



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

**Replacing two conserved amino acids, (Asn-52 to Val
and Tyr-67 to Phe), in the yeast Iso-1-cytochrome c
gene has little effect on the function of the protein.**

Francis Awuor McOdimba

A Thesis in the Department of Biology

**Presented in partial fulfillment of the requirements for the
Degree of Master of Science at
Concordia University
Montreal, Quebec, Canada.**

August, 1994.

© F.A. McOdimba, 1994.



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

THE AUTHOR HAS GRANTED AN
IRREVOCABLE NON-EXCLUSIVE
LICENCE ALLOWING THE NATIONAL
LIBRARY OF CANADA TO
REPRODUCE, LOAN, DISTRIBUTE OR
SELL COPIES OF HIS/HER THESIS BY
ANY MEANS AND IN ANY FORM OR
FORMAT, MAKING THIS THESIS
AVAILABLE TO INTERESTED
PERSONS.

L'AUTEUR A ACCORDE UNE LICENCE
IRREVOCABLE ET NON EXCLUSIVE
PERMETTANT A LA BIBLIOTHEQUE
NATIONALE DU CANADA DE
REPRODUIRE, PRETER, DISTRIBUER
OU VENDRE DES COPIES DE SA
THESE DE QUELQUE MANIERE ET
SOUS QUELQUE FORME QUE CE SOIT
POUR METTRE DES EXEMPLAIRES DE
CETTE THESE A LA DISPOSITION DES
PERSONNE INTERESSEES.

THE AUTHOR RETAINS OWNERSHIP
OF THE COPYRIGHT IN HIS/HER
THESIS. NEITHER THE THESIS NOR
SUBSTANTIAL EXTRACTS FROM IT
MAY BE PRINTED OR OTHERWISE
REPRODUCED WITHOUT HIS/HER
PERMISSION.

L'AUTEUR CONSERVE LA PROPRIETE
DU DROIT D'AUTEUR QUI PROTEGE
SA THESE. NI LA THESE NI DES
EXTRAITS SUBSTANTIELS DE CELLE-
CI NE DOIVENT ETRE IMPRIMES OU
AUTREMENT REPRODUITS SANS SON
AUTORISATION.

ISBN 0-315-97673-X

Canada

Abstract

Replacing two conserved amino acids, (Asn-52 to Val and Tyr-67 to Phe), in the yeast iso-1-cytochrome *c* gene has little effect on the function of the protein.

Francis A. McOdimba, 1994.

Tyr-67 and Asn-52 are among the conserved residues in the amino acid sequence of eukaryotic cytochromes *c*. These residues, together with Thr-78 are hydrogen bonded to a buried water molecule, (Wat-166), which is also a conserved structural feature in all naturally occurring eukaryotic cytochromes *c* whose three dimensional structures have been determined.

Previous studies have suggested that during oxidation-reduction states Wat-166 plays a role in shifting both the side chains and the main chain at the bottom of the protein molecule. Replacement of Tyr-67 in rat cytochrome *c* with Phe and Asn-52 with Ile in yeast cytochrome *c* have been shown to result in more thermally stable proteins than the wild type. The Asn-52 to Ile mutation also results in exclusion of Wat-166 with a substantial re-organization of hydrogen bonding in that region. Other replacement at position 52 using Ala for Asn result in a functional protein.

The objective of this project was to replace the two conserved amino acids around the heme environment in the yeast iso-1-cytochrome *c* with a view of destabilizing Wat-166. The two amino acids (Tyr-67 and Asn-52) were replaced with Phe and Val on the wild type iso-1-cytochrome *c* gene in a multi-copy pING4 expression plasmid vector which already had a Cys-102 to Thr mutation. Yeast cells carrying the triple mutant cytochrome *c* gene were

propagated on media with glycerol as the source of carbon. These mutant cells had similar growth patterns as those carrying the wild type gene in the absence of glucose, suggesting that the extra mutations did not have any effect on the expression of a functional cytochrome *c* in yeast.

Cytochromes *c* were extracted and purified from yeast cells carrying the mutant and wild type genes. The total protein yields were comparable in both cases. The spectrum of the oxidized form of the purified mutant protein showed a shift in the 695nm peak to 705nm, indicating that the integrity of the methionine sulfur bonding with the heme.

Kinetic assays using polarographic method with purified beef heart cytochrome oxidase showed similarities between the wild type and mutant proteins in the characteristic two activity sites on the oxidase with similar $V_{\max 1}$ & $V_{\max 2}$ and K_{m1} around the micro molar range and K_{m2} around the nano molar range for both proteins, suggesting that the mutations did not affect the conformational structure of the cytochrome *c* molecule.

ACKNOWLEDGEMENTS

I would like to thank Dr. Jack A. Kornblatt for financial support throughout my thirty-two months' study period in Canada and for his strict supervision of this work. For the numerous advice on techniques in molecular biology I would like to thank Professor M.J. Kornblatt, Drs. Yue Huang, Pamela Hanic-Joyce, Paul Joyce and Reginald K. Storms. I would like to acknowledge Ms Edith Munro for the encouragements and the "little tricks" that sometimes worked and Mr. Hans Schroeder for his pleasant companionship in the lab. To all the staff in the Biology office: I say "shukran" for all your help. I would like to thank Njeri Kangethe for "being there" whenever you were needed. I am indebted to my wife Terry for enduring all the lonesome moments while I was away. Finally, I would like to dedicate this work to the memory of my late brother Isaac whose funeral I was unable to attend. This work was supported by NSERC grants through Dr. J. Kornblatt.

TABLE OF CONTENTS

List of Figures.....	ix
List of Tables.....	ix
List of abbreviations.....	x

CHAPTER ONE - INTRODUCTION

1. Cytochromes.....	1
1.1. Cytochrome <i>b</i>	1
1.2. Cytochrome oxidase.....	2
1.3. Cytochrome <i>c</i>	2
1.4. Interaction of cytochrome <i>c</i> with its redox partners.....	4
1.4.1. Interaction of cytochrome <i>c</i> with cytochrome oxidase.....	4
1.4.2. Interaction of cytochrome <i>c</i> with cytochrome <i>bc₁</i>	5
1.5. Yeast cytochrome <i>c</i>	5
1.5.1. Amino acids that form the heme environment in cytochrome <i>c</i> molecule.....	6
1.5.2. Amino acids that are involved in protein folding.....	6
1.5.4. The role of Tyr-67 in cytochrome <i>c</i> molecule.....	7
1.5.5. The role of Asn-52 in cytochrome <i>c</i> molecule.....	8
1.6. The water molecules in cytochrome <i>c</i>	9
1.7. The Asn-52Val/Tyr-67Phe/Cys-102/Thr mutant.....	10

CHAPTER TWO - MATERIALS

2.1. The pING4 expression vector.....	12
2.2. Design of oligonucleotide primers.....	12
2.3. <i>E. coli</i> cells.....	13
2.3.1. CJ236 strain.....	13
2.3.2. MC1066 strain.....	14
2.3.3. DH 5 α 2671 strain.....	14
2.4. The yeast cells, GM3-C2 strain.....	14

CHAPTER THREE - METHODS

3.1. Preparation of competent <i>E. coli</i> cells.....	15
3.2. Preparation of competent GM-C2 yeast cells.....	15
3.3. Preparation of single stranded (ss) DNA templates.....	16
3.3.1. Preparation of phage plasmid DNA.....	16
3.3.2. Extraction of ssDNA from viral proteins.....	17
3.4. Phosphorylation of the oligonucleotides.....	18
3.5. <i>In vitro</i> mutagenesis.....	19
3.5.1. Annealing of oligonucleotide primers to the templates.....	19
3.6. Transformation of MC1066 <i>E. coli</i> cells with synthesized DNA.....	20
3.7. Restriction endonuclease screening of possible mutants	21
3.8. DNA sequencing.....	22
3.8.1. Preparation of double stranded DNA for sequencing.....	22
3.8.2. Annealing reaction.....	23
3.9. Transformation of GM3-C2 yeast cells with mutant DNA plasmids..	24
3.10. Curing yeast cells of the plasmids.....	25
3.11. Growth of yeast cells.....	25
3.12. Purification of cytochromes <i>c</i>	26
3.13. Spectral analysis of Cytochromes <i>c</i>	28
3.14. Kinetic assays.....	29

CHAPTER FOUR - RESULTS

4.1. Restriction enzyme digest.....	30
4.2. DNA sequence analysis.....	31
4.3. Growth of cells carrying cytochrome <i>c</i> genes.....	33
4.3.1. Yeast cells cured of the plasmids.....	33
4.3.2. Cell growth rate assays.....	34
4.3.3. Cell growth yields.....	35
4.4. Total cytochromes <i>c</i> purified from yeast cells.....	36
4.5. Spectral characteristics of purified cytochromes <i>c</i>	37
4.6. Kinetic assays.....	38

CHAPTER FIVE - DISCUSSION

5.1. Mutagenesis.....	42
5.2. Characterization of yeast cells carrying mutant cytochrome <i>c</i> gene.....	43
5.3. Spectral characteristics.....	44
5.4. Kinetic assays.....	45
5.5. The heme environment around Wat-166.....	46

CHAPTER SIX - APPENDIX

6.1. Media.....	50
6.2. Solutions.....	52
6.3. Antibiotics.....	55
6.4. Buffers.....	56

<u>CHAPTER SEVEN - REFERENCES</u>	59
---	----

LIST OF FIGURES

Figure 1: EcoRI Digestion of wild type and mutant DNA plasmids.....	30
Figure 2: Amino acid and nucleotide sequences of cytochromes <i>c</i>	31
Figure 3: Calibration curve for correlating yeast cell counts with optical densities.....	33
Figure 4: Cell growth rate assays.....	34
Figure 5: Cell growth yields.....	35
Figure 6: Absorption spectra of reduced and oxidized cytochrome <i>c</i>	37
Figure 7a: Kinetic assays of wild type yeast iso-1-cytochrome <i>c</i> with cytochrome oxidase.....	39
Figure 7b: Kinetic assays of mutant (Asn-52Val/Tyr-67Phe/Cys102Thr yeast iso-1-cytochrome <i>c</i> with cytochrome oxidase.....	40
Figure 8: The structure of the heme environment of mutant (N52V/Y67F/C102T) yeast iso-1-cytochrome <i>c</i>	41

LIST OF TABLES

Table 1: Purification of cytochromes <i>c</i> from yeast cells.....	36
Table 2: Kinetic analysis of wild type and mutant cytochrome <i>c</i> activity with purified beef heart cytochrome oxidase.....	41

ABBREVIATIONS

LB - Luria-Bertani media

MW - molecular weight

O.D. - optical density

PEG - polyethylene glycol

PNK - polynucleotide kinase

SC - synthetic complete media

SD - synthetic dextrose media

TAE - tris-acetate buffer

TBE - tris-borate-EDTA buffer

TE - tris-EDTA buffer

TEMED - N,N,N',N'-Tetramethylenediamine

TMPD - N,N,N',N'-Tetramethyl-p-phenylene diamine dihydrochloride

YP - yeast extract - peptone media

YPD - yeast extract - peptone - dextrose (glucose) media

YPG - yeast extract - peptone - glycerol media

YT - yeast extract - tryptone media

CHAPTER ONE

INTRODUCTION

1. Cytochromes

Cytochromes are a group of proteins present in both prokaryotes and mitochondria of eukaryotes. They are involved in the transfer of electrons in oxidation-reduction reactions during respiration or photosynthesis. They have a prosthetic heme group which is a porphyrin derivative complexed with an iron atom that alternates between the reduced ferrous state (Fe^{2+}), with no unpaired electrons and no charge, and the oxidized ferric state (Fe^{3+}) in which the iron has a single unpaired electron and a charge of +1, (Dickerson & Timkovich, 1975),.

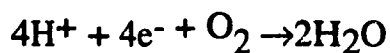
In yeast, cytochrome *c* transfers electrons from cytochrome *bc₁* to cytochrome oxidase as well as to a few other proteins. It is this transfer of electrons to the oxidase which subsequently transfers them to oxygen that allows yeast cells to grow on non fermentable substrates like glycerol, thereby allowing oxidative phosphorylation to occur.

1.1. Cytochrome *b*

Cytochrome *b* is a protoheme protein that is found in a membrane-bound form in the mitochondria where it can be extracted and solubilized with detergents such as cholate or deoxycholate. The protein forms a complex with cytochrome *c₁* (Complex III), which receives electrons from Complex II and transfers them to cytochrome *c*. Reduced cytochrome *b* has an absorption spectra that shows an α -peak between 555-567nm, a β -peak at 526-546 and an α -peak between 408-449, (Dickerson & Timkovich, 1975).

