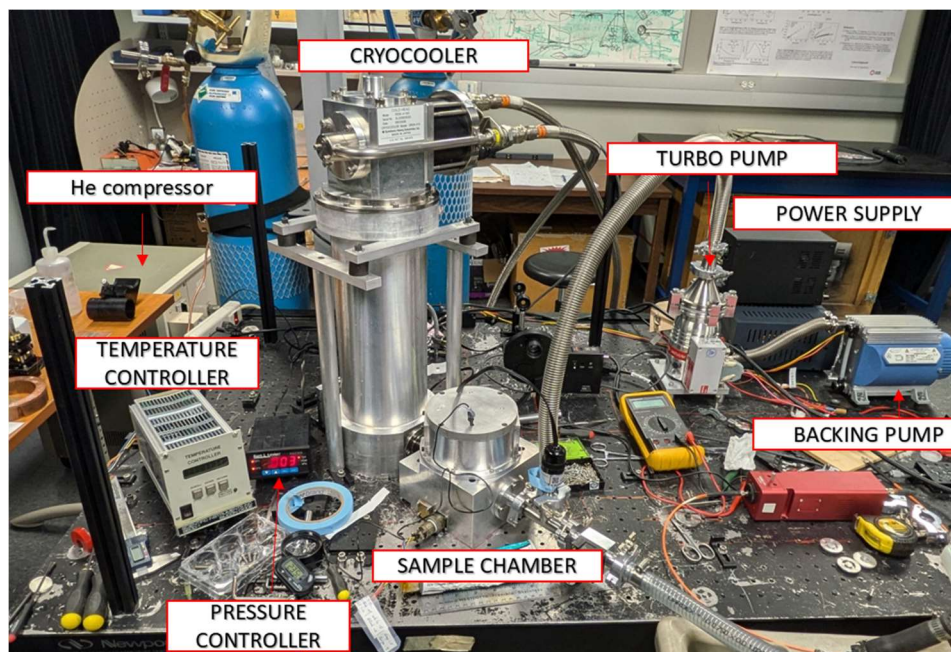


Figure 4-15: Image of the cryosystem.

Overall, the total cost of the system was about 20,000 CAD, including the cost of mistaken design solutions, which in today's prices is equivalent to five 60-liter vessels of liquid helium or 40-50 days of experiments employing a liquid helium cryostat. It could also be compared to about 100,000 USD price of a Montana Instruments cryosystem.

Acknowledgements:

Joe Cosco (Cryomech/BlueForce), Masuyama (Oshima National College for Maritime Technology), Toshiba, Sumitomo who offered valuable free advice despite being aware that our systems are out of warranty and that we are doing things with them that are normally done by certified service engineers.

5. Protein Energy Landscapes, Energy Transfer, and Light-Induced Conformational Changes in Photosynthetic Pigment-Protein Complexes

5.1 Previous Research

In this chapter, we will briefly discuss the experimental results for Cytochrome *b₆f* and LH2 pigment-protein complexes that are relevant for understanding the background of the original work presented in the subsequent sections. Note that the purpose of these studies is not to explore the biochemical function of these pigment-protein complexes (e.g. electron or proton transfer in Cytochrome *b₆f*) but to focus on protein dynamics and utilize these complexes as model systems. As stated in Section 1, small structural changes are part of non-photochemical quenching and may affect energy transfer pathways.

5.1.1 Conformational Dynamics and Cooperative Effects

A 2015 study by Zazubovich- group (Ref. [80]) investigated the protein energy landscapes and energy transfer within the dimeric Cyt *b₆f* complex using non-photochemical hole burning (NPHB) and fluorescence line narrowing (FLN) spectroscopies. A key finding was that the distribution of the barriers between protein substates followed a "glass-like" $1/\sqrt{V}$ dependency rather than a Gaussian distribution. This finding is significant because it is characteristic of glassy systems, which raised a concern that the observed dynamics might not be from the protein itself but from the surrounding frozen solvent matrix.

The study also found that the parameters of the energy landscape barrier distributions were in quantitative agreement with those of other photosynthetic pigment-protein complexes. The researchers provided evidence of nearly perfect spectral memory and a correlation between the barrier heights of the ground and excited states of individual pigment-protein systems. The entities responsible for the observed light-induced conformational changes were calculated to have an md^2 value (see eq. 38) on the order of $1.0 \times 10^{-46} \text{ kg} \cdot \text{m}^2$, suggesting they are likely protons or small groups of atoms undergoing sub-angstrom shifts. However, to account for all spectral hole burning and recovery data (the λ - and barrier distributions did not match), the attempt frequency had to be reduced to approximately 100 MHz, and this was interpreted as evidence of cooperative effects involving multiple TLS. (Similarly to the case of beats upon adding several waves of similar frequency, one could imagine that spectral shifts occur only when multiple motions in individual TLS are all in phase.) The integrity of the protein complex during the experiment was confirmed by observing excitation energy transfer between chlorophyll molecules of adjacent monomers. However, the hypothesis about observed dynamics belonging to the outside glassy solvent still needed verification.

5.1.2 Probing the Role of the Solvent and Detergent

To address the hypothesis that the dynamics were related to the surrounding glassy matrix, a subsequent 2017 study explored the effects of fully deuterated solvent and different detergents on the energy landscape of Cyt b_6f derived from the NPHB experiments on Cyt b_6f . It was proposed that if the pigments were reporting on the solvent's dynamics (a process known as "slaving" [81]), deuterating the solvent would affect the tunneling parameter, λ . This would cause

a significant increase in λ and lead to a substantial slowing down of the burning and recovery kinetics. (Note that the goal of these experiments was not to replace any protons inside of the protein with deuterons. In fact, every effort was made to minimize the deuteration of the protein.) However, the experimental results showed minimal difference in burning and recovery kinetics between protonated and deuterated samples, with no more than a 1% change in the mass of the tunneling entity. (Figure 5-1) At least 3% difference would be expected, in the case of -OH (or -OD) group rotation in the solvent, for example. Note that since λ is in the exponential in Eq. 42, even very small changes of λ cause noticeable effects. The 1% difference could be ascribed to small (unintended and unknown to us) differences in sample preparation affecting electron-phonon coupling S rather than λ (See section 2.3). This strongly suggested that the chlorophylls within the Cyt b_6f complex are well-screened from the external environment and that the observed $1/\sqrt{V}$ barrier distribution truly reflects the dynamics of the protein itself.

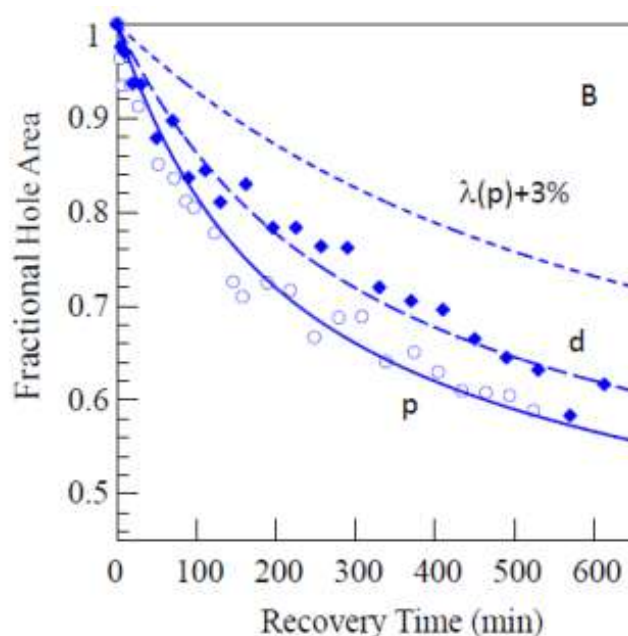


Figure 5-1: Recovery at fixed burn temperature for Cyt b_6f . Open circles and solid line correspond to OGP-solubilised sample in regular (protonated) solvent. Long-dashed line represents the fit to

the data for the deuterated solvent / OGP- solubilised sample, corresponding to 0.8% increase of λ . Short-dashed blue line depicts recovery expected upon deuteration if the observed dynamics were due to $-OH$ groups of the solvent (mass increase by 1/17, increase of λ upon deuteration by 3%).

While deuteration had little effect, significant differences in hole growth kinetics (HGK) were observed when Cyt b_6f was solubilized with different detergents. Samples prepared with n-dodecyl β -D-maltoside (DM) showed a stronger electron-phonon coupling and/or shorter excited-state lifetimes (more chlorophyll quenching) compared to those prepared with n-octyl β -D-glucopyranoside (OGP). (Figure 5-2)

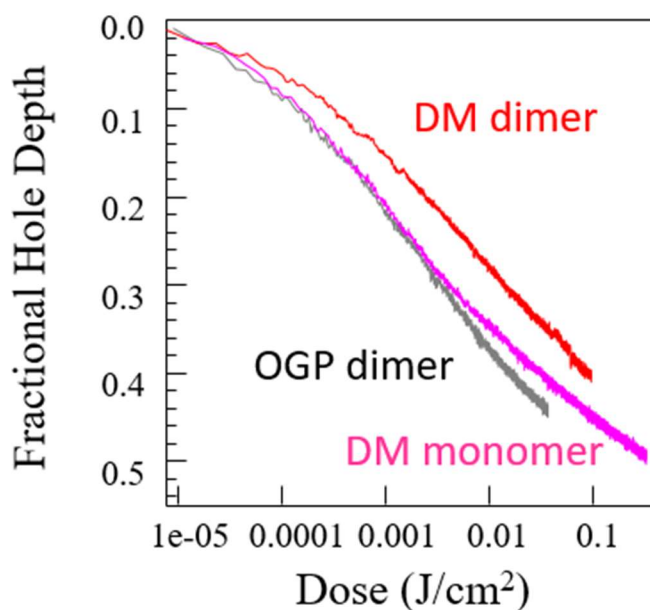


Figure 5-2: HGK curves of dimeric form of Cyt b_6f in both DM and OGP and monomeric form of Cyt b_6f in DM . 50%-deep holes observed in OGP solubilized dimer and DM-solubilized monomer correspond to $S \sim 0.7$.

Notably, the monomeric form of Cyt b_6f solubilized with DM behaved more like the OGP-solubilized dimer than the DM-solubilized dimer. This suggests that the DM micelle may not be in

direct contact with the chlorophyll but rather stabilizes the overall dimer structure, which increases the quenching probability. Stronger electron-phonon coupling in DM-solubilized samples is in agreement with larger Stokes shift (*Figure 5-3*). These findings further confirm that the intrinsic dynamics of the Cyt b_6f protein, not the surrounding solvent, are responsible for its observed spectral properties. They also highlight the crucial role of sample preparation and in particular detergents in influencing the protein's conformational dynamics

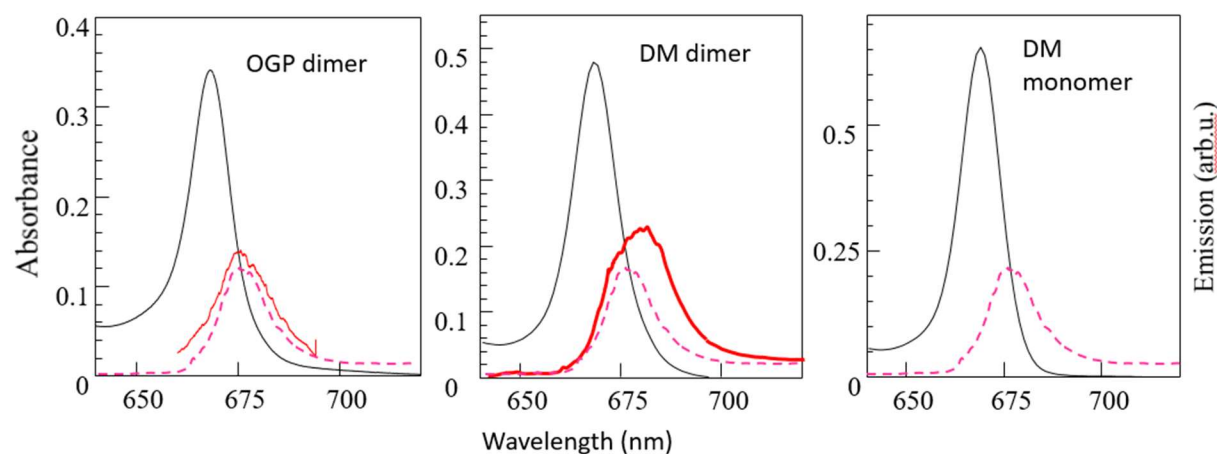


Figure 5-3: Absorption and fluorescence excitation spectra of various Cyt b_6f samples . Cyt b_6f monomer solubilized with DM detergent is more similar to OGP-solubilized dimer than to DM-solubilized dimer. Dashed red curve is the same in all frames and represent monomer emission.

However, the mismatch between the width of λ - and barrier distributions mentioned before persisted and has not been satisfactorily addressed by these studies.

5.2 Evidence of Simultaneous Spectral Hole Burning Involving Two

Tiers of the Protein Energy Landscape in Cytochrome b_6f .

This chapter is based on the paper “*Evidence of Simultaneous Spectral Hole Burning Involving Two Tiers of the Protein Energy Landscape in Cytochrome b_6f* ”, published by G. Shafiei, A. Levenberg, M.A. Lujan, R. Picorel, and V. Zazubovich in the *Journal of Physical Chemistry B* 123 (2019) 10930-10938. [16]

Some results in this paper were included in my MSc Thesis (May 2018) but between that time and the submission date substantial changes have been introduced. I contributed to all experiments, most of data analysis and paper writing.

Abstract: Cytochrome b_6f , with one chlorophyll molecule per protein monomer, is a simple model system whose studies can help achieve better understanding of non-photochemical spectral hole burning (NPHB) and single-complex spectroscopy results obtained in more complicated photosynthetic chlorophyll-protein complexes. We are reporting new data and proposing an alternative explanation for spectral dynamics that was recently observed in Cytochrome b_6f using NPHB. The relevant distribution of the tunneling parameter λ is a superposition of two components that are nearly degenerate in terms of the resultant NPHB yield and represent two tiers of the energy landscape responsible for NPHB. These two components likely burn competitively; we present the first demonstration of modeling a competitive NPHB process. Similar values of the NPHB yield result from distinctly different combinations of barrier heights,

shifts along the generalized coordinate d and/or masses of the entities involved in conformational changes m , with md^2 parameter different by a factor of 2.7. Consequently in Cytochrome b_6f the first (at least) ten hours of fixed-temperature recovery and thermocycling experiments preferentially probe different components of the barrier- and λ -distributions encoded into the spectral holes. Both components most likely represent dynamics of the protein and not of the surrounding buffer/glycerol glass.

5.2.1 Introduction

Spectral hole burning has been used for many years to explore energy landscapes and low-temperature dynamics in glasses, polymers and proteins [82], [83], [84] information about these landscapes can be obtained by observing the hole burning process, the recovery of the spectral holes and their broadening at fixed (burn) temperature as well as recovery and broadening upon temporarily elevating the temperature (thermocycling) [20], [26], [83], [85], [86]. Consistent analysis of the data obtained for the same pigment / host system in all these experiments allows for determining the shapes and the parameters of the distributions of barriers on the energy landscape and for making inferences about the nature of the entities whose structural rearrangement is responsible for non-photochemical spectral hole burning (NPHB) [87] or for spectral shifts observed in single molecule or single protein complex experiments [88], [89]. In the case of NPHB, the pigment molecule is not chemically photo-transformed; it is the pigment's environment that experiences some conformational change triggered by the optical excitation of a pigment [84]. This change can be represented by crossing a barrier on a protein energy landscape [90], for simplicity represented by a double-well potential (TLS, two-level system [91], [92], insert in *Figure 5-A*). The barriers are much lower when the pigment is in the excited

electronic state, so they could be crossed with reasonable probability ($\sim 10^{-4}$) within the nanosecond or sub-nanosecond excited state lifetimes. (It is assumed that NPHB occurs while the pigment is in the singlet excited state.) At low enough temperatures, below 10 K, barriers are crossed predominantly by tunneling, with the (downhill) transmission rate expressed as [80], [93], [94], [95]

$$k_0 = \Omega_0 \exp(-2\lambda)(n(\Delta, T) + 1), \quad (57)$$

where Ω_0 is the constant attempt frequency and $n(\Delta, T) = 1/(\exp(\Delta/k_B T) - 1)$ is the population number of phonons satisfying the energy balance condition, T is temperature and Δ is asymmetry of the TLS. The tunneling parameter λ represents a composite “barrier strength for the purpose of tunneling” and is usually reported in NPHB papers [80], [84], [93], [94], [95], [96], [97] as

$$\lambda = d\sqrt{2mV}/\hbar \quad (58)$$

Eq. 58 corresponds to the case of rectangular barriers. Here V is the height of the barrier above the energy of the tunneling particle, d is the barrier thickness, and m is the mass of the entity that rearranges during the transitions between two conformational states of the protein (or other amorphous solid). At higher temperatures thermally-activated barrier-hopping with the rate $k_0 = \Omega_0 \exp(-V/k_B T)$ may contribute to the NPHB process.

The parameters of the ground state barriers determine how long it takes the spectrum to return to the pre-burn state (usually multiple hours to days at 5 K). In the simplest possible case where the recovery at the lowest (burn) temperature was completed within just several hours

and the barrier distribution had only one Gaussian component, joint analysis of the fixed temperature (burn temperature) recovery data (yielding the ground-state distribution of λ encoded into the spectral hole) and the thermocycling data (yielding the respective distribution of barriers V) allowed for determining the value of the product md^2 and making inferences about the nature of the tunneling entities [87].

Pigment-protein complexes involved in photosynthesis have been the focus of intensive research for decades as they are important from the renewable energy perspective and exhibit a broad variety of exciting phenomena, including protein dynamics, excitation energy transfer, charge transfer, excitonic effects and even quantum coherencies. Cytochrome b_6f (Cyt b_6f), with its one chlorophyll a (Chl a) per protein monomer [98], is a very simple pigment-protein complex, and exploring NPHB in Cyt b_6f offers insights that may be useful for analyzing NPHB and single complex spectroscopy data for other, multi-pigment, complexes. The function of this Chl a , not directly involved in electron transfer in Cyt b_6f , is still a matter of debate. For instance, it was proposed that Chl's phytol tail can sense the passage of plastoquinone through the inter-monomer quinone exchange cavity and transmit a signal through perturbation of the G helix to the n-side of the complex, where it could affect the conformational state of the kinase for the light-harvesting chlorophyll protein (LHC) [99], [100]. Excited state lifetimes of about 200 picoseconds have been observed in Cyt b_6f at room temperature [101], [102], with matching 20-fold reduction of the fluorescence yield, compared to Chl a in methanol. This effect was attributed to protective quenching of the Chl a excitations by electron transfer from a nearby aminoacid, likely Tyr105. However, the widths of the spectral holes observed in Cyt b_6f at 1.9 K suggest lifetimes longer than 700 ps [80], and possibly several ns, taking into account the $\sim T^{1.3}$

dependence of the hole widths expected below 2 K in amorphous solids [86]. At 77 K only four-fold decrease of the fluorescence yield has been observed [103]. Thus, at liquid helium temperatures this lifetime-shortening mechanism appears inactive. In the case of Cyt *b₆f* our earlier NPHB data suggested “glass-like” $\sim 1/\sqrt{V}$ barrier distribution [80]. We also noticed that while the shapes of the distributions derived from the fixed-temperature recovery and thermocycling experiments on Cyt *b₆f* (and in CP43, where all results suggested Gaussian distribution [93]) were qualitatively consistent, their widths were not compatible. To resolve this problem, we proposed a radical reduction of both the attempt frequency Ω_0 and tunneling parameter λ , attributable to the cooperative effects [80]. However, we were unable to find a combination of parameters that would result in a satisfactory agreement with the experiment. Here we report additional observations, alternative explanation for our earlier results and the first simulations of NPHB and subsequent hole recovery involving two competing channels.

5.2.2 Experimental Methods

The sample was prepared by Dr. Rafael Picorel from Zaragoza, Spain, who has been long time collaborator in our research. Samples of chloroplastic Cyt *b₆f* were isolated from market spinach. [104] As cytochrome is originally a transmembrane protein, therefore isolated cytochrome had to be solubilized with the help of a detergent. Two different types of detergent were used in samples employed in our experiments. For the first batch we used *n*-octyl β -D-*glucopyranoside* (OGP) to disperse and stabilize the protein complex in micellar system. [93] For the second batch we utilized *n*-dodecyl β -D-maltoside (DM), and sample purification was done as explained in [80]. Molar Extinction Coefficient of 25 mM⁻¹·cm⁻¹ (at 554 nm), 21 mM⁻¹·cm⁻¹ (at 563 nm), and 75 mM⁻¹·cm⁻¹ (at 578 nm) were used for OGP and DM, respectively.

1-cm^{-1} (at 668.5 nm) were used for the Cyt *f*, Cyt *b*, and Chl, respectively to assess purity, quality and concentration of purified Cyt *b₆f*. [105] In our experiments we use glycerol-water-buffer to ensure formation of transparent matrix at cryogenic temperatures and to protect the complexes. Some precaution was taken to carry out the experiments in deuterated environment to minimize possible exchange of the protein protons with deuterons, thus all samples were stored at -80°C and unfrozen only immediately prior to the experiment. For experiments on Cyt *b₆f* in deuterated environment we have purchased d_8 -glycerol and D_2O from C/D/N Isotopes (Dorval, QC). To dissolve the pure Chl *a* (from Sigma-Aldrich) we used deuterated Methanol from C/N/D. Then we have dissolved <100 μL of methanol/ Chl *a* in ~ 1 mL of water-glycerol. Figure 5- 4 shows a cartoon of a Cyt *b₆f* complex surrounded by the detergent micelle in the solid (frozen) buffer-glycerol matrix.

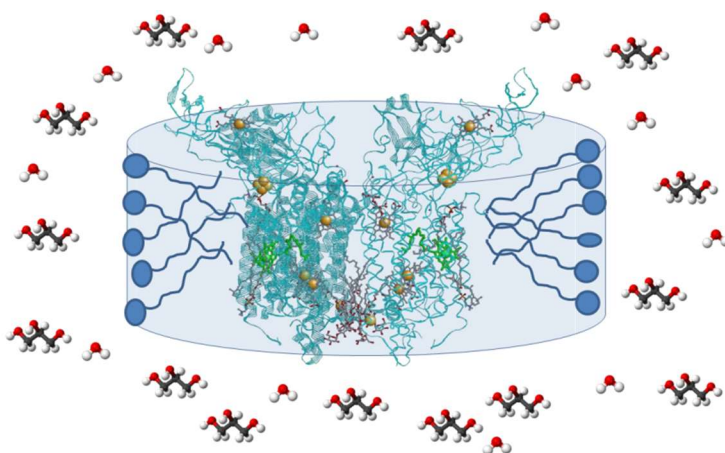


Figure 5-4: Cytochrome *b₆f* Complex solubilised with the help of detergent in water glycerol matrix. [106]

Absorption spectra were measured either with a Cary 5000 spectrophotometer with the resolution of 0.1 nm or with a home-built VIS-NIR spectrophotometer based on a Jobin-Yvon

HR640 Single Grating Monochromator Spectrograph with bandwidth of about 0.04 nm.

Broadband emission spectra were measured using Acton 2356 spectrograph with Princeton Instruments Pixis 1340x100 back-illuminated CCD. A 633 nm He-Ne laser was used for excitation. High-resolution spectra were measured with Sirah Matisse-DS frequency-stabilized tunable dye laser (bandwidth ~ 0.15 MHz). A 6 W 532 nm Spectra Physics Millennia was used as a pump laser. The samples were placed in a 4 by 10 mm cross-section plastic cuvette surrounded by an aluminum mask inside a liquid helium bath / flow cryostat (Ukrainian Academy of Sciences) with temperature stabilized to better than 0.1 K. High-resolution experiments were performed in fluorescence excitation mode, with vertically polarized excitation and with fluorescence detected with a Hamamatsu photomultiplier / photon counting module equipped with a long-pass filter (AELP700, Omega Filters, VT) at 90° with respect to excitation. This ensures optimal collection of light emitted by the excited molecular dipoles. The intensity of the excitation light was stabilized by BEOC laser power controller (Brockton, MA) and regulated with absorptive neutral density filters.

Thermocycling experiments involve producing a spectral hole at low (burn) temperature, e.g. 5 K, temporary increasing the temperature and allowing some systems to cross their respective barriers and return to their pre-burn state, measuring the hole spectrum again at low (burn) temperature, increasing the temperature to a slightly higher values than in the previous cycle, and so on. Simulations of competitive NPHB and recovery were performed on a personal computer with 32 GB of RAM.

