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YEAST ISO-1-CYTOCHROME C HEME CREVICE POLARITY STUDIED BY SECOND DERIVATIVE SPECTROSCOPY

Hans Schroeder

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in
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of
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Table of Contents

Chapter 3
Results

Chapter 4
Discussion

List of Figures

List of Tables

CHAPTER 1
INTRODUCTION

1.1. General Introduction:

Cytochrome *c* is a small (12.8Kda), water soluble, mitochondrial electron transfer protein made up of a single polypeptide chain (104-108 amino acids), and a covalently attached heme group. Its primary function is to shuttle electrons from cytochrome reductase to cytochrome oxidase thereby completing an essential step of the mitochondrial electron transfer pathway. Cytochromes *c* also play an important role in photosynthetic processes, and nitrate and sulfate reductions by bacteria (Lemberg and Barrett, 1973). Although cytochrome *c* and its relatives remain some of the most intensively studied proteins, mechanisms of electron transfer have yet to be elucidated.

Several high resolution crystal structures have aided considerably in identifying oxidation state dependent changes in different proteins. In particular, recent interest has focussed on an internally bound water molecule (Water 166) of eukaryotic cytochrome *c*, which mediates two distinct hydrogen bonding networks in the oxidized and reduced state, respectively. Site directed mutagenesis and X-ray crystallography have provided a means to study the function of this water molecule by altering its hydrogen bonding partners, and the volume of the cavity it occupies. Thus, it has become possible to study the properties of cytochrome *c* when water is excluded from the cavity, or when as many as two water molecules occupy the internal cavity. Water bound within the heme crevice is important for other reasons as well. First, the polarity of the heme environment is thought to control the midpoint potential of the protein. The midpoint potential can be thought of as the driving force with which the reduced

cytochrome wants to give up its electrons. Thus, modulation of the heme crevice water should effect the heme crevice polarity and therefore the reduction potential of the protein.

Perhaps the greatest obstacle in studying the interaction of water molecules with specific amino acid molecules is that there are few techniques to study polarity changes of this type. Polarity is most easily studied by fluorescence. In normal iso-1-cytochrome *c*, there is a single tryptophan residue that is removed from the heme crevice. Although tryptophans make good reporter groups of their environment polarity, energy transfer from the excited tryptophan to the porphyrin is exceptionally efficient in cytochrome *c*. This energy transfer is then quenched by the heme iron of the porphyrin. The five tyrosine residues, on the other hand, are generally inefficient for examining polarity, as their fluorescence emmision specta does not shift appreciably with changes in residue polarity (Lakowicz, 1983).

Thus, any technique that could look at the polarity of the heme crevice and the changes associated with the movement of the internal water molecule would be a considerable asset to cytochrome research.

1.2. Specific Purpose:

This project focussed on three main goals. The first was to find a suitable technique that would allow us to look at polarity changes associated with the perturbation of a water molecule (Water 166). The second, was to modify or extend this technique such that it could be applied to the study of Water 166. The final goal, which evolved from the previous two, was to show that this technique could be used to monitor the effects of perturbants on the cytochrome *c* protein.

1.3. Yeast Iso-1-C Heme Crevice Structure (Berguis and Brayer, 1992):

The yeast iso-1-cytochrome *c* heme crevice consists of a protoporphyrin IX heme prosthetic group that is covalently attached to the protein. The final two coordination sites of the iron are provided by methionine 80 and histidine 18. The heme crevice of oxidized and reduced cytochrome *c*'s vary in their hydrogen bonding patterns. Central to these changes, is a single water molecule whose movement in response to oxidation state creates two distinct hydrogen bonding networks. In the reduced state of yeast iso-1-*c*, this single water molecule (Water166) is constrained within a cavity of approximately 10Å^3. In this state, water 166 is thought to stabilize the electron rich reduced state of the heme iron. It accomplishes this by orienting the positive end of its dipole towards the heme iron atom. Water 166 is maintained in space by three hydrogen bonds each involving the water molecule itself and three other partners. These are asparagine 52, tyrosine 67 and threonine 78. On oxidation, water 166 shifts 1.7Å towards the heme iron atom thus changing the water-heme distance from 6.6Å to 5Å. This is presumably due to an electric field induced response of the oxidized heme iron. The end result is a cavity of approximately 25Å^3, with the water molecule located close to the heme iron atom. As a result of the movement of this water, the hydrogen bond between asparagine 52 and water 166 is broken as is the hydrogen bond between tyrosine 67 and methionine 80. Similar hydrogen bonding patterns have been shown for horse (Bushnell et al., 1990), and tuna cytochrome *c* (Takano and Dickerson, 1981a,b). A diagram of the iso-1-cytochrome *c* heme crevice is shown in Figure 1. Water 166 has been omitted for clarity.

Apart from creating and maintaining two distinct hydrogen bonding networks, this internal water molecule is important from other standpoints. First, either the removal or the incorporation of an additional water molecule, causes a rather large decrease in the midpoint potential of the protein, yet electron transfer rates remain unperturbed. Also, mutations that perturb the internal water molecule generally lead to more stable proteins. Evolution however, has conserved these residues and water 166 for millions of years. It is no wonder that the role of this water molecule is the focus of many intensive research efforts.

1.4. Y67F Heme Crevice Structure (Berguis et al, 1994):

The high resolution structure of the Y67F mutant shows several peculiar changes in comparison to the wild-type structure. The most notable is the incorporation of a second water molecule into the heme crevice in a position approximately equal to where the hydroxyl group of tyrosine 67 is found in the wild-type protein. Although there is an incorporation of an additional water molecule, the conformational differences between this mutant and the wild-type protein seem to be localized to the mutation site and the pyrrole A proprionate group, whereas little perturbation of the protein backbone is observed. In the mutant's reduced state, water 300 is located approximately 3.3Å from phenylalanine 67, and water 166 shifts approximately 1Å downwords to accomodate the inclusion of the other water molecule. Water 300 is found to hydrogen bond to methionine 80, threonine 78 and water 166. Water 166, on the

Figure 1.
WILD-TYPE ISO-1-CYTOCHROME C

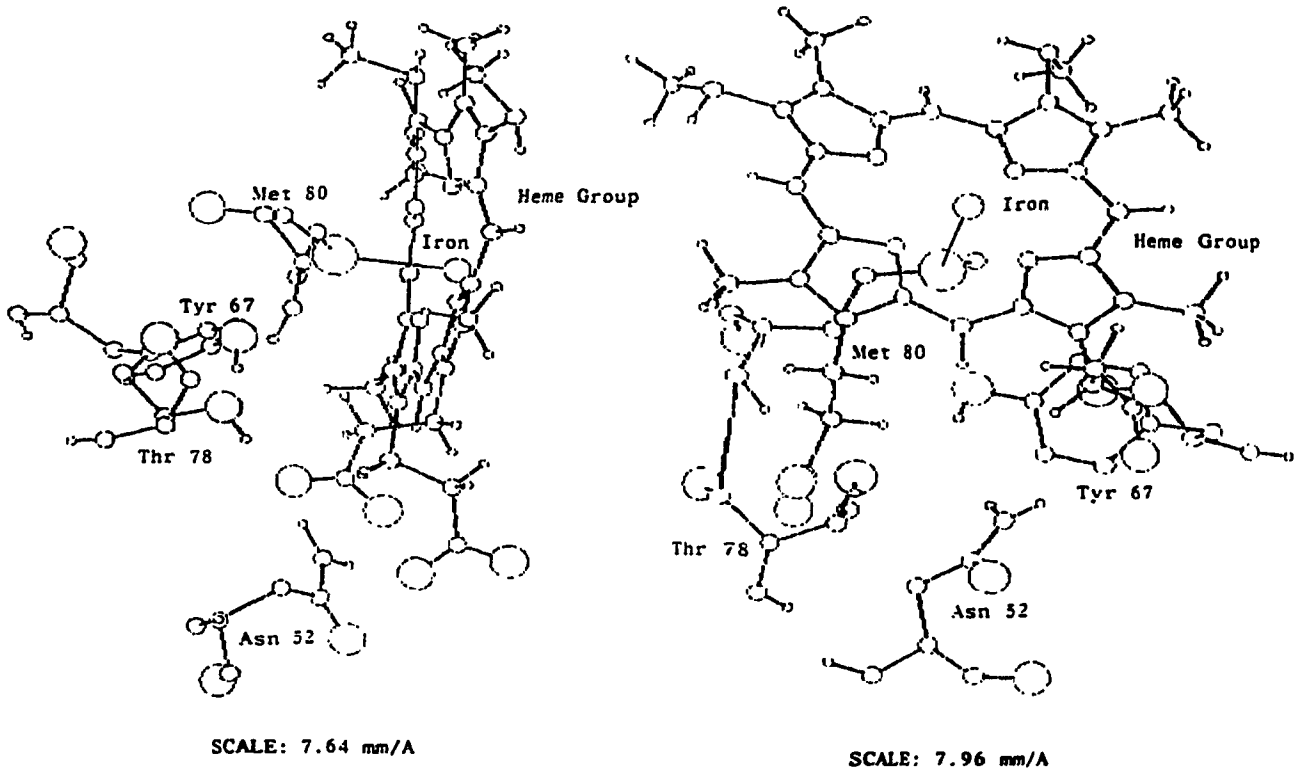


Figure 1. Wild-Type Iso-1-c Heme Crevice. Two views showing the cytochrome c heme crevice. Water 166 has been left out for clarity. Plots were generated using Quanta®, a molecular modelling system.

other hand, goes on to hydrogen bond to water 300, threonine 78 and asparagine 52. Also, in the oxidized state, neither water 300 nor water 166 could be resolved in the x-ray diffraction pattern, presumably as a result of these waters undergoing greater dynamic motion than in the reduced counterpart due to a shift in the side chain of asparagine 52. In this case, the authors indicate that the shift in asparagine 52 is responsible for the breakage of the hydrogen bond between this residue and water 166. Also, the shift in this side chain expands the heme cavity from 35Å^3 in the reduced protein to 60Å^3 in the oxidized protein. Analysis of solvent accessibility to the heme group shows that the average exposure to solvent in both oxidation states is smaller for this protein than the wild-type protein (7.7% exposed as opposed to 8.8% for the wild-type protein). Despite a decrease in the heme crevice exposure, the Y67F protein shows a drop of 56mV (to 236mV) in its midpoint potential.

1.5. N52I and N52I Y67F Heme Crevice Structure (Berguis et al, 1994):

The most notable difference between the N52I and N52I Y67F structures is the complete exclusion of any water molecules from the heme cavity. In the reduced state of the protein, replacing asparagine 52 with isoleucine decreases the size of the heme cavity to approximately 10Å^3 and leads to the expulsion of water 166. The abolition of this water molecule causes several hydrogen bonds to break and others to reform. For instance, tyrosine 67 shifts to hydrogen bond to threonine 78. In the oxidized state however, the magnitude of shift that tyrosine 67 undergoes is so small that the authors believe that a hydrogen bond cannot be formed between tyrosine 67 and threonine 78. As for the Y67F mutant, another important aspect of this mutation is that the hydrogen bond from tyrosine 67 to

methionine 80 is lost. In the N52I Y67F protein, the authors report that there are even fewer hydrogen bonds formed and that in both the oxidized and reduced structures, the internal cavity is reduced to approximately 8Å^3. Like the Y67F protein, these mutations cause the midpoint potential to drop by approximately 56mV to yield a redox potential of 234mV.

1.6. N52A Heme Crevice Structure (Berguis, Thesis):

In agreement with the Y67F mutant, the N52A mutant cytochrome exhibits two water molecules in its heme crevice yet tyrosine 67 remains intact. Also, the mutation does not dramatically affect the folding of the polypeptide chain. In fact, 55 residues whose side chains are buried in the interior of the protein are reported to have virtually identical conformations as seen in the wild-type structure. Mutation of asparagine 52 to alanine causes an increase in the internal heme cavity size (to approximately 70Å^3). The author indicates that this should be large enough to allow for the incorporation of a second water molecule (Water 300). Water 166 is reported to be in a position virtually identical to wild type protein, whereas the position of water 300 occupies a position analogous to where asparagine 52 is found in the wild-type protein. Again, some changes are localized around the pyrrole A propionate region. Calculation of the heme solvent accessibility shows that the heme group is only slightly more exposed than the wild-type protein (9.5% as opposed to 8.8% in the wild-type protein). Finally, the midpoint potential of the N52A mutant is reduced by 33mV (down to 256mV) when compared to the wild-type protein.

1.7. Second Derivative Spectroscopy:

Second derivative spectroscopy is a powerful, simple and nondestructive analytical tool in its ability to resolve overlapping bands in the normal spectrum (Mach et al, 1989; Servillo et al, 1982; Oliver and Green, 1975). Particular attention has focussed on the spectral contribution of three aromatic amino acids (tyrosine, tryptophan and phenylalanine) and methods to carry out their quantitative estimation (Balestrieri et al., 1978; Ichikawa and Terada, 1979; Servillo et al., 1982). The success in these areas has furthered this technique as being useful in detecting microenvironment changes associated with these aromatic residues. Ragone et al. (1984), has shown that at neutral pH, the degree of exposure of tyrosyl residues could be readily determined even in the presence of a high content of tryptophanyl residues. The following information has been taken from the work of Ragone et al., (1984). Any additional citations will be referenced accordingly.

The second derivative spectroscopy theory presented by Ragone was established by examining three model compounds (N-acetyl tyrosinamide, N-acetyl tryptophanamide and N-AcPheOEt), each which mimics the true connectivity of the chromophore as it would be found in an amino acid sequence. N-acetyl tryptophanamide has an extinction coefficient of 5690 $M^{-1}cm^{-1}$ at 280.8nM (Wetlaufer, 1963), whereas N-acetyl tyrosinamide has an extinction coefficient of 1490$M^{-1}cm^{-1}$ at 275.5nM (Edelhoch, 1967). As such, the absorption of phenylalanine and tyrosine residues is generally masked by the larger absorption of the tryptophanyl residues.