1.2. Cytochrome oxidase

Cytochrome oxidase is a multi-sub-unit terminal protein of the respiratory chain that catalyzes the transfer of electrons to oxygen to form water, in a process that results in the synthesis of adenosine triphosphate (ATP):



The enzyme couples this reaction to the translocation of protons across the membrane barrier (Wilkstrom, *et al*, 1981). Cytochrome oxidase is found as part of the plasma membrane in prokaryotes while in eukaryotes it is localized to the inner mitochondrial membrane. Some of the sub-units of the eukaryotic enzyme are encoded on the mitochondrial DNA while others are encoded in the nucleus (Capaldi, 1990). The enzyme contains two non-covalently attached heme groups and two protein bound copper ions (named Cu_A and Cu_B) per minimum catalytic unit. The protein from bovine heart also contains one zinc and one magnesium atom per monomer, (reviewed in Kornblatt, *et al*, 1990) as well as one additional Cu per dimer. Reduced cytochrome oxidase (a, a₃) has an α-peak at 605nm, a β-peak at 517nm and a Soret peak at 445nm, (Dickerson & Timkovich, 1975).

1.3. Cytochrome c

Cytochrome *c* is a small soluble cytosolic protein whose relative molecular mass is about 12,400 daltons. The protein is encoded for by a nuclear gene (Sherman, *et al*, 1966), and is synthesized on the cytoplasmic ribosomes (Clark-Walker & Linnane, 1967), in an apo-form which is then translocated to the inner mitochondrial membrane where the enzyme cytochrome *c* synthetase covalently attaches it with heme to form a holo-cytochrome *c*, that is finally trimethylated at Lys-77, (Delange, *et al*, 1970; Sherman & Stewart,

1971; Hennig, *et al*, 1983). Synthesis of cytochrome *c* is repressed by the presence of glucose in a mechanism that involves control of the amount of messenger RNA (Zitomer & Hall, 1976; Zitomer & Nichols, 1978).

Cytochrome *c* is a water soluble protein, thus making it possible to purify and crystalize. The protein from mammalian heart consists of a single polypeptide chain of 108 (104 from yeast) amino acid residues that are covalently attached to a heme group. It appears spherical in shape with a diameter approximately 34 Angstroms, (Dickerson & Timkovich, 1975). Its heme group is surrounded by tightly packed hydrophobic side chains while the iron atom is bonded to a sulfur atom of Methionine-80 and a nitrogen atom of a Histidine-18.

The protein has a relatively high affinity for electrons. In the oxidized state the protein has an α and β absorption peaks at 540nm and a Soret band at 409nm. In addition, there is a small peak at 695nm associated with the bonding between the sulfur of Met-80 and the heme, (Shechter & Saludin, 1967). In the reduced form it has an α -peak at 550nm, a β -peak at 521 and the Soret peak at 415nm, (Dickerson & Timkovich, 1975).

High-resolution three-dimensional crystal structures of cytochrome *c* from a number of species such as rice (Ochi, *et al*, 1983); tuna (Takano & Dickerson, 1981a,b); horse (Bushnell, *et al*, 1990); yeast iso-2-cytochrome *c*, (Leung, *et al*, 1989), and yeast iso-1-cytochrome *c*, (Louie *et al*, 1988a; Louie & Brayer, 1990) have been determined and found to be highly homologous with each other. X-ray diffraction, nuclear magnetic resonance

(NMR), (Gao, *et al*, 1991) as well as spectrophotometry have shown that tyrosine ionization curves of the reduced and oxidized protein are different, (Dickerson *et al*, 1971; Dickerson *et al*, 1975).

1.4. Interaction of cytochrome *c* with its redox partners

Cytochrome *c* interacts with ubiquinol-cytochrome reductase *bc₁*, (complex III), cytochrome *c* peroxidase and cytochrome oxidase, through a ring of positively charged amino acids including lysines, arginines and a trimethyl-lysine. This is due to the fact that these redox partners are negatively charged on their sites, (Margoliash and Bosshard, 1983) while cytochrome *c* is positively charged at the surface.

1.4.1. Interaction of cytochrome *c* with cytochrome oxidase

Cytochrome *c* in either reduced or oxidized state binds with cytochrome oxidase, (Dickerson, *et al*, 1971; Kornblatt and Laberge, 1988). This binding allows the enzyme to accept one electron from cytochrome *c* while it delivers four to oxygen. In the process, cytochrome *c* acts both as an electron donor and a conformational effector for the enzyme, (Kornblatt, *et al*, 1990).

Conformational changes in cytochrome oxidase are thought to make the enzyme function as a proton pump. As the enzyme catalyzes the oxidation of ferro-cytochrome *c* to ferri-cytochrome *c*, and the reduction of oxygen to water, it generates a proton electrochemical potential across the inner mitochondrial membrane which is subsequently used to drive the synthesis of ATP, (Wikstrom, *et al*, 1981). It has been suggested that electron transfer from cytochrome *c* to the oxidase takes place either across the edges or

directly across the parallel planes of the two proteins, (Wikstrom, *et al*, 1981; Kornblatt, *et al*, 1990).

In the presence of cytochrome *c* turnover of the enzyme may result in the formation of a conformer of the enzyme known as the pulsed form while when cytochrome *c* is absent an "oxygenated" form of the enzyme is formed. In an anaerobic condition electron transfer to the enzyme results in the formation of a reduced form of the enzyme (Kornblatt & Luu, 1986).

1.4.2. Interaction of cytochrome *c* with cytochrome *bc₁*

Studies using singly modified 4-carboxy-2,6-dinitrophenyl-lysine (CDNP-L) to define the interaction domain on cytochrome *c* for mitochondrial complex III showed that the sites of interaction for complex III and the oxidase are nearly identical, (Speck, *et al*, 1979). In another study that was set to determine if cytochrome *c₁* is the binding subunit of the complex III, Bosshard, *et al*, (1979), suggested that the same surface area of cytochrome *c* is in direct contact with the *bc₁* complex and with *c₁*.

1.5. Yeast cytochrome *c*

The yeast system has been used in many mutagenesis experiments that have attempted to determine the roles played by the invariant amino acids of cytochrome *c*. Wild type as well as many mutants of yeast cytochrome *c* have been isolated and functionally characterized (Hampsey, *et al*, 1986).

Two isozymes of cytochrome *c* occur in baker's yeast (*Saccharomyces cerevisiae*) of which iso-1-cytochrome *c* is the more common one accounting for 95% of the total complement of cytochrome *c* in the yeast

cells while iso-2-cytochrome *c* accounts for the remaining 5%, (Smith, *et al*, 1979).

The genes coding each of the two isotypes of cytochrome *c* have been isolated and the complete DNA sequence determined and found to be completely corresponding to the previously determined amino acid sequence, (Lederer, *et al*, 1972; Smith, *et al*, 1979; Montgomery *et al*, 1980, Moore and Petigrew, 1990). The coding region of the gene contains no intervening (nontranslatable) sequences, (Smith, *et al*, 1979).

1.5.1. Amino acids that form the heme environment in cytochrome *c* Molecule:

There are a number of amino acids that form the immediate heme environment. These include the invariant Leu-32 located in the lining of the heme crevice and flanking the heme moiety, Tyr-48 located at the floor of the heme crevice and an invariant Trp-59 located near the floor of the heme crevice. Trp-59 functions to stabilize the heme by forming a hydrogen bond with the heme propionate 7 and Leu-68. Leu-68 forms the right side hydrophobic heme pocket along with other residues that determine the hydrophobicity of the heme environment such as the conserved Tyr-67, Asp-52 and Thr-78, (Hampsey, *et al*, 1986).

1.5.2. Amino acids that are involved in protein folding

There are residues that are involved in the proper folding of the protein, including an invariant Gly-6 and a conserved Gly-29 located in a tightly packed tertiary structure where bulky side chains can not be accommodated.

Any replacement of these amino acids could result in loss of function due to the disruption of the backbone structure of the protein. There are a number of other residues including Pro-30, Tyr-67, Asn-70, Pro-71, Lys-79, Leu-94 and Leu-98 which are also involved in directing the local folding of the protein backbone into proper conformation, (Hampsey, *et al*, 1986; Koul *et al*, 1979; ten Kortenaar *et al*, 1985; Wallace, *et al*, 1989).

Iso-1-cytochrome *c* from yeast has five tyrosines, (Tyr-46, Tyr-48, Tyr-67, Tyr-74 and Tyr-97); seven asparagines, (Asn-31, Asn-52, Asn-56, Asn-62, Asn-63, Asn-70 and Asn-92); 4 phenylalanines (Phe-4, Phe-16, Phe-42 and Phe-88) and three valines (Val-20, Val-29 and Val-57) in its amino acid sequence, (amino acid sequences according to the yeast cytochrome *c* nomenclature).

1.5.3. The role of Tyr-67 in cytochrome *c* molecule:

Tyr-67 is one of the conserved amino acids in eukaryotes and appears to be important in the function of cytochromes-*c*. The role of this residue to the function of cytochrome *c* has been studied using several techniques including chemical modification (McGowan & Stellwagen, 1970); semi-synthesis method (Koul, *et al*, 1979; ten Kortenaar, *et al*, 1985; Wallace, *et al*, 1989; Fraunhoff & Scott, 1992); and more recently, by site-directed mutagenesis (Luntz, *et al*, 1989; Schejter, *et al*, 1992 and Berghuis, *et al*, 1994a).

Studies have shown that mutating this amino acid would remove the hydroxyl group from the residue, thereby making the heme environment more hydrophobic, (Koul, *et al*, 1979; ten Kortenaar, *et al*, 1985; Wallace, *et al*, 1989). This mutation is therefore expected to cause a major structural effect on Wat-166 located adjacent to the Tyr-67 position as well as the proper folding of the protein due to such mutations.

In two studies where Tyr-67 of rat cytochrome *c* was replaced with Phe it was found that the mutant protein was more stable than the wild type, (Luntz, *et al*, 1989; Wallace, *et al*, 1989); the original presumption was that stability arose due to the increase in hydrophobicity in the region. A high resolution three dimensional crystal structure of a mutant yeast iso-1-cytochrome *c* in which this residue was replaced with Phe has also been determined (Berghuis, *et al*, 1994a). However, in their study, Berghuis, *et al*, (1994a), observed that in the reduced form of the protein, this mutation resulted in the Wat-166 being retained around the cavity and only slightly displaced to 1 Angstrom away from its original site. They also observed that this slight displacement created a cavity which was previously occupied by Wat-166 but was subsequently filled by another water molecule, (Wat-300), from nearby, suggesting that the region became more hydrophilic instead.

1.5.4. The role of Asn-52

Previous studies in yeast iso-1-cytochrome *c* have shown that replacement mutations of Asp-52 with Ile resulted in exclusion of Wat-166 with a substantial reorganization of hydrogen bonding, (Hickey, *et al*, 1991; Berghuis, *et al*, 1994b). Other studies have also shown that this mutation

results in a more thermostable protein than the wild type, (Das, *et al*, 1989). However, Das, *et al*, (1989) also found that other replacements at this position using and Gly, which has the smallest side chains, resulted in less functional proteins. There is only one organism, *Monochrysis lutheri*, in which Asn at position 52 is replaced with Val, (Laycock, 1972). So far there are no literature reports on the replacement of Asn at position 52 with Val and therefore little is known about the effect of this mutation.

1.6. The water molecules in cytochrome c

In the oxidized form of cytochrome *c* there are a total of 49 water molecules, 27 of which are located at the outer surface and 22 at the inner surface of the molecule according to the assignment by the crystallographers, while in the reduced form there are 53 water molecules (Takano & Dickerson, 1981). Of these water molecules, 3 are buried inside the protein, one between Asn-52, Tyr-67 and Thr-78 to the lower left of the heme; a second inside the 20s loop and a third one below the buried heme propionate. The one that is buried between Tyr-67, Asp-52 and Thr-78, (Wat-166), is also hydrogen bonded to these amino acids, (Takano & Dickerson, 1981). This water molecule is a conserved structural feature in all eukaryotic cytochromes *c* whose three dimensional structures have been determined (Bushnell, *et al*, 1990).

In their studies, Takano & Dickerson, (1981), suggested that during the oxidation-reduction states of the heme this water molecule plays a role in shifting of both side chains and the main chain at the bottom of the protein molecule. This chain differential orientation helps to stabilize the molecule by positioning and orienting the dipole moment of water 166. It has also

been proposed that this water molecule sets the value of the midpoint reduction potential through a hydrogen bond interaction with the sulfur atom of Met-80 ligand, (Takano & Dickerson, 1981; Berghuis & Brayer, 1992). These studies have also indicated that upon oxidation of cytochrome *c* this Wat-166 shows a large displacement towards the positively charged heme iron atom (Takano & Dickerson, 1981; Berghuis & Brayer, 1992).

1.7. The Asn-52Val/Tyr-67Phe/Cys-102Thr mutant

The mutations that were carried out in this study aimed at displacing Wat-166 with a possibility of filling the cavity at its position by introducing amino acids with bulky and hydrophobic side chains. Site-directed oligonucleotide mutagenesis was used to induce mutations on the plasmid pING4; a multicopy expression vector that replicates in both *E. coli* and yeast cells, (Inglis, *et al*, 1991).