5.2.3 Results

Figure 5-5 presents an example of the hole growth kinetics (HGK) data (Frame A, closely resembling data from [78]) as well as the data on fixed-temperature (5 K) recovery of spectral holes of different initial depths (Frame B). The data in the insert of Figure 5-5B suggests the presence of nearly perfect spectral memory [78], [79] – the spectra recover by pigment-protein systems returning to the pre-burn conformation, with shallower holes exhibiting faster recovery (see insert in Frame B). The recovery data is inconsistent with the Gaussian shape of the λ - or V -distribution (dashed lines). Recovery of a 30%-deep hole at 5 K was once followed for 30 hours (main frame of Figure 5-5B; open symbols). Solid blue symbols and solid blue line in the main frame are the same as in the insert.

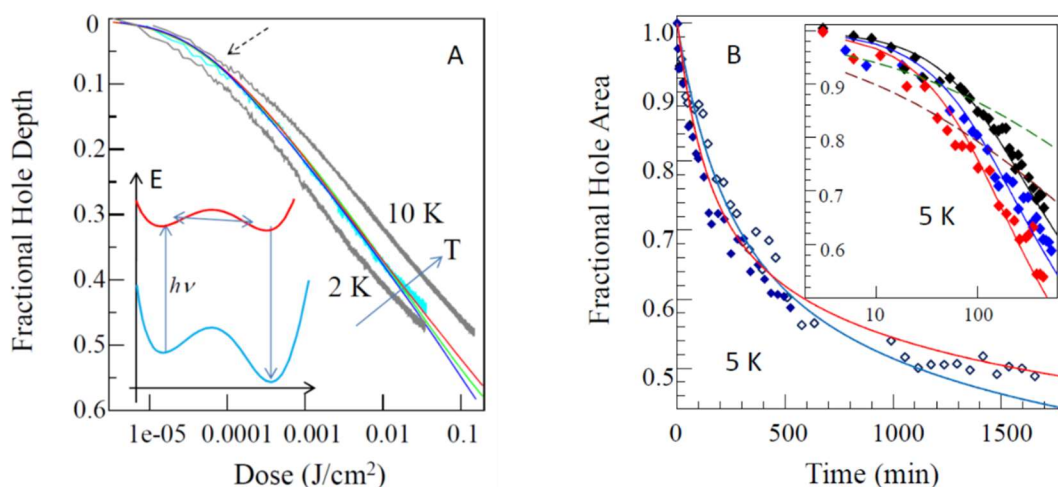


Figure 5-5: Frame A: HGK curves for 2, 5, and 10 K from [80]. Dashed arrow indicates the region in the beginning of the burn where at 10 K and above the burning is faster than tunneling-based model predicts, likely due to the onset of burning via barrier-hopping [80]. Also presented are the fits to the 5 K data (light blue). Blue: single-tier fit from [80]. Red: two components, both $\sim 1/\sqrt{V}$. Green: two components, dominant $\sim 1/\sqrt{V}$, another is Gaussian. Insert depicts the TLS model representing a pigment-protein system. Frame B: Fixed-temperature (5 K) recovery of two 30%-deep spectral holes (open and closed symbols) and the fits assuming presence of spectral memory and $\sim 1/\sqrt{V}$ barrier distribution. Blue curve is the fit resulting from the single-tier model [80], red curve is obtained using two-tier model, see text. Insert: Fixed-temperature (5 K) recovery data from 15 for 20% (red), 30% (blue) and 40%-deep (black) holes with fits employing $\sim 1/\sqrt{V}$ model

(solid curves) and Gaussian λ distribution model (dashed lines) with parameters following from the fit to the HGK data.

Figure 5-6A shows the results of two experiments with some period of fixed-temperature (5 K) recovery followed by thermocycling, both starting with a hole of 30% fractional depth. This is far from the saturated depth of about 50%. The insert represents the temperature profile of the first experiment (black squares in the main frame). In this case the hole was allowed to recover at fixed temperature (~ 5 K) for an hour before thermocycling was initiated. Increasing the temperature to 8, 9, 10 and 11 K for one minute and then returning to 5 K did not result in a faster hole recovery compared to the recovery at 5 K (circles and smooth blue curve). A notable acceleration of thermally-induced recovery occurred in the temperature range around 11-15 K, resulting in a step in the experimental data, with the magnitude of about 10-15% of the original hole area (left vertical arrow). In the second experiment (blue circles) the hole was allowed to recover at 5 K for ten hours before thermocycling was initiated. Examples of spectral holes from this dataset are shown in Figure 5-6B. Recovery at 5 K was the fastest during the first hour. From the second to the tenth hours at 5 K the hole experienced significant recovery (light blue spectra in Figure 5-6B), comparable in magnitude to the thermocycling-induced step described above. However, subsequent thermocycling still resulted in a recovery step at around 11-15 K (right vertical arrow in Figure 5-6A; blue to red spectra in Figure 5-6B), with approximately the same magnitude as observed for the black dataset. This indicates that ten hours long recovery at 5 K did not include the pigment-protein systems responsible for that step, but these systems were easily accessible with a small temperature increase.

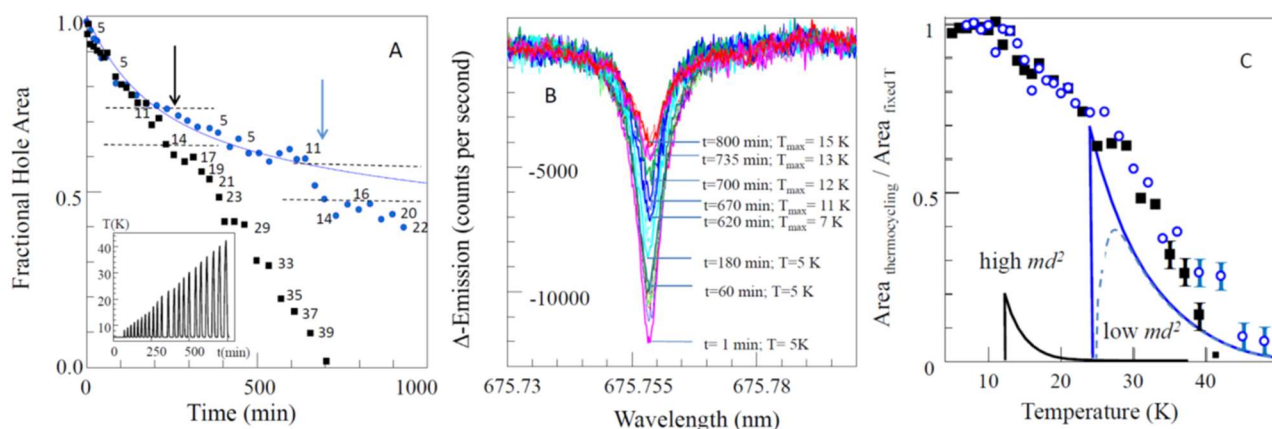


Figure 5-6: Frame A: Recovery of a 30% hole with thermocycling started one hour (black squares) and ten hours (blue circles) after the end of burning. Solid blue curve is the fit to fixed-temperature recovery data. Vertical arrows indicate the fast recovery step at 11-15 K in two datasets. Horizontal dashed lines assist in estimating the magnitude of the step. Frame B: Examples of the raw hole spectra from the experiment where thermocycling started after 10 hours of fixed-temperature recovery (blue circles in Frame A). Initial fractional hole depth was 30%. All spectra were measured at 5 K, T_{max} refers to the maximal temperature of the cycle conducted before measuring the respective spectrum. Frame C: The difference between thermocycling results (black squares in Frame A) and a fit to the fixed-temperature recovery represented as a function of temperature (black squares) and a similar dataset from the experiment with deuterated sample 26 (blue circles), illustrating repeatability of the effect (acceleration of recovery upon thermocycling to 11-15 K, resulting in a step in the data) and independence on deuteration of the solvent. Solid black and blue curves represent the barrier distributions encoded into the holes at the end of the burn / beginning of the recovery. Dashed blue curve indicates what is left of the "blue" distribution after the first three hours below 11 K. The black component remained untouched during the first 10 hours at 5 K.

This step cannot be attributed to some experimental artifact related, for example, to the unusual performance of the cryostat at this particular temperature, as it was repeatable in Cyt b_6f and not observed in CP43 [64] while using the same experimental setup. Thus, there most likely are two distinct contributions to the hole recovery (and burning), characterized by significantly different V and md^2 values, as opposed to a single- md^2 barrier distribution with both attempt frequency and

mean of the λ -distribution being drastically reduced, as was proposed for Cyt *b₆f* earlier [80], [93], [94]. *Figure 5-6C* depicts, as a function of temperature, the fraction of the evolution of spectral holes that cannot be accounted for by the hole recovery of the same duration at 5 K. It was obtained by dividing the thermocycling data in *Figure 5-6A* (black squares) by the fit to fixed-temperature recovery (smooth blue curve in *Figure 5-6A*). Data for a sample with deuterated solvent is shown for comparison.

Figure 5-7 summarizes the two-component model qualitatively explaining the data in *Figure 5-5* and *Figure 5-6* as well as in Refs [80], [93], [94] and [107]. $f(\lambda)$ and $g(V)$ are the distributions of tunneling parameter λ and barrier height V , respectively. NPHB is simultaneously (and possibly competitively, see below) probing two distinct barrier distributions with two different generalized coordinates and with overlapping ranges of NPHB yields (combinations of excited-state λ and Ω_0) – one with lower barriers V and higher md^2 (black) and the other with lower md^2 and higher V (blue). The lower md^2 higher V tier (blue) may feature either systematically higher values of NPHB yield (i.e. lower λ , as shown in *Figure 5-7*) or just broader NPHB yield distribution ensuring that the lower- λ end of the combined distribution features lower md^2 and so there is no significant burning by barrier-hopping in the excited state at 5 K. In thermocycling experiments the lower-barrier higher- md^2 part (black) is the first to recover (note the switching of the black and the blue curves between parts A, C, E and B, D, F of *Figure 5-7*).

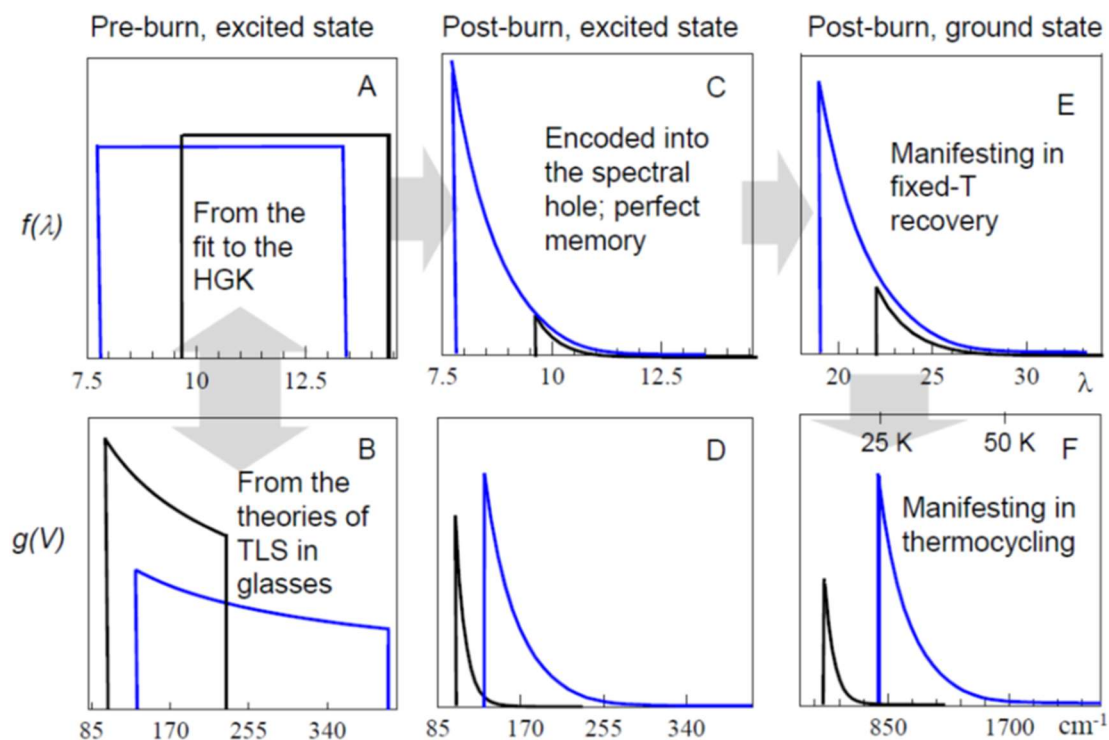


Figure 5-7: Likely relationship between two distributions (and sub-distributions encoded into the holes in the case of a perfect spectral memory) affecting NPHB process and hole recovery in Cyt b_6f . $f(\lambda)$ and $g(V)$ are the distributions of tunneling parameter λ and barrier height V , respectively. Frames A and B depict full pre-burn λ -distributions and barrier distributions, respectively. Frames C and D represent partial distributions encoded into the holes (with pigment in the excited state). Frames E and F represent the ground-state distributions manifesting in recovery experiments. The true full pre-burn λ -distributions for the ground state (not shown) would look like A and B but with the horizontal scale from E and F, respectively. Blue: lower md^2 higher- V component. Black: higher md^2 lower- V component. Note the switch between “blue” and “black” components between upper and lower rows of the figure. $\lambda_{gr}/\lambda_{exc}$ is slightly different for two tiers, see Frames C and E. $\lambda_{gr}/\lambda_{exc}$, blue=2.46 and $\lambda_{gr}/\lambda_{exc}$, black=2.30. See text for more details.

It is a common practice to fit the data such as presented in Figure 5-6C to the (derivative of the) barrier distribution $g(V)$ [20], [26], [85]. However, more rigorous procedure involves modeling such data using Eq. 59 and the temperature change profile $T(t)$ of the actual experiment (see insert in Figure 5-6A for an example of such profile). During the recovery the spectral holes evolve in such manner that partial λ -distributions encoded into the holes (such as those in Figure 5-7E) are multiplied by

$$q(\lambda_{gr}, V_{gr}, t, T) = \exp(-\Omega_0 t [(2n_{gr}(\Delta_{gr}) + 1) \exp(-2\lambda_{gr}) + 2 \exp(-\frac{V_{gr}}{k_B T})]) \quad (59)$$

This function was adopted from [87] and modified to include both tunneling (the first term in the square brackets) and barrier-hopping (the second term) [80], [93], [94]; $n_{gr}(\Delta_{gr})$ is the average population number for relevant phonons, determined by the asymmetry of the ground-state TLS. Eq. 59 describes the probability that burnt systems with given λ_{gr} and V_{gr} will remain unrecovered after time t at temperature T . The $q(\lambda_{gr})$ is a relatively sharp (but not infinitely sharp, as in earlier, simpler models [80], [93], [94], [87]) step-like function with zero at low λ_{gr} and one at high λ_{gr} . With the increase in time t this function is shifting towards higher λ_{gr} and V_{gr} , cutting more off the post-burn λ -distribution, and it shifts more within the same time with the increase of T . Attentive reader may notice that Eq. 59 contains extra factors of 2 in front of the $n_{gr}(\Delta_{gr})$ and the hopping probability, compared to similar equation from [80], [93], [94], [107]. This modification reflects the fact that recovery of the spectral hole involves evolution of the system towards equilibrium, which must occur with the rate that is the sum of the “uphill” and “downhill” rates [108]. For barrier-hopping, for the sake of simplicity it was assumed that as long as the barrier height is much larger than the asymmetry, the $1 \rightarrow 2$ and $2 \rightarrow 1$ rates for barrier hopping are nearly equal. Spectral hole recovers because the ensemble of the TLS evolves towards equilibrium. There is nothing fundamentally special about spectral holes, they are just a particular case of an ensemble of the pigment/ environment TLS being out of equilibrium [109]. A sub-ensemble of the pigment-protein systems with similar electronic transition energies does not “know” if it belongs to some spectral hole or not while its populations are evolving towards equilibrium.

The data from the thermocycling experiment following ten hours of fixed-temperature recovery (blue squares in *Figure 5-6A*, *Figure 5-6B*) suggests that one minute at 12 K had larger effect on

the sub-distribution of the barriers responsible for this particular recovery step (black distribution in *Figure 5-6C* and *Figure 5-7*) than 600 minutes of recovery at 5 K and therefore, approximately,

$$600 \text{ min} \cdot \exp(-2\lambda_{gr})(2n_{gr}(12K) + 1) \leq 1 \text{ min} \cdot 2\exp(-\frac{V_{gr}}{k_B 12})$$

Here we made an assumption that the ground-state attempt frequency is the same for tunneling and barrier-hopping. Equality corresponds to a situation when after ten hours of recovery at 5 K the q -curve (Eq. 59), moving towards the larger λ_{gr} , has reached exactly the $\lambda_{gr,min}$ of this sub-distribution. In the case of the thermocycling, $V(T_{max})=k_B T_{max} \ln(t_{max}\Omega_0)$ [80], [93], [94], where $t_{max} \approx 60$ sec is the time spent at the peak cycle temperature T_{max} . Due to the logarithm, the conversion between the temperature at which a subset of systems recovers and the respective barrier heights has weak dependence on the attempt frequency. Thus, for a broad range of reasonable Ω_0 around 10^{11} - 10^{13} Hz, the systems recovering at ~ 12 K (black distributions in *Figure 5-7*) feature barriers of $\sim 370 \text{ cm}^{-1}$ with at most $\pm 10\%$ uncertainty and $\lambda_{gr,min} \approx 25$ for these systems. Additionally, the other tier of the energy landscape (blue), responsible for the observable hole recovery at 5 K, must feature somewhat smaller $\lambda_{gr,min} \approx 20$. These values of λ_{gr} are in reasonable agreement with the data reported for hyperquenched glassy water [97].

Note that the above estimate does not require any knowledge of the excited state TLS parameters that could be obtained from the fit to the HGK (*Figure 5-5A*). Given the two-tier model shown in *Figure 5-7*, one should consider if the NPHB is competitive (both channels are available for each system) or one or another NPHB channel exists in a given individual pigment-protein system. The latter assumption would imply that our samples contained two distinct sub-populations of pigment-protein systems with very different md^2 . It is unlikely that two distinct local environments of a Chl a molecule, with different conformational changes available to them,

would exist in Cyt *b₆f* where all Chls are located in identical protein pockets [98]. NPHB in the latter scenario can be easily modeled as a sum of burning into two independent components.

The competitive NPHB on two different tiers of the protein energy landscape can be modeled assuming that NPHB yields for two competing hole burning processes are

$$\phi_1(\lambda_1, \lambda_2) = \frac{\Omega_{01} \exp(-2\lambda_1)}{\Omega_{01} \exp(-2\lambda_1) + \Omega_{02} \exp(-2\lambda_2) + \tau_1^{-1}}; \quad \phi_2(\lambda_1, \lambda_2) = \frac{\Omega_{02} \exp(-2\lambda_2)}{\Omega_{01} \exp(-2\lambda_1) + \Omega_{02} \exp(-2\lambda_2) + \tau_1^{-1}} \quad (60)$$

where Ω_0 are the attempt frequencies and τ_1 is the fluorescence lifetime that may be shortened by intersystem crossing, energy transfer or charge transfer. The total NPHB yield will be

$\phi(\lambda_1, \lambda_2) = \phi_1 + \phi_2$. Contributions of the two tiers of the energy landscape to any spectral hole

would scale as ϕ_1 to ϕ_2 . The HGK curves behave as in the case of a single-component λ -

distribution with significantly smaller width. For instance, a good fit to 5 K HGK curve in *Figure*

5-5A can be produced with a combination of two box-like λ -distributions, one ranging from 7.8

to 13.2 and another from 9.5 to 14.7. For the sake of simplicity and comparability, the same value

of the attempt frequency as in [80], [93], [94], $7.6 \cdot 10^{12} \text{ s}^{-1}$ was used for both tiers. The first (blue)

λ -distribution is significantly broader than a single λ -distribution that was required for fitting the

same HGK data (7.8 to 12.0 [78], i.e. 4.2 units versus 5.4 above). Note that in the non-competitive

burning scenario, involving two distinct populations with different conformational changes, the

pre-burn distributions will be narrower. Two partial distributions produced with the above

parameters and encoded into the 30% holes are actually those shown in *Figure 5-7E* and *F*. Since

there is spectral memory (shallower holes recover faster in *Figure 5-5B*), these partial

distributions are probed in the recovery experiments, not the full ones in *Figure 5-7A* and *Figure*

5-7B. The area under the black curve is about 10% of the total area under both curves combined,

in agreement with the magnitude of the recovery step observed at around 11-15 K. The two-component model offers slightly better fit to the fixed-temperature recovery data, especially at long times, and yields $\lambda_{gr}/\lambda_{exc}=2.46$. We are not aware of a physical reason why different tiers of the energy landscape should feature exactly identical $\lambda_{gr}/\lambda_{exc}$ ratios; so far this ratio was used as a free parameter to arrive at the best fit to the fixed-temperature recovery data [80], [93], [107]. Therefore, we allow for somewhat different $\lambda_{gr}/\lambda_{exc}$ for “blue” and “black” tiers of the energy landscape. Smaller $\lambda_{gr}/\lambda_{exc}$ for the “black” tier allows for somewhat smaller value of md^2 and better fit to the HGK data at temperatures higher than 5 K. However, $\lambda_{gr}/\lambda_{exc}$ should not be significantly smaller for the “black” tier than for the “blue” tier: If there were more overlap between the two ground-state λ -distributions (imagine shifting black curve in *Figure 5-7E* further to the left), the “black” tier of the landscape would contribute significantly to the tunneling-dominated fixed-temperature recovery at earlier times, and after the first ten hours at 5 K significant portion of this component would be recovered. The minimal value of $\lambda_{gr}/\lambda_{exc,black}$, i.e. the value where the fixed-temperature recovery does not yet become faster for fixed $\lambda_{gr}/\lambda_{exc,blue}=2.46$, is 2.30. *Figure 5-8* demonstrates the fits to fixed-temperature recovery and thermocycling data, obtained utilizing the temperature change profiles of the real experiments and yielding $md^2_{blue}=1.5\cdot10^{-46}$ kg·m² and $md^2_{black}=4.0\cdot10^{-46}$ kg·m² (or 9 amu·Å² and 24 amu·Å², or 32 and 85 amu·r_{Bohr}², respectively). The fits to the thermocycling data capture most of the details; possible reasons for the remaining discrepancies are considered in the Discussion section.

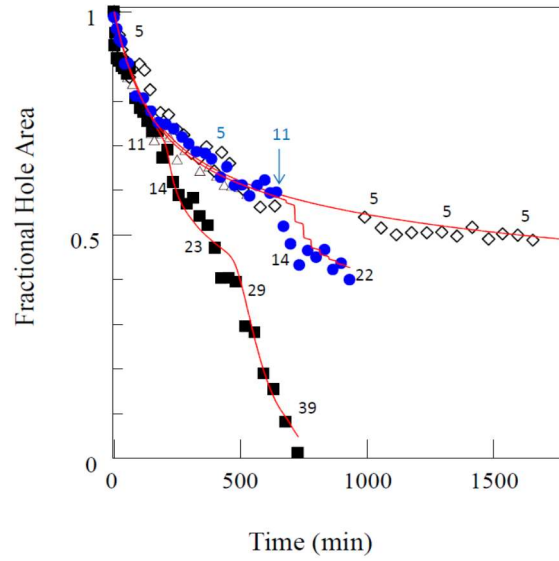


Figure 5-8: Fixed-temperature (5 K) recovery data (open symbols) from Fig. 5-5B and thermocycling data (filled symbols) from Fig. 5-6A with the fits (red curves) obtained with md^2 blue = $1.5 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ and md^2 black = $4.0 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ using the temperature change profiles of the respective experiments. Competitive NPHB. Numbers indicate temperatures in Kelvin for some data points. $\lambda_{gr}/\lambda_{exc}$, blue = 2.46 and $\lambda_{gr}/\lambda_{exc}$, black = 2.30. See text for more details.

It was impossible to achieve a satisfactory global fit to the available data when the pre-burn λ -distributions were Gaussian. We have verified that also in the case of competitive NPHB the dominant contribution encoded into the spectral holes (blue) is not the one derived from a Gaussian. However, given the uncertainties of the thermocycling data, one could argue that the second distribution (black) could still be Gaussian. A satisfactory fit to the 5 K HGK data in Figure 5-5A (good fit for the first 40%, smooth green curve) could be obtained starting from a box-like λ -distribution in the 7.8-13.0 range (similar to the blue distribution in Figure 5-7) plus a Gaussian λ -distribution peaked at 11.7 and with standard deviation $\sigma_\lambda = 1.0$ (replacing the black distribution in Figure 5-7). For 30%-deep hole this resulted in the “black” tier contributing about 13% to the hole. Note that the lower- λ tail of the Gaussian (black) component reached further than the λ_{min} of the “blue” component. In order to achieve sufficient separation between the

ground-state distributions encoded into the holes, and to allow for no or almost no recovery of the “black” component in the first ten hours at 5 K, the $\lambda_{gr}/\lambda_{exc}$ value for the “black” tier must not be smaller than for the “blue” tier, unlike in the case when one starts from two box-like λ -distributions. This requires a higher value of md^2 of at least $4.5 \cdot 10^{-46}$ kg·m² to ensure that the “black” tier still experiences recovery upon thermocycling at a correct temperature, which in turn leads to the earlier onset of spectral hole burning via barrier hopping, and poorer fits to the HGK data, particularly at 10 K and above.