Second derivative spectroscopy provides a convenient means of increasing the spectral resolution between overlapping tyrosine and tryptophan residues. In the second derivative spectrum of N-acetyl tryptophanamide

solutions, two maxima are centered around 287 and 295nM and two minima at 283 and 290.5nM. The position of these maxima and minima are only minimally affected by changing the polarity of the solvent. N-acetyl tyrosinamide exhibits a slightly different second derivative spectrum, having a minimum centered around 283.5nM and two maxima at approximately 280 and 289.5nM in dilute buffer, pH7 (Servillo et al., 1982). However, these positions are dependent on the polarity of the chromophore microenvironment. In fact, the more polar the surrounding media, the further the spectrum is blue shifted (shifted towards lower wavelengths). When both tyrosine and tryptophan chromophores are present in solution, their individual second derivative spectra can undergo constructive interference in some places and destructive interference in others. However, because the extinction coefficient of the tryptophan residues is so high, the maxima and minima still occur around 287, 295, 283 and 290.5nM respectively, but vary in magnitude. This is observed even when the molar ratio between tyrosine and tryptophan is five or more. From these observations, the ratio between two peak to trough distances (the peak to peak distances between the maxima at 287nM and the minimum at 283nM (termed the A value) and the peak to peak distance between the maximum at 295nM and the minimum at 290.5nM (termed the B value)), gives a rough indication of the polarity of the tyrosine microenvironments. Phenylalanine need not be taken into account because its spectrum occurs at a much lower wavelength, and so it does not contribute any absorption to the region being analysed by the peak to trough heights.

The absorbance at any wavelength of a mixture of N-acetyl tyrosinamide and N-acetyl tryptophanamide can be predicted on the basis of the Beer-Lambert Law. Using Ragone's nomenclature:

Absorbance = $\varepsilon_{Trp} C_{Trp} + \varepsilon_{Tyr} C_{Tyr}$

The absorbance differences at the two pairs of wavelengths are:

$A_1 = A_{287nM} - A_{283nM}$

$A_2 = A_{295nM} - A_{290.5nM}$

Dividing one by the other and rearranging the terms yields:

$$\frac{A_1}{A_2} = \frac{\Delta\varepsilon_{1\,Trp}/\Delta\varepsilon_{2\,Trp} + (\Delta\varepsilon_{1\,Tyr}/\Delta\varepsilon_{2Trp})(C_{Tyr}/C_{Trp})}{1 + (\Delta\varepsilon_{2\,Tyr}/\Delta\varepsilon_{2\,Trp})(C_{Tyr}/C_{Trp})}$$

The Beer-Lambert Law is not affected by differentiating two times. Therefore the ratio (R) of the peak to peak distances in the second derivative spectrum is:

$R = A/B = A_1"/A_2" = (Ax+B)/(Cx+1)$

Where X= the molar ratio between tyrosine and tryptophan,

$A = (\Delta\varepsilon_{1\,Tyr}"/\Delta\varepsilon_{2\,Trp}")$

$B = (\Delta\varepsilon_{1Trp}"/\Delta\varepsilon_{2\,Trp}")$

$C = (\Delta\varepsilon_{2\,Tyr}"/\Delta\varepsilon_{2\,Trp}")$

$\Delta\varepsilon"$ is the difference in the molar extinction coefficient at the two fixed pairs of wavelengths. From the equations above, it can be readily be seen that the R value depends on the molar ratio (x) of tyrosine and tryptophanamide and also on the solvent composition, because the values of A and C are expected to change due to spectral shifts that occur in various solvents. The B value ($\Delta\varepsilon_{1\,trp}"/\Delta\varepsilon_{2\,Trp}"$) was found to not change appreciably when the tryptophan chromophore was placed in a variety of solvents (p-dioxane, $CHCl_3$, n-C_3H_7OH, ethylene glycol, 6M Guanidine hydrochloride and H_2O). This is of fundamental importance, as the spectra of tryptophan should not shift with changes in the polarity of the solvent, otherwise the changes in the R value could not be attributed to tyrosine residues alone. The resistance of the tryptophan spectra to changes in polarity was observed in their study. In contrast, the numerical values of A and C should be strongly dependent on the properties of the solvent, since both contain extinction coefficient terms for tyrosine.

Proteins exhibit the same general characteristics as solutions of N-acetyl tyrosinamide and N-acetyl tryptophanamide (ie. two maxima centered around 287 and 295nM and two minima around 283 and 290.5nM). Furthermore, the position of these maxima and minima are almost unaffected by perturbing agents (denaturation salts, etc). A spectrum of the ultra-violet region of the oxidized and reduced wild-type protein and its converted second derivative are presented in Figures 2 and 3, respectively.

The degree of exposure has been calculated for several proteins using the equation:

$\alpha = (R_n-R_a)/(R_u-R_a)$

where R_n is the R value ratio (A/B) in the native form of the protein and R_u is the ratio (A/B) for the denatured protein. R_a is the R value for a mixture containing the same molar ratio of aromatic amino acids dissolved in a solvent which would most accurately reflect the interior of a protein matrix (ie. completely buried residues). This equation has accurately predicted the degree of exposure of tyrosine residues for a number of proteins including cytochrome *c*.

Although phenylalanine, tyrosine and tryptophan are the three principle residues absorbing in the near ultra-violet region, several other ultra-violet absorbing compounds do exist. For example, cysteines, histidines, prosthetic groups and turbidity all absorb in the ultraviolet region. However, histidine residues and disulfide bonds have much weaker absorption properties, so their contribution to the total near U.V absorbance of a protein solution is negligible if any aromatic amino acids are present (Wetlaufer, 1962). Also, light scattering and any other solution components which lack the sharp vibronic features of aromatic amino acids have a negligible contribution to second order derivative spectra (Mach et al., 1989).

The potential applications of second derivative spectroscopy extend well

Figure 2.

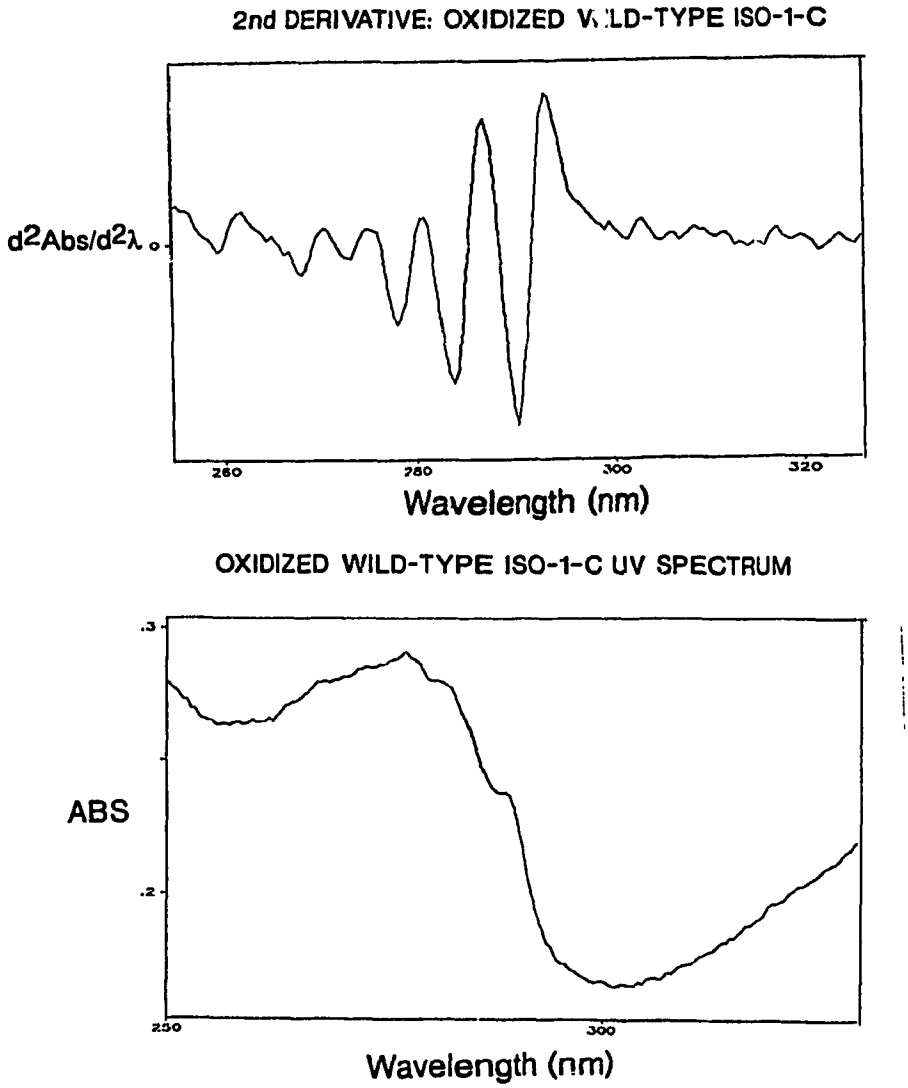


Figure 2. Oxidized wild-type iso-1-c second derivative profile (above) converted from its absorption spectrum (below).

Figure 3.

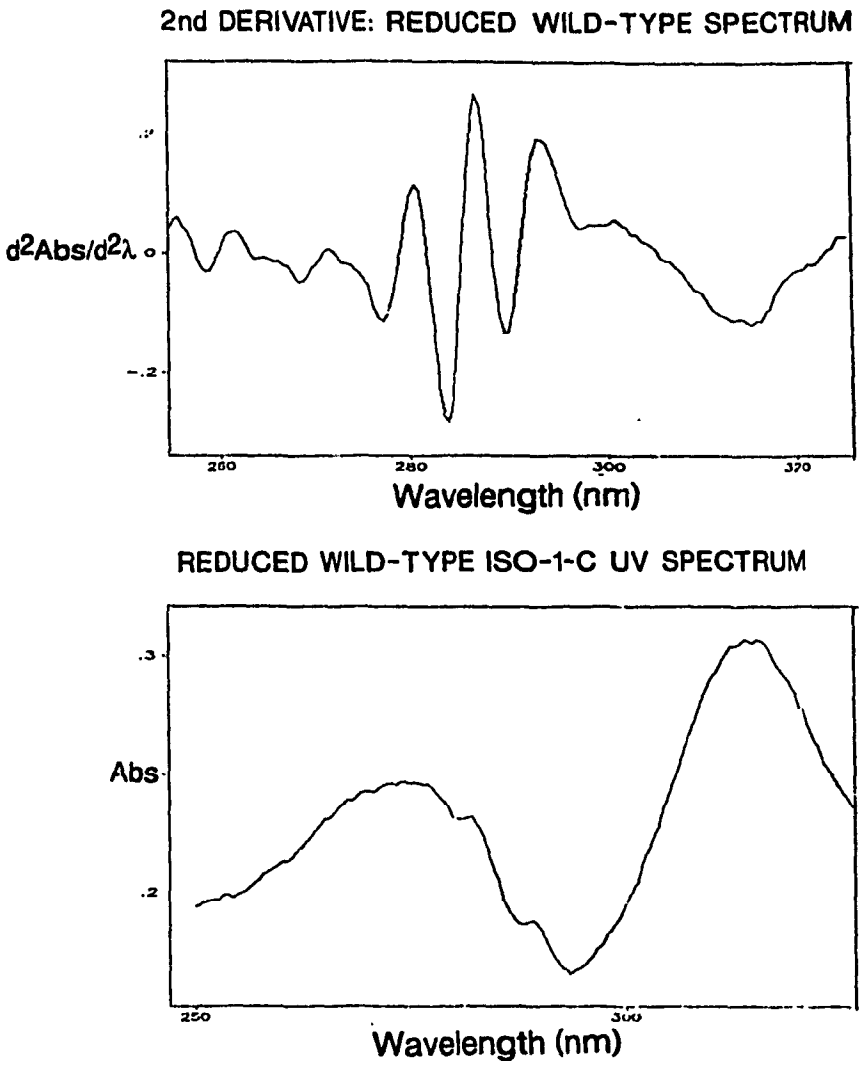


Figure 3. Reduced wild-type iso-1-c second derivative profile (above) converted from its absorption spectrum (below).

beyond the aromatic amino acid determination and calculation of tyrosine exposure. The technique has the potential for being used to monitor protein denaturation (this work), conformational states, measuring aggregation of molecular species, or as a probe for the exclusion or inclusion of solvents upon substrate binding. Indeed, Fischer and Sligar (1985), have shown that one tyrosine residue is linearly correlated with the percentage of ferric high spin cytochrome P-450$_{cam}$. Mach et al. (1991), analysed the kinetics of changes in the second derivative spectra bands of phenylalanine and tyrosine residues to obtain the conformational stability of Cu Zn-superoxide dismutase, the apoprotein, and its zinc substituted derivatives. Thus, the potential applications for this technique seem only to be limited by the imagination.

1.8. Protein Denaturation and Preferential Interactions with Solvent Components.

The three dimentional structure of a protein is determined by a variety of factors and is influenced by a delicate balance between stabilizing interactions and destabilization due to the loss of conformational entropy of the folded protein (Mathews, 1987). Forces that can stabilize protein structure include electrostatic interactions, dipole induced forces, Van der Waals interactions, hydrogen bonds and hydrophobic interactions. Electrostatic forces are due to charged interactions of molecules. The change in the energy between two charges: $\Delta E = Z_a Z_b \varepsilon^2 / D r_{ab}$ where Z is the charge on each atom, ε is the charge on an electron, D is the dielectric constant and r is the distance between the two charges (Creighton, 1984). The equation as written is only valid in the limit of distances greater than atomic radii and assumes a point charge model. Another

difficulty with this equation is that the dielectric constant in the interior of a protein between two charged groups may vary widely and is difficult to account for.