The plasmid DNA from *E. coli* cells that were transformed with mutagenesis products was digested with EcoRI restriction enzyme to screen for possible introduction of an extra enzyme cut site and the mutations were confirmed by nucleotide sequencing. Mutant plasmid DNA was used to transform GM3-C2 strain of yeast cells. The transformed yeast cells carrying mutants as well as the wild type cytochrome *c* genes were grown on media that had glycerol as the source of energy.

The growth of yeast cells carrying the plasmid with mutant cytochrome *c* was monitored on media with glycerol and compared with the cells carrying the wild type gene on the same plasmid and found to have similar rates. Both

wild type and mutant cytochromes *c* were extracted and purified from yeast cells. The total yields of cytochromes *c* from yeast cells with wild type and mutant genes were found to be comparable.

Kinetic studies using polarographic assays performed in a Clarke oxygen electrode with purified beef heart cytochrome oxidase, in the presence of Tris acetate buffer, N,N,N',N'-Tetramethyl-p-phenylene-diamine dihydrochloride, (TMPD) and ascorbate, showed the characteristic two activity sites on the oxidase with similar V_{max} s and K_m s for both the wild type and mutant cytochromes *c*.

CHAPTER TWO

MATERIALS

2.1. The pING4 expression vector

The pING4 is a 10.24 kbp plasmid vector derived from plasmid pYEP213, (Pielak *et al*, 1985). It contains the coding region and the upstream activation sequences of yeast iso-1-cytochrome *c*, (Inglis, *et al*, 1991) and thus confers to the yeast cells the ability to utilize glycerol as the sole source of carbon to support growth due to a functional cytochrome *c* gene. The plasmid also has the *fl* as well as the origin of replication (*ori*) in both yeast and *E. coli* and ampicillin resistance gene. It has two *HindIII*, two *PstI*, four *Avall*, two *PvuII*, two *MluI* and three *EcoRI* restriction endonuclease sites. Has a mutation on Cys-102 to Thr which has no structural or functional effect on the protein but prevents it from dimerizing during purification (Cutler, *et al*, 1987). The plasmid can be converted to single stranded DNA, (Baldari and Cesareni, 1985).

2.2. Oligonucleotides primers

The oligonucleotides primers used for *in vitro* mutagenesis were complementary to the coding strand of pING4 DNA. The oligonucleotide for Y67F position included one *EcoRI* restriction endonuclease site between position 68 and 69. The restriction site was used to screen for mutant DNA derivatives on agarose gel.

- i) 5'-AGT-CAA-^{*EcoRI*}GaA (67)-tTC TGA-CAT-3'.....Tyr-67 to Phe.
- ii) 5'-CTT-GAT-tAc(52)-GGC-ATC-3'..... Asn-52 to Val.

The oligonucleotides were synthesized at the Sheldon Biotechnology Centre, McGill University.

2.3. *E. coli* cells

2.3.1. CJ236 strain

- Has sex pili construction, therefore making it possible to be infected with helper phage.
- Genotype *dut*⁻, *ung*⁻, *thi*⁻, *relA*⁻, pCJ105(Cm^r), therefore has a deficiency in deoxyuridine triphosphate (*dut*⁻) and uracil N-glycosylase (*ung*⁻). The *dut*⁻ phenotype allows dUTP to accumulate inside the cell, leading to a higher concentration in these cells than in the normal *E. coli*, thus increasing the probability of uracil incorporation instead of dTTP. The *ung*⁻ makes the cells unable to remove uridylate once incorporated and uracil containing single stranded DNA can then be used in site directed mutagenesis, (Kunkel *et al*, 1987).

2.3.2. MC1066 strain

- Genotype *pyrF74::Tn5* (km^{r}), *leuB6*, *trpC9830*, *leu*, *hsdR*⁻, (*lacIP-OZYA*), *X74*, *galU*, *galK*, *strA*^r)
- Has the ability to degrade uracil-containing DNA thus selects for only double stranded DNA without uracil and was used for transformation with mutagenesis product.

2.3.3. DH5 α 2671 strain

- Genotype *F'**endA1 hsdR17*(rK-mK^+) *supE44thi-1* *recA1* *gyrA* (Na^{r}) *relA1* Δ (*lacZYA-argF* Δ) *U169d* Δ *80dlac* Δ (*lacZ*)*M15*) (Hanahan, 1983), was used for propagating DNA for sequencing, since the DNA from MC1066 persistently contained residual chromosomal DNA that interfered with sequencing reactions, and transformation of yeast cells.

2.4. The yeast cells, GM3-C2 strain

- Phenotype [*trp1-1 leu2-2, leu2-112 his4-519 cyc1-1 cyp3-1 (cyc7-)* *gal*⁻]).
- Carries deletion mutations that eliminate the gene for iso-1-cytochrome *c* and iso-2-cytochrome *c* from its chromosome, (Faye, *et al*, 1981).
- Has the ability to grow on synthetic-dextrose media without leucine and was used in the expression of both wild type and mutant iso-1-cytochromes *c* and for the functional characterization of the phenotypes of mutant genes.

CHAPTER THREE

METHODS

3.1. Preparation of competent *E. coli* cells

3 ml of liquid LB or 2x YT media was inoculated with one colony of each of the three strains and incubated at 37°C overnight with vigorous agitation. 2 x 250 ml of media pre-warmed to 37°C was inoculated with 500 µl of the overnight grown cells and incubated at 37°C while agitating until the optical density reached 0.4, (approximately 3.5 hours or about 5×10^7 cells/ml). The cells were chilled on ice and then centrifuged at 6,000 RPM in an IEC centrifuge. All subsequent stages were performed at 4°C. The supernatant was gently decanted and the pellet resuspended in 10 ml sterile 0.1 M CaCl₂, (Hanahan, 1983), using sterile 10 ml pipette to loosen the cells. The cells were transferred into 50 ml sterile centrifuge tubes and topped to two-thirds of the tubes with 0.1 M CaCl₂, mixed and incubated on ice for 30 minutes. The cells were then centrifuged for 5 minutes at 4°C at 6,000 RPM and the pellets gently resuspended in 5ml of 0.1 M CaCl₂ with 15% glycerol (made from sterile 1 M solution of CaCl₂ and 60% sterile glycerol solution). The cells were then transferred into 10 ml sterile tubes and gently mixed until the solution became homogenous. The suspensions were incubated on ice for 30 minutes and then aliquoted into 200 µl in sterile 1.5 ml pre-chilled eppendorf tubes on ice, labelled and immediately transformed with DNA or stored at -70°C.

3.2. Preparation of competent GM-C2 yeast cells

Overnight cultures of yeast cells from previously stored stock were grown in 1.5 ml media with glucose (YPD) without ampicillin at 30°C with agitation

overnight. 20 µl of the cell cultures were used to inoculate 100 ml YPD media in 2x 250 ml flasks and incubated overnight at 30°C with agitation. The cells were centrifuged at 5,000x g for five minutes at 4°C and the pellets resuspended in 10 ml of sterile 0.1 M lithium chloride in 1X TE buffer, (Ito, *et al*, 1983) and centrifuged again as above. The pellets were resuspended again in 1.2 ml of lithium chloride in TE buffer and incubated at 30°C for 30 minutes with agitation. The cells were aliquotted into 300 µl in sterile eppendorf tubes and 100 µl of 60% glycerol (15 % final concentration) was added. The cells were transformed immediately or stored at -70°C.

3.3. Preparation of single stranded (ss) DNA Templates

Single stranded DNA templates of pING4 plasmid were prepared in CJ236 *E.coli* cells using M13K07 helper phage, (an 8.7 kbp phage DNA with kanamycin resistance selectable marker), (Viera and Messing, 1987), following the Muta-Gene® Phagemid *in vitro* Mutagenesis Instruction Manual.

3.3.1. Preparation of phage plasmid DNA

Two sterile test tubes each containing 2 ml of 2x YT liquid media with 100 µg/ml Ampicillin were inoculated with *E. coli* CJ236 cells carrying pING4 plasmid. The cultures were incubated at 37°C overnight in a gyrating water bath. Two 250 ml flasks each containing 50 ml of 2x YT media were each inoculated with 1 ml of the bacterial cultures grown overnight. The cultures were incubated at 37°C for 3 hours while shaking until the optical density of the cell suspension reached 0.4. 50 µl of M13K07 (Muta Gene) or its derivative R408 helper phage (Strata Gene), corresponding to multiplicity of

infection of about 20 (20 phage particles per cell) were added to each flask and the cultures allowed to grow at 37°C for a further 1 hour to allow the helper phage to infect the cells. 50 µg/ml kanamycin (from a stock of 70 mg/ml) was then added to the cultures which were again allowed to grow for 4 to 5 hours. The cell cultures were then centrifuged at 17,000 g (12,250 R-PM) on Beckman J2 SH centrifuge with a J20 rotor for 15 minutes at 4°C. The supernatants were re-centrifuged for 15 minutes as above to remove all cell suspension before adding RNase (5 µg/ml) and allowing to digest for 30 minutes at room temperature. The DNA was precipitated with a final concentration of 3.5 M ammonium acetate in 20% polyethylene glycol (PEG 4000) and centrifuged at 17,000 x g for 15 minutes. The resulting pellet was dissolved in 200 µl of high salt concentration buffer containing 0.3 M NaCl, 0.1 M Tris pH8.0 and 1mM EDTA pH8.0. The suspension was transferred into 1.5 ml microfuge tubes and centrifuged for 5 minutes.

3.3.2. Extraction of ssDNA from viral proteins

The DNA above was immediately extracted from the phage particles with phenol; phenol-chloroform-isoamyl alcohol (50:49:1); chloroform-isoamyl alcohol (49:1) and finally with chloroform alone until there was no debris at the interface. The DNA was then precipitated at -70°C for 1 hour by adding one-tenth of the total volume of 10 M ammonium acetate and 2.5x vol/vol of cold absolute ethanol. The DNA was then centrifuged on a microfuge for 15 minutes at 4°C and washed once with 70% ethanol. The pellet was dried in a Speed Vac centrifuge (Sarvant) and then dissolved in 50 µl of 1x TE buffer. The concentration of single stranded DNA was estimated from the absorbance on the spectrophotometer at 260 nm using the formula: $\text{conc} = (120 \times$

mula: $\text{conc} = (120 \times \text{Ab}_{260}) \times \text{dilution factor}/\text{number of bases}$, (in picomoles per μl) and was found to be 0.63 pmol/ μl for pING4. 5 μl of the DNA sample were electrophoresed on agarose gel and found to migrate faster than the undigested pING4 plasmid or the phagemid DNA, although the bands were fairly faint. The DNA was either used immediately or stored at -20°C until required for mutagenesis .

3.4. Phosphorylation of the oligonucleotides

The oligonucleotides were phosphorylated in order to improve the frequency of mutagenesis, since they were synthesized without the 5'- phosphate group necessary for mutagenesis. This is necessary for ligation of the DNA during mutagenesis. The oligonucleotides were contained in ammonium hydroxide solution. Each sample was aliquoted into 100 μl and the ammonium hydroxide removed with 1-butanol and centrifugation on a microfuge for 5 minutes. The DNA was then vacuum dried on Speed Vac and each aliquot resuspended in 50 μl 10 mM Tris and 1 mM EDTA buffer. The concentrations of the oligonucleotide preparations were determined by measuring absorbance at 260nm on a Cary 2290 spectrophotometer and calculated by the formula:

$(W \times 38 \times 10^4 / N \times 33, \mu\text{l})$ to give 10 picomoles per μl . [W = no of O.D. units, N = Length of the oligo] (1 O.D. unit = 38 μg of DNA)]

The concentration of Y67F oligo was found to be 364 pmoles/ μl ; N52V was 316 pmoles/ μl . Before use the oligonucleotides were phosphorylated according to the method described in the Muta-Gene Phagemid *in vitro* mutagenesis instruction manual. 1 μl (4.5 units) of polynucleotide kinase (PNK), 1 μl

of 10 mM neutralized ATP, 3 μ l of 1 M Tris pH 8.0, 1.5 μ l of 10 mM $MgCl_2$ and 1.5 μ l of 5 mM dithiothreitol were added to 1 μ l of each of the oligonucleotide (equivalent to 364 pmoles of Tyr-67 to Phe; 316 pmoles of Asn-52 to Val and 488 pmoles of Asn-52 to Ile). The mixtures were incubated at 37°C for one hour and then at 65°C for 10 minutes to stop the reaction. The oligonucleotides were diluted to 6 pmoles/ μ l with 1x TE buffer and either used immediately or stored at -20°C for use in mutagenesis. The rest of the oligonucleotides were stored at -20°C unphosphorylated.

3.5. *In vitro* mutagenesis

Site-directed mutagenesis was performed according to the previously described method, (Zoller and Smith, 1987; Botstein and Shortle, 1985; Kunkel, *et al*, 1989), following the outline in the Bio-Rad's Muta-Gene Phagemid *in vitro* Mutagenesis instruction Manual (cat. number 170-3576) and using the pING4 expression vector, (Pielak *et al*, 1985; Inglis, *et al*, 1991).