5.2.4 Discussion

As demonstrated above, introducing simultaneous NPHB involving two tiers of the protein energy landscape that are partially overlapping in terms of λ but are separate in terms of the post-burn barrier distributions offered a natural solution to the problem of the mismatch between the widths of the λ - and barrier distributions deduced from the experimental results [80], [93]. Introducing competitive burning was helpful as well, as it allowed for starting from broader λ -distributions when modeling the HGK data. However, arriving at a single set of parameters that would explain all observations quantitatively still proved very difficult. Thus, below we consider possible avenues of further improving the procedures for modeling the NPHB and recovery data.

The Details of the NPHB Process and Implications for Spectral Memory

In the previous section we suggested that the recovery of the spectral holes should be interpreted as evolution of the ensemble of pigment-protein systems towards thermodynamic equilibrium. This resulted in additional factors of two in Eq. 59, taking into account that evolution towards equilibrium occurs with the rate that is the sum of the “uphill” and “downhill” rates [108]. Would similar argument be valid for the hole burning process, involving the excited state?

First, we stress that the following discussion is applicable to each of the “blue” and “black” components in *Figure 5-7*, one should not interpret it as if we were considering one of these components as memory-less and the other exhibiting perfect spectral memory. One can split the ensemble of the individual pigment-protein systems resonant with the laser into two sub-ensembles, one where upon excitation the individual systems end up in the lower well of the excited-state TLS and the other where they find themselves in the higher well of the excited-state TLS. Each of these two sub-ensembles will be out of thermodynamic equilibrium, and will evolve with the rate, which is the sum of the “uphill” and “downhill” rates for the excited state [108]. Can this evolution towards equilibrium argument still be valid given the short (nanoseconds) lifetime of the excited state? One could note that the tunneling model used in this and earlier NPHB works employed free-particle wavefunctions. In reality, the wavefunctions in each well of the TLS must be closer to those of the harmonic oscillator [110], [111] and the well populations would oscillate out of phase. Additionally, as demonstrated in [109], cooling of the sample in the beginning of the experiment (from RT to 5 K in two hours) may result in the ensemble being not in thermodynamic equilibrium prior to burning. Even in equilibrium, some systems will be found in the upper well of the ground-state TLS before burning. Thus, the recovery of any spectral hole may exhibit some small memory-less contribution and the respective distributions manifesting in hole recovery may contain both components. The magnitude of the memory-less component will depend on the pre-burn population of the higher well of the ground state TLS, i.e. on the ground state asymmetry, and on the history of the experiment / cooling procedure [107]. Since any component featuring spectral memory must be dominated by smaller barriers or λ [80], [93], [94] (*Figure 5-6C*), initial phases of recovery will still be determined by the component with memory.

Small memory-less contribution may provide somewhat more gradual recovery upon thermocycling, in better agreement with the experimental results.

According to *Figure 5-5A*, in the excited state tunneling dominates in the NPHB process until about 10 K. Thus,

$$\exp(-2\lambda_{ex,min})(2n_{ex}(10K) + 1) > 2\exp(-\frac{V_{ex,min}}{k_B 10K}) \quad (61)$$

And

$$-2\lambda_{ex,min} + \ln(2n_{ex}(10K) + 1) > -\frac{V_{ex,min}}{7} + \ln 2$$

The weak temperature dependence of the HGK (*Figure 5-5A*) indicates that the excited state TLS asymmetries are not lower than 4 cm⁻¹ (4.5 cm⁻¹ were used in our simulations). Excited state asymmetry as high as 27 cm⁻¹ was proposed for hyperquenched glassy water [97]. For large enough asymmetries both logarithm terms are smaller than other terms and partially cancel each other. Thus, $V_{ex,min}/\lambda_{ex,min} \gtrsim 14$ (cm⁻¹), which for $\lambda_{ex,min} \sim 7.8$ and 9.5 yields $V_{ex,min} \geq 110$ (blue) and 130 cm⁻¹ (black), respectively. For the lower- V_{gr} tier of the energy landscape (black) $\lambda_{gr}/\lambda_{ex} \sim 2.3$ and $V_{gr}/V_{ex} \leq 370/130 \sim 2.8$. These ratios of λ and V are clearly inconsistent with the quadratic dependence of V on λ (Eq. 58) indicating that more advanced models describing tunneling may have to be applied in this case. For the other tier (blue) $V_{gr}/V_{ex} \approx 6$ as this component exhibits recovery due to thermocycling starting from about 20 K. In other words, for this tier $V \sim \lambda^2$ is possible.

Eq. 58 was derived assuming rectangular barriers and free-particle wavefunctions outside of the barrier region. For more realistic inverted parabolic barriers and while $2\lambda \gg 1$, but still in the

approximation where the “outer walls” of the TLS are absent and reintroduced artificially via the attempt frequency,

$$\lambda = \frac{\pi}{4} d \sqrt{2mV} / \hbar \quad (62)$$

Another model, employing parabolic wells, was recently considered in [110]. The wavefunctions for the ground state of the harmonic oscillator were employed, the probabilities to find the system in each of the wells oscillated out of phase, and the probability of transition per unit time was

$$k_0 \approx \frac{2\nu}{\sqrt{\pi}} \delta \exp(-\delta^2) \quad (63)$$

$\delta = d / 2x_0$ is dimensionless, d is the full distance between the TLS minima, $x_0 = \sqrt{\frac{\hbar}{m\omega}}$ is the displacement of the harmonic oscillator at the lowest vibrational level, ν is the attempt frequency in Hz and ω is the same in rad/s. Comparing this with Eq. 57 suggests $\delta^2 = 2\lambda$. Overall, in this model the quadratic relationship between V and λ is preserved, but the pre-factor in the tunneling rate expression becomes dependent on λ as $\lambda^{3/2}$ (or $V^{3/4}$). Specifically, Eq. 63 can be manipulated to

$$k_0 = \frac{4\lambda\hbar}{\pi m d^2} \frac{2\sqrt{2\lambda}}{\sqrt{\pi}} \exp(-2\lambda) = \frac{1.27 * \lambda * 10^{12} \text{ Hz}}{m d^2} 1.6\sqrt{\lambda} \exp(-2\lambda) \quad (64)$$

where md^2 is in the units of $10^{-46} \text{ kg}\cdot\text{m}^2$. For $\lambda \sim 10$ -20, the pre-factor in Eq. 58 falls into the range of attempt frequencies reported elsewhere when md^2 is several times $10^{-46} \text{ kg}\cdot\text{m}^2$. One could generalize that $\lambda = Cd\sqrt{2mV}/\hbar$ where $C=1.0$ for rectangular barriers, $C=\pi/4$ for inverted parabolic barriers, $C \approx 1/4$ for parabolic wells, etc. Concerning the temperature dependence associated with Eqs. 63 and 64, the NPHB rate is weakly dependent on temperature, and the observed slowdown of the HGK (*Figure 5-5A*) is due to the increase of the homogeneous line width [80], [93], [94]. Thus, for low temperatures one should utilize the weak temperature dependence corresponding to tunneling assisted by phonon scattering [80], [93], [94], [95], [96], [97] [107] (see Eq. 57) rather than the exponential dependence suggested in [110]. What are the consequences of introducing $C < 1$? From Eq. 60 with $V \sim \lambda^2$ tunneling is the dominant NPHB mechanism when

$$\frac{\lambda_{\text{ex}}^2 \hbar^2 C^{-2}}{2md^2 k_B T} - 2\lambda_{\text{ex}} + \ln(n_{\text{ex}} + 1/2) > 0 \quad (65)$$

The last term is much smaller than the first two for $T=5$ -10 K, $\lambda_{\text{ex}} \gtrsim 10$ and reasonable excited state TLS asymmetries. At the first glance, as long as $C^2 > 1$, i.e. for any barriers but the rectangular ones, larger md^2 could be accommodated for the same λ_{ex} without introducing barrier hopping to the burning process. Unfortunately, this does not help one achieve better global fit to all data since for lower values of C larger values of md^2 are required to fit the thermocycling data.

5.2.5 Distributions

Earlier we reported a mismatch between the widths of the λ -distributions responsible for fixed-temperature recovery and thermocycling in Cyt *b₆f* [78]. Similar problem was in fact present also for CP43 complex [64]. Allowing for an additional distribution component (black) in Cyt *b₆f*

reduces this problem. (In the case of CP43, availability of different conformational changes may also result from the lowest-energy state being delocalized over several chlorophylls [112].)

Concerning the most probable shape of the “blue” component, providing dominant contribution to the recovery processes, the results presented in *Figure 5-5B* suggest that its pre-burn $f(\lambda)$ should be uniform / box-like (see *Figure 5-7A*). HGK and recovery results obtained for Cyt b_6f in fully deuterated buffer-glycerol environment indicate that there are very little differences in the hole burning and recovery dynamics [107]. We conclude that spectral dynamics on the energy landscape tier characterized by the blue curve in *Figure 5-7* definitely belongs to the protein, while the dynamics on the tier characterized by the black curve most likely belongs to the protein as well, but may with some small probability also be due to the movements of the –OH groups in the water/glycerol solvent surrounding the protein [107]. The latter is fairly unlikely because the respective deuteration-induced increase of λ by 3% [107] i.e. to $\lambda_{min, exc}=9.8$ would significantly reduce the contribution of the “black” tier to the holes and likely render the step at 11-15 K unobservable. However, according to *Figure 5-6C* the step at 11-15 K was still observed in thermocycling data of the deuterated-solution sample (blue circles). The values of md^2 deduced from the data effectively rule out tunneling of a single proton in a double-well potential associated with a hydrogen bond as a possible source of the observed dynamics. The md^2 in this case would be $0.7 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ ($4 \text{ amu} \cdot \text{\AA}^2$) or less, taking into account that separation of the potential wells in this scenario must be smaller than the typical hydrogen bond length.

Cooperative effects involving the whole hydrogen bonds network of the protein are still possible, since in this case m no longer has to be equal to the mass of a single proton. Alternatively, “blue” component could represent a rearrangement of some relatively light chemical group such as –CH₃, –OH, etc. Note that the Chl a central Mg is coordinated by a pair of hydrogen-bonded

water molecules, one ligated to the Mg atoms and the other hydrogen-bonded to the peptide side chain and backbone of the G helix of subunit IV. The mechanism involving rotations of a methyl group by 120 degrees is unlikely, as it has a characteristic signature in the NPHB data [113], [114] that we did not observe. Given the above values of md^2 , the possibility that one of the competing NPHB mechanisms observed in this work is related to protective quenching of Chl α excitations via electron transfer [102] also seems fairly unlikely.

5.2.6 Conclusions

In cytochrome b_6f the relevant distribution of the tunneling parameter λ is a superposition of two contributions, which are significantly overlapping in terms of the resultant NPHB yield and represent two tiers of the energy landscape responsible for NPHB. However, similar values of the NPHB yield result from distinctly different combinations of barrier heights, the shifts along the generalized coordinate and/or the masses of the entities involved in conformational changes. Specifically, md^2 of $(1.5 \pm 0.1) \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ (blue) and $(4.0 \pm 0.3) \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ (black) were deduced for these two tiers. Consequently, fixed-temperature recovery and thermocycling experiments preferentially probe different components of the barrier- and λ -distributions encoded into the spectral holes. The “blue” component likely represents the dynamics of the protein, while the “black” one may originate from several sources within the protein, as well as, though not likely, the –OH group dynamics of the solvent surrounding the protein. The protein dynamics may be cooperative. Both tiers feature barrier distributions of the $\sim 1/\sqrt{V}$ type (uniform / box-like λ -distribution) prior to burning. One should not assume that other orthogonal generalized coordinates with significantly different λ cannot exist on a real multi-well and multidimensional

protein energy landscape. They definitely can. However, landscape tiers with too small λ may yield only transient holes and/or hole broadening (also accessible using photon echo experiments [115], [116]), while the barriers of the landscape tiers with too large λ are very unlikely crossed with non-negligible probability when easier-burning landscape tiers are available. There are also indications that for the “black” tier of the energy landscape $V \sim \lambda^2$ (Eq. 58) is not valid. Further improvements in the interpretation and modeling of the NPHB data, including the data on hole recovery and thermocycling, require adopting more rigorous treatment of tunneling in biological systems, beyond the approximations used to obtain Eq. 58.

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5.3 Effects of Chlorophyll Triplet States on the Kinetics of Spectral Hole Growth

This chapter is based on the paper by A. Trempe, A. Levenberg, A. D. Gonzalez Ortega, M.A. Lujan, R. Picorel and V. Zazubovich, published in the *Journal of Physical Chemistry B* 125 (2021) 3278-3285.

I have contributed to all experiments, most of data analysis and paper writing.

Abstract: Spectral hole burning has been employed for decades to study various amorphous solids and proteins. Triplet states and respective transient holes were incorporated into theoretical model and software simulating non-photochemical spectral hole burning (NPHB) and including all relevant distributions, in particular the distribution of the angle between electric field of light E and transient dipole moment of the chromophore μ . Presence of Chlorophyll a triplet state with lifetime of several milliseconds explains the slowdown of NPHB (on the depth vs illumination dose scale) with the increase of the light intensity, as well as larger hole depths observed in weak probe beam experiments, compared to those deduced from the hole growth kinetics measurements (signal collected at fixed wavelength while stronger burning beam is on) in Cytochrome b_6f and chemically modified LH2. We also considered the solvent deuteration effects on triplet lifetime and concluded that both triplet states and local heating likely play a role in slowing down the HGK with increasing burn intensity.

5.3.1 Introduction

Spectral hole burning (SHB) has been widely used to recover narrow zero-phonon lines (ZPL) from the inhomogeneously broadened spectra of amorphous solids, such as glasses, polymers and

proteins, and to derive information encoded in these ZPL. [82], [89], [117]. This information includes data on various processes affecting the lifetimes of the excited states (e.g. pure dephasing, energy transfer, etc), as well as dynamics and energy landscapes of the surrounding amorphous solid. For chlorophylls in proteins, non-photochemical hole burning (NPHB) is the dominant SHB mechanism. Here the excited pigment remains intact but its local environment is altered which in turn alters the pigment's ZPL frequency. Such frequency shifts are also well known in single-molecule spectroscopy. [89], [117] At cryogenic temperatures these small conformational changes occur via tunneling. The simplest representation of the energy landscape of the protein is a double-well potential known as TLS (two-level system, *Figure 5-9A*).

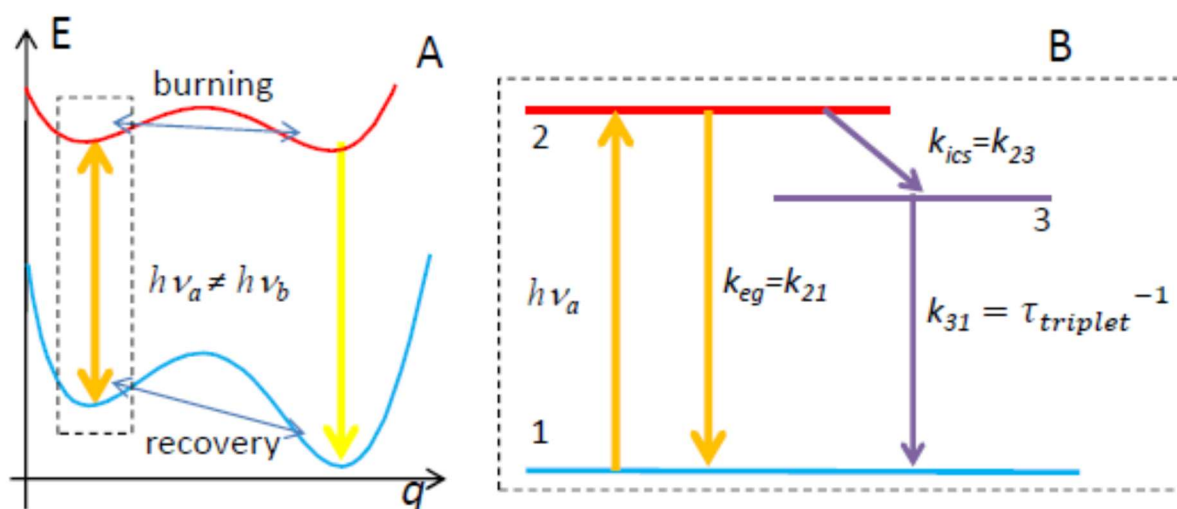


Figure 5-9: Blue: ground singlet state. Red: excited singlet state. Purple: triplet state. Frame A: The basis of NPHB. Excitation of the pigment molecule causes small conformational change in the immediate environment of the pigment molecules, represented by a double-well potential / TLS. As a result, transition frequency of the pigment gets modified. Frame B: Three-state energy diagram for one well of the TLS in Frame A. Definitions of rates relevant for subsequent discussion as well as intersystem crossing yield $\theta = k_{ics} / (k_{eg} + k_{ics})$. $\tau_1^{-1} = k_{eg}$ is the decay rate for the singlet excited state, including radiative and non-radiative processes, but not including intersystem crossing.

This model is also used to explain unusual thermal properties of various amorphous solids at cryogenic temperatures. [91], [95]. When narrow-band laser is used to illuminate the sample, a “hole” appears in the inhomogeneously broadened spectrum as a result of spectral shifts of the resonant molecules, i.e. a resonant decrease of absorption at the illumination frequency. The main component of this hole is the mirror image of the resonant ZPL. NPHB master equation [74] describes the absorption spectrum containing a persistent hole after illumination at ω_B of duration t :

$$D(\Omega, t) = \int d\omega L(\Omega - \omega) G(\omega) \int d\lambda f(\lambda) \int d\alpha \frac{3}{2} \sin \alpha \cos^2 \alpha e^{-P\sigma\phi(\lambda)L(\omega_B - \omega)t \cos^2 \alpha} \quad (66)$$

$G(\omega)$ is the site distribution function (SDF; the probability to find a chromophore with given ZPL frequency; it is Gaussian before any burning), $L(\omega)$ is the single-site spectrum (SSS) shape with area normalized to unity on the frequency scale; it contains narrow ZPL, broad phonon sideband (PSB) and intra-molecular vibrations. P is the photon flux, σ is the integral absorption cross-section, and ϕ is the NPHB yield that in the absence of triplet is given by

$$\phi(\lambda) = \frac{\Omega_0 \exp(-2\lambda)}{\Omega_0 \exp(-2\lambda) + \tau_1^{-1}} \quad (67)$$

Here Ω_0 is the attempt frequency, determining how often the systems try to cross the barrier between the two wells, and τ_1 is the excited state lifetime that includes both radiative and non-radiative processes but does not include pure dephasing. Alpha is the angle between the electric field $\mathbf{E}$ of light and transition dipole moment $\boldsymbol{\mu}$ of the chromophore. The distribution of α explicitly spelled-out in Eq. 66 corresponds to the case of random isotropic orientation of the chromophores and linearly polarized light. $f(\lambda)$ is the distribution of the tunneling parameter; λ

may be understood as a composite strength of the barrier for the purpose of tunneling and depends on the barrier height, shape and the effective mass of the tunneling entity. One particular modality of NPHB experiments involves monitoring the decrease of absorption or fluorescence signal in time while the laser is kept at fixed burn wavelength. The data obtained in this kind of experiment is called hole growth kinetics (HGK). HGK has been used to determine the distributions of the tunneling parameter in the excited state of the pigment / environment system, and, indirectly, also the parameters for the ground state barrier distributions (recovery while burning, [95], [97], post-burn recovery modeling [16], [80], [93], [107]).

In the course of our work over the years we have occasionally encountered evidence of intensity dependence of the HGK (plotted as fractional hole depth versus illumination dose, the product of intensity and time) as well as instances where the hole spectrum recorded after the HGK measurement was significantly deeper than what would follow from the HGK graph. (Fast hole recovery would yield the opposite effect.) These phenomena can be explained by local heating of the protein by several K: [78]: It is well known that the homogeneous ZPL width is strongly temperature-dependent due to coupling between electronic transition and small-barrier TLS and/or local vibrations. [117], [12], [64] This results in a reduction of the peak absorption cross-section of the ZPL and slowdown of the HGK with increasing temperature. Local heating may affect HGK if light energy being delivered to the chromophores and converted to heat is not further dissipated fast enough. Analysis of available low-temperature heat conductivity data for water, glycerol, other organic solvents and some proteins suggested that bulk heat conductivity values required for observing the above effects are unrealistically low and the interface between the protein and the surrounding solvent is the only possible bottleneck for heat dissipation. [78]

Alternatively, slowdown of the HGK with increased illumination intensity can be explained by introducing a three-state model, with one of these states being a long-lived triplet state. Here we are going to demonstrate that the mismatch between the hole depths obtained with different techniques is also explainable invoking triplet. *Figure 5-9B* represents the same singlet ground and excited states as in frame A (e.g. left well), as well as an additional triplet state. Chlorophyll (Chl) triplets can easily form in pigment-protein complexes involved in photosynthesis. Chls then can transfer triplet energy to molecular oxygen. This process has high yield and generates reactive singlet oxygen, which is damaging for pigment-protein complexes. Thus, photosynthetic organisms implement protective measures, for instance ensuring quenching of Chl triplets by carotenoids. [118], [119] For instance, triplet energy transfer from Chls to carotenoids is nearly 100% efficient in LHCII complex of Photosystem II (PSII) at room temperature (RT), while at 4 K this number is still 82%. [120]. On the other hand, broad shallow transient holes ascribed to triplet states were observed in CP43 [121] and CP47 [122] core antenna complexes at 5 K upon non-resonant excitation. Resonant and non-resonant transient holes were also observed in reaction centers (RC) including RC of purple bacteria (BRC) [123] and PSII. [124] However, in PSII triplet formation follows charge recombination, [125] while in BRC the bottleneck state is P^+Q^- . [123]. Triplet lifetimes on the order of tens of microseconds were observed for Chlorophylls at RT, [126], [127], while lifetimes of up to several milliseconds were reported at 5 K [128], [129], [130], [131], [132], [133] and triplet yields as high as 0.4 have been suggested. [116] Triplet lifetimes of 1.5-1.6 ms were reported in core complexes [125] and RC [117] of PSII. Bi-exponential kinetics with lifetimes of 0.6 and 2.0 ms and triplet yield of 0.16 were reported for CP47 antenna complex at cryogenic temperatures. [118].

It is known that triplet-related effects can slow down the HGK (because resonant molecules are not available for excitation and subsequent NPHB while they are in a triplet state) and that at high enough excitation ZPL experience saturation broadening. This broadening is not an ensemble effect and is present also for single-molecule ZPL. [134] Most papers on the effects of triplet states on SHB were primarily concerned with spectral diffusion and did not offer satisfactory explanation for all our observations. [120] [121] Müller et al. [120] provided easy to use expressions for broadened ZPL profile and site distribution function (SDF) modified by transient holes. They also engaged in some HGK modeling. However, it appears that they simply used saturation-broadened ZPL to model the HGK in the otherwise usual way, ignoring the fact that within the first $\tau_{triplet}$ (triplet lifetime) of burning some dynamic equilibrium is established between the populations of the three states in *Figure 5-9B* and resonant absorption is reduced before the first datapoint of the HGK curve is collected. Ref. [120] relies on an earlier paper by de Vries and Wiersma. [136]. The latter contains detailed treatment (based on the density matrix approach) of a) purely transient holes (probe beam is weak /non-saturating) or b) persistent hole shape after burning with intense beam has ended. In other words, it did not cover the situation where the probe beam is the same beam as the beam causing triplet-related saturation broadening, i.e. HGK measurement. Neither of these papers included the distribution of angles between transition dipole μ and electric field E of the polarized light. It appears that in order to engage in detailed quantitative analysis of HGK and hole recovery data such as were reported in [16], [80], [107], one needs to appropriately incorporate the triplet states and transient holes into our NPHB model. In reality, for sufficiently high flux P the absorbance at ω_B (proportional to the fluorescence count observed during the HGK measurement in fluorescence excitation mode) is affected by both transient hole and NPHB, with the former effect not explicitly included in Eq. 66.

In this manuscript we are going to incorporate the above effects into NPHB modeling [16], [80], [107].