Absolute net charges are not needed to cause stabilization or destabilization effects. Partial charges of opposite sign can lead to stabilization (at an appropriate distance). These are called dipole forces. Electronic dipoles are set up with atoms having different electronegativities, partially inducing a negative charge at one end of the molecule and a slight positive charge at the other. These dipoles can interact with other dipoles or with point charges. To summarize, between ions with net charges, the energy falls off as 1/Dr, between randomly oriented permanent dipoles the energy falls off as $1/Dr^6$, between an ion and a dipole induced by it, the energy falls of as $1/Dr^4$ and between a permanent dipole and a dipole induced by it, the energy falls off as $1/Dr^6$ (Fersht, 1985). Again, calculations of this sort are usually hampered by the unknown dielectric constant between the different regions of the protein that are interacting.

Van der Waals or London Dispersion forces are the small attractive forces that are a result of atoms being close to one another. It is often represented by a potential energy function of distance that includes the attractive force and another term for repulsion at short range (Creighton, 1984). The total potential energy can be calculated as follows:

Potential Energy= $A/r^{12} - B/r^6$

A is the Van der Waals radius, and B can be calculated from the Slater-Kirkwood equation. This equation takes into account the polarizability and the number of outer sphere electrons and is not discussed here. The inverse twelfth power is due the repulsive potential of two atoms that approach so closely that their electron clouds begin to interpenetrate (Fersht, 1985). The distance dependence of $1/r^6$ is due to the attractive dispersion forces that result from the mutual induction of electrostatic dipoles (Fersht, 1985). Although individual van der Waal

forces are quite weak, when they are summed over the entire protein surface, a significant contribution to protein stabilization may ensue (Fersht, 1985).

Hydrogen bonds occur when two electronegative atoms compete for the same hydrogen atom. The length and strength of a hydrogen bond depends primarily on electronegativity of the donor and acceptor group (Creighton, 1984), and on the orientation of the bond. Linear hydrogen bonds are usually stronger, but bending only causes small energy losses (Fersht, 1985). The distance at which hydrogen bonds form is usually around 2Å and gives rise to a stabilization of approximately 2 to 10 Kcal/Mol at room temperature (Creighton, 1984; Hagler, et al.,1974). Good hydrogen bond donors are of the order: N-H, O-H,>>>S-H>C-H while acceptors are: O=,-O-,-N=>>>-S-,-S- and π electrons of aromatic groups (Creighton, 1984).

Hydrophobic interactions provide one of the greatest contributions with regards to protein stability. Non-polar atoms do not interact favorably with polar solvents such as water and tend to coalesce among other nonpolar residues to minimize the contact with solvent. This is the reason why apolar amino acids are predominantly found buried in the interior of proteins.

If all of these forces are capable of contributing a net stabilizing effect to a protein. How is it then, that the the addition of thermal energy (an increase in temperature) or that the addition of some solvents is capable denaturing proteins? The answer to thermal unfolding is distinct from that of chemical unfolding and will be dealt with separately. With regards to chemical denaturation, the answer lies in how the denaturant molecules interact with the protein of interest in solution. The following description presents a rigorous analysis of how guanidinium chloride and other cosolvents act as a protein structures. These ideas are taken directly from the thermodynamic analysis presented by Timasheff, (1993). Readers interested in a more in-depth look into the

thermodynamics of solvent solute interactions are directed to this reference.

It is generally known that the denaturation of a protein by urea or by guanidinium chloride is accomplished through the direct binding of the denaturant to the protein. Tanford (1964, 1970), has shown that the thermodynamic facilitation of protein unfolding by denaturants is due to the free energy changes that accompany the transfer of the protein in the native and denatured from water to the denaturant solution. The thermodynamic control of equilibria by cosolvents can be described by three related thermodynamic parameters: the transfer free energy, the preferential interaction parameter and the preferential binding parameter.

The transfer free energy ($\Delta\mu_{2,tr}$) is defined by Timasheff to be the change in the interactions of the protein with the solvent system when it is transferred from pure water to the cosolvent system:

$\Delta\mu_{2,tr} = \mu_{2(cosolvent)} - \mu_{2(water)}$ where μ_i is the chemical potential of the ith component. The chemical potential is defined as:

$\mu_i = \mu_{i^o(P,T)} + RT \ln a_i$ where a_i is the activity of the ith component,

μ_{i^o} is the standard state chemical potential of the ith component at constant temperature and pressure. The activity is defined as:

$a_i = m_i\gamma_i$ where m_i is the molar concentration of the the ith component and γ_i is the activity coefficient. The subscripts are all presented in Scatchard notation. 1 represents the bulk solvent or water; 2 represents the protein; 3 represents the cosolvent or the solute added to the system. This notation will be used throughout the paper.

The preferential interaction parameter defines the perturbations of the chemical potentials of protein and cosolvent. The perturbation in the chemical potential of the protein is equal to the perturbation in the chemical potential of the cosolvent. Thus $(\partial\mu_2/\partial m_3)_{T,P,m2} = (\partial\mu_3/\partial m_2)_{T,P,m3}$. Timasheff (1993), further states

that is the gradient of transfer free energy with cosolvent concentration and the transfer free energy of the above partial differential equation by the following formula:

$$\mu_{2,tr} = \int_{0}^{M_3} (\partial\mu_2/\partial m_3)_{T,P,m2}\, dm_3.$$

The preferential binding parameter $(\partial m_3/\partial m_2)_{T,P,\mu 3}$ measures the amount of cosolvent that would have to be added to restore thermodynamic equilibrium when an infintesimal amount of protein is added.

$$(\partial m_3)/(\partial m_2)_{T,P,\mu 3} = - (\partial\mu_2/\partial m_3)_{T,P,m2} / (\partial\mu_3/\partial m_3)_{T,P,m3}$$

To think of denaturation using Tanford's model (1970) of transfer free energy, the standard free energy change of the reaction (ΔG^o) when the solvent is transferred from water to cosolvent is equal to the change in the chemical potential of the protein over the course of the reaction (ie. $\Delta G^o = \Delta\mu_{2,tr}$). Experimental results show that a lowering of the transfer free energy (between the denatured and native states) is responsible for the denaturation of proteins, whereas an increase yields protein stabilization. Typically, all protein denaturants bind preferentially to the denatured protein whereas protein stabilizers such as glycerol and certain sugars are preferentially excluded. An example of a denaturant and a stabilizer on the transfer free energy changes is shown in Figure 4. The illustration in Figure 4 shows that the free energy changes between the final and initial states is lowered for a denaturant as compared to a stabilizing compound. In the case of the denaturant, the protein need not be fully denatured to exhibit this decrease; thus, the interaction of the denaturant with the protein can be thought of as having lowered the free energy of the latter. If it were possible to view these plots after equilibrating in increasing amounts of denaturant, these levels would change based on the amount of denaturant present. The effect of solvent composition on the equilibrium constant and its relation to the preferential interaction parameter during the reaction is defined through the Wyman linkage relation:

$$\frac{\partial \ln K}{\partial \ln a_{3\,T,P,m2}} = \frac{(\partial \mu_3/\partial m_2)^R_{T,P,m3} - (\partial \mu_3/\partial m_2)^P_{T,P,m3}}{(\partial \mu_3/\partial m_3)_{T,P,m2}}$$

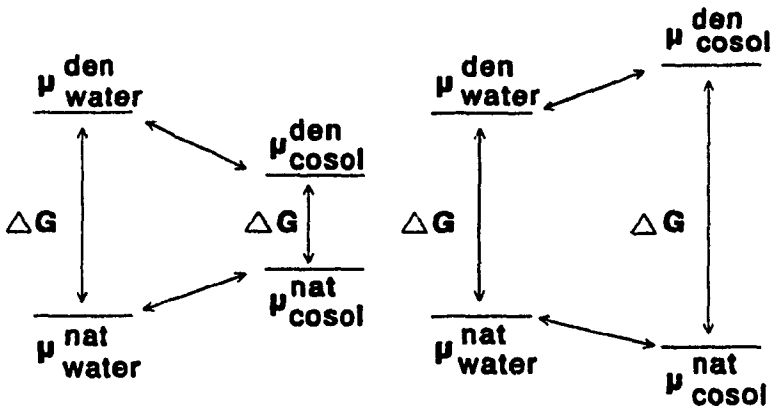
Where the superscripts R and P denote reactants and products, respectively. The subscripts denote performing the operation at constant temperature, pressure and mass of component. Combining the Wyman linkage relationship with the preferential binding parameter shows that the change in preferential binding during the course of the reaction is equal to the slope of the Wyman plot: $(\partial \ln K/\partial \ln a_3)_{T,P,m2} = \Delta(\partial m_3/\partial m_2)_{T,P,\mu 3}$

As a general rule, protein denaturants are preferentially bound to denatured proteins (Timasheff, 1992, 1993). This is in contrast to agents which stabilize protein structure. Solvents such as glycerol and certain sugars are preferentially excluded from the domain of the protein (ie. $(\partial m_3/\partial m_2)_{T,P,\mu 3}$ is negative) and as such, lead to an enhancement of stability (Gekko and Timasheff, 1981a,b; Arakawa and Timasheff, 1982a,b). An interesting twist to this line of reasoning is that many sugars and polyols are preferentially excluded from the domain of the protein yet do not stabilize protein structure. In fact, many of these compounds function as salting-out agents. This illustrates the complexity involved in understanding solute-solvent interactions.

The mechanism of thermal unfolding is dissimilar to the mechanism of chemical unfolding. As the temperature of a protein solution increases, intraprotein, protein-solvent and bulk solvent hydrogen bonds are broken and reformed (Hickey et al., 1988). Also, specific intraprotein electrostatic interactions are disrupted and replaced by solvent dipole-partial ionic charge attractions (Hickey, 1988). Thus a favorable enthalpy change from hydrogen bond formation drives thermal unfolding whereas the unfavorable entropy change due to a more ordered solvent structure dominates chemical unfolding (Hickey et al., 1988). Due

Figure 4.

THE EFFECTS OF COSOLVENTS ON THE FREE ENERGY OF FOLDED PROTEINS



LEGEND

Nat=Native State
Den=Denatured State
μ =Chemical potential of protein
△G=Free energy change

Figure 4. An illustration of how two different cosolvents (guanidinium chloride, left and 40% glycerol, right) affect the free energy of folded proteins.

to these differences, one should not expect free energy values determined by both these methods to be similar. However, the same relative trends should be observed when comparing a group of closely related proteins.

1.9. Oxidation-Reduction Potential Theory:

Many theories have been proposed to explain changes in the midpoint potential of a variety of cytochrome proteins, yet all fall short when tested experimentally by protein engineering techniques.

Early studies by Kassner (1972), suggested that oxidation reduction properties of cytochromes are determined primarily by the the polarity of the heme environment. This hypothesis was based on a method to measure redox potentials in non-aqueous solutions. Kassner found that as the polarity of the solution increased, the midpoint potential of the iron-porphyrin complex decreased and vice versa. Thus, the characteristics of the amino acid side chains that surround the heme iron and form part of the heme crevice should modulate the midpoint potential of the protein. Additional evidence for this hypothesis exists. For example, it has been showed that the potentials of other *c* type cytochromes from plants (Davenport, 1952), algae (Katoh, 1959), and bacteria (Kamen, 1955) are higher than that of iso-1-cytochrome *c* yet all have the same ligands as suggested by NMR spectroscopy (Krejcbek et al., 1971). Also, large cytochromes such as cytochrome *c* and cytochrome c_2 consist of 100-130 amino acids wrapped around the heme to completely envelope it except for a hydrophobic edge of the heme porphyrin ring (Takano, 1981a,b; Salemme, 1973). These cytochromes have midpoint potentials which are higher than those of small type cytochrome proteins that leave their heme group more exposed (ie. cytochrome *c551* from *Pseudomonas aeruginosa*).

Oxidation-reduction studies by Stellwagan (1978), seem to contradict those of Kassner, as it was shown that six heme proteins with almost identical heme apolarities had midpoint potentials that varied from 20 to 320mV. Instead, Stellwagan found that the E'_o value varied inversely with the exposure of the heme to the aqueous solvent according to the equation:

E'_o=-15(% exposure of heme) + 345mV

Thus, according to this equation, the midpoint potentials could vary from -1.155mV (maximally exposed) to 345mV when fully buried.

Apart from focussing on the polarity of the heme environment and its exposure, other studies have focussed on the heme propionate side chain. Moore (1983), proposed that the electrostatic interactions between a propionate side chain of the heme and a positive charge on the ferric iron assists in determining the midpoint potential and plays important roles in stabilizing conformational states. They further suggest that the differences in redox potentials between homologous cytochrome structures is due to differences associated with the the charge on the heme propionates. This theory was based on electrochemical experiments that measured midpoint potentials as a function of pH. These experiments suggested that ionizable groups, probably the heme propionate groups were responsible for the change in potential of the protein.