3.5.1. Annealing of oligonucleotide primers to the template

In microfuge tubes 0.3 pmoles of single stranded DNA template prepared above in 1 μ l 1x TE buffer was added to 6 pmoles of both Tyr-67 to Phe and Asn-52 to Val phosphorylated oligonucleotide primers each in 1 μ l of 1x TE buffer. To the template and primer mixtures were added 1 μ l 10x annealing buffer and then made upto 10 μ l with distilled water. Control reactions were set up in a similar manner but without the priming oligonucleotides. The microfuge tubes with the reaction mixtures were then placed in a 70°C water

bath which was then allowed to cool to 30°C over a period of 40 to 60 minutes at room temperature. The samples were then put on ice-water bath for 5 minutes. While the microtubes were still on ice-water bath, 1 µl of 10x synthesis buffer, 1 µl (3 units) of T4 DNA ligase and 1 µl of T4 DNA polymerase enzyme containing 1 unit, (0.5 µl diluted to 2.5 with enzyme dilution buffer), were added to each reaction mixtures. The reaction mixtures were incubated on ice for 5 minutes then at 25°C for another 5 minutes and finally at 37°C for 4 hours to allow the extension of the primers and synthesis of the double stranded DNA. The synthesized products were either used immediately for transforming *E. coli* MC1066 cells or stored at -20°C until required for transformation.

3.6. Transformation of MC1066 *E. coli* cells with synthesized DNA

Competent *E. coli* MC1066 cells were transformed with plasmids according to the method described by Hanahan (1983) with some modifications. 10µl of mutagenesis reaction products from the above step were mixed with 45µl of 5mM MgCl₂/5mM Tris pH 8 and 90µl of the competent cells. Controls with 1) double stranded DNA; 2) single stranded DNA without primers and 3) cells without transforming DNA were also prepared. The cell suspensions were gently mixed with pipette tips and chilled on ice for one hour. The cells were then treated at 37°C for 5 minutes to allow them to take in the foreign DNA. The cells were again chilled on ice for 5 minutes after which 500 µl of liquid LB media without antibiotics was added to each tube and incubated at 37°C to outgrow for 1 hour.

40 µl of each cell suspension was spread onto plates with LB-agar media with 100 µg/ml ampicillin. The remaining cells with DNA were also spread onto other plates with ampicillin. Half of the control cells without transforming DNA were spread on plates with media without ampicillin and the other half media with ampicillin. The culture plates were incubated at 37°C overnight. Transformed colonies from plates with cells carrying synthesized DNA were picked, re-plated on fresh LB agar media with ampicillin and incubated at 37°C overnight. The cells without the plasmid DNA as well as those transformed with ssDNA template without primers did not grow on media with ampicillin.

3.7. Restriction endonuclease screening of possible mutants

Mini prep DNA from transformed colonies was obtained by the alkaline lysis and phenol-chloroform extraction method, (Sambrook *et al* 1989). 2x 2ml cultures of transformed cells were grown overnight at 37°C. The cells were centrifuged in a microfuge for five minutes and 100 µl of solution I added. The cell pellets were vortexed for one minute to break up the cells before adding solutions II and III. The suspensions were centrifuged for five minutes and the supernatants collected in fresh microfuge tubes. The DNA was then precipitated by adding 1/10th v/v solution III and cold absolute ethanol. The suspensions were then centrifuged for 15 minutes in a microfuge at 4°C. The resulting DNA precipitate was then dissolved in 100 µl of 1x TE buffer. 100 µl of 10 µg/µl DNase free RNase was added and incubated at 37°C for 30 minutes. The DNA was subsequently extracted once with 1:1 phenol-chloroform and then chloroform, precipitated with 3 M potassium acetate and absolute ethanol and then centrifuged for 15 minutes

at 4°C. The resulting DNA precipitate was washed with cold 70% ethanol, dried in a Speed Vac and reconstituted in 20 µl 1x TE buffer. The DNA preparations were digested with EcoRI restriction enzyme and electrophoresed on a 1% agarose gel in 1x TBE buffer. The DNA that showed four bands on the gel, (fig 1.) was used to transform *E. coli* strain DH5α cells from which approximately 20 µg of DNA was obtained by the alkaline lysis, phenol chloroform mini-prep method and used for sequencing. DH5α cells were preferred because they gave higher and cleaner yields of DNA without the contaminating chromosomal DNA than the original MC1066 strain.

3.8. DNA sequencing

Double stranded DNA of both the wild type and mutant plasmids were sequenced in order to confirm the mutations. Sequencing of both the wild type and mutant pING4 DNA was done by the dideoxy method (Sanger, *et al*, 1977) using bacteriophage T7 DNA polymerase (Tabor & Richardson, 1987) provided in the T7 Sequencing kit (Pharmacia Biochemicals Co. Ltd).

3.8.1. Preparation of double stranded DNA for sequencing

Double stranded DNA was denatured according to the instructions outlined in the T7 Sequencing Kit instruction manual with minor modifications. 5 µl containing approximately 5 µg of DNA were mixed with 27 µl of water and 8 µl of 2 N NaOH in microfuge tubes, vortexed briefly and centrifuged for five seconds. To each microfuge tube 7 µl of 3 M sodium acetate pH 4.6, 4 µl water and 120 µl of cold absolute ethanol were added, mixed and the solutions were placed at -70°C for 30 minutes. The solutions in the microfuge

tubes were centrifuged for 15 minutes at 4°C and the pellets washed with cold 70% cold ethanol. After centrifugation for 15 minutes at 4°C the pellets were dried in a Speed Vac centrifuge for 5 minutes then dissolved in 10 µl water.

3.8.2. Annealing reaction

The sequencing primer (C-T: 5'-GGAAAAATGCAGAAACG-3') was diluted to 5 µg per µl with water. To the 10 µl of denatured DNA template above, 2 µl of the diluted prime was added to give a final concentration of 1 µg/µl. To the denatured template and primer mixture was added 2 µl of annealing buffer (provided in the sequencing kit). The solutions were vortexed gently, centrifuged briefly and incubated at 65°C for 5 minutes before being transferred to 37°C incubator for 10 minutes then to room temperature for at least 10 minutes.

The primer extension using T7 polymerase was done exactly according to the specification in the T7 Sequencing manual. For each DNA sample 3 µl of the template, 1 µl ³⁵S labeled dATP (New England Nuclear, Du Pont) and 2 µl diluted T7 DNA polymerase (diluted to 0.5 units/ml) were added together. The whole 6 µl of the pre-mixed enzyme was added to the annealed DNA template and primer, briefly centrifuged and incubated at room temperature for 5 minutes. Into four fresh microfuge tubes labeled A, C, G and T were added 2.5 µl of Short dATP, dCTP, dGTP & dTTP (T7Sequencing kit), respectively for each annealed reaction sample, ("Short" dNTP is normally used for sequencing DNA of not more than 300 bases). 4.5 µl of the corresponding annealed reactions were added to each dNTP tubes, mixed,

centrifuged briefly and incubated for 5 minutes at 37°C. To terminate the reactions 5 µl of stop solution (from the kit) was added to each reaction tube and centrifuged briefly. The samples were either heated to 80°C for 2 minutes and loaded directly onto the sequencing gel or stored at -20°C. The gel (6% acrylamide previously polymerized overnight by adding 10% ammonium persulfate and 30 µl of N, N, N', N'-Tetramethylethylenediamine (TEMED) per 100 ml gel solution) was pre-run for 45 minutes at 1200 volts before loading the samples. Electrophoresis was allowed to run for 4 hours at 2000 volts and at temperature between 45 and 50°C. The gels were dried on vacuum gel drier (Speed Vac) for one hour before exposure to a Kodak X-ray photographic film for 48 hours. The films were developed manually, air dried and the nucleotide sequences read.

3.9. Transformation of GM3-C2 yeast cells with mutant DNA plasmids

Yeast cells were transformed with both wild type and mutant plasmids using the alkali cation method of Ito, *et al*, (1983). 10 µg of wild type and mutant pING4 DNA were each used to transform 200 µl of freshly prepared competent GM-3-C2 yeast cells. The competent cells and DNA were added together in microfuge tubes along with 185 µg of carrier DNA (boiled and sonicated salmon sperm DNA), in 25 µl of TE buffer and incubated at 30°C for 30 minutes with agitation. 1.2 ml of 40% PEG 4000 in 0.1M lithium chloride diluted in TE buffer was added to each sample, vortexed briefly to mix and then incubated at 30°C for 30 minutes with agitation. The cells were heat shocked at 42°C for 15 minutes, incubated at room temperature for 15 minutes and pulse-centrifuged for 5 seconds. The cells were washed twice with sterile distilled water, centrifuged for 10 seconds and the pellets

resuspended in 500 µl of water. The cells with DNA as well as controls without DNA but treated in the same way were spread on SD Leu⁻ plates and incubated at 30°C for two days to select for the GM3-C2 strain. One colony of transformed cells with the wild type and mutant plasmids each was transferred to YPG media plates and incubated for two days at 30°C to select for the cells carrying the cytochrome *c* gene in the plasmids.

3.10. Curing yeast cells of the plasmids

A colony from each group of cell cultures was inoculated into 2 ml of liquid media with dextrose, (glucose) (YPD) with 100 µg/ml ampicillin and incubated at 30°C for two days. The cultures were sub-inoculated into fresh 2 ml YPD liquid media and incubated at 30°C for a further two days. The cultures were then spread onto YPD media plates with 100 µg/ml ampicillin and incubated at 30°C for one day. Twenty four distinct colonies were picked and transferred onto another YPD plate and incubated overnight at 30°C. Cells from each of these colonies were transferred to SD Leu⁻ and YPG (glycerol) plates and incubated at 30°C for two days.

3.11. Growth of yeast cells

Yeast cells carrying both wild type and mutant plasmid DNA were grown for two days at 30°C on liquid media with glycerol. The cells were diluted to a maximum of 0.2 O.D. units at 600 nm with fresh media. Further dilutions were made as follows: 1/2, 1/4, 1/8, 1/10 and 1/20 after which the cells were counted on the Neuber chamber under the microscope. The cell counts were plotted against the predetermined O.D. readings, (Fig.3), in order to obtain a calibration curve correlating the actual cell counts with the O.D. readings.

20 µl of both wild type and mutant cells from the above cultures were used to inoculate duplicate test tubes of YP media with 0%, 0.0312%, 0.0625%, 0.125%, 0.250%, 0.5%, 1%, and 2% glycerol. The cultures were incubated at 30°C for two to three days until the cells reached stationary phase as judged microscopically by the absence of budding cells. Samples from each culture were diluted 20x in YP media and their absorbance determined on the spectrophotometer at 600nm and the absorbances matched with the concentrations on the correlation curve from which the total cell growth yields were obtained.

100 µl of both wild type and mutant cell cultures grown in YP media with glucose overnight were used to inoculate triplicate 250ml flasks, each containing 25 ml of liquid media with 3% glycerol. The growth rates of both groups of cells were monitored every hour for up to 8 hours by determining the absorbance at 600 nm of cell cultures diluted to a maximum of 0.2 O.D. units. The concentrations of cells per ml were determined from the corresponding cell count in the calibration curve.

3.12. Expression and purification of recombinant cytochromes *c* in yeast

Four ml cultures of yeast cells carrying the wild type and four ml of cells with mutant plasmids were pre-grown for two days at 30°C with agitation in YPG liquid media with 100 µg/ml ampicillin. The cultures were each transferred into 2x 150 ml YPG liquid media with ampicillin and incubated at 30°C overnight on a gyrating incubator. The cells were aseptically transferred to a 20 liters carboy fermenter with 12 or 14 liters of YPG media along with antifoam, 560 mg streptomycin and 140 mg tetracycline. The

incubated in a thermostatically controlled refrigerator/incubator at 30°C and allowed to grow for two days with large amount of air being bubbled into the fermenter while vigorously stirring. The cultures were boosted with lactic acid (pH 5.0) after two days and the cells allowed to grow for another two days. The cells were harvested, when the culture reached approximately 6 O.D. units, by centrifugation at 3,000 RPM in an IEC DPR-6000 centrifuge with swing rotor at 4°C for 10 minutes, in pre-weighed 1 liter centrifuge tubes. Cytochromes *c* were extracted by resuspending the cells in 1/2 v/w of 1 M NaCl in 12.5 mM potassium phosphate buffer pH 7.0 and 1/4 v/w of ethyl acetate in the presence of phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA as protease inhibitors, with vigorous stirring for 20 hours. β -2-mercaptoethanol was added to a final concentration of 1 mM to preserve the protein in a reduced form. The suspensions were centrifuged on an IEC B-20A centrifuge with fixed angle rotor, at 10,000 RPM for 15 minutes at 4°C. The supernatants were collected into measuring cylinders and extraction was repeated on the pellets until the resulting supernatant showed no indication of cytochrome *c* present by the colour of the resulting supernatants. The total volumes of the supernatants were measured and the total amounts of proteins were also determined from the reading of absorbance at 415 nm (extinction coefficient = 129 μ m) using a Varian Carry 2290 spectrophotometer. The supernatants were brought to 50% saturation with ammonium sulfate and centrifuged at 10,000 RPM at 4°C for 10 minutes to precipitate other cellular materials. The yields of the proteins were again monitored. The proteins were dialyzed in a dialysis tubing with MW cut-off: 6-8,000, diameter 14.6 mm (Spectra/Por), for two days against four changes of 8 liters of pre-chilled 12.5 mM potassium phosphate buffer, pH 7.0, in the presence of EDTA and

12.5 mM potassium phosphate buffer, pH 7.0, in the presence of EDTA and β -2-mercaptoethanol. The yields of the proteins after dialysis were monitored. The proteins were then chromatographed through DEAE-Sephacel column (Pharmacia Chemicals), which holds all other cytochromes as well as the negatively charged proteins leaving the positively charged cytochromes *c* to pass into the holding CM-Sepharose CL-6B (Pharmacia Chemicals). While in the holding column the proteins were washed with excess amounts of potassium phosphate buffer before eluting with 250 mM NaCl in 12.5 mM phosphate buffer pH 7.0 and the final yield of each protein determined.