5.3.2 Experimental Methods

The spinach dimeric Cytochrome b_6f samples were described in Ref. [12]; n-octyl β -D-*glucopyranoside* (OGP) detergent was used to solubilize the protein. LH2 light-harvesting complexes from *Rbl. acidophilus* were chemically modified using procedures similar to those described in Ref. [137] Bacteriochlorophylls *a* were replaced with Chlorophylls *a*. The process included two rounds of size-exclusion chromatography ensuring that all unattached pigments were removed. (See the next chapter for a detailed NPHB study of modified LH2) Prior to the experiments the protein solution was mixed with glycerol acting as a cryoprotectant and facilitating formation of a transparent glassy solid at low temperatures. In experiments involving deuterated solvent, D₂O-based buffer and d₈-glycerol (98% of all protons exchanged for deuterons) from C/D/N Isotopes, Dorval, QC, were employed. The time spent by the samples at room temperature was carefully minimized to ensure negligible deuteration of the protein itself and potential damage by glycerol. The samples were placed in a plastic cuvette with 4×10 mm cross-section inside a liquid helium optical cryostat. High-resolution spectra and HGK curves were measured in fluorescence excitation mode using SpectraPhysics / Sirah Matisse-DS frequency-stabilized tunable dye laser (bandwidth ~0.15 MHz). Fluorescence was detected with a Hamamatsu photon counting module through the combination of long-pass (AELP700; Omega Filters, VT) and short-pass (750 nm, ThorLabs) filters. Thus, in the case of the chemically modified LH2 we selectively probed only those Chls that failed at transferring energy to Bacteriochlorophylls (but were still attached to the protein).

5.3.3 Results

Figure 5-10 shows examples of normalized experimental HGK and persistent hole spectra indicating intensity dependence of the HGK and mismatch between the hole depth obtained from the HGK data and from the hole spectrum measured immediately after the HGK measurement was finished. These spectra were obtained for Cyt *b₆f* [12] and modified LH2. It is clear that a) the HGK depicted as a function of illumination dose (product of intensity and time) slow down with the increase of intensity and b) that for high-enough intensities there was a definite mismatch between the hole depths obtained from HGK and from post-burn spectra.

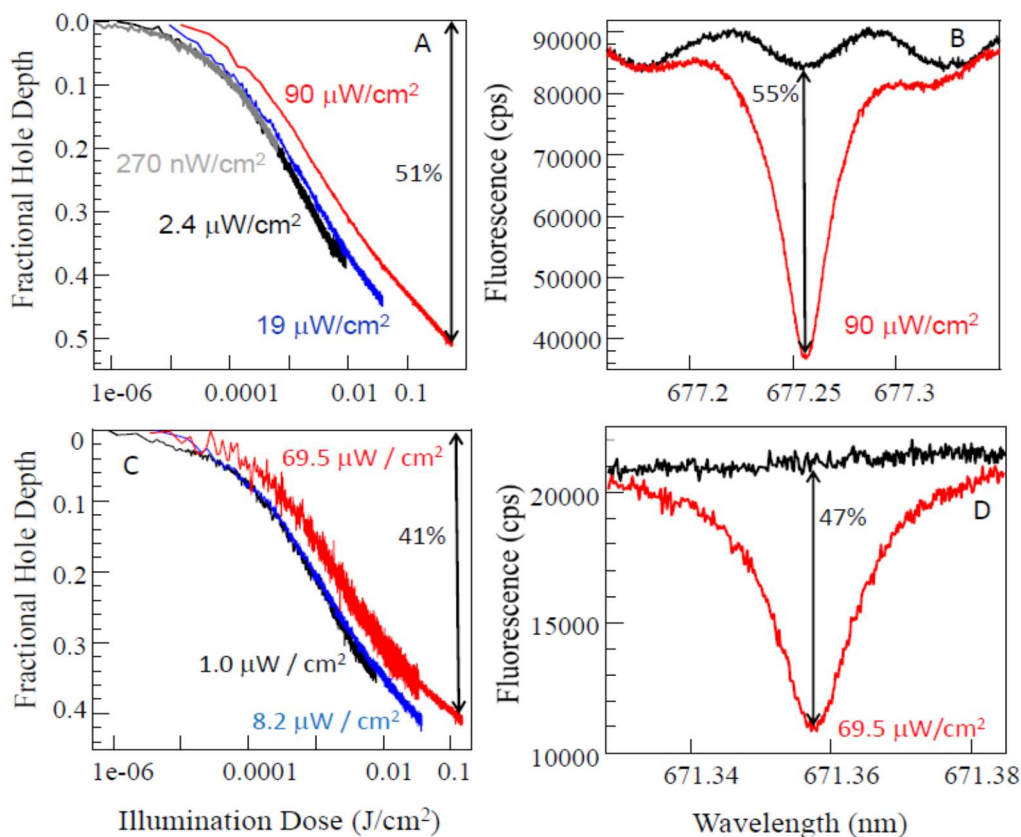


Figure 5-10 Frames A and B: Dependence of HGK on intensity and the spectral hole corresponding to the highest-intensity HGK for Cytochrome *b₆f*. Wave-like pattern is due to the etaloning effect of the cryostat windows. Frames C and D: Dependence of HGK on intensity and the spectral hole corresponding to highest-intensity HGK for Chl *a* in chemically modified LH2. All data belongs to samples in protonated solvent.

The solution to the modeling challenge outlined in the Introduction includes the following steps: First, we offer a brief overview of relevant equations from Refs. [120] and [121]. Wherever possible we will point out the equivalences between quantities employed Refs. [120] and [121] and those defined in this work. Applying the rotating wave approximation, de Vries and Wiersma [121] wrote down the set of equations of motion for the relevant density matrix elements:

$$\dot{\rho}_{11} = k_{21}\rho_{22} + k_{31}\rho_{33} + \frac{1}{2}i\chi(\overline{\rho_{21}} - \overline{\rho_{12}}) \quad (68)$$

$$\dot{\rho}_{22} = -k_{21}\rho_{22} - k_{23}\rho_{22} + \frac{1}{2}i\chi(\overline{\rho_{12}} - \overline{\rho_{21}}) \quad (69)$$

$$\dot{\rho}_{12} = (i\Delta - \frac{1}{T_2})\overline{\rho_{12}} + \frac{1}{2}i\chi(\rho_{22} - \rho_{11}) \quad (70)$$

$$\dot{\rho}_{33} = k_{23}\rho_{22} - k_{31}\rho_{33} \quad (71)$$

$$\rho_{11} + \rho_{22} + \rho_{33} = 1 \quad (72)$$

Here index 1 corresponds to the singlet ground state (blue in *Figure 5-9*), index 2 corresponds to the singlet excited state (red in *Figure 5-9B*) and index 3 corresponds to the triplet state (purple in *Figure 5-9B*). $\chi = \mu E \cos \alpha / \hbar$, where μ , E and α are transition dipole magnitude, electric field amplitude of the burning beam and angle between the $\mathbf{E}$ and $\boldsymbol{\mu}$ vectors, respectively. T_2 is affected by $2 \rightarrow 1$ processes as well as pure dephasing and determines the homogeneous line width Γ_{hom} which in turn affects the peak absorption cross-section ε_0 . $\Delta = \omega_B - \omega$ is detuning with respect to the burn frequency ω_B which is resonant with $1 \rightarrow 2$ transition. $k_{23} = k_{ics}$ is the

intersystem crossing rate, $k_{21} = k_{eg} = \tau_1^{-1}$ and $k_{31} = \tau_{triplet}^{-1}$. The steady-state population difference between ground state (1) and excited singlet state (2) will then be (Eq. 18 of Ref. [121])

$$\rho_{11} - \rho_{22} = \frac{\Delta^2 + \frac{1}{T_2^2}}{\Delta^2 + \frac{1}{T_2^2} + K^2} \quad (73)$$

Here $K^2 = \chi^2(2+A)/2T_2(k_{21}+k_{23})$ and $A = k_{23}/k_{31} = k_{ics} \tau_{triplet}$. Note that K^2 is proportional to E^2 i.e. to photon flux P , and to $\cos^2 \alpha$. One may also notice that Eq. 73 describes one minus a flux-dependent Lorentzian. This is the function by which one has to multiply the pre-burn SDF ($G(\omega)$ in Eq. 66) in order to obtain the SDF containing the transient hole. Without any illumination, $\rho_{11} - \rho_{22} = 1$, $\rho_{11} = 1$, $\rho_{22} = 0$ and $\rho_{33} = 0$ for any Δ . For zero detuning Δ (at ω_B) and very large photon flux, $\rho_{11} - \rho_{22}$ approaches zero. The fraction of the SDF remaining at ω_B after the transient hole is formed will be (Eq. 73 with zero detuning)

$$\frac{\frac{1}{T_2^2}}{\frac{1}{T_2^2} + K^2} = \frac{1}{1 + K^2 T_2^2} = \frac{1}{1 + P \cos^2 \alpha / P_s} \quad (74)$$

Here we made a transition to quantities from Ref. [135] that are more compatible with the Eq. 66.

$$P_s = \frac{k_{31}(k_{21} + k_{23})}{(2k_{31} + k_{23})\varepsilon_0} \approx \frac{k_{31}(k_{21} + k_{23})}{k_{23}\varepsilon_0} = \frac{1}{\tau_{triplet}\theta\varepsilon_0} \quad (75)$$

is so-called saturation photon flux. (As long as the triplet lifetime is much longer (milliseconds) than the singlet excited state lifetime (nanoseconds), $k_{31} \ll k_{23}$.)

$$\theta = k_{ics}/(k_{eg}+k_{ics}) = k_{23}/(k_{21}+k_{23}) \quad (76)$$

is the intersystem crossing yield and $\varepsilon_0 = \sigma L(\omega_{ZPL})$ is the peak absorption cross-section of unsaturated ZPL. Note that the full single-site spectrum (SSS) containing non-broadened ZPL, phonon sideband (PSB) and intra-molecular vibrations was used to obtain ε_0 from the integral cross-section σ (determined in the absence of saturation). If the remaining SDF at ω_B is described by Eq. 74, the fractional depth of the transient hole in the SDF is

$$\frac{\Delta G(\omega_B)}{G(\omega_B, P=0)} = 1 - 1/(1 + P \cos^2 \alpha / P_s) = (P \cos^2 \alpha / P_s) / (1 + P \cos^2 \alpha / P_s) \quad (77)$$

For low flux P or perpendicular $\mathbf{E}$ and $\boldsymbol{\mu}$ this fractional depth approaches zero, while for large flux and parallel $\mathbf{E}$ and $\boldsymbol{\mu}$ this quantity approaches one. In Ref. [135] the saturation-broadened Lorentzian line was written as

$$L(\Delta, a, P) = \frac{\varepsilon_0 \Gamma_{hom}^2}{\Delta^2 + (1 + \frac{P \cos^2 \alpha}{P_s}) \Gamma_{hom}^2}, \quad (78)$$

but without the angular dependence. For zero photon flux ($P=0$) the peak of this line is normalized to $\varepsilon_0 = \sigma L(\omega_{ZPL})$. It is obvious from Eq. 78 that saturation-broadened single-molecule ZPL has the width of $\Gamma_{hom} \sqrt{1 + P \cos^2 \alpha / P_s}$ and that the peak of the saturation-broadened ZPL is reduced by a factor of $1 + P \cos^2 \alpha / P_s$.

For modeling purposes α -distribution was taken into account directly, using additional round of numerical integration, and not employing the approximation from [95]. For every value of α between 0 and 90 degrees (with step of five degrees), we first considered a purely transient hole in the SDF, a result of the dynamic equilibrium that emerges within $\tau_{triplet}$ of several milliseconds,

much smaller time interval than one datapoint of the experimentally measurable HGK curves (50-500 ms). The small amount of persistent NPHB occurring in the first $\tau_{triplet}$ of illumination was neglected. This transient hole was used to pre-modify the SDF $G(\omega)$ utilized for subsequent persistent NPHB. This way we accounted for unavailability of a fraction of the chromophores that are in the triplet state for resonant excitation and subsequent NPHB. The transient hole in the SDF was assumed to be purely Lorentzian, on the grounds that for narrow enough ZPL (in the case of no EET or charge separation on a single-picosecond timescale) and small to moderate electron-phonon coupling the peak absorption cross-section for PSB is two orders of magnitude smaller than for the ZPL and illumination of the PSB is unlikely to cause saturation effects. In other words, for narrow ZPL there is no transient pseudo-PSB (spectral feature due to the ZPLs of the molecules excited via their PSB). For persistent hole and HGK modeling for every α we utilized two different single-site spectra: one with the ZPL saturation-broadened and another with the ZPL not broadened. Broadened-ZPL SSS with reduced peak absorption cross-section was always utilized in the exponential term in the NPHB master equation (Eq. 66). Modified SDF was convoluted with non-broadened SSS to obtain the spectral hole shape in the case of a weak (non-saturating) probe beam (this corresponds to the experiment where the hole spectrum was measured with low power after the HGK measurement had been finished; Frames B and D of *Figure 5-10* as well as experimental data from [135]). SDF modified by both transient and persistent hole was convoluted with SSS containing saturation-broadened ZPL to obtain the hole shape during the HGK measurement. Essentially, Eq. 66 was transformed into

$$D(\Omega, t) = \int d\omega \int d\lambda f(\lambda) \int d\alpha L'(\Omega - \omega, \alpha, P) G(\omega) [\rho_{11} - \rho_{22}] (\omega_B - \omega, \alpha, P) \quad (79)$$

$$\frac{3}{2} \sin\alpha \cos^2\alpha \exp[-P\sigma\phi(\lambda) L_B(\omega_B - \omega, \alpha, P) t \cos^2\alpha]$$

where $[\rho_{11} - \rho_{22}] (\omega_B - \omega, \alpha, P)$ is from Eq. 73, $L_B(\omega_B - \omega, \alpha, P)$ is the SSS (containing ZPL, PSB and intra-molecular vibrations) with broadened ZPL, and $L'(\Omega - \omega, \alpha, P)$ is the SSS with broadened or non-broadened ZPL, for HGK and spectral holes measured with weak (negligible saturation) probe beam, respectively. Note that the integral of $L_B(\omega, \alpha, P)$ was no longer normalized to one. This can be understood as a reduction of the integral absorption cross-section upon saturation. The NPHB yield was also reduced compared to the case of the two-state model covered by Eq. 67 (see the additional k_{ics} term).

$$\phi(\lambda) = \frac{\Omega_0 \exp(-2\lambda)}{\Omega_0 \exp(-2\lambda) + k_{ics} + \tau_{eg}^{-1}}. \quad (80)$$

We stress again that the absorbance or fluorescence signal during the HGK measurement represents the bottom of the spectral hole containing both persistent and transient components, both of them being saturation-broadened. *Figure 5-11 A-C* show HGK data simulated using the above model for three different burn intensities, 19, 90 and 270 $\mu\text{W}/\text{cm}^2$, $\theta = 0.2$ and $\tau_{triplet} = 2.0$ ms. The blue curves represent the evolution of the purely persistent holes measurable with small probe intensity after preceding burning or HGK measurement is stopped. This type of curves is conventionally used to fit the HGK data [93], [95], [97], [16], [80], [107]. Black curves represent the HGK curves containing both transient and persistent components.

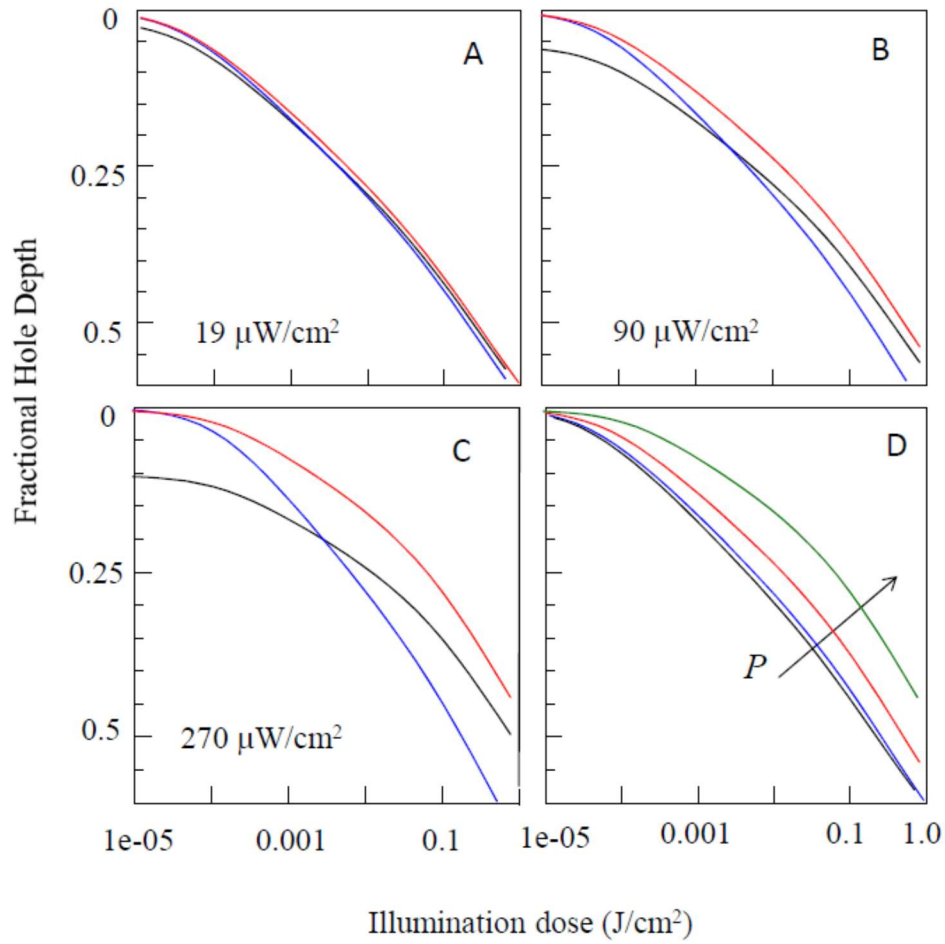


Figure 5-11: Frames A-C: HGK curves calculated for 19, 90 and 270 $\mu\text{W}/\text{cm}^2$, respectively. $\tau_{\text{triplet}} = 2.0 \text{ ms}$, $\theta = 0.2$. Other parameters can be found in the text and Table 1. Black curves: HGK including both transient and persistent components. Blue curves: HGK including only persistent component. Red curves: solid black curves renormalized to start from zero fractional hole depth and reflecting one's lack of knowledge, at the time of the HGK measurement, that/if transient hole is present. Frame D: HGK including both persistent and transient components and normalized to start from zero fractional depth (same as red curves from Frames A-C). Black, blue, red and green curves are for 4.3, 19, 90 and 270 $\mu\text{W}/\text{cm}^2$, respectively.

Black curves generally do not start from zero fractional depth, indicating the presence of the transient holes. For any dose, the depth of the purely transient component of the hole in the SDF is roughly the same percentage of the remaining SDF at ω_B . (This percentage increases with intensity.) The absolute magnitude of this purely transient component is decreasing with dose as

there is less and less SDF remaining at ω_B . On the other hand, in order to obtain the blue curve, reflecting the evolution of the observable persistent holes, the purely persistent component of the hole in the SDF is convoluted with the SSS containing narrow, not saturated ZPL. And to obtain the black curve, the hole in the SDF containing both persistent and transient components is convoluted with the SSS containing saturated broadened ZPL with reduced peak intensity. As a result, blue and black curves may eventually cross (Frames B and C).

In real HGK experiments, it is not known a priori if transient hole is present or how deep it is (results of this work can provide a decent estimate, as long as all model parameters are known with sufficient confidence). One usually assumes that the HGK starts at zero fractional hole depth. Red curves are black curves renormalized to start from zero depth. For low enough intensity (single $\mu\text{W}/\text{cm}^2$, lower intensities in *Figure 5-10* and *Figure 5-11*) triplet-related effects are negligible. For $19 \mu\text{W}/\text{cm}^2$ (*Figure 5-11A*) the effect is barely visible. In frames B and C red curves clearly correspond to smaller hole depths than the blue curves. The difference between blue and red curves increased with burn intensity. The 4-6% differences in depth observed in *Figure 5-10* for $70\text{-}90 \mu\text{W}/\text{cm}^2$ could be simulated with $90 \mu\text{W}/\text{cm}^2$, $\tau_{\text{triplet}} = 2.0 \text{ ms}$, triplet yield $\theta = 0.2$ and integral absorption cross-section of $4.3 \cdot 10^{-13} \text{ cm}^2\text{cm}^{-1}$ (*Figure 5-11B*). In Supporting Information of [151] it is explained in detail how $\sigma = 4.3 \cdot 10^{-13} \text{ cm}^2\text{cm}^{-1}$ was obtained. *Figure 5-11C* shows the results for higher intensity than used in *Figure 5-10*. Relatively small depths of the transient holes (beginning of black curves in *Figure 5-11A-C*) are a direct consequence of the α -distribution. Fractional depths of transient holes obtained for $E \parallel \mu$ were much larger. *Figure 5-11D* contains the observable HGK curves (same as red curves from frames A-C) demonstrating the presence of the intensity dependence of the apparent hole burning rate. In the above simulations it was also

assumed that non-saturated $\Gamma_{hom} = 0.0256 \text{ cm}^{-1}$, $\tau_1 = 3.125 \text{ ns}$, electron-phonon coupling $S = 0.72$ (and other electron-phonon coupling parameters of OGP-solubilized Cyt b₆f [16], [80], [107]) and that the distribution of the tunneling parameter λ was uniform between 7.8 and 13.1, with $\Omega_0 = 7.6 \cdot 10^{12} \text{ s}^{-1}$. 3.125 ns were used to keep the effective singlet excited state lifetime (affected by both k_{eg} and k_{ics}) at 2.5 ns [64], [92] with $\theta = 0.2$, i.e. to keep NPHB yields (Eq. 67) the same as in our earlier works for same values of λ .

Figure 5-12 shows the potential influence of deuteration of the solvent on the HGK curves, assuming that deuteration results in the decrease of the $P_s = (\tau_{triplet})^{-1} / \theta \varepsilon_0$, mostly due to the increase of $\tau_{triplet}$. It contains calculated HGK curves renormalized to start at zero depth (similar to the red curves in *Figure 5-11 A-C* for $\tau_{triplet} = 1.0, 2.0, 3.0, 4.0$ and 5.0 ms . Laser intensity in Frame A was $19 \mu\text{W}/\text{cm}^2$, the highest intensity for which we have experimental data on Cytochrome b₆f in both protonated and deuterated solvent (insert in Frame A). The difference between 1.0 ms and 5.0 ms curves (i.e. factor of five difference in $\tau_{triplet}$, not 25% [128], [130]) is clearly smaller than the difference between black and blue curves in the insert. Frame B shows results of simulations for $90 \text{ mW}/\text{cm}^2$, somewhat above the highest intensity employed for modified LH2. Here before the renormalization the fractional depths of the transient holes increased from about 3% for $\tau_{triplet} = 1.0 \text{ ms}$ to about 10% for $\tau_{triplet} = 5.0 \text{ ms}$. The insert shows respective experimental data, obtained with $69.5 \text{ mW}/\text{cm}^2$. The five-fold increase of $\tau_{triplet}$ is barely enough to reach some semblance between simulations and experiment.

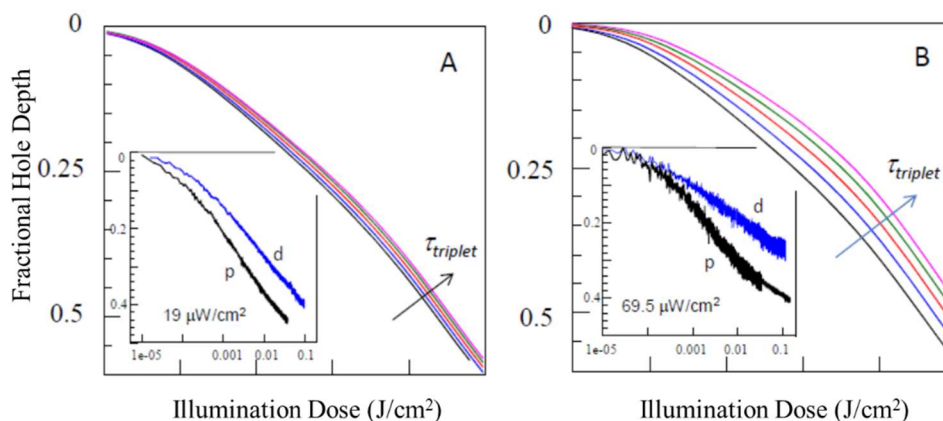


Figure 5-12: Calculated observable HGK curves (similar to red curves in Figure 5-11) normalized to start at zero fractional hole depth for $19 \mu\text{W}/\text{cm}^2$. Black, red, blue, green and magenta curves are for $\tau_{\text{triplet}} = 1.0, 2.0, 3.0, 4.0$ and 5.0 ms, respectively. Otherwise same parameters as for Figure 5-11, see text. The insert depicts experimental HGK curves for Cyt b_6f in protonated and deuterated solvent [107]. Frame B: simulation results for $90 \mu\text{W}/\text{cm}^2$. Other parameters were as above. The insert depicts experimental HGK curves for Chl a in modified LH2 in protonated and deuterated solvent for $69.5 \mu\text{W}/\text{cm}^2$.