On closer scrutiny, Moore and Williams (1979), proposed that five main factors control the midpoint potential of a protein:

1) The electrostatic charge of the ligand binding the heme. The higher the negative charge, the lower the reduction potential. 2) The donor power of the ligand binding the heme iron as accessed by pKa values. The higher the pKa, the lower the redox potential. 3) The ability of the ligand to function as an acceptor. This uses unsaturation as a guide to acceptor efficiency. For example, the greater the π acceptor power, the higher the redox potential. 4) Changes in the

spin state of the central metal. This factor can infuence factors 1 and 2 above. 5) Steric factors that may favor a particular oxidation state or a particular spin state of the central metal. Some of these proposals were made from the observations that proteins containing a particular porphyrin type which had two axial histidine or methionine ligands have lower midpoint potentials than proteins containing mixed ligands (Lemberg, 1973; Dickerson and Timkovitch, 1975; Mathews, 1972). According to the theory above, this should be expected since nitrogen is a better electron donor than sulfur and sulfur a better π electron acceptor than nitrogen. Also corroborating factor 2, it was shown that as the redox potentials of the protein increased, the chemical shifts (as determined through NMR spectroscopy) for the methionine methyl and the γCH resonances increased whereas that of the ßCH group decreased. These shifts were reported to be due to the ring current shifts of the heme group and indicated a shift in the Fe-S bond length. The investigators go on to show that a 0.1Å shortening of the Fe-S bond length (corresponding to a maximum 15% variation in the ring current) could cause a decrease in the redox potential of approximately 400mV. Thus the increase in the donor power of the ligand tends to stabilize the ferric iron and cause a decrease in the midpoint potential. Finally, there is a line of evidence that suggests that the midpoint potential depends on the effective net dielectric constant of the protein. The effective dielectric constant between two charges (q1 and q2) is equal to their product divided by the product of their electrostatic energy and their effective separation. In a similar approach, Churg and Washel (1986), have performed calculations by considering the protein matrix as a type of solvent for the heme iron and then calculated the difference between the electrostatic energy of the reduced and oxidized heme.

This background information is essential to understanding the results and discussion of this thesis, as all are inextricably linked the observed properties of

the protein in solution.

Chapter 2
Method and Materials

The site-directed mutagenesis protocols for the iso-1-cytochrome *c* protein are presented in greater depth elsewhere (McOdimba, 1994).

2.1. Growth of Yeast:

Yeast cells carrying a multicopy plasmid of the iso-1-cytochrome *c* gene were grown for two days at 30 degrees with agitation in 4ml of YPG medium (also containing 100μl/ml ampicillin) and then transferred to two 500ml flasks containing approximately 150ml of YPG medium. These cultures were grown for another two days with shaking at 30 degrees. These cells were then used to inoculate a 15L culture of YPG medium. All media were autoclaved prior to use to minimize contamination. At the time of inoculation of the 15L culture, tetracycline (140mg in 7ml 90% ethanol) and streptomycin (560mg in 7ml distilled water) was added to help control bacterial contamination. The streptomycin was sterile filtered through a Nalgene filter unit. Also, 1ml of sterile antifoam was added to control foaming. The 15L culture was grown for two days in a 20L carboy fermenter at 30 degrees with stirring and aeration. Air was passed through a sterile glass wool filtering unit before being delivered into the culture. The air flow was adjusted so that the culture bubbled vigorously. After two days of growth, another batch of sterile antibiotics, antifoam and lactic acid (170mL 85%w/w lactic acid and 170mL distilled water brought to pH 5 with solid sodium hydroxide pellets) was added and the culture was grown for another two days.

Yeast cells containing the N52I, N52I Y67F, Y67F and N52A mutations were provided by Dr. Guy Guillemette.

2.2. Isolation and Purification of Cytochrome *c*:

Throughout the entire purification scheme all solutions and suspensions were either kept on ice or at 4°C.

The yeast cultured was transferred to preweighed centrifuge bottles and centrifuged at 3000rpm for 10 minutes in an IEC DPR 6000 centrifuge with a swinging bucket rotor. The supernatant was discarded and the yeast pellet weighed. To this was added 0.5 volume(ml)/mass(g) of 1M NaCl (in 12.5mM Kpi,pH 7) and 1/4 volume to mass of ethyl acetate. Phenylmethylsulfonylfluoride (PMSF) was made up in 90% ethanol and added to the pellet containing solution (final concentration 0.3μg PMSF per ml suspension). Similarly, EDTA was added to an equivalent concentration. Finally, 1ml of 6M 2-mercaptoethanol was added. The resulting solution was stirred vigorously for approximately 20 hours. After allowing the cells to lyse, the suspension was centrifuged at 10000rpm for 15 minutes in an IEC B20A centrifuge equipped with a fixed angle rotor. The supernatant was decanted through cheesecloth and kept on ice. The yeast pellets in the centrifuge bottles were then broken up with a glass rod, washed and resuspended with a small amount of 1M NaCl and recentrifuged. If the supernatant had any trace of pink color, it was pooled with the previous fraction. Ammonium sulfate was then added slowly to 50% (0.337g/ml of supernatant). The solution was then centrifuged (10000rpm, 10minutes) and the supernatant again filtered through cheesecloth. The resulting solution was transferred to several dialysis bags (Spectra/Por MW Cut-off 6-8000, diameter 14.6mm) that were previously soaked in distilled/deionized water for approximately 10 minutes. The solutions were dialysed over a period of 48hours against 4X7L changes of 1mM EDTA, 1mM 2-mercaptoethanol, 12.5mm potassium phosphate buffer, pH7.

After checking the osmotic potential of the dialysate with a osmometer, the cytochrome solutions were chromatographed through a large DEAE Sephacel (Pharmacia Chemicals) column in series with a small CM Sepharose column (Cl-6B). After binding, the cytochromes were washed with an excess of 12.5mM potassium phosphate buffer, pH7. Cytochrome *c* was eluted from the CM column with 12.5mm Kpi, 500mM NACL, pH 7. The protein was subsequently repurifed by HPLC (BioRad) in the following manner. The protein was diluted with distilled water until its osmolarity was less than 75mOsm. This protein was then applied to the CM column and washed with approximately 100ml of 12.5mM Kpi pH 7 that had been distilled/deionized, filtered and degassed. The protein was then eluted in a linear gradient (0mM to 500 mM NaCl in 12.5mM potassium phosphate buffer pH7) over a 100 minute time period (flow rate: 1ml/min). The elution was monitored at 280nM to check for impurities. In all cases, none was evident. Fractions with an optical density greater than 0.1 at 280nM were pooled. As a further test of purity, the ratios of the Soret band to the absorbance at 280nM was calculated and compared for proteins with the same tyrosine/tryptophan ratio in both the oxidized and reduced states. A typical spectrum of wild-type Iso-1-*c* showing the Soret band and other spectral features is presented in Figure 5.

Dr. Guy Guillemette provided provided purified F82Y, W59F, Y48F W59F, and Y48F cytochrome proteins. All contained the C102T background mutation to prevent dimerization (as do ours). The horse porphyrin *c* protein was prepared by Dr. Jack Kornblatt.

2.3. Absorption and Second Derivative Spectroscopy:

All spectra were recorded on a Varian Cary 2290 UV-VIS Spectrophotometer. Temperature was maintained at 20±2°C with a Brinkmann

Figure 5.

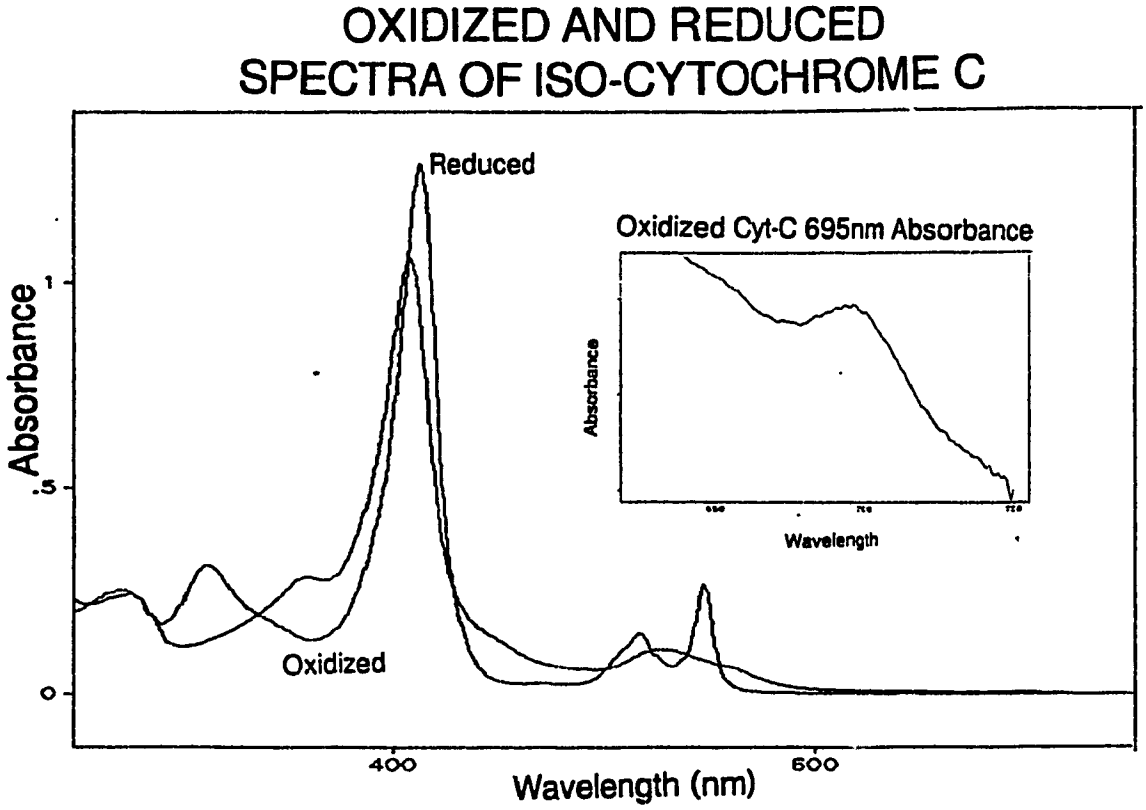


Figure 5. Oxidized and Reduced spectra of Iso-1-Cytochrome c. Inset: An expanded view of the oxidized 695nm peak caused by methionine 80 ligation to the heme iron.

Rauda RC3 circulating water bath. The spectrophotometer baseline was recorded from 750nm to 250nm using buffer (12.5mM potassium phosphate, 500mM NaCl, pH7) and the following parameters: Scan rate=0.5nm/sec; spectral bandwidth=0.5nm; response time=1sec. Prior to collecting spectra, cytochrome solutions were oxidized with a few small crystals of potassium ferricyanide, and applied to a pasteur pipette containing a small amount of CM Sepharose. The column containing the bound protein was then washed with buffer (12.5mM potassium phosphate pH7) and then eluted with 12.5mM potassium phosphate, 500mM NaCl, pH7. Two millilitres was then transfered to a Hellma quartz glass cuvette (4ml, 1cm light path). The initial cytochrome spectrum was recorded from 750nm to 250nm at 5nm per second and a sampling interval of 1nm (all other parameters remain constant). After this, 10 independent collections were performed from 330nm to 250nm using a scan rate of 0.5nm per second and a sampling interval of 0.5nm. Finally, the entire cytochrome spectrum was run again to ensure that there were no changes in the oxidation state of the protein. This was checked by two criteria. The first was that the maximum absorbance of the Soret band did not change in its wavelength position. The second was that the ratio of the Soret to the 550nm absorbance for the oxidized and reduced protein did not exhibit a variation greater than 5% when comparing these values after the second derivative calculations and before it. The same method was used to examine the reduced forms of the cytochrome solutions except that a few crystals of sodium dithionite was added instead of ferricyanide. Also, because of the tendency of the reduced proteins to become oxidized, two sets of five collections from 330nm to 250nm were used instead of the previous 10. Second derivative spectra were obtained by transforming the ten ultraviolet absorption collections with a built in second derivative function (using nineteen convolution points) from the spectrophotometer control program SpectraCalc®.

The porphyrin *c* protein was prepared in the same way except that no ferro or ferricyanide was added. Also, the chromatography was done in the dark to minimize destruction of the sample. The sample was exposed to light for only as long as needed to obtain the measurements.

The second derivative spectra for the oxidized proteins with cyanide were performed exactly as outlined before with the following exceptions: The concentration of cytochrome protein was such that the absorbance for the charge transfer band (695nm band) was approximately 0.06. The absolute spectrum was initially recorded from 750nm to 250nm using 2 mL of cytochrome solution. After this collection, 2 mL of freshly prepared cyanide solution (0.2M KCN in 12.5mM KCN, pH7 (prepared in fume hood) was added and allowed to incubate for a five minute period before measurements were taken. The entire spectrum was then rescanned. The binding of cyanide to the heme crevice was unsuccessful if there was evidence of a 695nm peak and a lack of any change in the second derivative spectrum. For cytochromes with their 695nm bands shifted, measurements were taken at the peak of this band.

2.4. Ionic Strength, pH, Polarity and Cosolvent Effects:

The effects of ionic strength were studied by dialysing HPLC purified yeast iso-1-cytochrome *c* in a fifty fold excess of the desired buffer at 4°C overnight. The buffers included 12.5mM potassium phosphate pH7, 100mM potassium phosphate pH7, 12.5mM potassium phosphate-100mM NaCl pH7, 12.5mM potassium phosphate-1M NaCl pH7, and 100mM potassium phosphate-1M NaCl pH7. Specta for oxidized and reduced cytochrome *c* were obtained as previously described. The experiment was repeated with the same buffers except at pH 6.

The effects of bulk solvent polarity on the second derivative spectrum of

wild-type iso-1-cytochrome *c* was assayed by incorporating glycine into the buffer solutions (see discussion for the effects of how glycine effects the polarity of solutions). The standard buffer compositions included 0.1M potassium phosphate buffer, 500mM NaCl with 0.5M, 1M, 1.5M and 2M glycine, respectively. Second derivatives were recorded at pH 6 and 7 as previously outlined.

The effects of a cosolvent (glycerol) were examined by dissolving wild-type iso-1-cytochrome *c* in potassium phosphate buffer such that the final glycerol concentrations were achieved: 10%, 15%, 20%, 25%, 30%, 35% and 40% volume/volume glycerol. Volumes were obtained by weighing appropriate amounts of glycerol (density of 100% glycerol is 1.126g/ml (CRC Handbook (1977)). All buffers were standardized at pH 7 at the appropriate ionic strength such that the dilution by glycerol ended in a final concentration of 12.5mM potassium phosphate buffer.