3.13. Spectral analysis of Cytochromes *c*

The spectra of both reduced and oxidized wild type as well as mutant cytochromes *c* were monitored on a Cary 2290 spectrophotometer interfaced with a computer with Spectra Calc software. The proteins were diluted 100 times with 12.5 mM phosphate buffer pH 7.0 to give a maximum absorbance at 415 nm of 1.2 O.D. units and then either fully reduced with sodium dithionite or oxidized with potassium ferricyanide, passed through CM-Sepharose mini-columns and washed with phosphate buffer to remove excess dithionite or ferricyanide. The proteins were put into 1 ml quartz cuvettes with 1 cm light path and the spectra monitored from 350 nm to 750 nm.

3.14. Kinetic assays

Kinetic assays were performed using the polarographic method in low ionic strength buffer, (25 mM Tris-acetate buffer pH 7.9 and 0.1% Tween 80 as detergent), with purified beef heart cytochrome oxidase in a Clarke oxygen electrode chamber (Yellow Springs Instruments) thermostated at 30°C. Reducing substances for both free and oxidase-bound cytochrome *c* to the oxidase were 4 mM ascorbate, (Fluka Chemika), and 0.4 mM N,N,N',N'-Tetramethyl-p-phenylene-diamine dihydrochloride, (TMPD), (Fluka Chemika) The initial concentration of the oxidase in the chamber was 40 nM. The rates of reduction of oxygen to water were monitored on a plotter (Servogor 120), with each addition of cytochrome *c* through an air lock system using Hamilton syringes. The concentrations of cytochromes *c* in the chamber ranged from 10 nM to 4 µM. The assays were performed in triplicates. Assays performed in 12.5 mM potassium phosphate buffer produced irreproducible data for reasons that were unclear.

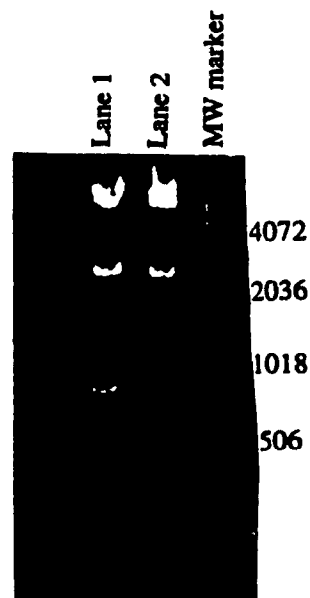
CHAPTER FOUR

RESULTS

4.1. Restriction enzyme digest

The results of the *EcoRI* digests on the mutant and wild type pING4 plasmid DNA are shown in figure 1. The smallest *EcoRI* restriction fragment in pING4 plasmid includes the coding region for cytochrome *c* while the newly introduced *EcoRI* restriction site is 220 bases down stream of this coding region.

Figure 1: *EcoRI* digests of wild type and mutant DNA plasmids. The DNAs were electrophoresed on a 1% agarose gel in 1x TBE buffer and stained with ethidium bromide. The wild type pING4 DNA (lane 1) shows three bands approximately 7.8 kbp, 2.3 kbp and 0.5 kbp. The mutant Asn-52Val/Tyr-67Phe/Cys-102Thr plasmid DNA, (lane 2), shows a reduced size third band and an extra fourth band at about 0.2kbp. Lane 3 is a 1 kb ladder molecular weight marker.



4.2. DNA sequence analysis

More than 200 nucleotide bases (206 for wild type pING4; 216 for Asn-52Val/Tyr-67Phe/Cys-102Thr) were read. The nucleotide sequences are shown in figure 2. The sequences show a replacement of bases complementary to the coding strand for asparagine at position 52 with those bases that are complementary to the ones coding for valine while those complementary to the ones coding for tyrosine at position 67 have been replaced with bases complementary to those coding for phenylalanine in the Asn-52Val/Tyr-67Phe/Cys-102Thr plasmid DNA.

Figure 2. Amino acid and nucleotide sequences of cytochromes c, wildtype and mutant (Tyr-67 to Phe:Asn-52 to Val). The mutations at positions 67 (showing the introduced *EcoRI* site) and 52 are underlined. The nucleotide sequences are complementary to the coding sequences of the Iso-1-cytochrome c gene.

	87	86	85	84	83	82	81	80	79	78
	Lys	Lys	Leu	Gly	Gly	Phe	Ala	Met	Lys	Thr
WT (C102T):	C-CTT-CTT-CAA-CCC-ACC-AAA-GGC-CAT-CTT-GGT-									
N52V/Y67F:	C-CTT-CTT-CAA-CCC-ACC-AAA-GGC-CAT-CTT-GGT-									
	77	76	75	74	73	72	71	70	69	68
	G.	Pro	Ile	Tyr	Lys	Lys	Pro	Asn	Thr	Leu
WT (C102T):	-ACC-AGG-AAT-ATA-TTT-CTT-TGG-GTT-AGT-CAA-									
N52V/Y67F:	-ACC-AGG-AAT-ATA-TTT-CTT-TGG-GTT-AGT-CAA									
	67	66	65	64	63	62	61	60	59	58
	Tyr	Glu	Ser	Met	Asn	Asn	Glu	Asp	Trp	Leu
WT(C102T):	-GTA-CTC-TGA-CAT-GTT-ATT-TTC-GTC-CCA-CAA-									
N52V/Y67F:	- <u>GAA-TTC</u> -TGA-CAT-GTT-ATT-TTC-GTC-CCA-CAA-									
	57	56	55	54	53	52	51	50	49	48
	Val	Asn	Lys	Lys	Ile	Asn	Ala	Asp	Thr	Tyr
WT (C102T):	-CAC-GTT-TTT-CTT-GAT-ATT-GGC-ATC-TGT-GTA-									
N52V/Y67F:	-CAC-GTT-TTT-CTT-GAT- <u>TAC</u> -GGC-ATC-TGT-GTA-									

57	56	55	54	53	52	51	50	49	48
Val	Asn	Lys	Lys	Ile	Asn	Ala	Asp	Thr	Tyr

WT (C102T):-CAC-GTT-TTT-CTT-GAT-ATT-GGC-ATC-TGT-GTA-
N52V/Y67F: -CAC-GTT-TTT-CTT-GAT-TAC-GGC-ATC-TGT-GTA-

47	46	45	44	43	42	41	40	39	38
Gly	Tyr	Gly	Glu	Ala	Gln	Gly	Ser	His	Arg

WT C102T):-CGA-ATA-CCC-TTC-AGC-TTG-ACC-AGA-GTG-TCT-
N52V/Y67F:-CGA-ATA-CCC-TTC-AGC-TTG-ACC-AGA-GTG-TCT-

37	36	35	32	33	32	31	30	29	28
Gly	Phe	Ile	Gly	His	Leu	Asn	Pro	Gly	Val

WT (C102T):GCC-AAA-GAT-ACC-ATG-CAA-GTT-TGG-ACC-AAC-
N52V/Y67F:-GCC-AAA-GAT-ACC-ATG-CAA-GTT-TGG-ACC-AAC-

27	26	25	24	23	22	21	20	19	18
Lys	His	Pro	Gly	Gly	Lys	Glu	Val	Thr	Gln

WT (C102T):-CTT-ATG-TGG-GCC-ACC-CTT-TTC-CAC-GG
N52V/Y67F: -CTT-ATG-TGG-GCC-ACC-CTT-TTC-CAC-GGT-GTG

17	16
His	Cys

WT (C102T):-
N52V/Y67F: -CA'T-TGT

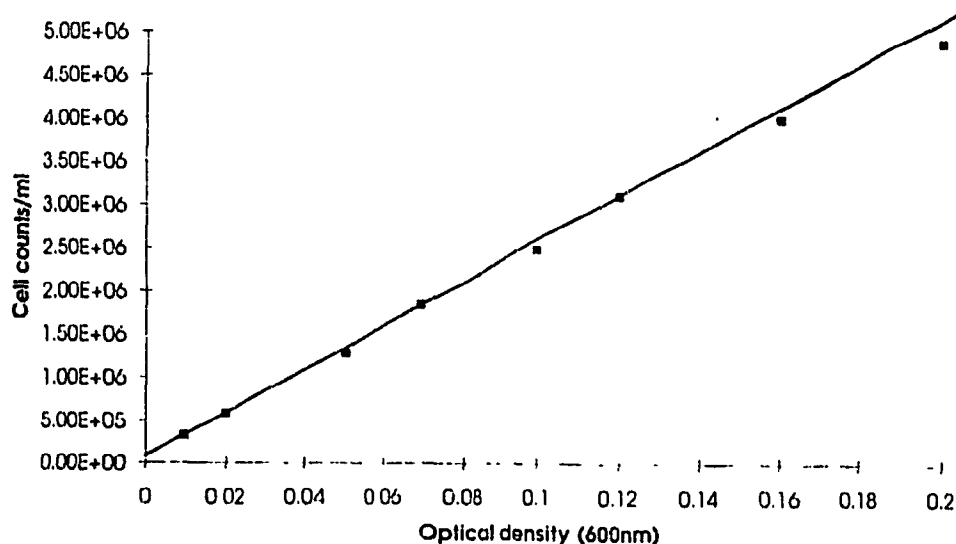
4.3. Growth of cells carrying cytochrome c genes

Yeast cells transformed with both wild type and mutant plasmids were grown at 30°C on media with glycerol as the source of energy. Plates with control cells without DNA did not have any colonies after two days while the plates with pING4 and the mutant plasmid DNA had over twenty colonies. The level of transformation was rather low for all the plasmids but were consistent with results from other workers, (Dr. Yue Huang, personal communication).

4.3.1. Yeast cells cured of the plasmids

Of the twenty-four colonies of yeast cells that were to be cured of the plasmid eight colonies of the cells presumed to be carrying Asn-52Val/Tyr-67Phe/Cys-102Thr mutant were unable to grow on media without leucine and on media with glycerol as the source of energy.

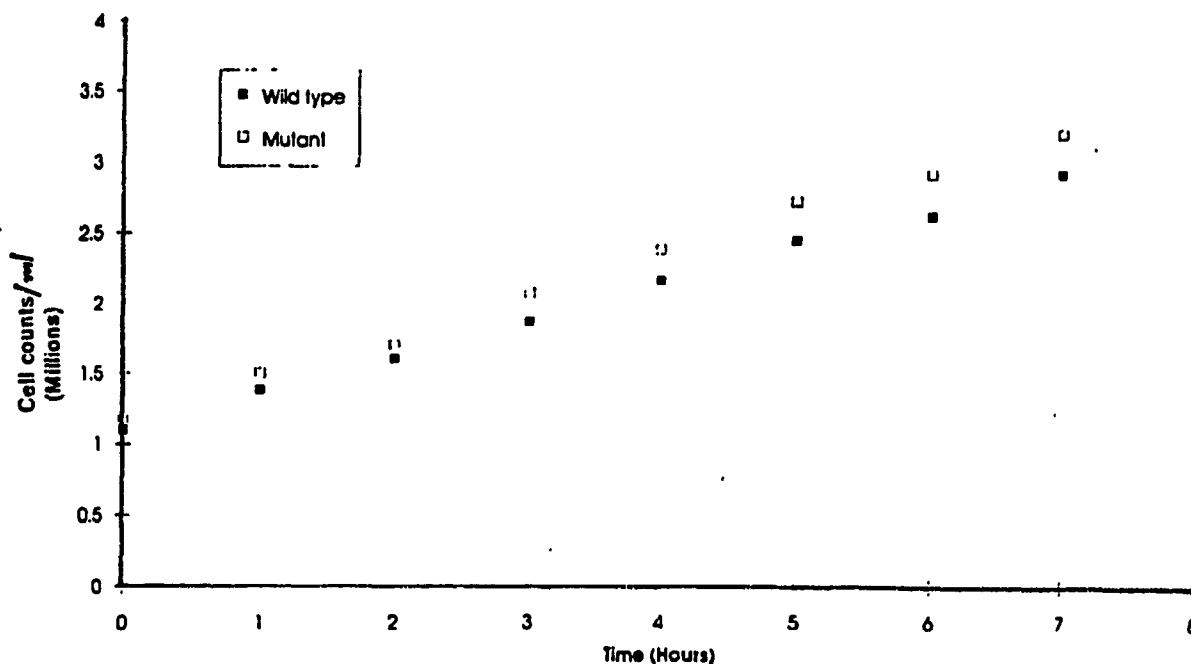
Figure 3: Calibration curve for correlating yeast cell counts with optical densities. The cells were counted on the microscope using a hemacytometer.