For comparison, we also explored if the above results are in agreement with broad transient holes observed in some pigment-protein complexes, as such data has not been reported for Cyt b_6f . Figure 5-13 depicts absorption spectra of Cyt b_6f with 445 nm (Genmitsu laser diode) light on (red) and off (blue), as well as enhanced difference spectrum (supposed transient triplet bottleneck hole; black). Illumination intensity was around $500 \text{ mW}/\text{cm}^2$ and temperature was 77 K. At 77 K persistent hole burning does not occur (persistent holes burnt at 5 K recover by about 45 K). [107] Measuring absorption spectra with a conventional UV-VIS-NIR spectrometer (Varian Cary 5000) while illuminating sample with intense light results in strong noise at all wavelengths due to scattering, with noise getting higher with the increase of absorbance. Several spectra were taken with light on and light off and averaged to reduce the noise. Individual light-on spectra

exhibited systematically lower peak OD than the light-off spectra. The carotenoid region turned out too noisy to make any conclusions.

The 5 K homogeneous ZPL width is 0.0265 cm^{-1} (0.79 GHz) and inhomogeneous width is $\sim 13 \text{ nm}$ or 290 cm^{-1} or 8370 GHz. The ratio of inhomogeneous to homogeneous width is about 10^4 , suggesting that about 900 mW/cm^2 instead of $90 \text{ }\mu\text{W/cm}^2$ would be required to produce $\sim 7\%$ deep broad triplet bottleneck hole (see *Figure 5-11B*). Thus, 2% deep transient hole with about 500 mW/cm^2 illumination is in reasonable agreement with the triplet lifetime and intersystem crossing yield estimates offered above, especially taking into account that triplet lifetimes may be somewhat shorter at 77 K than at 5 K.

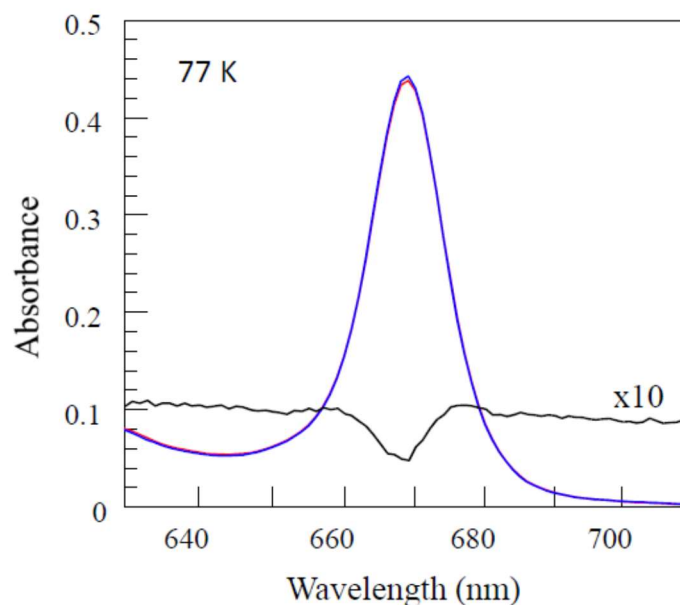


Figure 5-13: Absorption spectra of Cyt b_6f with 500 mW/cm^2 445 nm excitation on (red) and off (blue) as well as enhanced difference spectrum (black). The latter spectrum is shifted up for clarity.

5.3.4 Discussion

As mentioned in the Introduction, Chl *a* triplet-state parameters are strongly dependent on temperature. Triplet lifetimes on the order of tens of microseconds were reported at RT for several organic solvents in recent papers. [126], [127] These lifetimes depend on solvent, Mg coordination number, presence of triplet quenchers in the solution and other factors. Triplet lifetimes of up to several milliseconds were reported at 5 K [128], [129], [130], [131], [132], [133] and triplet yields as high as 0.4 have been suggested. [130] Triplet lifetimes of 1.5-1.6 ms were reported in core complexes [125] and reaction centers [132] of PSII. Bi-exponential kinetics with lifetimes of 0.6 and 2.0 ms and triplet yield of 0.16 were reported for CP47 antenna complex at cryogenic temperatures. [133] Thus, $\tau_{\text{triplet}} = 2.0$ ms and $\theta = 0.2$ used in this work are fairly realistic values. We did not attempt achieving perfect fit between simulations and experimental data by using slightly different parameters. However, we are confident that our ε_0 , τ_{triplet} and θ are correct within a factor of two. Note that ZPL at 5 K are narrower than 1 GHz for the lowest energy states of the antenna proteins (and Cyt *b₆f* or Chl *a* not transferring energy to BChl *a* in modified LH2 in this work), while inhomogeneous Q_y bandwidths of Chl *a* are on the order of several thousand GHz. For Cyt *b₆f* we verified that illumination at 445 nm with about 500 mW/cm² at 77K results in ~2% transient bleach of the Chl *a* Q_y band (*Figure 5-13*). Thus, observation of deeper transient bleaches in the antenna proteins ascribed to “triplet bottleneck” effects [121], [122] likely requires longer than average triplet lifetimes and/or higher triplet yields.

The hole burning modeling incorporating Chlorophyll triplet states with millisecond lifetimes readily yields significant intensity dependence of the HGK (*Figure 5-11D*) as well as

discrepancies between the hole depths obtained from the HGK and those observed after higher-intensity burning, by tuning a weaker probe beam (*Figure 5-11B, C*). One has to be mindful of these effects when fitting HGK data for the purpose of obtaining the distribution of the tunneling parameter λ in the excited state [93], [95], [97], [16], [80], [107]. Specifically, utilizing high-intensity HGK without taking triplet-related effects into account means that one is fitting the experimental data to the blue-type curve (*Figure 5-11A-C*), while it has to be fitted to the red-type curve. This error would result in overestimation of both mean and width of the λ -distribution. For example, let us assume that the experimental data is represented by the red HGK curve for 90 $\mu\text{W}/\text{cm}^2$ in *Figure 5-11B*. It can be fitted without invoking triplets while using uniform λ -distribution with $\lambda_{\min}=8.3$ and $\lambda_{\max}=13.8$, compared to 7.8 and 13.1 respectively, when triplet is present. These differences are significant, as typical errors of determining the parameters of the λ -distribution from experimental data are less than 0.1. This is equivalent to reducing the average NPHB yield by a factor of ~ 3.3 . (As stated above, singlet excited state lifetime of 2.5 ns was used when triplet was not included, to ensure same NPHB yield for the same λ .) Thus, invoking triplets allows for utilizing smaller λ and, as we pointed out earlier [12], utilizing smaller λ is beneficial for achieving better simultaneous quantitative agreement between simulations and experiments including HGK, recovery at fixed (burn) temperature and thermocycling. Various parameters used to model this selected HGK curve are summarized in Table 1.

Table 1. Modeling parameters employed for 90 $\mu\text{W}/\text{cm}^2$ HGK curve (red) in Figure 5-11B.

Parameter	No Triplet	Triplet included, $\tau_{\text{triplet}}=2 \text{ ms}$, $\theta=0.2$
Non-saturated SSS shape	See Ref. 11, $\Gamma_{\text{hom}} = 0.0256 \text{ cm}^{-1}$, $S_{\text{el-ph}}=0.72$	See Ref. 11, non-saturated $\Gamma_{\text{hom}} = 0.0256 \text{ cm}^{-1}$, $S_{\text{el-ph}}=0.72$
τ_{eg}	2.5 ns	3.125 ns
$\phi(\lambda=10, \Omega_0=7.6 \cdot 10^{12} \text{ s}^{-1})$	$3.92 \cdot 10^{-5}$	$3.92 \cdot 10^{-5}$
λ -distribution	Uniform, 8.3 to 13.8	Uniform, 7.8 to 13.1
Average ϕ	$4.8 \cdot 10^{-6}$	$1.6 \cdot 10^{-5}$

Small changes (by 25% for deuterated ethanol) in triplet lifetime due to the deuteration of the solvent were reported for Chl *a* [128], [130]. In a more extreme example, triplet lifetime for sulphonated aluminum phthalocyanine increased upon deuteration of aqueous solvent from 0.6 to 1.6 ms, with triplet yield increasing from 0.40 to 0.52 (decrease of saturation flux P_s by a factor of 3.5). Changes in simulated curves corresponding to decreasing P_s by a factor of 5 are not large enough to match the observed experimental results for modified LH2 and Cyt *b₆f* (Figure 5-12). Note that in Refs [128] and [138] Chl *a* and sulphonated aluminum phthalocyanine respectively were in direct contact with the deuterated solvent and were not screened from it by the protein. Fully exposing Chl *a* to water/glycerol mixture may cause other major effects that we did not observe: aggregation, strong fluorescence quenching, very poor persistent hole burning even for Chls that appear monomeric and emit at $\sim 680 \text{ nm}$ [64]. HGK for low enough burn intensities (single $\mu\text{W}/\text{cm}^2$ or lower) and hole recovery dynamics did not depend on solvent deuteration, indicating that the mass of the entities involved in tunneling did not change by more than several percent [64]. The Fluorescence Line Narrowing difference spectra were also

consistent with the presence of slow energy transfer between two Chl *a* molecules in the intact Cyt *b₆f* dimer [12]. Summarizing, it appears that experimentally observed deuteration effect is too strong to be explained by triplet-related effects alone and local heating / poor protein-solvent interface thermal conductance hypothesis [64] is still viable.

We note that sufficiently long-lived charge transfer (CT) states could affect HGK in a similar manner as triplet states. Chl-carotenoid and Chl-Chl CT states were proposed, along with other possibilities, as quenchers in LHCII antenna complexes of Photosystem II (Non-photochemical Quenching; NPQ; see [139], [140] for reviews). However, the lifetime of the carotenoid radicals observed in spinach thylakoids was on the order of 150 ps [141] and the lifetimes of Chl-Chl CT states ranged from 400 ps in *Arabidopsis* leaves to several ns in isolated LHCII [142]. The only scenarios where charge separation is long-lived enough involve actual reaction centers [123]. At least in Cyt *b₆f* formation of Chl-Chl CT states is impossible due to extremely weak inter-pigment coupling ($\sim 0.5 \text{ cm}^{-1}$) and Chl-carotenoid CT states are very unlikely as the closest distance between Chl *a* and carotenoid is as large as 14 Å (compared to about 5 Å in LHCII). To independently verify that Chl triplets are indeed responsible for the effects described here one could also monitor phosphorescence peaked at 960 nm [118]. Since Chl phosphorescence yields are $\sim 10^5$ times lower than fluorescence yields [118], detecting Chl phosphorescence simultaneously with a HGK measurement would be very challenging. We did not attempt that and instead relied on available literature that agrees on existence of Chl triplets with millisecond τ_{triplet} at 5 K but, to the best of our knowledge, contains no evidence for CT states with comparable lifetimes in the antennae.

Finally we note that the assumption that transient holes do not contain pseudo-PSB contributions may not be valid when the ZPLs are broad, ZPL and PSB peak absorption cross-sections are comparable and very high excitation intensities (hundreds of mW/cm^2) are used, for instance in the case of the reaction center of PSII [124] where the system gets to the triplet state from the charge separated state and the effective yield of charge separation is close to one. For practical purposes, our software is capable of including the full single-site spectrum in transient hole simulations instead of a Lorentzian.

5.3.5 Conclusions

This work represents, to the best of our knowledge, the first attempt to include triplet states into advanced modern NPHB modeling including various distributions, particularly the distribution of the angle between polarization of light and pigment's transition dipole. Modeling involving realistic low-temperature triplet lifetimes on a millisecond timescale produces results that agree well with the experiment - exhibiting the intensity dependence of the HGK curves as well as the mismatch of the hole depths deduced from HGK curves and hole spectra. The effects of solvent deuteration on Chlorophyll triplet lifetimes described in available literature are not strong enough to explain the differences in hole growth kinetics observed for pigment-protein complexes in deuterated and protonated solvent, indicating that additional effects may still be at play, in particular local heating of the protein due to poorer interface thermal conductivity between (protonated) protein complex and deuterated outside solvent [64].

Acknowledgments:

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5.4 Non-photochemical Spectral Hole Burning Study of Modified LH2 Complex from *Rbl. acidophilus*: Do Parts of the Pigment Molecules Affect Small Light-induced Structural Changes?

This chapter is based on the paper by A. Levenberg, A. Trempe, M. A. Lujan, R. Picorel and V. Zazubovich published in the *Journal of Physical Chemistry B*, 2026, 130, 3016-3028
I have contributed to all experiments, most of data analysis and paper writing.

Abstract. Nonphotochemical spectral hole burning (NPHB) experiments were performed on chemically-modified LH2 complex from *Rbl. acidophilus* in which some but not all B800 BChl *a* molecules have been replaced with Chl *a*, with the initial goal of utilizing Chl *a* as a local thermometer. The focus of the work eventually shifted to exploring low-temperature protein dynamics. Different sample batches contained different proportions of Chl *a* capable and incapable of excitation energy transfer (EET) to the BChl *a* molecules. This indicates that there were at least two sub-populations of Chl *a*, with only one of them featuring proper reconstitution of Chl *a* into the original B800 protein pocket. Nevertheless, the other, EET-incapable Chl *a* molecules, were clearly associated with the LH2 and were not located in solution. NPHB and hole recovery experiments reveal that relevant protein energy landscapes do not differ much between different subsets of Chl *a* despite different environments but they both differ from those of the original B800 BChl *a*. This suggests that small structural changes responsible for NPHB and shifts of spectral lines in single-complex experiments may involve pigment molecule itself or that structural changes in the immediate protein environment of the pigment are constrained differently by different pigment molecules. The NPHB dynamics did not depend much on deuteration of the solvent, except the slowdown of NPHB with the increase of light intensity was

more prominent. Attributing this effect to triplets alone would require unrealistic five-fold increase of the product of triplet yield and triplet lifetime. This observation lends support to the hypothesis that local heating of the protein complexes plays a role in spectroscopy experiments, particularly single-molecule spectroscopy ones where excitation intensities are higher.

5.4.1 Introduction

Over the years, high-resolution frequency-domain optical spectroscopy methods such as single-molecule spectroscopy and non-photochemical spectral hole burning (NPHB) have been successfully employed to study pigment-protein complexes involved in photosynthesis. [83], [89], [117] In all of these systems one can observe, even at cryogenic temperatures, the shifts of the spectral lines (i.e. the changes of the electronic transition energies) that can be both light-induced and light-independent. At cryogenic temperatures these shifts are particularly easy to observe as well as more informative since the absorption (or emission) spectra of individual pigment molecules feature narrow purely electronic lines (zero-phonon lines, ZPL, see *Figure 5-14*). Behind these shifts are small structural changes in the environment of the pigment. Some of these structural changes are involved in photoprotection [139], [143], [144], [145], [146], but they are a much more widespread phenomenon and have been observed in all pigment-protein complexes explored so far by means of either single-complex spectroscopy, or NPHB, or both.[89], [89], [117], [147], [148], [149], [150], [14], [16], [80], [86], [88], [93], [107], [122], [151], [152], [153], [154], [155]

The simplest model capable of qualitatively explaining results of NPHB and single-complex experiments is the Two-Level System (TLS) model, where the energy landscape of a protein is represented by a double-well potential as a function of some generalized coordinate. This model

was originally developed to explain anomalous low-temperature properties in glasses [91], [92] and was later applied to NPHB, see [117] for more details. The TLS model implies the existence of two conformational sub-states corresponding to slightly different optical transition frequencies of the pigment embedded in the protein (or other amorphous solid). This model can be generalized to Multi-Level (or more precisely - multi-well) Systems, MLS. The barrier(s) in the TLS/MLS can be crossed by either quantum-mechanical tunneling or conventional thermal activation (“barrier-hopping”) which have distinctly different temperature dependence [16], [80], [107], [93]. *Figure 5-14* depicts the mechanism behind NPHB and spectral line shifts in single-molecule experiments. When the photon is absorbed by a pigment molecule in configuration 1, the pigment is promoted to the excited state and then some small conformational change in the protein local environment may bring the system to configuration 2. After tunneling in the excited state, the system returns to the ground state and gets trapped in configuration 2. The parameters of the ground state determine how long it will take for the system to return to configuration 1. The barriers are much lower when the pigment is in the excited electronic state (or persistent hole burning would be impossible / holes would recover immediately), so they could be crossed with reasonable probability ($\sim 10^{-4}$) within the nanosecond or sub-nanosecond excited state lifetimes. At low enough temperatures, below 10 K, barriers are crossed predominantly by tunneling.

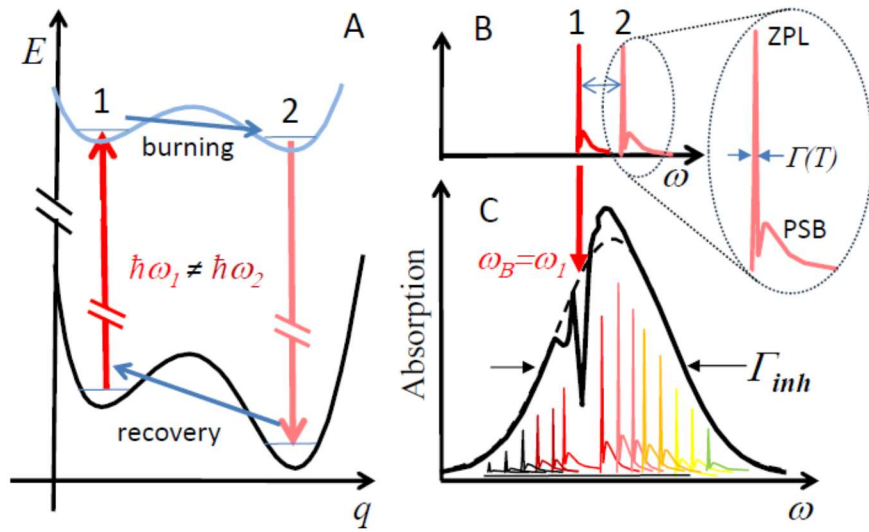


Figure 5-14: Frame A: Proposed mechanism behind NPHB and single-molecule line shifts based on double-well potentials (TLS). Before absorbing a resonant photon, the pigment–protein system is assumed to be in the left well (1). Upon absorbing a photon, the system finds itself in the left well / in the excited electronic state, where the barriers are lower. It has a reasonable chance to tunnel through the barrier within the several-nanoseconds lifetime of the excited electronic state and the system finds itself in configuration (2) represented by the right well. Subsequently, the pigment returns to the ground electronic state, while the environment still remains in configuration (2). Pigment’s transition energies are different in the left and right wells, i.e., a spectral shift is observed, as schematically indicated in Frame B. The single-molecule spectrum consists of zero-phonon line (with homogeneous width $\Gamma(T)$) and phonon sideband (PSB); additional features due to intra-molecular vibrations are not shown. The pigment-protein system may eventually return to the original (left) well in the dark. Frame C: In case a macroscopic sample with an inhomogeneously broadened spectrum with the site-distribution function of width Γ_{inh} is illuminated with a narrow-band laser, a sharp resonant spectral hole will be formed as a result of multiple molecules’ lines shifting out of resonance with the laser at wavelength λ_B . These shifted lines contribute to the “anti-hole”.

The NPHB quantum yield $\phi(\lambda)$ is then given by

$$\phi(\lambda) = \frac{\Omega_0 \exp(-2\lambda)}{\Omega_0 \exp(-2\lambda) + \tau_1^{-1}}, \quad (81)$$

Here Ω_0 is the attempt frequency, determining how often the systems try crossing the potential energy barrier between the two wells, and τ_1 is the fluorescence lifetime that may be purely radiative or be affected by energy transfer or other forms of quenching, depending on the

pigment-protein system. The tunneling parameter λ represents a composite “barrier strength for the purpose of tunneling” and is usually reported in NPHB papers [14], [16], [80], [93], [107], [117] as

$$\lambda = d \sqrt{2mV}/\hbar \quad (82)$$

Eq. 82 corresponds to the case of rectangular barriers. Here V is the height of the barrier with respect to the energy of the tunneling particle, d is the barrier thickness, and m is the effective mass of the entity that rearranges during the transitions between two conformational states of the protein (or other amorphous solid). Due to the amorphous character of the systems where NPHB can be observed, including proteins, V and λ are subject to distribution while m and d are considered fixed. The composite md^2 parameter can be determined from global fitting to hole growth and hole recovery data, including thermocycling [64], [66], [70]. In the case of a macroscopic sample the NPHB master equation [117] describes the absorption spectrum containing a persistent hole after illumination at ω_B of duration t :

$$D(\Omega, t) = \int d\omega L(\Omega - \omega) G(\omega) \int d\lambda f(\lambda) \int d\alpha \frac{3}{2} \sin\alpha \cos^2\alpha e^{-P\sigma\varphi(\lambda)L(\omega_B - \omega)t \cos^2\alpha} \quad (83)$$

Here $G(\omega)$ is the site distribution function (SDF; defined as the probability to find a chromophore with given ZPL frequency; usually Gaussian with width Γ_{inh} before any burning), $L(\omega)$ is the single-site spectrum (SSS) shape with area normalized to unity on the frequency scale; it contains narrow ZPL with width $\Gamma(T)$, broad phonon sideband (PSB) and intra-molecular vibrations. P is the photon flux, σ is the integral absorption cross-section and α is the angle between the electric field $\mathbf{E}$ of light and transition dipole moment $\boldsymbol{\mu}$ of the chromophore. The distribution of α

contained in Eq. 83 corresponds to the case of random isotropic orientation of the pigments and linearly polarized light. $f(\lambda)$ is the distribution of the tunneling parameter.

Recently we published several papers where Eqs. 81-83 were used to model both the hole burning per se and also the hole recovery at fixed temperature and upon thermocycling [16], [93], [93], [107]. Modeling the hole growth kinetics (HGK) with the help of Eq. 83 yields distributions of the tunneling parameter λ in the pigment-environment system with pigment in the excited electronic state. One can also determine the sub-distributions of λ of a subset of systems that have actually experienced burning and therefore contributed to the spectral hole. Modeling of hole recovery at fixed (burn) temperature yields the matching sub-distributions of λ in the ground electronic state, while thermocycling yields the corresponding ground-state sub-distribution of barriers V . By comparing the latter two distributions one can determine the md^2 parameter and make inferences about the nature of the entities involved in structural changes. Another important conclusion of the NPHB studies of Cytochrome b_6f was that V - and λ -distributions almost did not depend on the deuteration of the outside solvent [16], [80], [107]. This indicates that for energy landscape tier(s) affecting NPHB in Cyt b_6f (and most probably other chlorophyll-protein complexes as well) the protein dynamics are likely due to some small movements in the protein environment of the pigment molecules and not due to “slaving” to the dynamics of the solvent or the hydration shell [22], [81], [156]. Still, several questions remain debated, namely if the spectral jumps observed in pigment-protein complexes are light-induced or thermally-induced (i.e. in the latter case they must be occurring anyway and are merely observed in optical experiments) [14] as well as what is the mechanism (tunneling vs classical thermal activation) and temperature dependence of the rates of the shifts [155]. The exact

molecular nature of the small structural changes responsible for spectral shifts is also a matter of debate [80], [84], [150], [157], [158], [159], [160].

Another important question relevant for interpreting the results of the optical experiments is if pigment-protein complexes are dynamically heated by optical excitation, particularly in single-complex experiments featuring much higher light intensities than bulk / macroscopic sample NPHB experiments. Some evidence of such local heating can be found even in the NPHB experiments [107]. Namely, replacing the solvent surrounding the pigment-protein complex with the deuterated one results in a major slowdown of burning with the increase of the excitation intensity, without much change in the barrier parameters [107], [151]. However, an alternative explanation exists, where the slowdown of NPHB at higher light intensities is attributed to triplet-related effects [151]. It follows from Eq. 83 that the observable rate of spectral hole formation must be dependent on the shape (particularly the ZPL width $\Gamma(T)$) of the single-molecule spectrum $L(\omega)$. This width can be affected (in the absence of the excitation energy transfer, EET) by either triplet-related saturation effects or just by temperature-dependent pure dephasing-related ZPL broadening. In order to explore the possibility of the local heating it is tempting to choose a pigment-protein complex where relatively large amounts of energy are dissipated non-radiatively and to use some of the pigments in the complex as “local thermometers”, whose ZPL widths would respond to the increase of the effective temperature inside the complex while the other pigments are absorbing light energy, transferring that energy to each other and part of that energy being dissipated non-radiatively. At the first glance this is difficult to achieve. The “thermometer” pigments should absorb higher in energy so one could be confident that they would only probe dissipated vibrational energy (i.e. there would be no

excitation energy transfer, EET, to these pigments, no NPHB due to the excitations transferred to these pigments and no light-induced hole filling). On the other hand, in this case the “thermometer” pigments would themselves transfer energy to the lower-energy pigments in the complex and this would broaden their ZPL and reduce the accuracy of the temperature measurements. This would be the case for Chl *b* to Chl *a* EET in LHCII complex, for example [159], [160], [161]. Note that here we are assuming that thermal equilibration within a pigment-protein complex is occurring faster than energy dissipation into the surrounding environment.