2.5. Guanidinium Chloride Denaturation:

Oxidized cytochrome *c* with a minimum absorbance of 0.5 at 280nm was prepared as previously described. Two millilitres of this protein solution was placed in a quartz cuvette and aliquots of 6M guanidinium chloride, 12.5mM potassium phosphate, pH7 were added. Five spectra were collected after each addition of guanidinium chloride, converted to second derivative spectra and averaged. Additions were continued until there was no appreciable change in the peak to trough ratios of the second derivative spectra. Denaturation curves were obtained by plotting the fraction of denatured protein versus guanidinium chloride concentration.

2.6. Thermal Denaturation of Cytochrome Solutions:

Thermal denaturation of cytochrome solutions was performed by monitoring the changes in 695nM absorbance as a function of temperature. Temperature was monitored with a digital thermometer that measured the temperature of an adjacent cuvette jacket of the spectrophotometer. Two millilitres of oxidized cytochrome solution (approximate absorbance of 0.04 at 695nM) was initially equilibrated at 6°C. The temperature was then increased in small intervals, and the cytochrome solutions were allowed to equilibrate at each temperature for five minutes before the absorbance reading was taken. Denaturation curves were obtained by plotting the 695nM absorbance versus temperature.

2.7. Midpoint Potentials:

Midpoint potentials were determined by the method of mixtures (Wallace et al., 1986). Approximately 4ml of purified and concentrated N52V Y67F, N52I Y67F iso-1-cytochrome *c* and horse heart cytochrome *c* were dialysed overnight at 4°C in 1L of 40mM potassium phosphate buffer, pH7. The solution was allowed to come to room temperature before the proteins were removed from their dialysis bags. Once this was satisfied, the cytochrome solutions were removed from their dialysis bags, diluted to 20μM using the dialysate buffer, and thoroughly degassed before use. Prior to this, 422mg of potassium ferrocyanide ($K_4(Fe(Cn)_6 3H_2O$) and 329mg of ferricyanide ($K_3(Fe(Cn)_6)$) were weighed and dissolved separately in 10 ml of degassed nano-pure water. A 100μL aliquot of each ferricyanide containing solution was then mixed with a 900μl aliquot of the 20μM cytochrome solution and the absorbance was measured at 550nm in a

previously zeroed spectrophotometer. To the ferrocyanide-cytochrome c containing solution, 5μl of the ferricytochrome-cytochrome c containing solution was added. Again the absorbance at 550nM was measured. Further additions of 5, 2.5, 2.5, 5, 5, and 5μl were made and the the absorbance measurements were taken. The midpoint potentials were calculated by plotting the log of the ferrocytochrome c/ferricytochrome c ratio against the log of the ferro/ferricyanide ratio and extrapolating back to zero, the midpoint of the ferrocytochrome/ferricytochrome ratio. This value allows for a determination of the potential using the expression $E=E^{o'}-0.06$ log [ferrocyanide/ferricyanide], where $E^{o'}$ for the ferri/ferrocyanide couple was taken to be +0.43V.

Chapter 3
RESULTS

3.1. Protein Denaturation:

Guanidinium chloride denaturation of three iso-1-cytochrome proteins (Wild-type, Y48F and N52V Y67F) was followed by second derivative spectroscopy. Results depicted in Figure 6, show a two state denaturation for each protein. The fraction of protein unfolded was obtained from the absolute derivative values using the following formula:

$$\text{Fraction Unfolded } (f_u) = (X-X_n) / (X_d-X_n)$$

where X_n and X_d are the limiting values of the second derivative R value for the native and denatured state respectively, and X is the R value at any particular guanidinium chloride concentration. From these values, the unfolding equilibrium constant was calculated:

$$K_u = f_u / (1-f_u)$$

Thermodynamic analysis for a two state system (Schellman, 1978) indicates:

$$\text{Ln } K_u(C_3) = \text{Ln } K^\circ_u - \Delta b^\circ_{23} C_3.$$

With $\Delta G = -RT \text{ Ln } (K_u(C_3))$, the above expression becomes:

$$\Delta G_u(C_3) = \Delta G^\circ_u + RT\Delta b^\circ_{23} C_3$$

where

$\Delta G_u(C_3)$ is the free energy of unfolding in C_3 moles of denaturant

ΔG°_u is the free energy of unfolding in no denaturant

R is the gas constant (1.987 cal K^{-1} Mol^{-1})

T is temperature in Kelvin

C_3 is the concentration of guanidinium chloride

Figure 6.

GUANIDIUM CHLORIDE DENATURATION OF ISO-1-CYTOCHROME C's

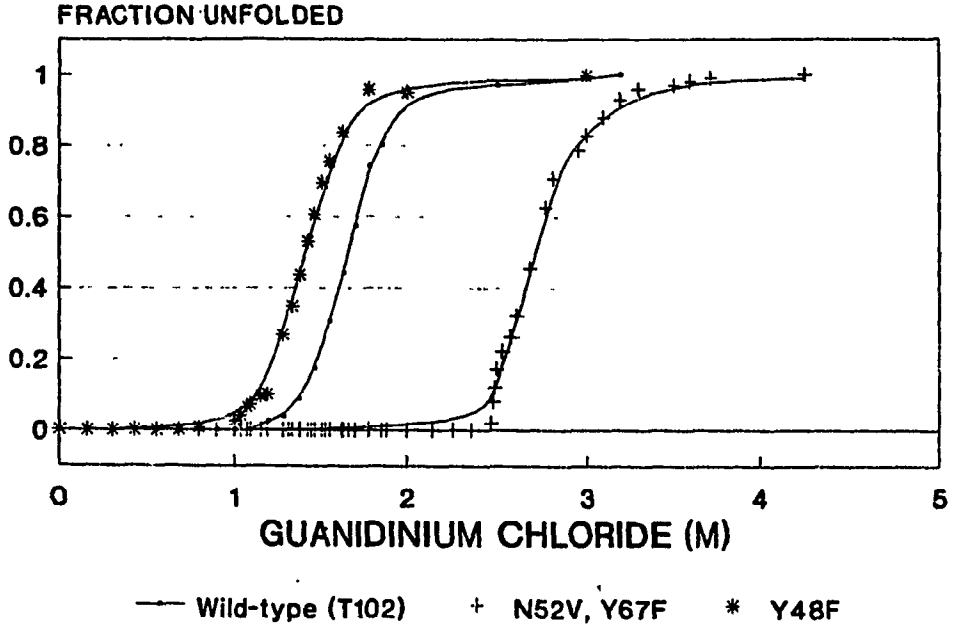


Figure 6. Two state denaturation curves obtained for three iso-1-cytochrome c proteins.

Δb°_{23} measures the cooperativity of the transition and is equal to $-(\partial \ln K_u) / (\partial C_3)_{T,P}$.

ΔG°_u and $RT\Delta b^{\circ}_{23}$ were obtained by plots of $\Delta G_u(C_u)$ versus guanidinium chloride concentration (C_3) using f_u values between 0.2 and 0.8 (Figure 7). The slope and intercept are equivalent to $RT\Delta b^{\circ}_{23}$ and ΔG°_u, respectively. At the mid-point of the denaturation, the ratio of unfolded to folded protein is unity, thus $\Delta G_u(C_3)=0$ Kcal/Mol and therefore

$$C_m = -\Delta G^{\circ}_u / RT\Delta b^{\circ}_{23}$$

The results of such thermodynamic analysis is presented in Table 1. From this data, it is easily seen that the proteins conform to the following order of stability:

N52V Y67F>>>Wild-type>Y48F

Thermal denaturations were performed on the same three proteins by monitoring the decrease in the 695nM absorbance as a function of temperature. Again the order of stability paralleled that obtained for the guanidinium chloride denaturation studies. No thermodynamic analysis was performed on these results because regression analysis was needed to calculate the theoretical absorbance of the unfolded protein at all temperatures (Hickey et al., 1988). At high temperatures, the proteins precipitated and hence, an insufficient number of data points could be collected at the end of the assay (Figure 8).

3.2. Ionic Strength, pH, Polarity and Cosolvent Effects on the Second Derivative:

The ionic strength, pH, polarity and cosolvent dependence on the average R value of wild-type iso-1-cytochrome *c* as determined by second derivative

Figure 7.

FREE ENERGY vs. GUANIDINIUM CHLORIDE FOR ISO-1-CYTOCHROME C's

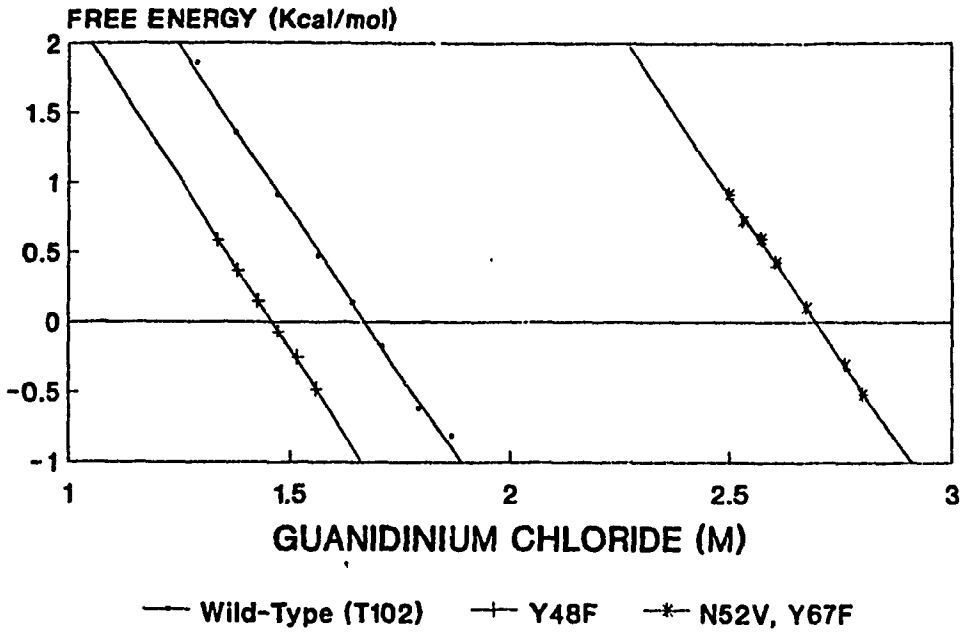


Figure 7. Analysis of the transition regions from the two state transition curves obtained in Figure 6.

THERMODYNAMIC RESULTS OF CHEMICAL DENATURATION

Construct	ΔG°_{u} (Kcal mol^{-1})	C_m (M)	$RT\Delta b^{\circ}_{23}$ (Kcal L/M^2)
Wild Type Iso-1-c	8.2	1.66	4.95
N52V Y67F	12.6	2.67	4.72
Y48F	7.15	1.46	4.89

Table 1. Thermodynamic analysis of the transition zone of three cytochrome proteins. C_m is the molar concentration of guanidinium chloride to reach the midpoint of the transition region. ΔG° is the free energy of unfolding of the proteins. $RT\Delta b^{\circ}_{23}$ shows the cooperativity of the unfolding transitions. Estimated errors are as follows: C_m ±0.1M/L; $-RT\Delta b^{\circ}_{23}$ ±0.1 kcal L/M^2; ΔG°_{u} ±0.5 kcal/M.

Figure 8.

695nM THERMAL DENATURATION OF ISO-1-CYTOCHROME C's

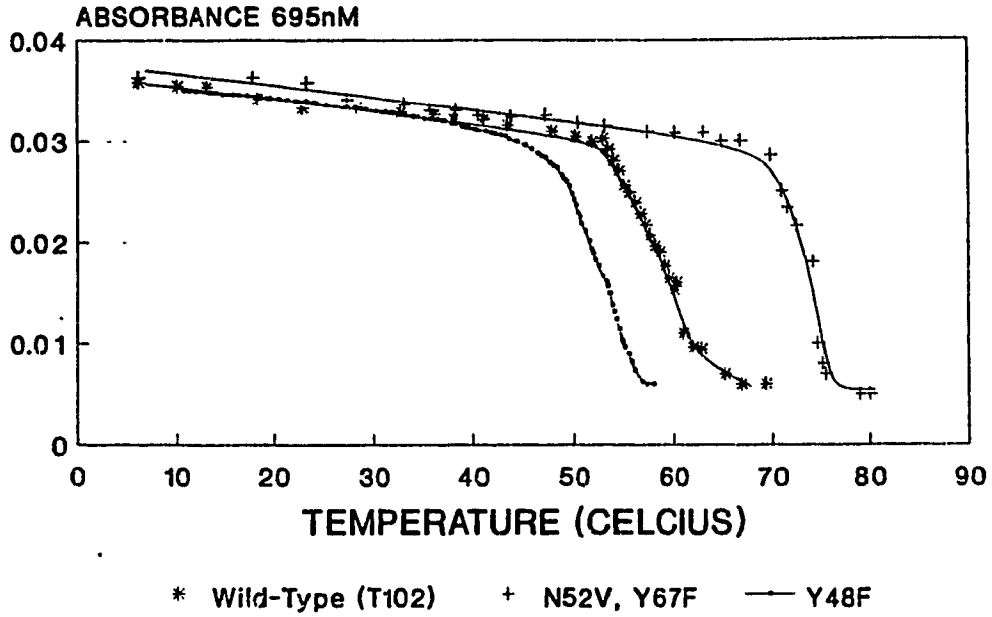


Figure 8. Iso-1-Cytochrome c thermal denaturation measured by size of the 695nm band as a function of temperature.

spectrophotometry is presented in Table 2. Ionic strength did not seem to effect the average R values of the cytochrome proteins in the range studied herein. Also showing no effect was the switch in pH from 7 to 6, and the modulation of the polarity of solution by the incorporation of glycine. Conversely, glycerol did affect the average R value, but only at relatively high glycerol concentrations.