4.3.2. Cell growth rate assays

The results of the growth rates of yeast cells carrying both wild type and mutant plasmids on media with glycerol is shown on figure 4. Each determined O.D. was correlated to the cell counts using the calibration curve, (Fig. 3). The data obtained was subjected to a linear regression analysis, which showed that the rates of growth of both cell groups were similar under growth restricting media conditions.

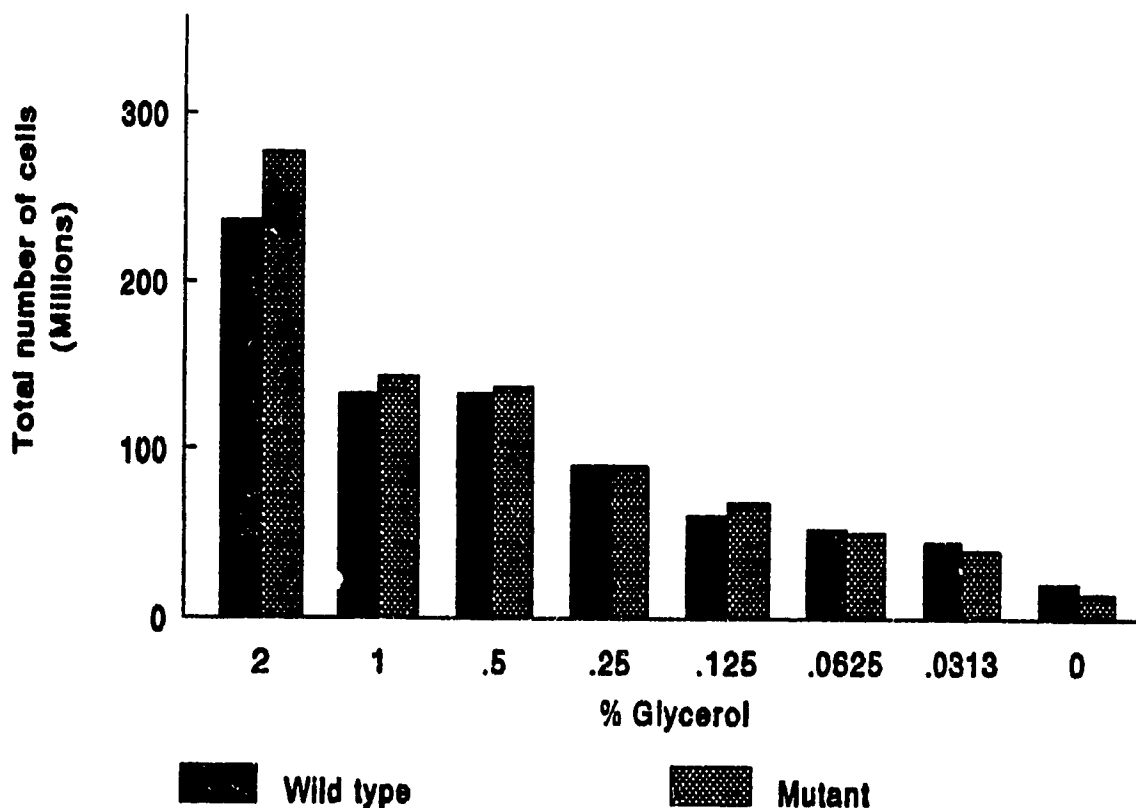
Figure 4: Cell growth rate assays. Yeast cells were grown as described in the methods section and the concentrations determined from the calibration curve.



4.3.3. Cell growth yields

The total yields of yeast cells carrying both wild type and mutant plasmids are shown in figure 5. Equal amounts of cells were used to inoculate 2ml YP media with varying amounts of glycerol as the growth limiting factor. Cultures were grown for three days to stationary phase. However, the cells with mutant plasmid reached stationary phase on the second day but the assays were performed the following day when the cells with wild type plasmid reached stationary phase. The results show that the total number of cells carrying mutant plasmid was slightly higher than the number of cells with wild type plasmid.

Figure 5: Cell growth yields. Yeast cells were grown for three days until cultures of both wild type and mutant cells reached stationary phase



4.4. Total cytochromes *c* purified from yeast cells

The total yield of the proteins after each purification step is shown in Table 2. The cultures were grown on media containing glycerol for four days with lactate boost on the second day. Cytochromes *c* were extracted from the cells and chromatographed through DEAE and CM-Sepharose columns. The proteins were eluted from the CM-Sepharose columns with 250 mM NaCl in 12.5 mM potassium phosphate buffer pH 7.0. The total yield of the protein from 12 liters of wild type cell culture was 26 mg, while the total yield from 14 liters of mutant cell culture was 31 mg.

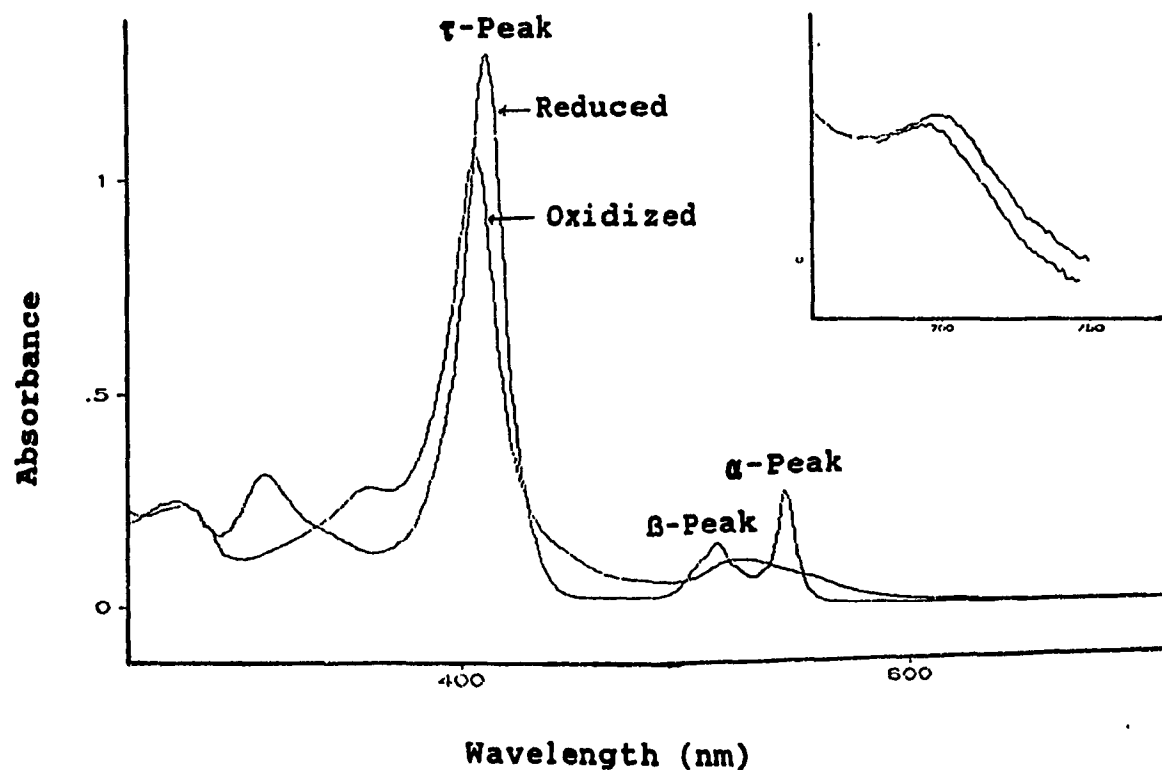
Table 1: Purification of cytochromes *c* from yeast cells. The wild type (Cys-102Thr) was grown in 12 liters while the mutant (Asn-52Val/Tyr-67Phe/Cys-102Thr) was grown in 14 liters of culture.

Cells	Weight of cells	Weight of proteins - crude extract	Weight of proteins after precipitation	Weight of proteins after dialysis	Weight of proteins after chromatography
Wild type	165 g	121.2 mg	47.8 mg	29.2	26 mg
N52V/ Y67F/ C102T	178 g	148.7 mg	63.8 mg	34.7 mg	31 mg

4.5. Spectral characteristics of purified cytochromes *c*

The absorption spectra of both reduced and oxidized cytochromes *c* are shown in figures 6. The figure shows the spectra of the mutant Asn-52Val/Tyr-67Phe/Cys-102Thr in the oxidized and reduced forms. In the reduced forms the spectra show the specific characteristic α -peak at 550 nm, the β -peak at 521 nm and the Soret peak at 415 nm. In the oxidized forms the spectra show a shift in the 695 band in the mutant protein to 701 nm, (inset).

Figure 6: Absorption spectra of reduced and oxidized cytochrome *c*. Inset: the 695 nm band of oxidized form of wild type overlayed on the spectra of the mutant. The spectra were collected with a Carry 2290 spectrophotometer as described in the methods section.



4.6. Kinetic assays

The kinetic activity between cytochrome oxidase and both wild type and mutant cytochromes *c* are shown in figures 7 a & b. Transformed to the Eadie-Hofstee-Scatchard plots (insets), the data show the characteristic high and low affinity kinetic phases on the oxidase, (Ferguson-Miller, *et al*, 1976; Garber, *et al*, 1988; Cooper, *et al*, 1990; Sinjorgo, *et al*, 1983 & 1986), for both wild type and mutant proteins.

The corresponding V_{\max} and K_m s of the activity with oxidase are shown in table 2. In wild type yeast iso-1-cytochrome *c* the low affinity activity had K_m of $1.1 \times 10^{-5}M$ and V_{\max} of 488, while the high affinity activity had a K_m of $2.2 \times 10^{-8}M$ and V_{\max} of 93. In the mutant Asn-52Val/Tyr-67Phe/Cys-102Thr the low affinity activity had a K_m of $8.3 \times 10^{-6}M$ and V_{\max} of 300, while the high affinity activity had a K_m $2.9 \times 10^{-8}M$ and V_{\max} of 102.

Figure 7a: Kinetic assays of wild type yeast iso-1-cytochrome *c* with cytochrome oxidase. Inset: Eadie-Hofstee-Scatchard plots of the activity.

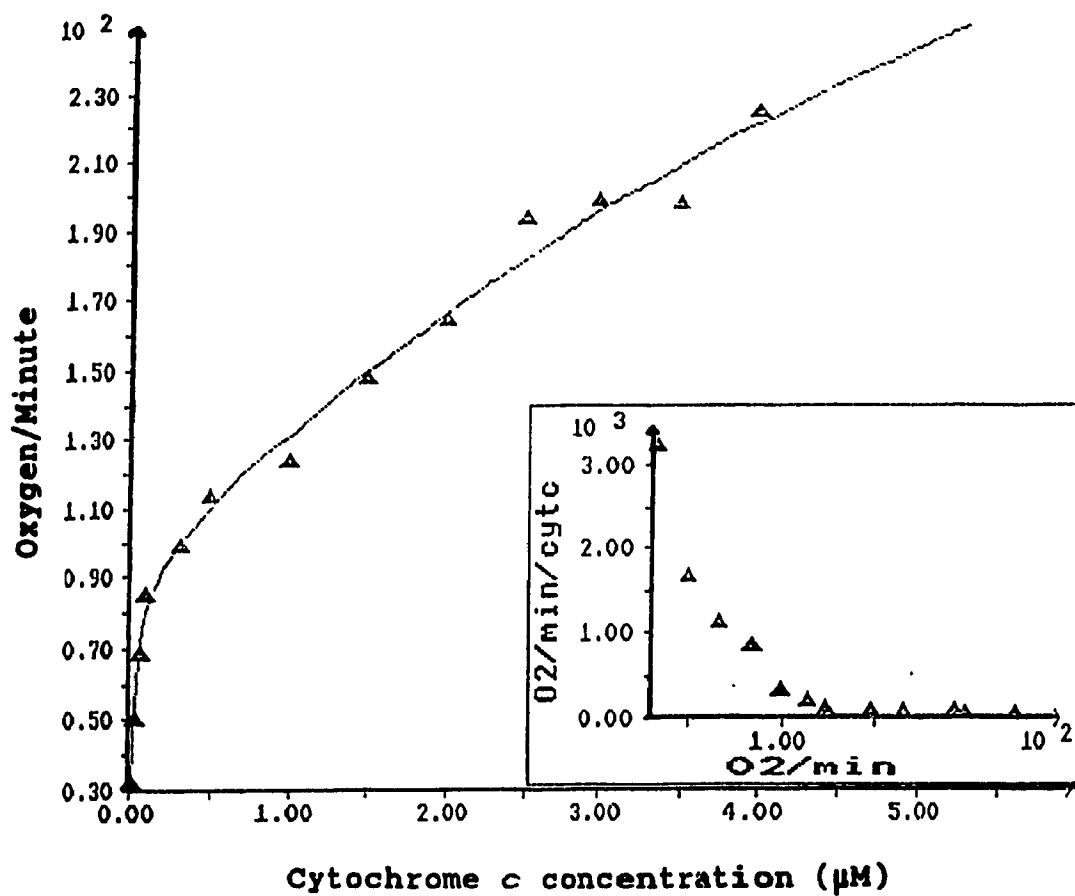


Figure 7b: Kinetic assays of mutant (Asn-52Val/Tyr-67Phe/Cys-102Thr yeast iso-1-cytochrome *c* with cytochrome oxidase. Inset: Eadie-Hofstee-Schatchard plots of the activity.