To address these contradictory requirements, we developed a chemically modified version of the light harvesting complex 2 complex (LH2) [14], [88], [153], [154], [162], where some but not all of the B800 BChl *a* molecules (absorption peak at ~804 nm at cryogenic temperatures) were replaced with Chl *a* molecules (absorption peak at ~670 nm). The latter would be serving as local “thermometers”. This was expected to reduce relevant spectral overlaps and slow down the EET from the “thermometer” pigments. The LH2 complex of purple bacteria is one of the most thoroughly explored pigment-protein complexes. In particular, multiple papers were devoted to single-complex spectroscopy of LH2, including but not limited to Refs. [88], [153], [154], [162]. LH2 is especially suitable for local heating experiments due to large fraction of energy dissipated non-radiatively upon B800 to B850 EET and relatively low fluorescence yield [163]. The chemical procedures for pigment replacement in LH2 have also been reported previously [137], [164], [165], [166], [167], [168]. *Figure 5-15* shows the desired pigment arrangement in the modified LH2 complex and the proposed scheme of the local heating experiment. As it turned out, we have accidentally achieved imperfect pigment reconstitution (not into the original B800 protein pocket), which would be even more beneficial for our purposes, as it further reduced EET from

(one fraction) of the Chls a to the BChl a . Although the originally designed local heating experiment was inconclusive due to too low broadband 800 nm illumination intensity, we still present some other evidence of local heating as well as the guidelines for future experiments. More importantly, based on the exploration of spectral dynamics of BChl a and Chl a in different environments in LH2 complex by means of NPHB, we argue that the small structural changes responsible for spectral shifts (see above) involve parts of the pigment molecule itself or are somewhat constrained by the pigment molecule.

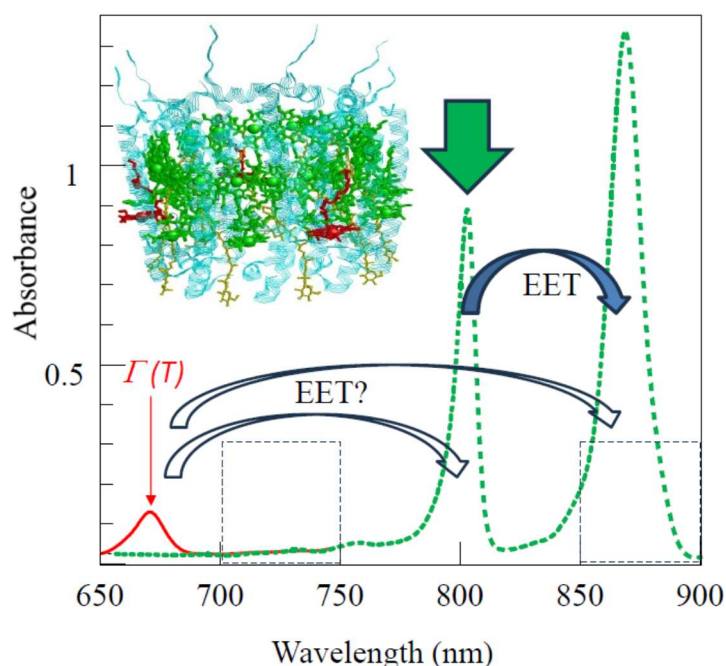


Figure 5-15: Desired structure of a modified LH2 complex and the background of the originally proposed experiment. Green: Bchl a . Red: Chl a . Cyan – protein. Yellow: carotenoids. Upon being illuminated at around 800 nm (green arrow), B800 Bchl a molecules absorb light and transfer energy to the B850 manifold. In this process, significant amount of energy has to be dissipated non-radiatively. EET from Chls a (red) to Bchls a has to be less likely. Relatively low-intensity burning in the Chl a region (red arrow) is used to determine local temperature via ZPL width $\Gamma(T)$ or HGK measurement. Also shown are detection windows for fluorescence resulting from excitation of EET-incapable (700-750 nm) and EET-capable (>850 nm) Chls a (dashed rectangles), see text below for more details.

5.4.2 Experimental Methods

LH2 complexes from *Rbl. acidophilus* (formerly *Rps. acidophila*) have been modified as in [137], [166], with some modifications. In [137], [166] removal of B800 BChls *a* was achieved by washing the chromatography column with Triton GB-10 detergent at pH = 4.75. However, here it was achieved by washing the complexes with N-dodecyl β -D-maltoside (DM) at the same pH, with no previous detergent treatment. The native LH2 was bound onto a cationic chromatography column in the presence of 0.1% (w/v) DM at pH = 4.75. The material out of the chromatography column showed a small but detectable band at around 770 nm (likely due to free BChl *a* in detergent micelles). The column was washed with buffer with DM until the 770 nm contribution was very low. In the case of LH2 from *Rbl. acidophilus* where B850 is very stable and the B800 is quite unstable, the preparation after this step consisted of complexes with an entire B850, and almost none B800 or B770, ready for reconstitution experiments. Then the material was eluted with 0.5 M NaCl and dialysed for two days against a buffer with 0.05 % (w/v) DM. After this long period of dialysis in the presence of DM some disrupted BChls *a* absorbing at 770 nm recovered their native interactions within the protein complex, absorbing now at 800 nm. Thus, the material after dialysis contained native B850, some disrupted BChls *a* absorbing at 770 nm, some occupied B800 sites, and with most of the B800 sites empty and ready to receive new BChls *a* or Chls *a* during the reconstitution experiments. We took advantage of the different MW of DM micelles (around 50 kDa) and LH2 protein micelles (over 120 kDa). By using Amicon filter with 100 kDa MWCO we were able to wash most if not all the DM micelles while retaining the micelles containing LH2 proteins. Three different batches have been produced, which differed somewhat in Chl *a* / BChl *a* ratios and in the number of times (1 or 2) they were purified using size-exclusion chromatography ensuring that all unattached pigments were removed. For the purpose of optical experiments at

liquid helium temperatures the LH2 complexes were solubilized with 0.05% (w/v) LDAO detergent and dissolved in the mixture of buffer (20 mM TRIS-HCl, pH= 8.0) and glycerol (66%). The components of deuterated solvent (D₂O and glycerol with 98% H to D replacement) were obtained from CDN isotopes, Dorval, QC. The time spent by the samples at room temperature was carefully minimized to ensure negligible deuteration of the protein itself and potential damage of LH2 by glycerol. (Glycerol is known to gradually extract B800 BChls *a*.) Estimates of the number of different pigments per modified LH2 complex can be found in Table 2.

The samples were placed in a plastic cuvette with 4×10 mm cross-section inside a liquid helium optical cryostat (Ukrainian Academy of Sciences). Between consecutive days of the measurements the samples got heated to about 120 K overnight; samples very briefly reached room temperature and were stored at –80°C between different rounds of experiments. High-resolution spectra and hole-growth kinetics (HGK) curves in the Chl *a* range were measured in fluorescence excitation mode using Spectra-Physics / Sirah Matisse-DS frequency-stabilized tunable dye laser (bandwidth ~0.15 MHz). Spectra-Physics model 3900 Ti-Sapphire tunable laser was used to perform NPHB on the original B800 band of LH2 at around 800 nm. BEOC laser power stabilizer was employed. Fluorescence was detected with a red-optimized Hamamatsu PMT photon counting module through different combinations of filters. In order to selectively detect emission from the EET-incapable fraction of Chl *a* molecules (see below), long-pass (AELP700; Omega Filters, VT) and short-pass (750 nm, ThorLabs) interference filters were used. In order to selectively measure emission following excitation of the EET-capable fraction of Chl *a* molecules or of the original BChl *a* molecules the 850 nm long-pass (Omega) was used. These ranges are shown using dashed boxes in *Figure 5-15*. Broadband absorption spectra were measured with

Varian Cary 5000 spectrophotometer. Emission spectra were measured with Princeton Instruments / Acton 2356 spectrograph with Pixis 1340x100 back-illuminated CCD camera. Thermocycling involves burning a spectral hole at some low temperature (e.g. 5 K), elevating the temperature slightly for a minute to allow for crossing of some barriers, lowering the temperature to 5 K again, measuring the hole spectrum, briefly elevating temperature a bit further, and so on. For measurements of local heating a lamp filtered through a 10 nm FWHM, 805 nm peak bandpass filter (Omega) was used. Additional 750 nm longpass and 850 nm shortpass filters were used to further protect the Chl a Q_y region and the detector. This resulted in intensity of $760 \mu\text{W}/\text{cm}^2$ for all wavelengths reaching the sample combined, or about $5 \mu\text{W}/\text{cm}^2 / \text{cm}^{-1}$ in terms of intensity per frequency interval.

5.4.3 Results and Discussion

5.4.3.1. *Absorption spectra of LH2 complexes*

Figure 5-16 shows the 5 K absorption spectra of two different batches of modified LH2, one with regular / protonated water-glycerol solvent (blue) and another with deuterated solvent (red). Also shown are the absorption spectrum of intact unmodified LH2 (dashed green) and the spectrum of monomeric BChl a in water-glycerol-LDAO detergent solution with Q_y band peaked at 778 nm (black) from [169]. As can be seen, different batches of modified LH2 featured different numbers of remaining B800 BChls a but similar number of Chls a . The absorption spectra of the modified LH2 samples also feature an enhanced shoulder to the blue of the B800 band (at ~ 780 nm). The spectra of modified LH2 in this spectral region can be approximated by a sum of properly normalized B800 band of the original LH2 and the spectrum of monomeric BChl a in solution [169]. We have excluded the possibility that free-floating BChls a in solution could

contribute to this spectral feature, as concentration and purification of the modified LH2 involved size-exclusion chromatography. Free BChls *a* and Chls *a* in solution would also be removed when replacing regular (not deuterated) solvent with deuterated solvent, yet the 780-nm shoulder (as well as the Chl *a* band at 670 nm, see below) persisted. Emission spectra (Figure 5-25) of modified complexes also exhibited a weak band around 785 nm, indicating that pigments responsible for that shoulder are poor energy donors for the rest of the LH2. The insert focuses on the Chl *a* region. The spectrum of one batch in regular solvent (blue) is peaked at 670.8 nm while the spectrum of another batch (dissolved in deuterated solvent, red) is peaked at 672.1 nm. We argue that the difference between these two Chl *a* absorption spectra can be attributed to different proportions of Chls *a* located in two somewhat different environments. While for Chl *a* the spectra of these two contributions overlap, in the case of BChls *a* these fractions absorb at 780 and 800 nm.

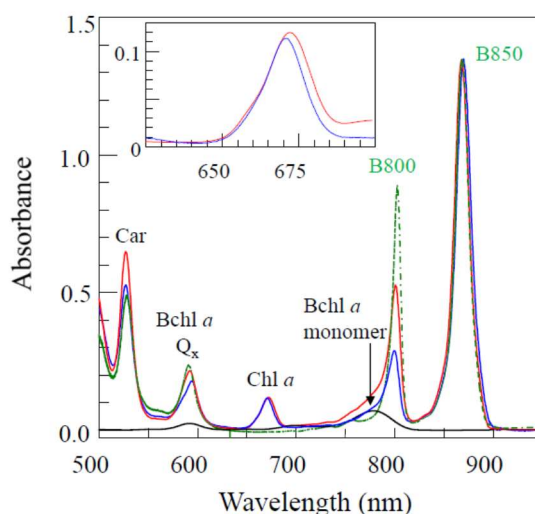


*Figure 5-16: 5K absorption spectra of intact and modified LH2 complexes in protonated and deuterated solvent. Dashed green: intact original LH2. Blue: modified LH2 in regular water-glycerol solvent. Red: modified LH2 (different batch) in deuterated water-glycerol solvent. Black: vertical black arrow: spectrum of BChl *a* in water-glycerol matrix from. [169] The LH2 spectra were normalized to equal absorbance at the peak of the B850 band. The insert shows the details of the Chl *a* spectral region.*

One can also examine the excitonic shift of the B800 band in the modified LH2 systems. Excitonic coupling between BChls *a* in native LH2 induces a red shift in the absorption spectra both for B850 and, less obviously, B800 bands. However, modification of LH2 results in a distribution of complexes with various combinations of BChls *a*, Chls *a*, and empty sites in the B800 ring. In the intact B800 ring, excitonic coupling between neighboring BChls *a* is relatively weak (20-25 cm⁻¹) due to large Mg...Mg separation of 21.2 Å between the adjacent B800 BChl *a* molecules. The coupling is even weaker for second-nearest neighbors (4–7 cm⁻¹), and negligible for pigments that are further apart. Thus, removal of some B800 BChls *a* must result in a decrease of average couplings and a blue shift of the B800 band. The absorption spectra indeed indicate a shift of the B800 band between native and modified complexes, from 803 nm (green) to 801 nm (red) to 800 nm (blue), which correlates with a reduction of the number of B800 BChls *a* remaining in their pockets and associated reduction in average excitonic coupling among the remaining BChls *a* in B800. Interestingly, BChl *a* Q_x demonstrates the red shift in modified complexes relatively to the native ones. At a first glance the latter resembles the signature of BChl *a* oxidation [170], [171], [172]. However, such oxidation must also result in the bleaching and/or shifts of the B850 band and in the formation of the Acetyl-chlorophyll (AcChl) band at 696 nm. Neither of these effects were observed in this work. Besides, oxidation of BChl *a* inside the LH2 complex requires prolonged high-intensity illumination at ambient conditions. Our samples were not exposed to such illumination and were exposed to ambient temperatures as little as possible.

5.4.3.2. *Broadband Fluorescence Excitation Spectra*

Figure 5-17 shows fluorescence excitation spectra of the Chl *a* region of the samples from three different batches used in this study. They featured similar Chl *a* and B850 absorption but different B800 absorption. As can be seen, they featured fairly different ratios of intensities of 700-750 nm and >850 nm emission, indicating different fractions of EET-capable and EET-incapable Chl *a* present in different batches of our samples. The slight spectral shift between the fluorescence excitation spectra of the two fractions is in agreement with the slight shift of the Chl *a* absorption spectra in the insert of *Figure 5-16*. The spectrum of EET-incapable fraction (red) is peaked at 672.7 nm while the spectrum of EET capable fraction (blue) is peaked at 669.0 nm. Thus, the Chl *a* Q_y absorption spectra in *Figure 5-16*, peaked between these two wavelengths, can indeed be interpreted as the sums of different amounts of EET-capable and EET-incapable Chls *a*. Note that the deuterated-solvent sample exhibiting the smallest fraction of EET-capable Chls *a* is likely a coincidence, as replacing the solvent represented an additional chance to remove any Chls *a* that are not associated with LH2 protein. The spectra were corrected for transmission of neutral density filters used to attenuate the laser beam but not for detector spectral response or possible differences in the emission quantum yields of EET-incapable Chls *a* and B850 Bchls *a*. Therefore, one should not quantitatively convert the integral intensity ratios of these spectra into the ratios of EET-capable and EET-incapable Chls *a*.

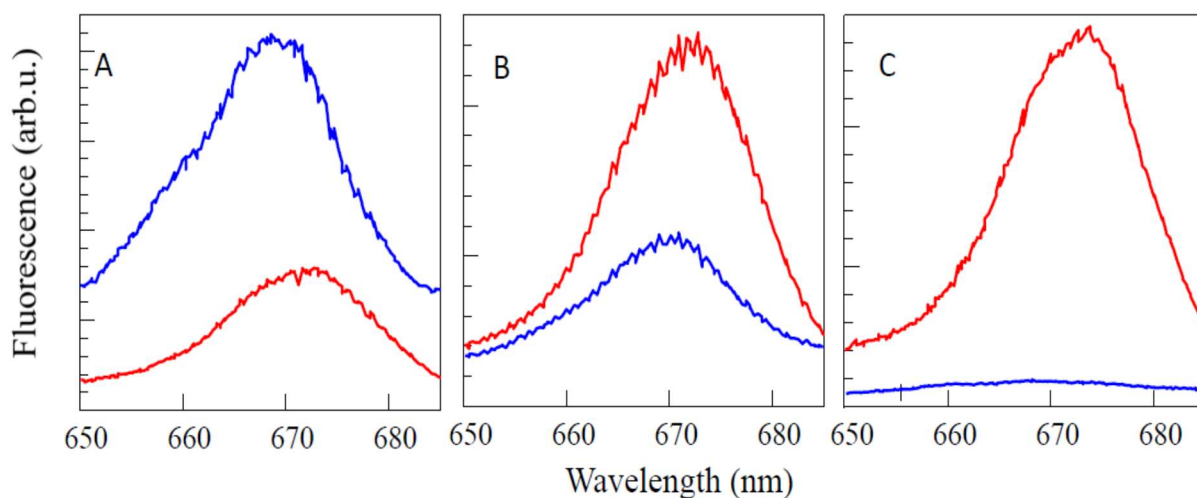


Figure 5-17: Chl *a* – region fluorescence excitation spectra of three different modified LH2 samples used in this study. Red: detection at 700-750 nm (EET-incapable fraction). Blue: detection at >850 nm (EET-capable fraction). A: Protonated sample with dominant EET-capable fraction. B: Protonated sample with dominant EET-incapable fraction, but also significant EET-capable fraction; blue curve in Figure 5-16; C: sample with almost no EET-capable Chls *a* (deuterated solvent: red curve in Figure 5-16). Here the solvent was also replaced with the deuterated one.

5.4.3.3. Spectral Hole Growth and Hole Recovery

As stated above, analysis of the spectral hole growth yields information on the barriers on the protein energy landscape and the distribution of the tunneling parameter λ . In the context of the proposed local heating experiments it is also important to understand the behavior of the “thermometer” in the absence of additional illumination. In order to measure the hole growth kinetics (HGK) curves and obtain the excited-state λ -distributions, one monitors the signal while the sample is illuminated with constant intensity at fixed burn frequency. The fractional hole depth, $1 - D(\omega_B, t)/D(\omega_B, 0)$ (with $D(\omega_B, t)$ defined by Eq. 83), is the fraction of absorption at ω_B that has been burned away after time t . Employing fractional quantities allows one to compare results obtained under the broader range of experimental conditions, for different samples, etc.

5.4.3.3.1 HGK for EET-incapable Fraction

Figure 5-18A shows HGK hole growth kinetics (HGK) curves obtained at different temperatures for Chl *a* in EET-incapable fraction of the sample as well as the dependence of the homogeneous ZPL width on temperature. The burn wavelength was around 671 nm. The illumination power was low enough ($\sim 1 \mu\text{W}/\text{cm}^2$) to ensure that triplet-related effects were negligible. [151] Several HGK curves are shown for 7.8 K, 4.7 K and 2.1 K to demonstrate repeatability between measurements performed on different days. An additional longer HGK curve was measured at 2.1 K but it featured some irregularities probably due to unstable temperature or Helium level passing the cryostat windows (not shown). Nevertheless, this long burn at 2.1 K resulted in $\sim 45\%$ deep hole shown in the insert. The deepest holes burned at 5 K reached 48%. This indicates that electron-phonon coupling is moderate ($0.6 < S < 0.8$) in this system. The Δ -FLN spectrum obtained with 680 nm excitation (Figure 5-26) features fairly narrow phonon sideband, also indicating moderate multi-phonon contributions in agreement with $S \approx 0.7$. The faction-specific SDF parameters were determined by fitting the fluorescence excitation spectra in Figure 5-17 using the single-site spectrum shape from the Δ -FLN spectrum and $S = 0.7$. The 5 K HGK data could be fitted while employing either Gaussian ($\lambda_0 = 9.63$; $\sigma_\lambda = 1.15$) or box-like ($\lambda_{\min} = 7.9$, $\lambda_{\max} = 11.6$) λ -distributions, with Gaussian distribution providing slightly better fit. These parameters are fairly typical for intact pigment-protein complexes naturally containing Chls *a* [14], [80], [93], [107], [147], [151]. On the other hand, Chl *a* in water-glycerol mixture burns much more poorly [107], indicating either significantly higher range of the tunneling parameter λ or reduced lifetime due to quenching. Thus, these results represent additional evidence that EET-incapable Chls *a* in our modified LH2 are not located in the solvent and are associated with the protein.

Each datapoint in the temperature dependence of the homogeneous hole width (*Figure 5-18B*) was obtained by measuring several spectral holes while adding illumination time / dose and extrapolating the hole width to zero illumination dose. The shape of the dependence is typical for pure dephasing in pigment-protein complexes and likely is a combination of $T^{1.3}$ (due to TLS) and exponential (due to vibrations) terms [117] The 2.1 K hole width corresponds to the lifetime of about 530 ps. However, due to the expected $T^{1.3}$ temperature dependence of the homogeneous linewidth, the true lifetime must be longer (i.e. it would likely be more similar to the radiative lifetime of several nanoseconds), reflecting low probability of this fraction of Chls a to transfer energy to BChls a . For EET-capable Chls a the 5 K hole width was $\sim 1.9 \text{ cm}^{-1}$ (see below), i.e. out of range of *Figure 5-18B*, and the same temperature dependence would be superimposed on this EET-determined baseline. It is clear that, in case of EET-incapable fraction of Chls a , the HGK or a width of a shallow hole can indeed be used for thermometry purpose with the precision of about 1 K.

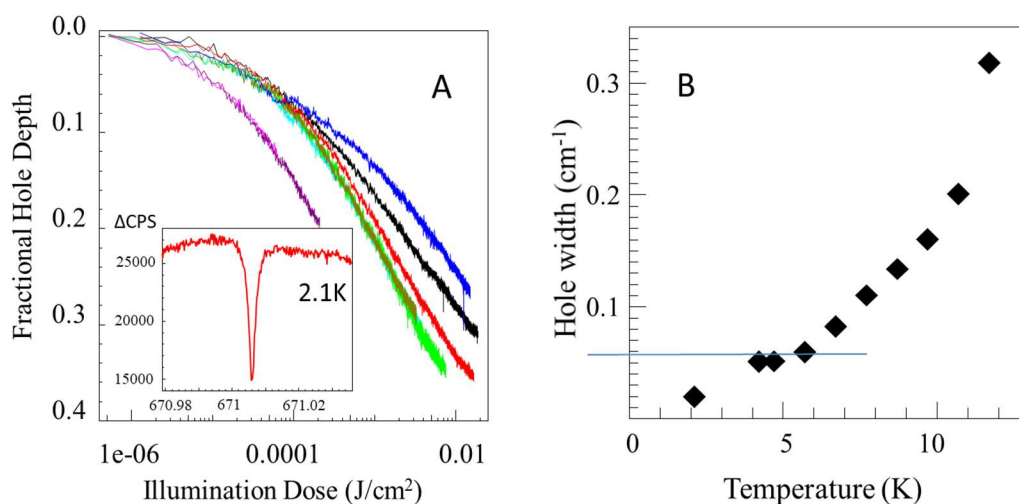


Figure 5-18: EET incapable Chl a. Frame A: the HGK curves for $T = 2.1 \text{ K}$ (purple and magenta), 4.7 K (several curves in different shades of green), 5.7 K (red), 7.7 K (black and grey) and 9.7 K (blue) for EET-incapable Chls a . The insert depicts a 45%-deep spectral hole that resulted from a long burn at 2.1 K . B: The dependence of the homogeneous zero-phonon hole width (twice the ZPL width; extrapolated to zero dose) on temperature.