3.3. Second Derivative Spectroscopy of Cytochromes *c*:

The average polarity of the tyrosine microenvironments was measured by second derivative spectroscopy in a series of cytochrome *c* proteins (Table 3). The oxidized and reduced wild-type iso-1-cytochrome *c* spectrum and its corresponding second derivative spectra are shown in Figures 2 and 3, respectively. Similar second derivative measurements were made for the proteins in the presence of 0.1M KCN, an ion which binds in the heme crevice replacing methionine 80 ligation to the heme iron in the oxidized form of the protein. Again, these results are summarized in Table 3. The R values obtained were found to be independent of the concentration of the protein used, although scans of the ultra-violet range with a minimum absorbance value lower than 0.15 yielded increased background noise in the second derivative spectrum. Also, ionic strength of the buffer solutions was not found to alter the R values, nor did changing the buffer pH from 6 to 7 (data not shown). In most cases, the reduced forms of the cytochromes have higher R values than their oxidized counterpart. Also, those with tyrosine 67 intact, showed the greatest change in the R values upon going from oxidized to reduced state. The two mutant proteins W59F and Y48F, W59F do not produce second derivative spectra comparable to the other cytochrome

The Effects of Ionic Strength, Polarity, pH and Cosolvent Addition on the Second Derivative Spectrum

Polarity Effects

Glycine (M)	ΔR (Second Derivative)	ΔR with pH (6-7)
0.5M	no	no
1M	no	no
2M	no	no

Ionic Strength Effects

Buffer Comp.	ΔR (Second Derivative)	ΔR with pH (6-7)
12.5mM Kpi	no	no
100mM Kpi	no	no
12.5mM Kpi 100mM NaCl	no	no
12.5mM Kpi 1M NaCl	no	no
100mM Kpi 1M NaCl	no	no

Effects of Glycerol

Volume Glycerol (% Vol/Vol buffer)	SecondDerivative (R)
10	0.82
15	0.80
20	0.81
25	0.79
30	0.73
35	0.71
40	0.65

Table 2. The effects of polarity, buffer ionic strength, solution pH and cosolvent addition on the second derivative spectrum. Buffer are 12.5mM Kpi, pH 7 unless indicated. All effects are for the wild-type iso-1-cytochrome c protein.

Second Derivative Values for Iso-1-Cytochrome c's

Construct	R (oxidized)	R (Reduced)	R (+0.1M KCN)
Wild-Type	0.81	1.66	1.49
Y67F	1.22	1.31	D.N.B.
N52I Y67F	1.13	1.32	D.N.B.
N52V Y67F	1.42	1.16	1.15
Y48F	0.66	1.48	1.34
F82Y	0.73	1.56	1.41
Horse c	0.73	1.15	0.91
Porphyrin c	0.77*	-	-
W59F Y48F W59F		Cannot be compared	
N52A	0.92	1.23	D.N.B
N52I	1.28	0.8	D.N.B
Wild-Type	0.81	1.66	1.49

Table 3. R values obtained by averaging ten second derivative R values. All cyanide additions were made to the fully oxidized protein. (*) Not actually in oxidized form due to lack of heme iron. (D.N.B) Did not bind in the heme crevice as shown by the retention of the 695nM peak and no change in the average second derivative R value. (-) Were not or could not be determined. The error for the oxidized R values was found to be ±0.04 while that for the reduced values is ±0.1.

proteins. This is due to the removal of the tryptophan amino-acid. They do however, show a second derivative spectra for the average of all the tyrosine amino acids, which shift in response to oxidation state (see figures 9 and 10).

3.4. Midpoint Potential of N52V Y67F:

The midpoint potential of the N52V Y67F iso-1-cytochrome *c* protein was determined to be 244mV. For comparison, the midpoint potentials of horse cytochrome c and N52I Y67F iso-1-*c* were found to be 271mV and 246mV, respectively. All proteins exhibited a slope of approximately one and a high correlation coefficient. The midpoint potential data is presented in Table 4., and is graphically illustrated in Figure 11.

Figure 9.

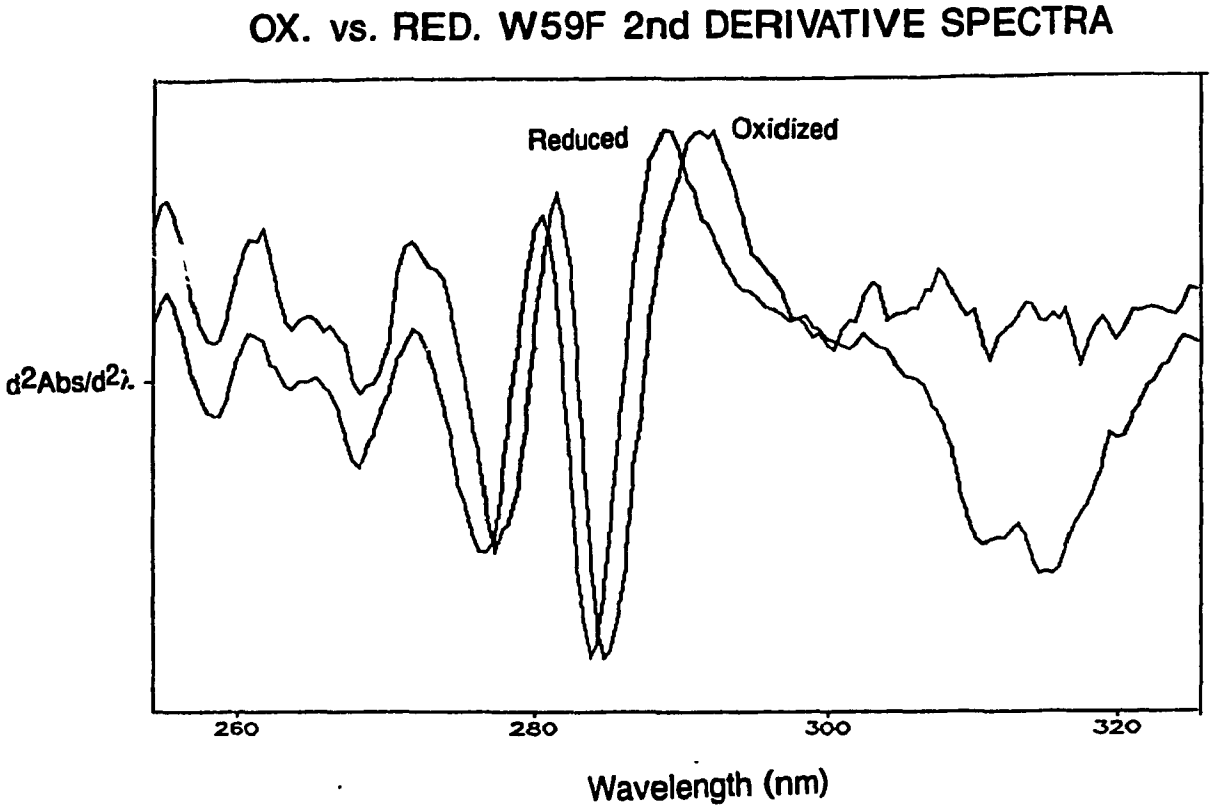


Figure 9. Oxidized and reduced second derivative spectra of W59F. The reduced form is blue-shifted (shifted to lower wavelengths) relative to the oxidized form of the protein.

Figure 10.

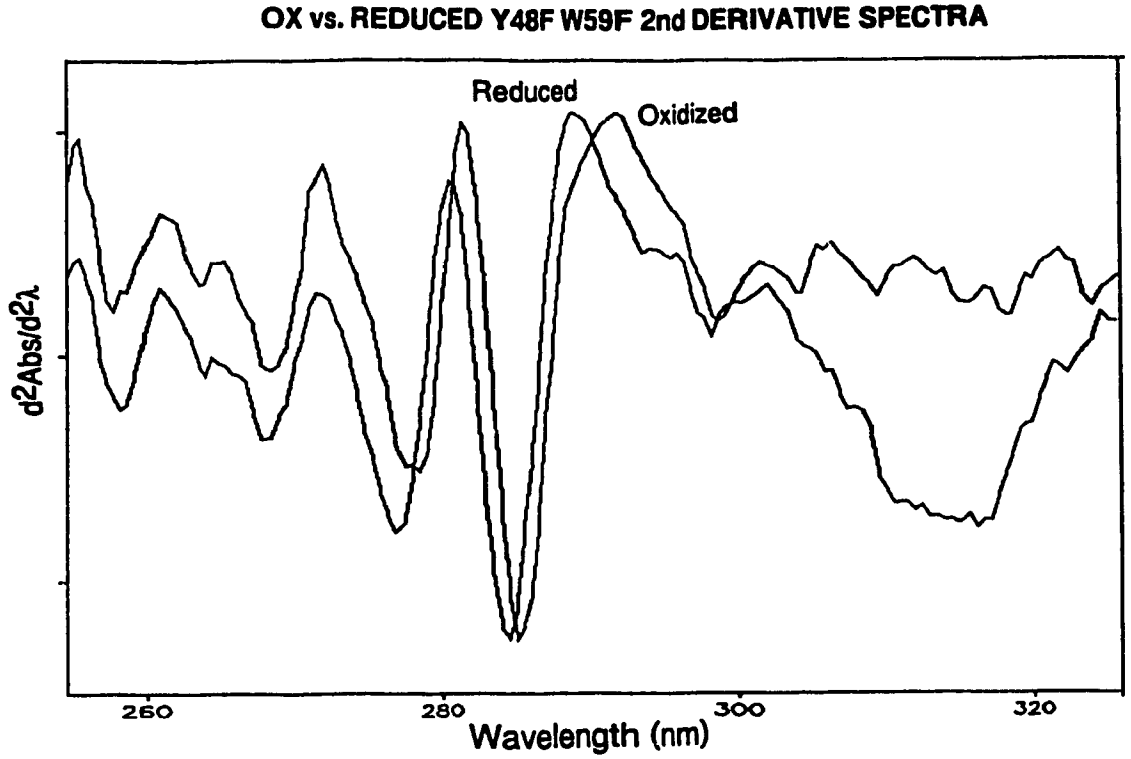


Figure 10. Oxidized and reduced second derivative spectra of Y48F W59F. The reduced spectrum is blue-shifted relative to the oxidized spectrum.

Midpoint Potentials of Cytochromes *c*

Cytochrome c	Midpoint (mV)	Slope	Correlation
Horse	271	1.01	0.99
Horse	273	0.96	0.99
Horse	269	1.06	0.99
N52V Y67F	244	1.02	0.99
N52V Y67F	244	1.01	0.99
N52V Y67F	243	1.04	0.99
N52I Y67F	249	0.95	0.99
N52I Y67F	243	1.02	0.98
N52I Y67F	245	1.01	0.94

Table 4. Midpoint potential data obtained by the method of mixtures. Slope refers to the slope of the data (see figure11.) Correlation refers to the correlation in the data points. Both double mutants are of the yeast iso-1-*c* species.

Figure 11.

MIDPOINT POTENTIALS OF CYTOCHROMES

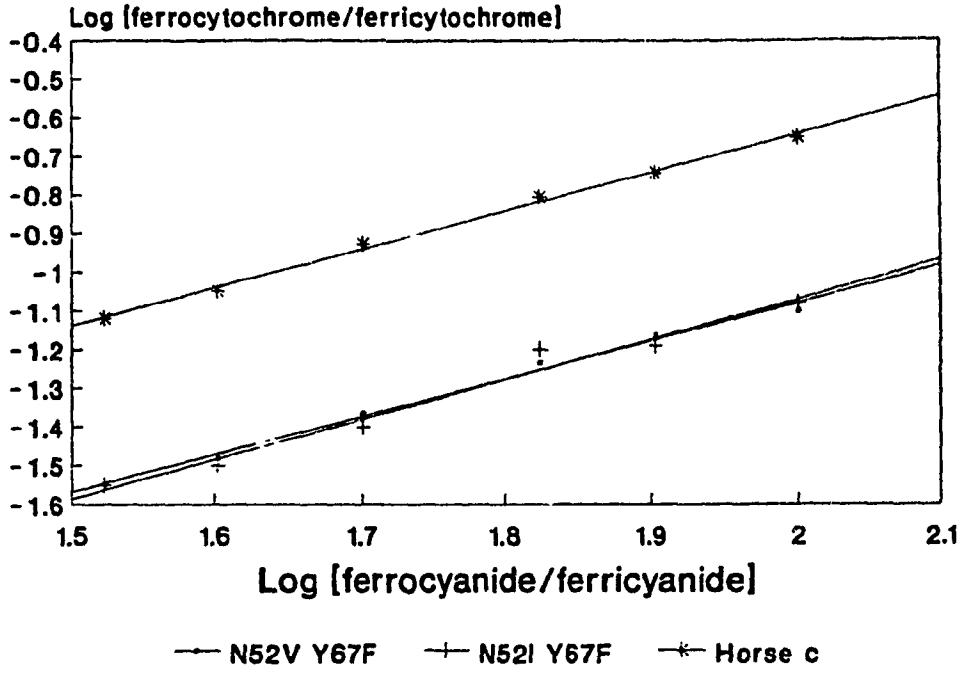


Figure 11. Plots of the log ferro/ferricytochrome ratio versus the log of ferro/ferricyanide ratio for three iso-1-cytochrome c's according to the method of mixtures (see text for explanation).