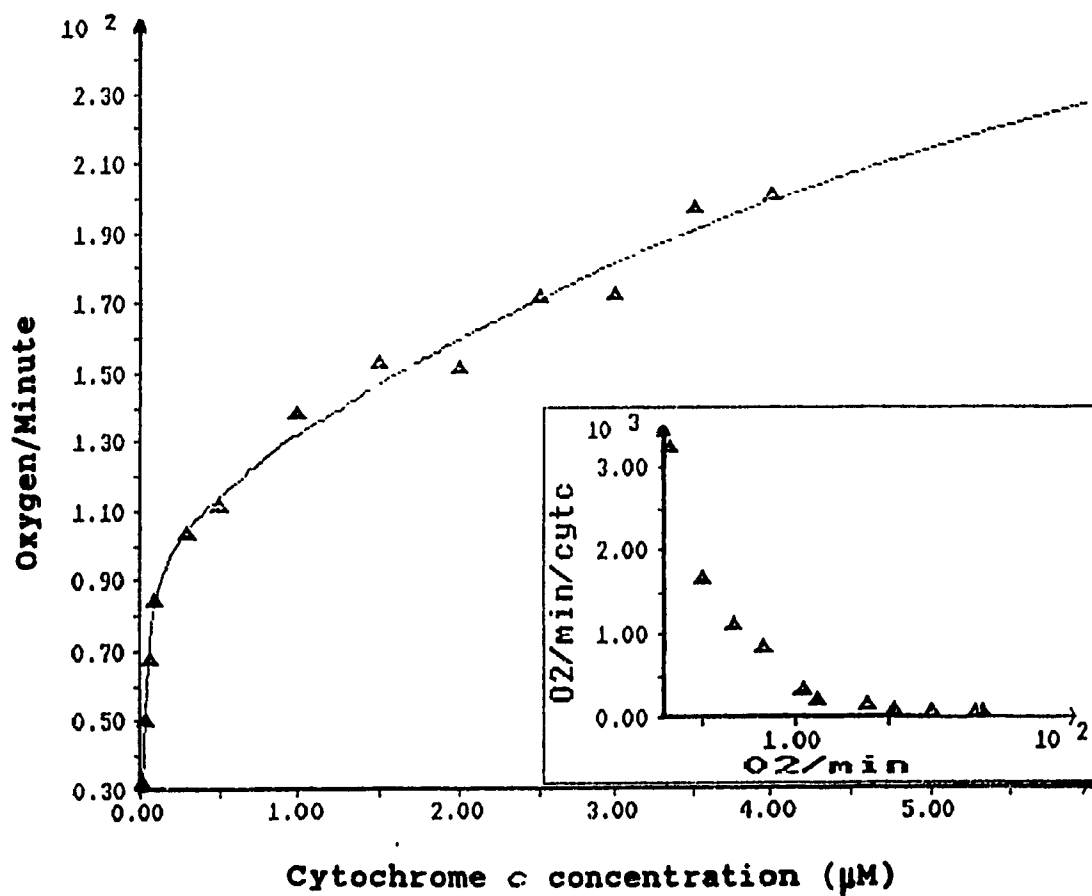
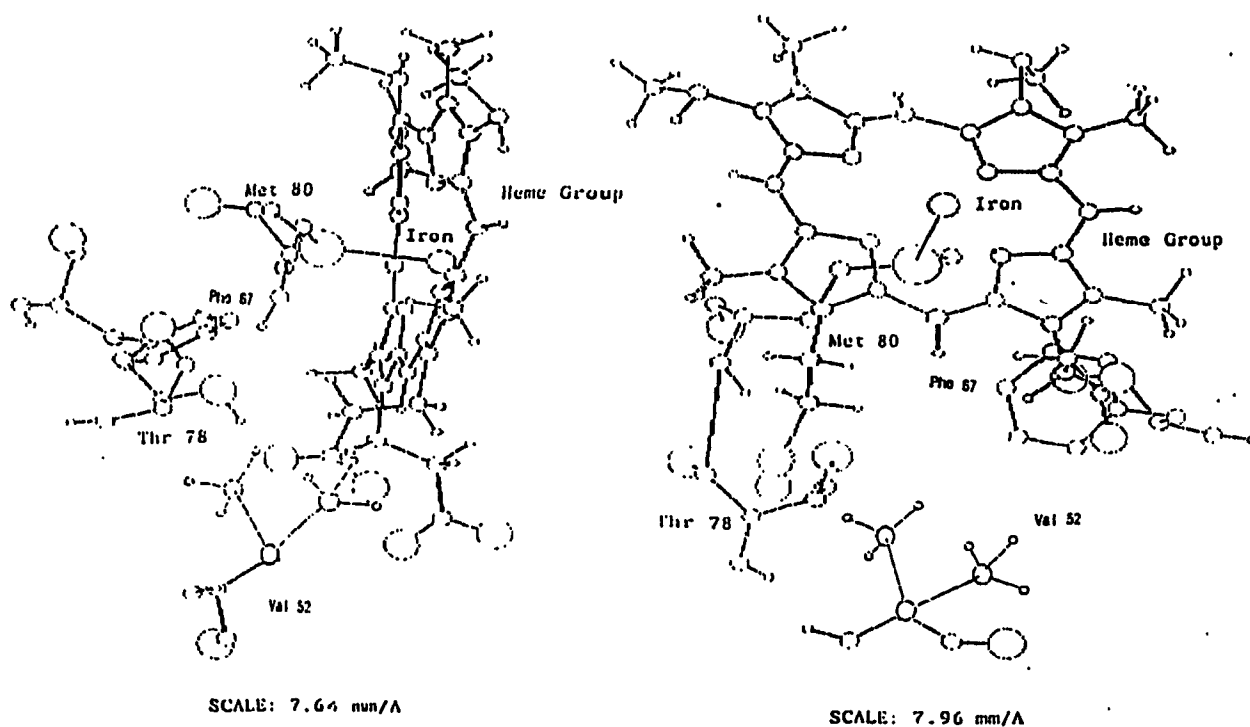


Table 2: Kinetic analysis of wild type and mutant cytochromes *c* activity with purified beef heart cytochrome oxidase. The assays were performed in 25 mM Tris-acetate buffer pH 7.9 with 0.1% Tween 80 in the presence of 4 mM ascorbate and 0.4 mM TMPD. The starting concentration of the oxidase in the chamber was 40 nM. The standard errors for V_{max2} in both cases were more than 50% while the corresponding V_{max1} had standard errors less than 10%.

	V_{max1}	K_{m1} (μ M)	V_{max2}	K_{m2} (μ M)
Wild type(C102T)	92.9	0.022	488	11
Mutant(N52V/Y67F/C102T)	102	0.029	300	8.3

Figure 8: The proposed structure of the heme environment of mutant (N52V/Y67F/C102T) yeast iso-1-cytochrome *c*.



CHAPTER FIVE

DISCUSSION

5.1. Mutagenesis

The mutation changing Tyr-67 to Phe involved replacing a thymine base with an adenine in the middle of the codon at position 67 (GTA to GaA). It was also apparent from the nucleotide sequence of yeast iso-1-cytochrome c gene that creating a silent mutation at position 66 (CTC to tTC) would provide a restriction site for the enzyme *EcoRI* but otherwise cause no change in the amino acid since CTC and TTC are an alternate complimentary bases to the codons for leucine. This silent mutation became a useful marker in screening for mutant plasmid DNA for possible introduction of an extra *EcoRI* site, in the Asn-52Val/Tyr-67Phe/Cys-102Thr mutant.

Upon digestion with *EcoRI* restriction enzyme, the mutant Asn-52Val/Tyr-67Phe/Cys-102Thr plasmid migrated on the 1% agarose gel with four bands, indicating an introduction of an extra *EcoRI* site. Both the wild type and the mutant plasmid DNAs were sequenced and the sequences confirmed the nucleotide base changes indicating that the required mutations were present in the plasmid. The mutant plasmid DNA was used to transform yeast cells strain GM3-C2. Transformants were selected on their ability to grow on media without leucine, (Faye, *et al*, 1981). Selected yeast cells were then transferred to media with glycerol.

5.2. Characterization of yeast cells carrying mutant cytochrome *c* gene

During glucose fermentation cytochrome *c* is not required. It has been found that in the presence of glucose, synthesis of cytochrome *c* is inhibited, (Zottimer & Nicholls, 1978). Cytochrome *c* is absolutely required for the growth of yeast cells in media with non fermentable substrate such as glycerol. For this reason, only the cells with a functional gene expressing cytochrome *c* can grow on media with glycerol. Yeast cells carrying the mutant plasmid were able to grow on this media, indicating that the cytochrome *c* expressed by this mutant gene was functional.

When grown on rich media with glucose, yeast cells tend to lose the plasmid carrying cytochrome *c* gene. The cells carrying the plasmid pING4 can therefore be cured of the plasmid by growing them on media with glucose as the source of energy, (Faye, *et al*, 1981). Curing the cells of the plasmid was done in order to ensure that the yeast carried cytochrome *c* gene on the plasmid. The cells that cannot be cured are considered to be contaminants carrying the gene in their chromosomes, (Faye, *et al*, 1981).

Individual colonies of yeast cells grown on glucose media were transferred back to glycerol containing media. After three generations of growth under non-selective conditions one third of the colonies were not able to grow on glycerol media, suggesting that they had lost the plasmid and were therefore not expressing cytochrome *c* required for respiratory electron transfer chain to be completed.

The results obtained from transformed cells cured of the plasmid by growing them on media with glucose as a source of carbon is an indication that the expression of cytochrome *c* enabling the cells to grow on YPG media was due to the transforming plasmid and not as a result of reversion in the yeast chromosomal gene back to wild type. The conclusion arising from these observations is that the mutant gene was the one expressing a functional cytochrome *c*.

Yeast cells carrying both wild type and mutant cytochrome *c* genes were grown on media with glycerol. The growth rates of cells carrying both wild type and mutant genes were monitored for eight hours. The growth curves show no significant difference between the cells with wild type and those with mutant genes as the cells carrying the mutated plasmid grew on YPG media as well as the wild type. However, the total growth yields were significantly different, since the cells carrying the mutant plasmid reached the stationary phase in two days while those carrying wild type plasmid reached their stationary phase on the third day. These results suggest that the cells carrying the mutant gene had a somewhat faster growth rate. The data shown in figure 5 were collected on the third day after both groups of cells reached stationary phase. The total yields of purified wild type and mutant cytochrome *c* were however comparable.

5.3. Spectral characteristics

The absorption spectra of reduced and oxidized forms of both wild type and mutant cytochromes *c* were determined and the results showed a shift in the 695nm band in the oxidized form of the mutant to 705nm. The 695 nm band

is related to the integrity of the bonding between the sulfur atom of Met-80 and the heme iron. A shift in this band is therefore suggestive of a structural change around the heme ligand (Schejter and Saludjian, 1969), in this case, attributable to the loss of hydrogen bonding between Met-80 and Tyr-67 that exists in the wild type protein. These results suggest that the mutations caused structural changes in the heme environment which appear to affect Met-80 and heme ligand.

5.4. Kinetic assays

Kinetic studies with beef heart cytochrome oxidase have shown that two activity sites of interaction on the enzyme with K_m of about $6 - 5 \times 10^{-7}$ M and K_m about $3.5 - 8 \times 10^{-5}$ M. These sites are normally demonstrated at low ionic strength, (Nicholls, 1965; cited in Garber, *et al*, 1988; Ferguson-Miller *et al*, 1976; Rieder & Bosshard, 1978; Cooper, 1990; Sinjorgo, *et al*, 1984; 1986). Other kinetic studies using yeast iso-1-cytochrome *c* with beef heart mitochondrial particles and performed in 25 mM Tris-acetate buffer pH7.9, have also shown the two kinetic phases with the low affinity K_m of 7.7×10^{-7} M and high affinity K_m of 6×10^{-9} M, (Garber, *et al*, 1988).