5.4.3.3.2 HGK for EET-capable Chl *a* Fraction and the Original B800 BChls

Figure 5-19 shows 5 K HGK for EET-incapable Chls *a* (red, one of the curves from Figure 5-18), EET-capable Chls *a* (blue), and the BChl *a* B800 band of the unmodified (light green) and modified (dark green) LH2 (Frame A), as well as spectral holes measured for EET-capable fraction of Chl *a* (Frame B). The best fit to EET-incapable Chl *a* HGK involving single-tier barrier / Gaussian λ -distribution featured $\lambda_0 = 9.63 \pm 0.05$ and $\sigma_\lambda = 1.15 \pm 0.05$. The original B800 BChl *a* curves (green) matched those from [14] (Figure 5-27). The best fit involving single-tier barrier / λ -distribution featured $S=0.5$ [14], $\lambda_0 = 10.2 \pm 0.1$ and $\sigma_\lambda = 1.2 \pm 0.1$. The same line width distribution as in [14], based on [173], peaked at $3 \text{ cm}^{-1} / 1.75 \text{ ps}$ was utilized here. Note that NPHB tends to probe predominantly the longer-times (narrower spectral holes) end of the line width distribution, therefore the distribution from [14], [173] is in agreement with $\sim 2.3 \text{ ps}$ B800-B850 EET times obtained with NPHB. Despite the actual HGK curves being nearly identical, $\lambda_0 = 10.3 \pm 0.2$ and $\sigma_\lambda = 0.7 \pm 0.2$ were reported in [14]. We attribute these minor discrepancies to two main factors. First, our modeling software has evolved since 2010 and many additional features potentially affecting the calculated curves (e.g. recovery while burning, lognormal PSB shapes [174], etc) have been introduced [16], [80], [93], [107]. Second, in [14] the LH2 HGK curves were fitted with two - three different contributions to NPHB (corresponding to different generalized coordinates in Figure 5-14) as opposed to one here.

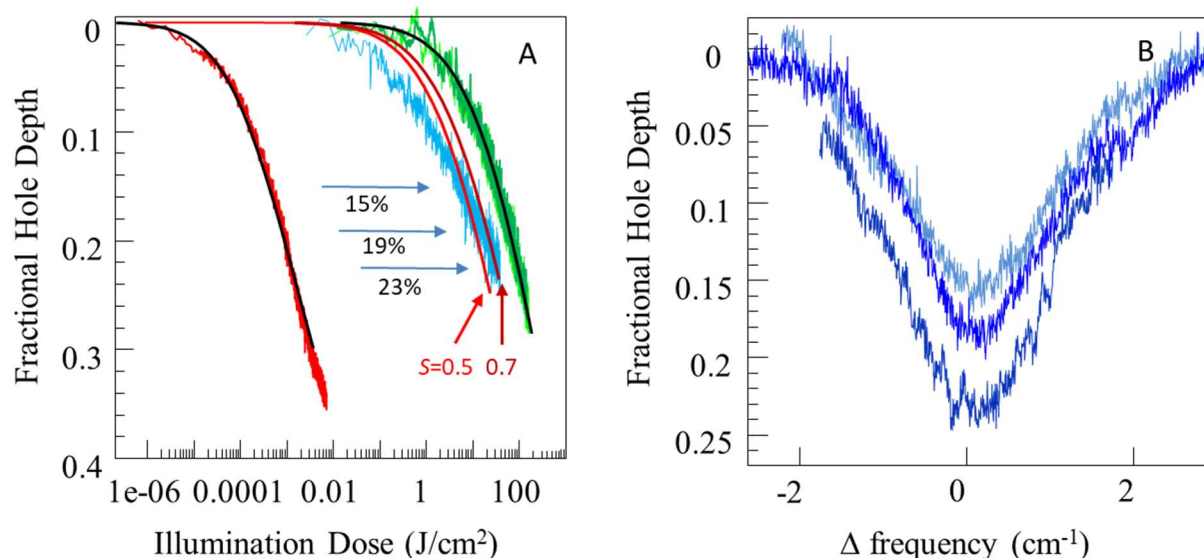


Figure 5-19: Frame A: 5 K HGK of EET-incapable Chls a (red), EET-capable Chls a (blue) and BChls a of the remaining B800 band of the modified LH2 (dark green) and non-modified LH2 (light green). Smooth curves represent modeling/fitting results; see text for parameters. B: 15%, 19% and 23%-deep spectral holes (see arrows in Frame A) measured for EET-capable Chls a .

The noisy blue curve in *Figure 5-19A* was obtained for EET-capable Chls a while detecting fluorescence at >850 nm. It features somewhat faster burning than the original B800 band and much slower burning than the EET-incapable Chls a , in agreement with fast EET on a picosecond-scale (but slower than in the original LH2). This curve appears “flatter” than the other HGK curves, indicating that the dispersion of NPHB rates is higher for this sample than for both EET-incapable Chls a and the original B800 BChls a . This increased dispersion could originate from a broader λ -distribution or a broader line width distribution or both. In the light of the recovery results below, significantly broadened λ -distribution is unlikely. It is quite possible that the EET time distribution has been broadened due to the additional EET channel from the Chls a to the remaining neighboring B800 BChls a , on top of the EET directly to the B850 BChls a . Chl a , BChl a and empty B800 pockets (see Table 2) are distributed randomly within the B800 ring of our modified LH2 and

therefore Chl *a* may have two, one or zero nearest-neighbor B800 BChl *a*. There is also a variety of combinations involving next-nearest neighbors. The ~ 0.085 nm or ~ 1.87 cm⁻¹ width of the 15% hole in Frame B corresponds to 5.7 ps EET time. Assuming that in the zero-dose limit the hole would be somewhat narrower (corresponding to longer EET time), this result is in good agreement with the 8.3 ps EET time of [168]. Note that 8.3 ps EET times were obtained in [168] despite using a sample with B800 BChls *a* fully removed and replaced with Chls *a* (or other chlorophylls) and not partially removed and replaced as in this study. As a result, Chl *a* to B800 EET was impossible in [168] and 8.3 ps corresponded to Chls *a* transferring energy directly to the B850 manifold. Thus, fast Chl *a* to B850 BChl *a* EET in the modified LH2 complexes likely involves so called extended Förster mechanism [173], [175], [176]. An alternative mechanism has also been suggested where the carotenoids contribute to the coupling between the B800 and B850 BChl *a* molecules [177], [178], [179], [180]. The EET from Chls *a* to B800 BChls *a* in our samples is definitely possible, but, given poor spectral overlaps, Förster approximation yields EET time distributions centred at about 60 ps for Chl *a* to B800 BChl *a* EET at 5K (calculated using the EET-time distribution generating feature of our NPHB modeling software [181]). In the case of two Bchl *a* nearest neighbors being present the average de-excitation time of a Chl *a* due to EET to B800 BChls *a* alone could be shortened to 30 ps. Returning to the extended Förster mechanism, we adopted a rescaled EET time distribution from [173] with the peak shifted from 1.75 to 5.3 ps and modeled the respective HGK data (blue) using the same λ -distribution as for the original B800. Creating a better line width distribution including all of the above considerations is outside of the scope of this work, but it is clear that a broader line width (EET time) distribution would indeed result in a better fit to the experimental data.

5.4.3.4. *Effects of Solvent Deuteration.*

Figure 5-20 shows a comparison of low-intensity (triplet-free) [151] HGK obtained for EET-incapable Chl *a* fraction in samples with regular and deuterated solvent. The regular solvent sample data includes several HGK curves obtained at 671.3 nm, i.e. near the peak of the respective absorption band, see Figure 5-16. Multiple curves are presented to demonstrate the minimal differences between HGK curves obtained on different days under otherwise identical experimental conditions. The sample experienced excursions to 120-140 K between different days, fully erasing the holes. The dark blue curve was obtained for deuterated-solvent sample at 4.9 K and 673.2 nm, taking into account the red shift of the deuterated sample absorption of EET-incapable fraction, see Figure 5-17. The black curve was obtained at slightly shorter wavelength (671.3 nm, same wavelength as for protonated sample, which is to the blue with respect to the absorption maximum of the deuterated-solvent sample) and slightly higher temperature of about 5.35 K. The difference between dark blue and black curves is consistent with the difference in burn wavelength and temperature. The difference between HGK in protonated- and deuterated-solvent samples is small and corresponds to differences in mean excited-state λ of less than 1%. For comparison, 3% difference would be expected from tunneling involving –OH (–OD) groups in the solvent and increase of λ by a factor of $\sqrt{2}$ would be expected from proton/deuteron tunneling along the hydrogen bond. Observed curves are in much better agreement with the assumption that the small difference in HGK is due to slightly broader λ -distribution (hence faster burning in the very beginning) and possibly slightly larger electron-phonon coupling in the case of the sample with the deuterated solvent. These may be a result of the differences in previous preparation steps for this particular sample batch, not necessarily of solvent replacement with deuterated one. Therefore, EET-incapable Chl *a* molecules in modified LH2 appear to be well

isolated from the solvent and to be reporting mostly on the protein dynamics, or perhaps a combination of the protein and detergent micelle dynamics. However, this protein dynamics would not be the dynamics of the original B800 protein pocket.

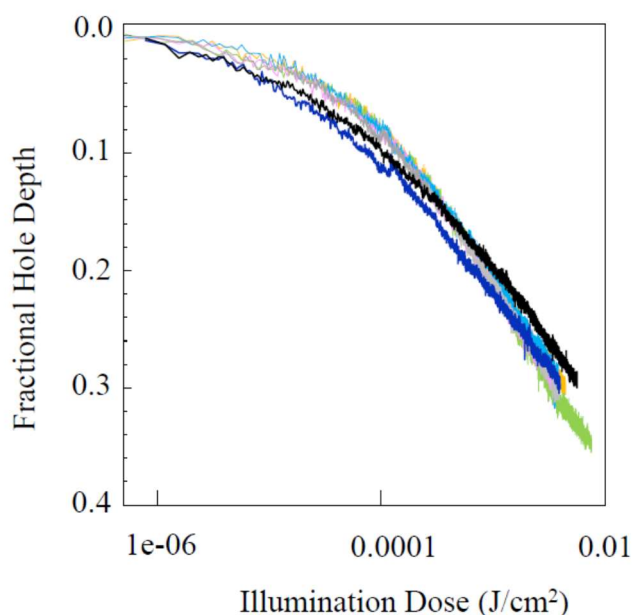


Figure 5-20: Low-intensity ($\sim 1 \mu\text{W}/\text{cm}^2$) HGK curves obtained for modified LH2 samples in protonated-and deuterated solvent. Dimmed curves of different colors were obtained on different days at about 4.8-4.9 K at 671.3 nm in regular/protonated solvent sample. The dark blue curve was obtained for deuterated-solvent sample at 4.9 K at 673.2 nm, in agreement with the red shift of the deuterated sample absorption in Figure 5-16. The black curve was obtained at slightly shorter wavelength (671.3 nm, less ZPL contribution at that wavelength) and slightly higher temperature of about 5.35 K.

Figure 5-21 shows comparison between higher-intensity HGK for samples with regular/protonated and deuterated solvent. Low-intensity HGK for the protonated solvent sample is also shown for comparison. As was demonstrated for both Cyt *b₆f* and modified LH2 in [151], explaining such a large difference with triplet effects alone would require at least 5-fold increase in the product of Chl *a* triplet lifetime and triplet yield upon solvent deuteration. Smaller changes (by about 25% for deuterated ethanol) in triplet lifetime due to the deuteration of the solvent

were reported for Chl *a* [128], [130]. Note, however, that Chls *a* in our modified complexes are not likely to be exposed to the deuterated solvent in the first place. Furthermore, fully exposing Chl *a* to water/glycerol mixture may cause other major effects that we did not observe such as aggregation, strong fluorescence quenching, and very poor persistent hole burning even for Chls *a* that appear monomeric and emit at ~ 680 nm. [107] Therefore, the observed deuteration effect on higher-intensity HGK (light red) quite possible still represents evidence of local heating. Enhanced local heating of the protein in deuterated solvent might be due to poorer interface thermal conductivity between (protonated) protein complex / detergent micelle and deuterated solvent [182], [183].

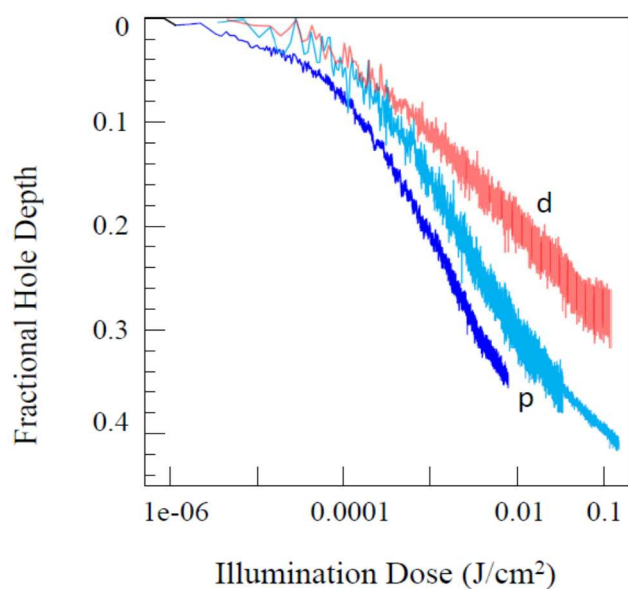


*Figure 5-21: Low intensity HGK (blue, $\sim 1 \mu\text{W}/\text{cm}^2$) and higher-intensity HGK (light blue, $69.5 \mu\text{W}/\text{cm}^2$) of the sample with regular / not deuterated solvent. Light red: high-intensity HGK ($69.5 \mu\text{W}/\text{cm}^2$) for sample in deuterated solvent. All curves were obtained for EET-incapable fraction of Chls *a*, detection between 700 and 750 nm.*

5.4.3.5. Local Heating Experiment

The data in *Figure 5-21* allows one to estimate the intensity of 800 nm illumination necessary to observe local heating in an experiment described in *Figure 5-15*. Note that it is beneficial to use a deuterated-solvent sample for local heating experiments as in the light of the results presented above lower intensities of 800 nm illumination could be used to observe comparable effects. Assuming that $69.5 \mu\text{W}/\text{cm}^2$ intensity at 670 nm is required to reliably produce measurable local heating by a couple Kelvin and using $\sim 5.45 \cdot 10^{-12} \text{ cm}^2$ as 5 K Chl *a* peak absorption cross-section corresponding to 1 GHz ZPL width (*Figure 5-18B* and Ref [80]), a resonant Chl *a* molecule is absorbing light energy at the rate of about $380 \cdot 10^{-18} \text{ W}$. The 5 K B800 BChl *a* peak absorption cross-section corresponding to 2 cm^{-1} ZPL width (in the original B800 band) would be $\sim 1.35 \cdot 10^{-13} \text{ cm}^2$. Thus, intensity of $2.8 \text{ mW}/\text{cm}^2$ would be required to achieve the same energy absorption rate. First, it is obvious that this intensity is orders of magnitude lower than was employed in single-complex experiments on LH2, which was on the order of W/cm^2 [88], [153], [154], [155]. Second, one may recognize that 800 nm excitation has to be broadband in an experiment described in *Figure 5-15* as no correlation is expected between Chl *a* and BChl *a* ZPL wavelengths within each individual modified LH2 complex. In other words, using a narrow-band laser for B800 excitation may result in different individual LH2 complexes being illuminated at 800 nm and 670 nm. Thus, required broadband illumination intensity per frequency interval must be on the order of $1.4 \text{ mW}/\text{cm}^2 / \text{cm}^{-1}$. Given that one modified LH2 complex still possesses several BChl *a* molecules, the above estimate for (broadband) intensity per frequency interval can be reduced several times. The estimates so far did not take into account that the fraction of absorbed energy that is being non-radiatively dissipated is likely smaller for EET-incapable Chl *a* than for B800-pocket BChl *a*. As stated in the introduction, the fluorescence yield of LH2 is fairly low [163] and

even in the case of the perfect yield the difference between the energy of the absorbed and emitted photons would be much higher for intact LH2 (800 nm or 12500 cm^{-1} – 890 nm or 11235 cm^{-1}) than for EET-incapable Chl *a* (670 nm – 680 nm). Still, one would need at least a hundred $\mu\text{W}/\text{cm}^2 / \text{cm}^{-1}$ for this kind of experiment to succeed. The broadband source available to us at the time of these experiments provided only about $5 \mu\text{W}/\text{cm}^2 / \text{cm}^{-1}$ around 800 nm and, not surprisingly, we were not able to observe any local heating with this illumination, *Figure 5-22*. Further studies involving more intense broadband 800 nm illumination are required to settle this issue.

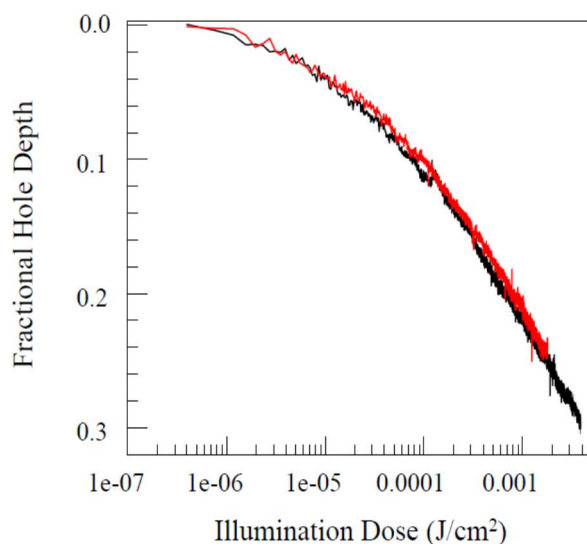


*Figure 5-22: Attempt at measuring illumination-intensity dependence of HGK of the (EET-incapable) Chls *a* in the deuterated sample of our modified LH2 . $T=4.9 \text{ K}$, $\lambda_B=673.2 \text{ nm}$. Black: the B800 BChls *a* were not illuminated. Red: the B800 BChls *a* were illuminated, potentially allowing for local heating.*

5.4.3.6. Hole Recovery

Figure 5-23 presents fixed-temperature recovery at 5K, including holes burned into EET-capable and EET-incapable fractions of Chl *a* as well as the original B800 BChl *a*.

Fractional hole area is defined as the fraction remaining at a given time after burning has concluded; it is one immediately after the end of burning. Presenting the results as a function of the hole area allows one to account for possible hole broadening due to spectral diffusion involving small spectral shifts (smaller than homogeneous hole width). The global fit to various NPHB data including hole recovery is obtained as follows: First, hole-growth kinetics (HGK) curves (obtained with as low intensity as possible, to avoid triplet-related effects and/or local heating; see *Figure 5-18A*) are fitted to determine the parameters of the excited state λ -distribution(s). One can also calculate (with the help of the software employing Eq. 83) the sub-distributions of excited-state λ that are encoded into the hole of the same depth as the hole whose recovery has been measured in an experiment (e.g. 30% for EET-incapable Chls *a*). Next, these excited-state sub-distributions are multiplied by an empirical factor of around 2.3 - 2.5 [16], [80], [93], [107] to obtain the respective ground-state λ sub-distributions. The exact values of this factor are likely specific to the particular design of the pigment's protein pocket. Thus, this factor could be dependent on d , m or V in yet unknown ways. This simple rescaling is justified by the assumption of the "perfect spectral memory", with pigment-protein systems returning to the initial conformation (the hole is refilled by the same systems that have been burned, not other systems randomly wandering around the MLS-style landscape) and with a strong correlation between ground and excited state barriers (what burns first recovers first). These assumptions have been confirmed by analyzing recovery of the holes of different fractional depth burned at the same wavelength in the same pigment-protein system [80], [93]. The fixed-temperature recovery curve is calculated based on the ground-state sub-distribution of λ . If there is noticeable recovery (e.g. in the case of EET-incapable Chl *a* fraction, red symbols in *Figure 5-23*) the simulated recovery is very sensitive to the above scaling factor. One also can distinguish between Gaussian and box-like

λ -distributions. Dashed red curve is the simulated recovery for the case of box-like λ -distribution providing a fair fit to the HGK data in *Figure 5-18*. Thus, the fixed-temperature recovery is also in better agreement with relevant λ -distributions being Gaussian. Once the appropriate scaling factor is found, one should vary the md^2 parameter to obtain the barrier height distributions that manifest in recovery upon thermocycling (see below). Good fits to fixed-temperature recovery involving single NPHB mechanism for EET-incapable Chls *a* (two datasets represented by red circles) required the ratio of ground- and excited-state λ of $\lambda_{gr}/\lambda_{ex}=2.48\pm0.01$.

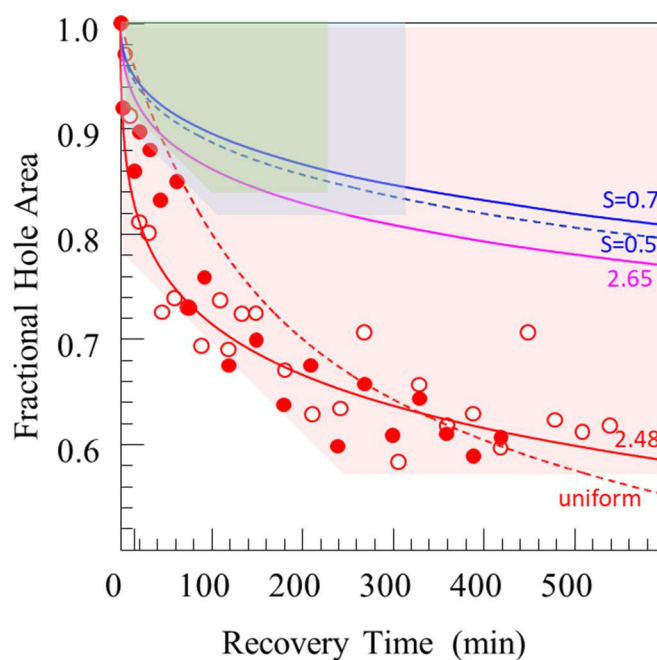


*Figure 5-23. Red circles: Two datasets for fixed-temperature recovery for EET-incapable Chls *a*. Red solid curve represents simulated recovery of a hole starting from a Gaussian excited-state λ -distribution with $\lambda_{0,ex}=9.63$, $\sigma_{\lambda,ex}=1.15$ (see) and $\lambda_{gr}/\lambda_{ex}=2.48$. Dashed red curve represents simulated recovery in case one starts from a box-like λ -distribution. For EET-capable Chls *a* (blue) and the original B800 BChl *a* (green) only the maximal recovery ranges are shown (assuming no hole broadening). The magenta curve is the simulated recovery with Gaussian excited-state λ -distribution with $\lambda_{0,ex}=9.63$, $\sigma_{\lambda,ex}=1.15$ and $\lambda_{gr}/\lambda_{ex}=2.65$. Blue solid and dashed curves represent simulated recovery for Gaussian excited-state λ -distribution with $\lambda_{0,ex}=10.2$, $\sigma_{\lambda,ex}=1.2$ (as measured for the original B800 BChls *a*), $\lambda_{gr}/\lambda_{ex}=2.48$ and Huang-Rhys factor $S=0.7$ or 0.5 , respectively.*

Due to small (if any) fixed temperature recovery we show only the possible ranges of recovery for EET-capable systems (blue for Chl *a* and green for B800 BChl *a*). The maximal recovery was estimated assuming no hole broadening, based on the changes of hole depth alone (see *Figure 5-28* for the examples of raw hole spectra). In case of some hole broadening due to spectral diffusion, the true recovery (the change of the hole area) would be even smaller. One can simulate recovery within the reported range using scaling factors $\lambda_{gr}/\lambda_{ex} \geq 2.65$ (magenta curve). As this empirical factor is a property of the system containing pigment and its immediate environment, somewhat different values might indeed be possible for EET-capable and EET-incapable fractions of Chls *a*. However, instead of increasing this factor one could just assume slightly higher range of the tunneling parameter λ in both excited and ground state. Ultimately, the fixed-T recovery is extremely sensitive to the parameters of the ground-state λ -distribution (or, rather, partial distribution actually encoded into the hole) [16], [107] and the same ground-state λ -distribution can be obtained by either increasing $\lambda_{gr}/\lambda_{ex}$ or by increasing both λ_{gr} and λ_{ex} . The differences observed in *Figure 5-18* correspond to the differences in λ_{gr} of just a few percent. This estimate is in good agreement with the shift of the excited state λ_0 from 9.63 for the EET-incapable Chls *a* to 10.2 for the original B800 BChls *a*. Two blue curves in *Figure 5-18* were obtained starting from the latter parameters of the λ -distribution, $\lambda_{gr}/\lambda_{ex} = 2.48$ and $S=0.7$ (as for EET-incapable Chls *a*) or $S=0.5$ (as for B800 BChls *a*). These curves are well within the possible range of recovery. According to Eq. 82, a 6% increase of λ corresponds to 6% increase of d or 12% increase of m or V , or some combination of smaller increases of several of these parameters.