Chapter 4
Discussion

4.1. Second Derivative Spectroscopy as an Analytical Tool:

Ragone et al., (1988) have shown that the ratio of peak to trough heights in the second derivative spectrum is indicative of the number of tyrosines and tryptophans present and on the polarity of the tyrosine microenvironents. In order to determine whether the R values obtained from the second derivatives are truly indicative of the average polarity of the tyrosine microenvironments, it should be possible to denature the cytochrome proteins with guanidinium chloride and obtain the characteristic two state denaturation curves typically seen in fluorescence, and circular dichroism studies (Hickey et al.,1988; Ramdas et al.,1986; Hickey et al.,1991). Figure 6 shows that three cytochrome c proteins, N52V Y67F, wild type iso-1-*c* and Y48F, all undergo two state denaturations as predicted. The order of protein stability was also as predicted by other denaturation assays using similarly engineered proteins. Studies on the N52I protein (Das et al.,1989; Hickey et al.,1991; Berguis et al., 1994) show a dramatic increase in the thermostability of the cytochrome. Additionally, in the N52I Y67F protein, Guillemette (unpublished results) has shown that the alkaline transition occurs at pH 11 in the N52I Y67F protein, whereas in the wild-type protein the heme iron ligand bond is broken at pH 8.5 (Pearce et al.,1989). Previous to this, Saigo (1986), had shown that the resistance to this alkaline transition is a measure of protein stability. Similarly, temperature, urea denaturation (Luntz et al.,1989) and alkaline transition results (Guillemette, unpublished results) show that the Y67F mutant cytochrome *c* is more stable than the wild-type protein. Thus, it was not surprising that the N52V Y67F shows a similar increase in

stability to the N52I Y67F protein.

The Y48F mutation, on the other hand, was arguably thought to destabilize the protein. In the wild-type protein, tyrosine 48 is thought to be involved in burying the heme propionate A group in the interior of the protein (Davies et al., 1983). Replacement of this tyrosine with phenylalanine would remove the hydroxyl group capable of hydrogen bonding to the heme proprionate. Since it is usually highly unfavorable to bury a charged group in a protein matrix without hydrogen bonding or charge stabilization, this mutation should lead to a less stable protein. This destabilization was confirmed by our second derivative denaturation studies.

The N52V Y67F and Y48F proteins have different initial R values, reflecting changes in polarity due to different tyrosine residues, but on denaturation, yielded R values which lie within experimental error of each other (data not shown). This was expected since these two proteins should have all four of their tyrosine residues in approximately identical environments when they are fully denatured.

Analysis of the transition regions was accomplished by plotting free energy versus guanidinium chloride concentration. The midpoints for the denaturation of the cytochromes were determined to be 1.66M, 1.46M and 2.67M for wild-type, Y48F and N52V Y67F, respectively. This corresponds to ΔG° values of 8.2 Kcal/Mol for the wild-type, 7.15 Kcal/Mol for Y48F and 12.6Kcal/Mol for N52V Y67F. Comparasin of these values to those obtained in other experiments indicate that these values are somewhat high. Denaturation results obtained using fluorescence (Ramdas et al., 1986) have shown that the denaturation midpoint using guanidinium chloride was 1.07M for the wild-type protein, with a respective free energy of unfolding of 3.6 Kcal/Mol. Similar results were obtained by other circular dichroism and fluorescence studies (Hickey et al, 1988).

However, one critical difference that exists between the forementioned studies and our own is that in the other studies, cysteine 102 was reacted with methyl methanethiosulfonate, converting the free sulfhydryl to a -SCH_3 group to prevent dimerization. Our proteins all contain a threonine group instead of cysteine to prevent dimerization. This might possibly explain the increase in stability of our proteins. Indeed, replacement of cysteine 102 with alanine increases the denaturant midpoint by 0.22 M compared to the chemically blocked cysteine and increases the free energy of unfolding to 4.7Kcal/Mol (Hickey et al, 1991). Treatment of the cysteine 102 sulfhydryl group with small inorganic molecules causes an increased sensitivity of the iso-1-cytochrome to proteolysis (Montonaga et al., 1965), and that this increase in sensitivity to proteolysis may be explained by a partial unfolding of carboxyl terminal helix of the cysteine 102-SCH_3 protein. Thus, it is conceivable that the threonine 102 background is responsible for the increase in the stability over the methyl methanethiosulfonate blocked proteins.

The slopes of the free energy plots are all approximately equal in the three proteins studied. This would tend to indicate that the proteins all show the same cooperativity of unfolding through the transition region.

In general, these studies clearly show that Y48F is slightly less stable than the wild-type cytochrome, whereas both are much less stable than the N52V Y67F cytochrome *c* protein. The same order was confirmed by thermal denaturation studies, measuring the absorbance of the oxidized protein at 695nM as a function of temperature. The 695nM band is indicative of methionine 80 ligation to the heme iron and has been a probe for the integrity of the heme crevice (Kaminsky et al., 1973; Greenwood and Palmer, 1965). Although it may be argued that the thermal denaturation of the protein in this manner is dissimilar to denaturation of the entire protein by guanidinium chloride denaturation, both

methods should yield similar results with respect to protein stability. Thus second derivative spectroscopy was corroborated to be a technique that accurately reflects the average polarity of the tyrosine microenvironments.

4.2. Attempts to Perturb the 2nd Derivative spectrum of cytochrome *c*:

In solution, the native form of yeast iso-1-cytochrome *c* has some tyrosine residues that are partially exposed to solvent, and some tyrosine residues which are buried and are incapable of interacting with bulk water. Those that are partially exposed undergo a perturbation in the absorption spectra, which shifts them to lower wavelengths and therefore leads to an increase in the R value. This is presumably thought to be due to the high polarity of the solvent (water). Interestingly enough, the bulk polarity of a solution can be modulated through various techniques. Douzou (1977), has shown that increasing the temperature of a solution decreases its dielectric constant and therefore the polarity of the solution. This is due to an increase in the thermal motion (translational, rotational and vibrational) of the water molecules. When the temperature is lowered, water is better able to hydrogen bond in a unique pattern that maximizes dipole-dipole interactions. If the solution is heated, the extensive hydrogen bonding network becomes increasingly more disordered and therefore causes a decrease in polarity. Another way to modulate the polarity of a solution is to add a zwitterion such as glycine. Again Douzou (1977), has shown that the dielectric increment for glycine is $22.5M^{-1}L$ when placed in 100% water. If the dielectric constant for water to begin with is 80.4, the addition of 1 mole of glycine to a 1L solution should raise the dielectric constant of the resulting solution to:

$D = D_0 + C\delta D = 80.4+1(22.5) = 102.9$

When this type of addition was made to cytochrome protein solutions, changes in the R values were expected to increase. However, even up to near saturating levels of glycine, no perturbation of the protein second derivative was observed. In contrast to this result, when the proteins were immersed in 40% volume/volume glycerol solutions (a *non structure perturbing* solvent at this concentration), the R values decreased. This was expected since the addition of glycerol to water lowers the dielectric constant and the molar polarizability of the medium. At 20°C, a 40%v/v glycerol solution changes its dielectric constant from 80.4 to 68.6 (Douzou, 1977). This apparent paradox can somewhat be explained by preferential interaction parameters. Timasheff (1978), has shown that zwitterions such as glycine are preferentially excluded from the domain of the protein or the protein is preferentially hydrated by water. As such, the polarity of the first solvation layer surrounding the protein would be different from the polarity of the bulk solution. Timasheff has also shown that glycerol is preferentially excluded from the domain of the protein. Even though glycerol promotes preferential hydration, this does not necessarily mean that it is excluded from the entire domain of the protein. It may interact strongly with the protein in some places and be completely excluded in others. Even though this may be the case, glycerol is probably able to penetrate the primary solvation layer of the protein in different regions and perturb the absorption spectra of partially exposed tyrosine chromophores.

The effects of glycerol on the average R value of the protein could be attributed solely to a decrease in polarity (or polarizability) and not due to a change in the activity of solution (ie. pH). Douzou (1977), has shown that compared to a dilute (0.01M) phosphate buffer pH7, a 50% volume/volume glycerol:buffer ratio has a pH of 7.15 at 20°C. Thus, at the ratios of glycerol used, the activity changes due to cosolvent addition were thought to be negligible.

Ionic strength effects were examined to see whether an increase in osmotic pressure could perturb the internal water molecule or lead to its exclusion from the heme cavity. However, because the R values remained unperturbed, it seems highly unlikely that osmotic pressure can functionally displace water 166 from the heme crevice. An additional line of evidence that would tend to support this argument is provided by the method of cytochrome *c* crystallization. Cytochrome *c* is crystallized in up to 90% saturated ammonium sulfate, a solution which would surely exert substantial osmotic pressure effects. The fact that water 166 can still be resolved in the X-ray diffraction pattern suggests that this range of osmotic pressure is unable to remove this water molecule.

pH studies on the second derivative spectra showed no appreciable difference between pH 6 and 7. At pH's as low as 7, cytochrome *c* is reported to be in an equilibrium with its alkaline isomer. Alkaline isomerization is the replacement of the methionine 80-iron bond with a ligand of yet unknown nature. As this type of effect could have serious effects on the second derivative signal, the experiment was repeated at pH6 to ensure that the ferric iso-1-*c* population contained only the Met 80 S-Fe bond. The observation that the second derivative does not change clearly shows that the equilibrium for the alkaline isomer:

Fe-S-Met 80 $\leftrightarrow$ Fe-X

lies so far to the left that the amount of protein in the alkaline isomer form was thought to be negligible. No other pH studies were performed above pH7 due to the possibility of a significant amount of alkaline isomerization. Also, tyrosine 67 has a much reduced pKa. Therefore, at pH's where this residue or a proportion of these residues become ionized, the second derivative spectra could not be interpreted. Similarly, pH's lower than 6 were not attempted because of the potential change of the spin state of the heme iron. Also, at exceedingly low pH's (between pH 4 and 2), cytochrome c is reported to resemble a molten globule

state; below this, the protein denatures. (Kornblatt, personal communication).

4.3. Midpoint Potential of N52V Y67F:

The midpoint potential results show that there is no appreciable difference between the potential of the N52V Y67F and N52I Y67F iso-1-cytochrome *c* proteins while the horse heart cytochrome protein exhibited its characteristic potential of 270mV. Both yeast proteins exhibited a potential of approximately 245mV. Previous investigations have shown that the midpoint potential for the N52I Y67F protein was 234mV (Guillemette et al., in preparation). However, these measurements were performed by cyclic voltammetry at pH 6 and different ionic strengths. Given that they have also shown that the Y67F, and N52I Y67F proteins exhibit the same midpoint potential of 234mV, it was not unreasonable to expect the N52V Y67F protein to exhibit the same midpoint potential as the N52I Y67F protein.

The N52V Y67F protein is currently being crystallized. This will allow us to see whether the removal of a single methyl group (from isoleucine to valine) at position 52 is capable of expanding the heme crevice cavity enough to allow the incorporation of a water molecule. If this is the case, then the effect of this water molecule on the midpoint potential can be examined more closely.

4.4. Second Derivative Changes due to Replacement of Tyrosine and Tryptophan Residues

Wild-type iso-1-*c* displays marked changes in its R value with respect to oxidation state, from 0.81 to 1.66 upon changing from oxidized to reduced form. This tends to indicate that the average polarity of tyrosines is greater in the reduced form of the protein. Although distinct oxidation state dependent structural changes occur, it seems unlikely that the increase is due to surface tyrosine residues becoming increasingly exposed in the reduced state. In order for Ragone's theory to accurately predict the exposure of tyrosine residues in cytochrome *c*, the spectral contribution from tyrosine 67 must indicate that it is completely buried. In the reduced form, Ragone's theory becomes questionable. Thus the R values obtained could have to do with polarity changes as a result of electrostatic reorganization of the heme crevice water molecule and the associated hydrogen bonding network it creates. It was possible to look at oxidation state dependent changes in residues other than tyrosine 67 by simply mutating tyrosine 67 to phenylalanine, with or without mutating other residues. This was accomplished by the following three mutant cytochrome proteins.

4.5. Y67F; N52I Y67F, N52V Y67F:

Tyrosine 67 is a completely buried residue. As such it is generally excluded from interaction with bulk solvent molecules. Thus mutation of tyrosine 67 to phenylalanine should result in an increase in the resulting average tyrosine polarity and therefore, an increase in the R value should be observed. To illustrate this point in more tenable terms, think of the R value as being the average of four numbers. A large R value mean a lot of polarity, whereas a low

value means little polarity. Now, assume that two numbers are assigned large values and two numbers low values (these represent exposed and buried residues, respectively). The average of these four numbers (the R values) represents the average polarity. Now, if one of the buried residues were changed to an another amino-acid, the R value becomes the average of three numbers- two large and one small. Thus the weighted average shifts to a higher value. This is similar to what is observed when tyrosine is mutated to phenylalanine. N52I Y67F, N52V Y67F, and Y67F all show an increase in their R (oxidized) value when compared to the wild-type protein. Furthermore, the differences between their reduced and oxidized R values is probably indicative of oxidation state polarity changes of the remaining tyrosine residues (excluding tyrosine 67). All three of the proteins generally show small changes in R values which could correspond to the movement of a partially exposed tyrosine to a more fully exposed position or just an increase in polarity surrounding one of the tyrosine chromophores. Finally, the change in R value for the wild-type protein is approximately twice the order of magnitude of the proteins without tyrosine 67 present. This is highly suggestive that tyrosine 67 plays a major role with respect to changes in the R values between different oxidation states, and that second derivative spectroscopy is capable of measuring changes associated with this internal water molecule (Water 166). Interestingly enough, when cyanide binds in the heme crevice of the oxidized proteins, the resulting R values increase to become more like the reduced values. Whether this is due to aberrent hydrogen bonding patterns being created, or just due to the effect of the negative charge of the cyanide ion (like the reduced iron atom) is uncertain.