Figure 5-24 depicts the results of the thermocycling experiments, with the fractional area presented as a function of temperature. The temperature-based distributions are proportional to the distributions of barriers V , with the proportionality coefficient of about 30, depending on the attempt frequency Ω_0 and the time spent at the top temperature of the cycle, about one minute [16], [80], [93], [107]. Here the datasets for EET-capable (blue) and EET-incapable (red) Chls a are similar within the experimental error margin, while the BChl a dataset (green) is significantly different. While for all Chls a spectral holes fully recover by 45 K, the barriers are higher in the case of BChl a , there is almost no recovery up to about 20 K (presenting additional evidence for almost no recovery in fixed-temperature recovery experiments) and some molecules persist in the burned configuration beyond 60 K. (See *Figure 5-29* for the examples of the hole spectra measured at 5 K after temporary raising temperature to specified levels.) This is despite the fact that two fractions of Chls a are in significantly different local environments / locations within the complex, resulting in drastically different EET times, while one (EET-capable) fraction of Chls a and the original BChls a are in a very similar local environment. Note that thermocycling results in full hole recovery by 45 K also in other complexes containing Chl a , including CP43 [93] and Cytochrome b_6f [16], [80], [107].

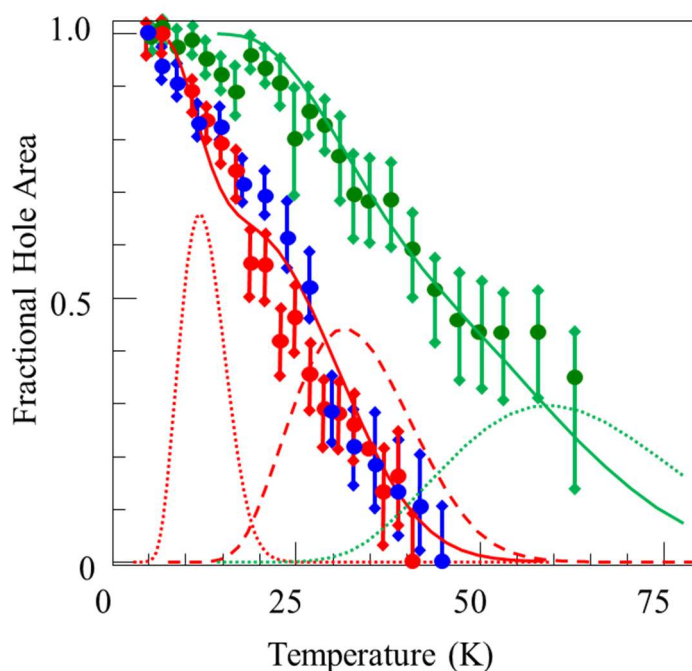


Figure 5-24. Thermocycling-induced recovery of $\sim 30\%$ -deep spectral holes burnt at ~ 806 nm in BChls *a* in regular LH2 (green), at ~ 671 nm in EET-incapable Chls *a* in modified LH2 (red), and at ~ 671 nm in EET-capable Chls *a* in modified LH2 (blue). The area is normalized to one at the point after some fixed temperature recovery takes care of the fastest/ smallest λ_{gr} systems (first ~ 30 minutes) and before the start of the thermocycling. Red dashed curve is the partial barrier distribution also responsible for fixed-temperature recovery, for $md^2 \approx 1.5 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$. Red dotted curve is the second partial barrier distribution, not affecting fixed-temperature recovery, for $md^2 \approx 4.0 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$. Red solid curve represents the fit to the thermocycling-induced recovery of holes burned into the Chl *a* region. Green dotted curve is the second partial barrier distribution for B800 BChls *a*, $md^2 \approx 0.8 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$. Green solid curve represents the fit to the thermocycling-induced recovery of holes burned into the B800 band.

The ratio $\lambda_{gr}/\lambda_{ex} = 2.48$ obtained from the analysis of fixed-temperature recovery of EET-incapable Chls *a* can be used to generate the ground-state barrier distribution and fit (a part of) the respective thermocycling data. Red dashed curve represents the ground-state barrier sub-distribution encoded into 30% holes in EET-incapable Chl *a* fraction corresponding to $\lambda_{gr}/\lambda_{ex} = 2.48$ and $md^2 \approx 1.5 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$. Fully fitting the thermocycling data would require implementing the model involving competitive burning with two different NPHB mechanisms, as introduced for

Cytochrome *b₆f* [16]. In this model different combinations of V and md^2 result in overlapping distributions of excited-state λ , ensuring that one or another light-induced structural change may occur with comparable probabilities. A two-tier NPHB model for LH2 would also be in agreement with [14], [88]. A good fit to the thermocycling-induced recovery data (solid red curve) can be obtained with two contributions, featuring $md^2 \approx 1.5 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ (dashed red curve) and $md^2 \approx 4.0 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ (dotted red curve). These values are very close to those observed in Cytochrome *b₆f* [16] suggesting that the respective NPHB mechanisms may be universal for Chl *a*-containing protein complexes. Note that the area under the dotted red curve is somewhat smaller than the area under the dashed red one. Furthermore, the dashed component had experienced some recovery prior to the start of the thermocycling experiment. This suggests that the range of λ is shifted to slightly higher values for the dotted component and as long as $\lambda_{gr}/\lambda_{ex}$ for this component is not lower than 2.48, this component would not contribute to the first several hours of fixed-temperature recovery [16]. The quality of the thermocycling data is not sufficiently good to state that with certainty, but it is possible that the barriers are indeed 12% higher for the EET-capable fraction of the Chls *a*, particularly in the temperature range covered by the $md^2 \approx 1.5 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ (dashed) component. Thus, minimal differences in thermocycling-induced recovery do not contradict the large differences in fixed-temperature recovery.

For B800 BChls *a*, the thermocycling-induced recovery is also in agreement with a two-component NPHB model, but the details are very different. Namely, a good fit to the experimental data can be obtained while preserving the (dashed) $md^2 \approx 1.5 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ component (perhaps shifting it to $1.6\text{--}1.7 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$) but removing the $md^2 \approx 4.0 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ component (dotted red curve) and replacing it with a new $md^2 \approx 0.8 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ component (dotted green

curve). The value of $0.8 \cdot 10^{-46} \text{ kg} \cdot \text{m}^2$ is subject to a relatively large error, as the data quality in this temperature range is poor. With that in mind, and given the limitations of the NPHB model used in this work (Eq. 82 based on rectangular barriers and free-particle wavefunctions) we did not attempt the time-consuming simulations with two λ -distributions and an additional line-width distribution that would be required for the original B800 band and EET-capable Chls *a* in the B800 protein pockets (future work).

Thus, based on the large differences in barrier distributions between Chl *a* and BChl *a* in the same protein pocket, we conclude that some components of the pigment molecule itself, and in particular the side groups different between BChl *a* and Chl *a*, might be contributing to the light-induced spectral shifts / are participating in the generalized coordinate in *Figure 5-14*. Alternatively, one could suggest that pigment molecules constrain some motions in nearby protein and affect the heights of the respective barriers on the protein energy landscape. Note that Saga et al. [165] attributed differences in affinity of Bchl *a* and Chl *a* to the B800 binding pocket to differences in distortion of tetrapyrrole π -macrocycle in chlorophyllous pigments (since the π -macrocycles of Chl *a* is more rigid than that of BChl *a*) and / or to difference in the 3-substituents, namely vinyl and acetyl groups in Chl *a* and BChl *a*. The 3-acetyl group in B800 BChl *a* is hydrogen-bonded with Arg20 in the β -polypeptide of LH2, likely resulting in the Q_y shift to 800 nm for BChl *a* [184]. With respect to spectral shifts observed in single-complex experiments, it has been estimated that relevant TLS are located about 0.4 nm from the BChl *a* molecule [154], [162]. Differences in orientation and conformational freedom between B800 Bchls *a* of three different species of purple bacteria were addressed in [162]. One should note that B800 pockets are somewhat different in these three species, while pigments are the same (Bchl *a*). In this work

it is the pigment that is different, while the protein pocket residue composition stays the same (between Bchl *a* and EET-capable Chl *a* fraction). Furthermore, the distributions of the spectral shifts reported in [162] were not analyzed in terms of illumination intensity, reflecting the (incorrect) understanding at the time of that publication that spectral shifts in single-molecule experiments are occurring anyway and are merely observed using optical experiments. In reality, vast majority of the shifts observed in [162] must have been light-induced; i.e. they represented the single-molecule analog of NPHB. Summarizing, directly correlating results of this work and single-complex results of [162] would be very difficult.

Finally, we address the possible origins of the EET-incapable Chl *a* fraction. As stated above, there are multiple reasons to believe that these Chls *a* are strongly associated with the LH2 complex. Size-exclusion chromatography and replacement of solvent with the deuterated one were expected to remove free pigments in solution, yet the sample with the deuterated solvent contained the largest fraction of the EET-incapable Chls *a*. There are quantitative similarities between NPHB and recovery behavior of the two Chl *a* fractions, with one of them clearly being located in the original B800 protein pocket. The respective barrier and λ -distribution parameters are typical for Chls *a* in the intact pigment-protein complexes. On the other hand, the NPHB behavior of Chl *a* in water-glycerol mixture is very different from that in the proteins [107]. One may ask why then the EET is so poor for these pigments if they are still located within a relatively small distance from the BChls *a*. One can speculate that since the EET for the other Chl *a* fraction, located in the proper protein pocket, is very fast – indicating either extended Förster mechanism [173], [175], [176] or carotenoid-assisted EET [177], [178], [179], [180] - even a small displacement of Chl *a* from the optimal position could have drastic effects on the EET. Orientation

of these Chls *a* (i.e. of their transition dipoles) may have also become unfavorable. It is possible that Chls *a* in question are strongly but non-specifically bound on the surface of the protein but inside the detergent micelle.

5.4.4 Conclusions

Chemically modified LH2 samples with some but not all B800 BChls *a* replaced with Chls *a* feature at least two fractions of Chls *a* located in distinct local environments. One of these environments represents an intact B800 protein pocket and here fast (<10 ps) EET to the B850 manifold is possible. The other fraction is likely comprised of Chls *a* strongly bound to the protein but not located in the original B800 protein pocket and are incapable of fast EET (fastest limit >530 ps, most likely several nanoseconds). Surprisingly, the barriers of the protein energy landscapes manifesting in the optical experiment are very similar for those two fractions, and different from those of the original B800 BChls *a*. Furthermore, the barriers of both Chl *a* fractions are similar to that in other Chl *a* containing protein complexes. On the other hands, fixed-temperature hole recovery is similar for Chls *a* and BChls *a* in the B800 protein pockets, with behavior of EET-incapable Chls *a* being different. This difference, however, can be attributed to just several percent difference in the tunneling parameter λ , i.e. a several percent change of V , d or m . The data for all Chls *a* and BChls *a* can be reconciled assuming that both pigments feature two competing NPHB mechanisms, but while one of these mechanisms appears preserved across both pigments, the second component is drastically different. These observations likely indicate that the small structural changes responsible for spectral shifts involve parts of the pigment molecule itself or, alternatively, occur in the protein but are somewhat constrained by the pigment

molecule (to different degree by different pigment molecules). This would narrow down the search space for relevant groups of atoms in the protein to the vicinity of the pigment, and specifically to the vicinity of the atom groups that are different between BChl *a* and Chl *a*. The research on spectral dynamics of LH2 can be expanded by introducing other chlorophyll-like molecules as a replacement for the B800 BChls *a* or deliberately adding pigments non-specifically bound to the LH2.

Finally, we conclude that local heating of the pigment-protein complexes (especially LH2) by the exiting laser beam is quite possible, especially in single-complex spectroscopy experiments. Estimates for conditions required for conclusive experiments on local heating have been obtained in the course of this work and further experiments will be performed in the future. Note that we assumed that heat dissipation and thermal equilibration within an LH2 complex are occurring faster than energy transfer from LH2 into the surrounding environment. While this may be true, more rigorous treatment of local heating should include the possibility of different energy dissipation pathways within the complex, depending on the location of the originally excited BChl *a* pigment and the “thermometer pigment”.

5.4.5 Supplementary Information

Estimates of pigment content in different sample batches:

Table 2: Estimates of the numbers of Chls *a* and BChls *a* per LH2 complex in different sample batches.

Integrals	820 -900	695-820 (BChl) Total/B800/780	620-695 (Chl)	B850/B800 ratio	Number of BChls total / intact B800	Number of Chls, both fractions
Non-modified LH2	26.69	12.47	0	2.1	8.6	0
Protonated, <i>Figure 5-17B</i>	30.14	7.9/4.9/3.0	2.49	6.15	4.7/ 2.9±0.3	3.0±0.3
Deuterated, <i>Figure 5-17C</i>	29.44	13.27/8.96/4.31	2.81	3.29	8.2 / 5.5±0.5	3.4±0.3*

*The third sample (Figure 5-17A) featured very similar band intensity ratios at RT as the first protonated-solvent one; no 5 K spectrum is available. However, most of its 3 Chl *a* per complex were located in the proper B800 pockets, allowing for fast EET.*

**most of them likely not in the B800 pocket*

Estimates above are based on comparing integral intensities of various bands and assuming that B850 band is due to 18 BChls *a* per complex. The region around the B800 band of the modified samples was decomposed into the sum of the B800 band of the non-modified sample and the BChl *a* monomer spectrum peaked at about 780 nm (see *Figure 5-16*). The latter represents BChls *a* that are associated with the LH2 protein but do not have normal interactions with the B800 protein pocket / are displaced.

The ratio of the oscillator strengths of BChl *a* and Chl *a* of ~2 was taken from [185]. This paper reports the squares of the transition dipole moment of several chlorophylls for a broad range of the index of refraction, and these squared moments are based on integrated spectra, not peak extinction coefficients as in some other works.

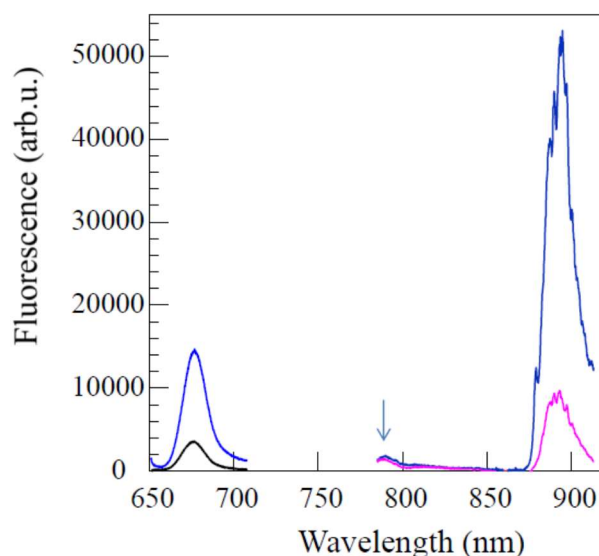


Figure 5-25. Emission spectra (obtained for deuterated solvent sample, red in Figure 5-16; see also Figure 5-17C). Black: excitation at 532 nm. Blue: excitation at 550-650 nm (lamp). Magenta: excitation at 650-720 nm (lamp). Arrow indicates emission of BChl *a* outside of B800 pockets (absorption at ~780 nm) that were excited either via EET from the Chls *a* to BChls *a* or via BChl *a* absorption tails due to intra-molecular vibrations. Poor quality of the IR bands is due to CCD etaloning.

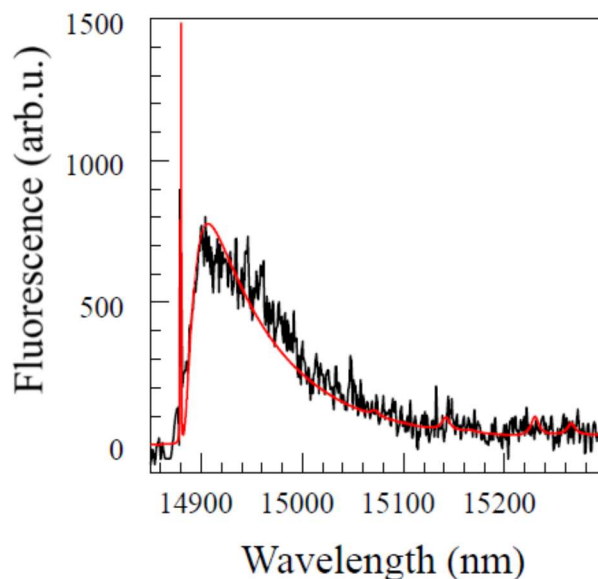


Figure 5-26: A 5K Δ -FLN spectrum obtained at the red edge of the Chl *a* band of the modified LH2 sample and the modeled spectrum obtained with the parameters used in this work for Chl *a* band, i.e. $S=0.7$ and a lognormal phonon sideband [174] with $s=0.82$ and $\omega_c=49$ (peak 25 cm^{-1} ; and half-widths of 15.5 and 40.7 cm^{-1} for ZPL side and opposite side, respectively) and with intra-molecular vibration modes.

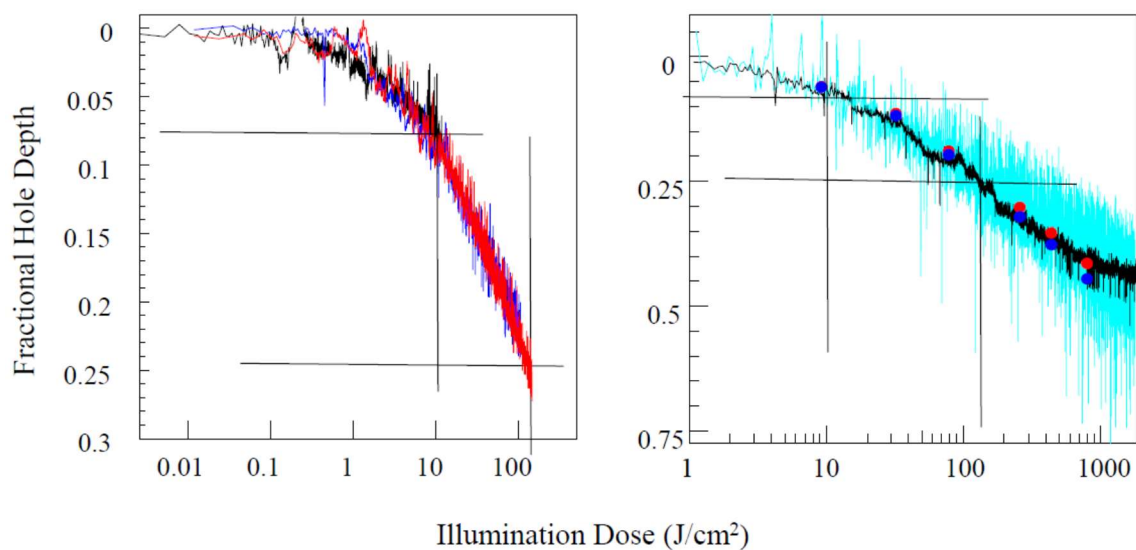


Figure 5-27: Three ~ 805 nm HGK obtained for non-modified LH2 sample at 5 K in this work (left) and several matching HGK reported in [14] (right). The crosshairs are at 140 J/cm^2 and 25% fractional depth and at 10 J/cm^2 and 7.5% fractional depth. .

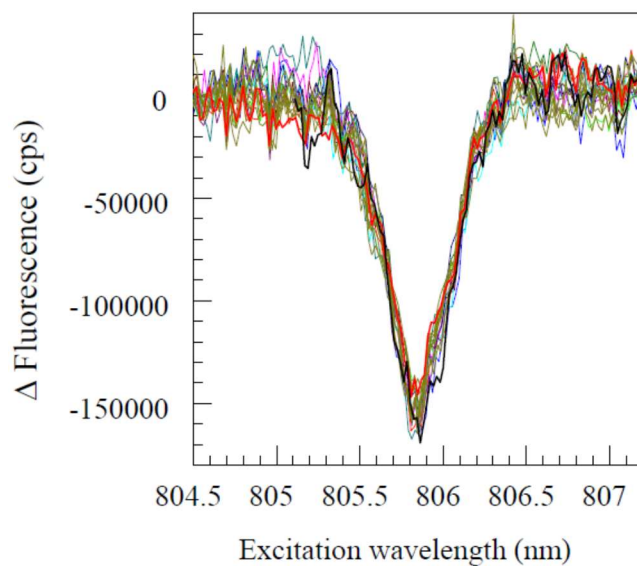


Figure 5-28: Examples of spectral holes in the course of fixed-temperature (5 K) recovery for the original non-modified LH2. Black: initial hole, a minute after the end of hole burning; red: after 4 hours of recovery.

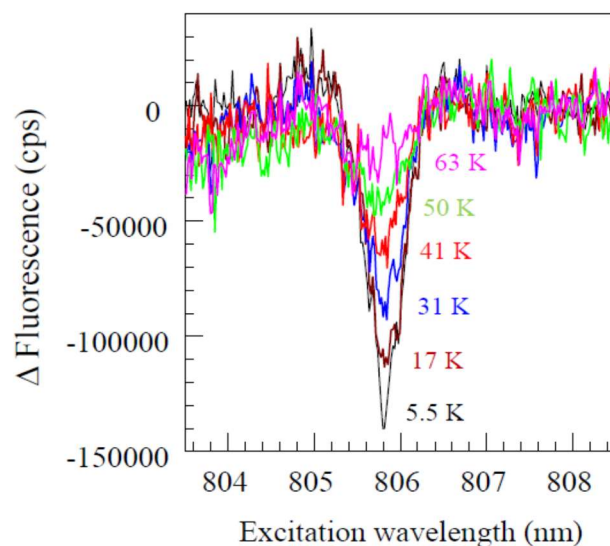


Figure 5-29: Examples of spectral holes after cycling to various temperatures for the original B800 band of the non-modified LH2 sample.

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6. Future work

The research and technological advances presented in this work provide a foundation for several promising research avenues. The following sections outline the most pertinent areas for further investigation, many of which leverage the specialized cryogenic system detailed in Chapter 4 to explore low-temperature spectral dynamics.

6.1 Local heating in LH2 with sufficient illumination of B800

As was demonstrated in the previous chapter (see section 5.4.3.4), exploring local heating effects in modified LH2 likely requires intensities on the order of hundreds of $\mu\text{W}/\text{cm}^2 / \text{cm}^{-1}$ to succeed. Recently we have purchased diode light source with the emission band matched to the B800 band and with power of 1W to continue experiments on local heating effect in modified LH2 complexes.

6.2 Spectral dynamics in LH2 - obtaining better quality data.

We are looking forward to carrying out experiments in order to confirm that pigment degrees of freedom (perhaps some hydrogen bond between Chl and protein) are part of the generalized coordinate in our TLS; and also to obtain more information on the shapes and parameters of the barrier distributions in the original and modified LH2. We should complete thermocycling experiments to explore ground states tunneling parameters and barrier distribution. Conducting

the ultra-long recovery experiments are required to observe recovery dynamics that is clearly distinguishable from experimental errors.

6.3 LH2 complex reconstitution with various pigments

The LH2 project can be further expanded by introducing other chlorophylls (e.g., Chl *b*, divinyl Chl *a*) into modified LH2. This would explore the effects of such substitutions on protein dynamics and determine whether pigment molecule side groups are indeed part of the Two-Level Systems/Multiple-Level Systems (TLS/MLS) responsible for Non-Photochemical Hole Burning (NPHB).

6.4 Non-specifically bound pigment dynamics

As shown above, the EET-incapable Chls in modified LH2 are likely still associated with the protein. Thus, instead of trying to replace the pigments in their original pockets, one may explore spectral dynamics for pigments that are non-specifically bound to proteins (but still inside the detergent micelle).

6.5 HGK and HR experiments on WSCP

Recently, our group has performed MD simulations on Water-Soluble Chlorophyll-Binding Protein (WSCP) and identified possible microscopic movements responsible for spectral dynamics [8].

Namely, rotations of alanine and serine side groups are good candidates for the origin of small structural changes observable in NPHB experiments in a variety of pigment-protein complexes. As NPHB data of the type and quality required for comparison are not yet available for WSCP, similar experiments need to be performed as have been recently reported for Cytochrome *b₆f* and modified LH2 complexes. These involve monitoring both the burning and recovery processes of

spectral holes at different temperatures, from 4 K to about 60 K. The Reppert group at Purdue has recently produced a series of WSCP mutants. The purpose of these mutations was to explore whether one can theoretically predict the differences in Chl *a* transition energies associated with different compositions of the protein pocket. Some of these mutants involve the replacement of serines or alanines by other amino acids (or the replacement of other amino acids by serine or alanine) and are therefore suitable for exploring whether the addition or removal of serine and/or alanine opens, closes, or modifies the parameters of various NPHB channels. These studies could be accompanied by MD simulations on these mutants to determine energy landscape features associated with possible rotations of these modified serines and alanines. On the other hand, MD simulations similar to those performed for WSCP can also be carried out for LH2 and cytochrome *b₆f*.

6.6 The effects of hydrostatic pressure on spectral and protein dynamics.

Relatively low hydrostatic pressure (supplied at cryogenic temperatures via liquid helium) is sufficient to observe spectral shifts (which are proportional to pressure), provided that the lines or holes are narrow enough, such as in the absence of energy transfer. Local compressibilities can be derived from these shifts. The key question is whether we can use pressure to constrain any specific movements in a protein, particularly those responsible for NPHB, to an observable degree.

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