4.6. W59F, Y48F W59F:

These two mutant proteins were characterized to ensure that the increase in the R values between the oxidized and reduced wild-type protein are due to shifts in tyrosine residue polarity and not due to some aberration in the microenvironment of the tryptophan residue. Three observations argue strongly for this. First, the denaturation curves show a two state denaturation model. As the protein begins to unfold, tyrosines partially buried would be gradually exposed to solvent, increasing the R values in the second derivative. Tryptophan 59 is a buried residue. If it were to suddenly become exposed (and have its absorption spectrum shift to the blue), the R values should decrease. Secondly, thermal denaturations of iso-1-cytochrome *c* are often performed by monitoring the decrease in absorbance at 287nm as a function of temperature (Hickey et al.,1991; Das et al.,1989; Hickey et al.,1988). Donovan (1973), reports that tyrosine has a nonvanishing difference solvent perturbation extinction coefficient. When iso-1-cytochrome c unfolds, the tyrosine residues are increasingly exposed to solvent and their molar difference extinction coefficient at 287nm decreases (Hickey et al.,1991), presumably as a result of the blue shift of the tyrosine spectra. Since the tryptophan spectrum overlaps the tyrosine spectra and has a larger molar extinction coefficient, if tryptophan were to shift in response to polarity changes in its microenvironment, the absorbance changes at 287nm could not be attributed to the movement of only tyrosines into a more polar environment. This would cast serious doubt on this method of determining protein stability. However, it is not immediately apparent why tryptophan should not shift with changes in the polarity of its microenvironment, as solutions of n-acetyl-tryptophanamide in different concentrations of glycerol clearly show a red shift of the spectrum, and that this red shift is dependent on the amount of glycerol in

solution (data not shown). The only possible explanation for this is that n-acetyl tryptophanamide shows aberrent behavior in solution. The final line of evidence that the change in R values is due only to tyrosines is suggested by the second derivative spectra of W59F and Y48F W59F in both oxidized and reduced form. As previously mentioned, the R value obtained is dependent on the ratio of the tyrosine to tryptophan in the protein. Since mutation of this residue produces proteins that do not contain tryptophan, their second derivative spectra cannot be directly compared with protein spectra in which tryptophan is present. However, the second derivative does give an indication of the average polarity of the tyrosine residues. This is based purely on the position of the second derivative spectrum. As figures 9 and 10 show, the average exposure for the tyrosines is blue shifted for the reduced forms of the proteins. Furthermore, since the magnitude of the shift is approximately the same in both proteins, it seems highly unlikely that tyrosine 48 undergoes changes in its microenvironment in response to oxidation state.

4.7. Horse *c* and Horse Porphyrin *c*:

Horse heart cytochrome *c* contains four of the five tyrosines in identical sequence position to the yeast iso-1-*c* species. Only tyrosine 46 is replaced by phenylalanine in the horse species. The high degree of sequence similarity creates the same heme crevice hydrogen bonding patterns with an analogous water molecule. As for wild-type iso-1-*c*, the horse protein clearly shows an increase in the R value as the protein goes from the oxidized to reduced form. The magnitude of the change is smaller than that observed for the wild-type species. This may be due to the lack of a tyrosine residue at position 46 in the horse heart cytochrome species. Porphyrin *c* is identical in sequence to horse

heart cytochrome *c* except that its heme iron atom has been removed. The porphyrin cytochrome is reported to be unperturbed in its tertiary structure (Erecinska, 1975). Indeed, the R values obtained in the second derivative show that the average polarity of the tyrosine microenvironment is unchanged by this treatment. This result would tend to indicate that the charge on the heme iron in the oxidized state does not affect the polarity of region surrounding tyrosine 67. If the charge on the heme iron does not directly affect the polarity of the tyrosine 67 microenvironment, it is plausible that the changes in the R values in the wild-type iso-1-*c* protein is due predominantly to the movement of the internal water molecule (Wat166) in response to oxidation state.

4.8. Heme Crevice Polarity, Mid-Point Potential and the Modulation of Wat 166:

In order to compare R values of different proteins in which the heme crevice water molecule is perturbed the following criteria must be satisfied. First, tyrosine 67 must be present. This is the reporter group whose contribution to the second derivative spectrum would be affected by the perturbation of water 166. Second, the proteins being compared must have the same ratio of tyrosines and tryptophans and must have these residues in identical sequence position as the wild-type structure. Third, the mutations must not alter the the global three dimensional structure of the protein. Finally, tryptophan 59 must be intact. There are four proteins that satisfy these criteria. The wildtype protein has one water molecule in the heme crevice whereas N52I and N52A have zero and two water molecules respectively. These proteins all exhibit variations in the R values obtained in the oxidized and reduced states. In order to fully comprehend the

changes that might influence the heme crevice polarity and, the resulting R values, it is necessary to examine the nature of polarity itself.

A functional implication of second derivative spectroscopy is that the spectra of tyrosine residues shift to the blue in response to an increase in polarity of the chromophore's microenvironment. In a protein, a buried tyrosine residue is typically in a hydrophobic environment and thus the polarity surrounding the residue is quite low. As the protein denatures, the buried residue gradually becomes exposed to solvent, which in most cases is water. Water differs greatly from the interior of a protein matrix because of the unique hydrogen bonding network it is capable of forming in solution. This network gives water a high dielectric constant and molar polarizability. In essence, water is a highly polar solvent. Therefore, as the tyrosine chromophore gradually becomes exposed to solvent, it is exposed to an increasingly polar environment. This causes a blue shift of its spectrum. It is this nature of these tyrosine chromophores that allows second derivative spectroscopy to accurately determine the average exposure of tyrosines in a protein. Cytochrome *c* can differ from most proteins because tyrosine 67, although buried, is in a highly polarizable region due to the internally bound water molecule. Tyrosine 67 also forms part of a heme crevice and interacts with several highly polar residues including several hydroxyl groups, amide groups and a sulfur group. Finally, it is in close proximitry to a heme iron atom that is capable of electron rich or electron poor oxidation states. Thus, in addition to solvent exposure, polarity must be thought of to include the three dimensional electron distribution surrounding the chromophore of interest. This might help explain why there is such a large difference between the R values of the oxidized and reduced states of wild-type iso-1-cytochrome *c*.

According to second derivative theory, a large R value implies much polarity. If tyrosine 67 is responsible for a majority of the shift in R value it is

tempting to conclude that the polarity of the heme crevice in the wild-type protein is greater in the reduced state than in the oxidized state. Similar conclusions could be drawn for the N52A protein and exactly the opposite could be concluded for the N52I protein (ie. the heme crevice of the oxidized protein is more polar than the reduced protein). This is exactly opposite what one would predict based on their midpoint potentials. Based on electron transfer theory, an increase in the hydrophobicity of the heme crevice should drive the midpoint potential to a higher value (Kassner, 1972). Conversely, decreasing the hydrophobicity or increasing the polarity of the heme crevice (ie. increasing solvent exposure) should decrease the driving force with which the cytochrome wants to give up electrons thus yielding a decrease in the electrochemical potential of the protein. To explain the differences between experiment and theory, a close examination of the hydrogen bonding patterns of the heme crevice is needed.

A possible explanation for the observed differences in the second derivative R value between oxidized and reduced wild type iso-1-cytochrome *c* may lie with the nature and strength of the hydrogen bonds that occur between tyrosine 67 and other substituents (Figure 12). In the cases where tyrosine 67 is present and involved in hydrogen bonds with water 166 and methionine 80, the observed R values are larger than when tyrosine 67 hydrogen bonds to a single partner. For instance, in the reduced wild-type protein, the hydroxyl group of tyrosine 67 donates a hydrogen bond, and this is accepted by the lone pair of electrons on methionine 80. At the same time, the lone pair of electrons on tyrosine 67 hydrogen bonds to a water 166 hydrogen group. In this case, the average R value is 1.66. In the oxidized form of the protein, the hydrogen bond between tyrosine 67 and methionine 80 is broken due to the reorientation of the dipole of water 166. Now, only one hydrogen bond is formed with tyrosine 67, and this occurs with water 166. The R value for this state of the wild-type protein is

Figure 12.

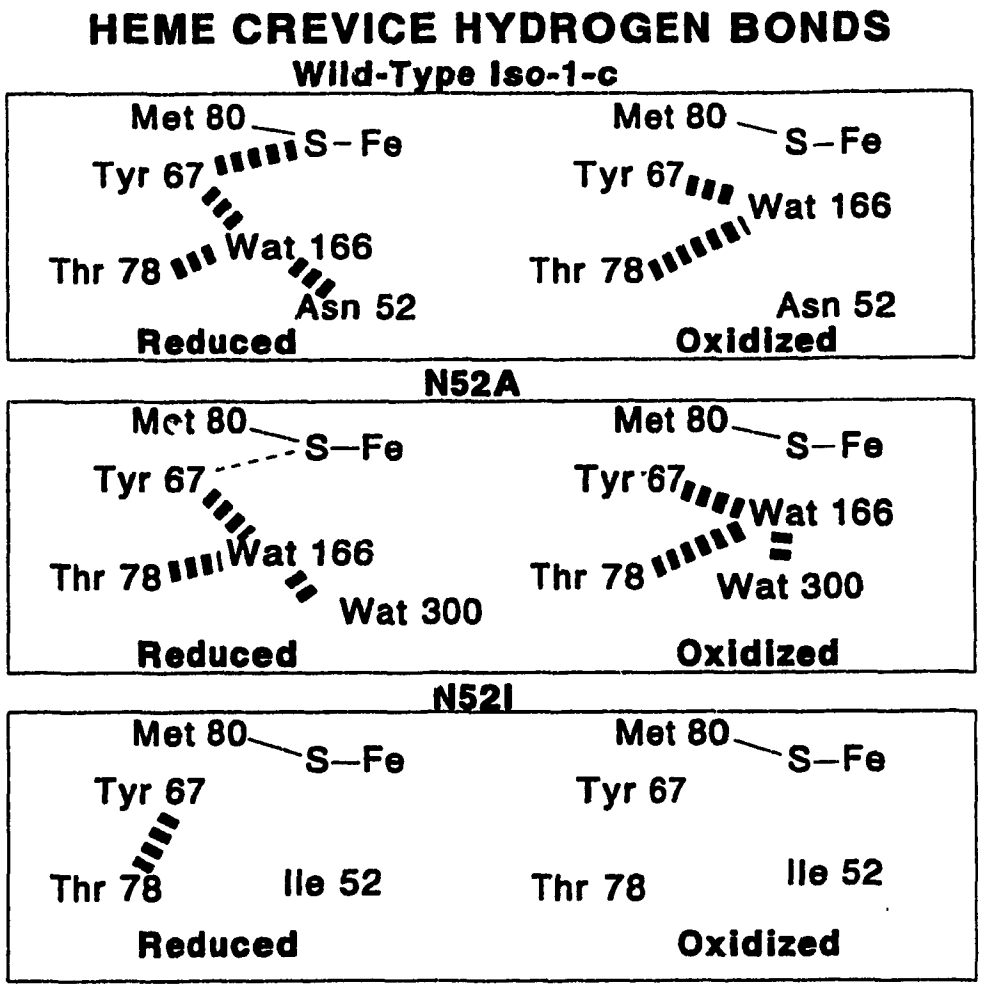


Figure 12. Diagram of the heme crevice hydrogen bonds associated with tyrosine 67 and water 166 for the oxidized and reduced forms of three iso-1-cytochrome c's.

0.81. Now lets examine the N52A protein. In the reduced form, the same type of hydrogen bond network is set up as seen in the wild-type protein with one important difference. In the N52A mutant, the tyrosine 67 hydroxyl group functions less as a hydrogen acceptor and more as a hydrogen donor group to water 166, and this causes the tyrosine 67-methionine 80 hydrogen bond to weaken (Berguis, unpublished). Note the R value for this state is only 1.23 as compared to 1.66 for the wild-type protein, where the hydrogen bond between tyrosine 67 and methionine 80 is stronger. Additional evidence that supports this proposal is presented by the reduced N52I protein. In this structure, water 166 is removed from the heme crevice. Also, the hydrogen bond from tyrosine 67 to methionine 80 is broken in favor of a hydrogen bond to threonine 78. In this case, the R value was determined to be 0.80. The oxidized forms of the wild-type and N52A protein have one hydrogen bond formed between themselves and water 166 and have relatively close R values (0.81 wild-type and 0.92 N52A). Tyrosine 67 from oxidized N52I on the other hand does not hydrogen bond to any ligands and this might explain the aberration in its R value (1.28). The last piece of evidence that would suggest that these hypotheses are true stems from the fact that n-acetyl tyrosinamide residues are blue-shifted when placed in solutions of increased polarity. This makes sense, for in solution, the hydroxyl group of the phenyl ring can act both as a hydrogen bond donor (the hydrogen of the hydroxyl) and as a hydrogen bond acceptor (the lone pair of electrons on the hydroxyl group). Furthermore, as the polarity of the solutions is increased, the strength of the hydrogen bonds formed in solution should increase. This would be due to an increase in the charge separation due to the electronegative atoms. For instance, the hydrogens of a water molecule may exhibit an increased positive charge whereas the oxygen would retain a greater net negative charge. Thus, obeying the fundamental law of electrostatics, the force between charges in this situation

should be greater. This in turn would lead to an even greater blue shift of the spectra. This is the hypothesis for the difference between the oxidized and reduced second derivative R values for yeast iso-1-cytochrome *c*.

4.9. Conclusions:

Second derivative spectroscopy has been shown to be a good technique to monitor polarity changes associated with tyrosine residues in iso-1-cytochrome *c*. This includes global polarity changes associated with the denaturation of iso-1-cytochrome *c* by guanidinium chloride and regional polarity changes examined by engineering proteins to displace or include an additional water molecule in the heme crevice. Tyrosine 67 seems to exhibit more a more polar spectrum in the reduced form of the protein as compared to the oxidized form of the protein. This is thought to be due to changes in the hydrogen bonding patterns of this amino-acid with water 166.

In conjunction with mutagenic approaches, second derivative spectroscopy promises to provide a wealth of information which is currently unattainable through other techniques.

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