The results obtained in this study also show similar kinetic activities with low affinity K_m s within the micro molar range, (8×10^{-6} to 1.1×10^{-5}) and the high affinity within the nano molar region (2.2×10^{-8} to 2.9×10^{-8}), with both the wild type and mutant cytochromes *c*. Polarographic assays were performed in 25 mM Tris-acetate buffer. Many attempts were made to perform the experiments in 12.5 mM phosphate buffer. However, the data repeatedly showed only one site of activity due to the fact that phosphates

compete with the substrate in binding to the oxidase (Garber, *et al*, 1988). The data showed no significant differences between the wild type and the mutant proteins in the reaction kinetics of cytochrome *c* and the purified oxidase. The results are very similar to those cited above for the wild type mammalian and fungal proteins.

The fact that the two kinetic sites could still be demonstrated on the oxidase with the mutant cytochrome *c* suggests that the mutations did not cause any conformational change in the protein. However, there are indications of clear changes in the hydrogen bonding around the heme region, as suggested by the shift in the 695 nm band and the other data obtained in this study.

5.5. The heme environment around Wat-166

The role of Tyr-67 in the function of cytochrome *c* has been studied extensively, (McGowan & Stellwagen, 1970; Koul *et al*, 1979; ten Kotenaar, *et al*; Luntz *et al*, 1989; Schejter *et al*, 1992). Replacing Tyr-67 with non polar residues to removes the hydroxyl group from the region thereby making the heme environment more hydrophobic, (Koul *et al*, 1979; ten Kortenaar *et al*, 1985; Wallace, *et al*, 1989). Such mutations would be expected to affect Wat-166 located adjacent to the site of the mutation. Considering the role of Tyr-67 the mutation might also affect the proper folding of the protein.

The findings by Luntz, *et al*, (1989), and Wallace, *et al*, (1989), that replacing Tyr-67 with Phe in cytochrome *c* resulted in a more stable protein is consistent with the suggestion that the region becomes more hydrophobic. However, Berghuis *et al*, (1994a), showed that the reduced form of the mutant iso-1-cytochrome *c* still had the Wat-166 which was only slightly displaced to about 1 Angstrom away from its original site. This slight displacement created a cavity which was subsequently filled by another water molecule, (Wat-300), from nearby. This implies that the region becomes even less hydrophobic due to the additional water molecule despite the loss of a hydroxyl group and the introduction of a hydrophobic amino acid residue in the region.

In contrast, previous studies in yeast iso-1-cytochrome *c* have shown that mutations of Asp-52 to Ile resulted in exclusion of Wat-166 with a substantial reorganization of hydrogen bonding, (Hickey, *et al*, 1991). The mutant protein was also found to be more stable than the wild type, (Das, *et al*, 1989). The disruption of the hydrogen bond network due to this mutation leads to the shifting of the side chain of Tyr-67 towards the side chain of Thr-78 with which its hydroxyl group forms a hydrogen bond, (Berghuis, *et al*, 1994b). This reorganization in hydrogen bonding was observed in both oxidation states of the mutant protein. Indeed, Berghuis, *et al*, (1994b), showed that in both oxidation state structures of Asn-52Ile/Tyr-67Phe/Cys-102Thr mutant, only a few hydrogen bonds are formed and the Wat-166 is missing, since the size of the cavity that it normally occupies is reduced to about 8 Å³ which is less than the space required to accommodate a water molecule. In all the studies cited above the proteins have been crystallized

and their three dimensional structures have been determined.

The mutations carried out in this study were similarly targeting the location of Wat-166. The crystal structure of the Asn-52Ile/Tyr-67Phe/Cys102Thr was not available when this study was initiated. Replacing Asn-52 with Val and Tyr-67 with Phe in the heme environment, (Fig. 8) was expected to result in exclusion of Wat-166 and possibly, the inclusion of Wat-300. The figure was derived from the structure of wild type cytochrome c molecule (3YCC) from the protein data bank (PDB) file. The molecule was transferred in Quanta (MSI), and mutated through CHARMM. Valine residue is smaller than the isoleucine used in the previous experiments by a single methyl group. The Asn-52V/Tyr-67Phe mutation should leave a slightly larger cavity than in Asn-52Ile/Tyr-67Phe that may well be enough to accommodate a water molecule. The question that arises from this hypothesis is, whether the water molecule could still exist in the cavity with only one residue, (Thr-78), contributing in the hydrogen bonding instead of the normal three in the wild type protein.

The mutant protein has been sent for x-ray crystallography and determination of its three dimensional structure will undoubtedly shed more light on the overall structure and hydrogen bonding in the region and possibly reveal the presence or absence of Wat-166 in the cavity. However, even in the absence of the three dimensional structure the possibility of a water molecule being retained in an otherwise hydrophobic environment with very few hydrogen bonding, (Rashin, *et al*, 1986), is unlikely and it is possible to speculate that Wat-166 has been removed by these mutations. One should

not lose sight of the fact that the Tyr-67Phe mutation incorporates a second water molecule, (Berghuis, *et al*, 1994a), despite the hydrophobic nature of the mutation.

The mutant protein has been subjected to second derivative spectroscopy, a technique that yields information about the polarity around tyrosine residues, (Schroeder & McOdimba, 1994; Schroeder, 1994, thesis in preparation). In addition, its chemical and thermal stability have also been studied, (Schroeder, 1994, thesis in preparation).

In conclusion, the experiments conducted in this study have shown that yeast iso-1-cytochrome *c* can still function without the two conserved amino acids and that replacement of Asn-52 with Val and Tyr-67 with Phe does not affect the conformational structure of the protein. It is possible that the presence of these residues and their hydrogen bonding network to the buried Wat-166 are not absolutely required for the overall function of cytochrome *c*. The presence of Wat-166 buried in this region, apart from determining the mid point potential when the protein goes from one oxidation state to another, does not seem to be significantly important in the overall function of the protein in yeast cells and its activity with cytochrome oxidase.

CHAPTER SIX

APPENDIX

6.1. MEDIA

6.1.1. LURIA-BERTANI (LB) Media

Bacto-tryptone_____10 g

Bacto-yeast extract_____5 g

NaCl_____10 g

Dissolve in 1 liter of distilled water, divide 500 ml into 100 ml aliquots in milk dilution bottles. Add 7.5 g (1.5%) Bacto agar to the remaining 500 ml in a 2 liter flask. Autoclave for 20 minutes at 121°C. Add ampicillin to 20 µg/ml to the media when required.

6.1.2. 2x YT Media

Bacto-tryptone_____8 g

Bacto-yeast extract_____5 g

NaCl_____2.5 g

Dissolve in 450 ml distilled/deionized water. Adjust the pH to 7.0 with 5N NaOH. Make upto 500 ml. Add agar to 1.5% if necessary. Autoclave as above and add ampicillin to 20 µg/ml when necessary.

6.1.3. YP Media

Yeast extract_____5 g

Bacto peptone_____10 g

Dissolve in 500 ml distilled water, bring the pH to 6.0 with NaOH and autoclave for 20 minutes. To prepare YPD add sterile glucose (dextrose) to 3%. To prepare YPG add sterile 60% glycerol to 3%.

6.1.4. SD Leu⁻ Media

10x SD (Synthetic dextrose):

Yeast nitrogen base without amino acids_____5.1 g

Ammonium sulfate_____15 g

Dextrose_____60 g

Prepare YNB and ammonium sulfate in 150 ml and autoclave.

Prepare dextrose in 150 ml and autoclave. Add equal volumes of each solution together.

25x Synthetic complete media (SC)

Weigh: adenine - 96 mg; uracil - 96 mg; tryptophan - 96 mg; arginine - 96 mg; methionine - 96 mg; aspartic acid - 100 mg; tyrosine - 144 mg; isoleucine - 144 mg; lysine -144 mg; threonine - 200 mg; histidine - 240 mg; phenylalanine - 240 mg; glutamic acid - 480 mg; valine - 720 mg and serine - 1.8 g. Dissolve in 200 ml water. Filter sterilize. (Note: not all the amino acids go into solution, some precipitates will not dissolve).

To prepare SD Leu⁻ agar plates:

Bacto agar_____10 g

Water_____430 ml

Autoclave and cool to 60°C. Add 50 ml of 10x SD and 20 ml of 25x SC.

Pour into culture plates immediately.

6.2. Solutions

6.2.1. Calcium chloride

Prepare 1M CaCl_2 :

CaCl_2 _____14.7 g

Water_____100 ml.

Autoclave as above and use as 0.1M solution in sterile distilled water.

6.2.2. Glycerol

Prepare 60% glycerol

Glycerol_____60 ml

Distilled water_____40 ml

Autoclave as above and use as 15% in the preparation of competent E.coli cells as final 0.1M CaCl_2 or as 3% in the YPG media for growing yeast.

6.2.3. 10 M Ammonium acetate

Ammonium acetate_____77.08 g

Water_____100 ml

Autoclave and store at room temperature.

6.2.4. 1 M Sodium chloride

Sodium chloride_____8.44 g

Water_____to 1000 ml

Store at room temperature.

6.2.5. 1 M Magnesium chloride

Magnesium chloride_____9.52 g

Water_____100 ml

Autoclave and store at room temperature.

6.2.6. 1 M Lithium chloride

Lithium chloride_____4.24 g

Water_____100 ml

Autoclave and store at room temperature.

6.2.5. 50 % Polyethylene glycol (PEG 4000)

PEG 4000_____50 g

Water to_____100 ml

Autoclave and store at room temperature.

6.2.6. 0.5 M EDTA

EDTA (disodium salt)_____18.61 g

Water_____80 ml

Adjust pH to 8.0 with NaOH pellets. Make up final volume to 100 ml.

Autoclave and store at room temperature.

6.2.7. 10 M Sodium hydroxide

Sodium hydroxide pellets_____40 g

Water_____100 ml

Store in plastic or polycarbonate container at room temperature.

6.2.8. Solutions for alkaline lysis extraction of DNA mini- preps

Solution I: 50 mM Glucose

25 mM Tris pH 8.0

10 mM EDTA pH 8.0

Prepare 100 ml, autoclave and store at 4°C.

Solution II: 0.2N NaOH (make from 10 M solution)

1% SDS (make from a 10% solution)

Freshly mix in sterile distilled water, from stock 10N NaOH and 10% SDS, just before use.

Solution III: 5 M Potassium acetate

Potassium acetate_____60 ml

Glacial acetic acid_____11.5 ml

Distilled water_____28.5 ml

This results in a 3 M potassium and 5 M acetate. Mix, autoclave and store at 4°C.

6.2.9. Lactic acid solution

85% w/w_____170 ml

Distilled water_____170 ml

Raise the pH to 5 using NaOH pellets, autoclave for 30 minutes and cool to 30°C. This is enough for boosting 14 liters of yeast culture.

6.3. Antibiotics

6.3.1. Ampicillin stock solution

Prepare 10 mg/ml stock solution

Ampicillin_____100 mg

Distilled water_____10 ml

Filter 1 ml through a 0.45 filter into each sterile eppendorf tubes. Label and store at -20°C. Use at 20 µg/ml final concentration.

6.3.2. Tetracycline solution: (20mg/ml)

Tetracycline hydrochloride (Sigma)_____280 mg

Ethanol, 90%_____14 ml

This is enough for initial inoculation and boosting of a 14 liter yeast culture.

6.3.3. Streptomycin solution: (80 mg/ml)

Streptomycin sulfate (Sigma)_____1.120 g

Distilled water_____14 ml

Filter sterilize through 0.22 µ filter. This is enough for initial inoculation and boosting of a 14 liter yeast culture.

6.3.4. Chloramphenical solution

Chloramphenical_____340 mg

90% ethanol_____10 ml

Dissolve and store at -20oC. Use at 170 µg/ml

6.3.5. Kanamycin solution

Kanamycin_____100 mg

Water_____10 ml

Filter sterilize and store at -20°C. Use at 50 µg/ml.

6.5. Buffers

6.5.1. 5x Tris/Borate/EDTA (TBE) buffer

Tris base_____54 g

Boric acid_____27.5 g

0.5 M EDTA_____20 ml

Water_____to 1 liter.

Autoclave. Dilute to 1x with distilled water for use in both agarose and sequencing gel electrophoresis.

6.5.2. Phosphate buffer

1 M potassium phosphate, dibasic_____174.18 g

Distilled water_____to 1 L

1 M potassium phosphate, monobasic_____136.09 g

Distilled water_____to 1 L.

To prepare 12.5 mM phosphate buffer pH 7.0 add 61.5 ml of dibasic and 38.5 ml of monobasic solutions. Add distilled or deionized water to 8 liters.

6.5.3. Tris acetate buffer

25 mM Tris acetate:

Tris acetate _____ 6.05 g

Glacial acetic acid _____ 1.43 ml

6.5.4. 1 M Tris buffer

Tris base _____ 12.1 g

Water _____ 80 ml

Adjust pH to either 7.4 or 8.0 with 1 N HCl.

Make up to 100 ml with water, autoclave and store at room temperature.

6.5.5. 1x TE buffer

Tris buffer _____ 10 mM

EDTA _____ 1 mM

Autoclave and store at room temperature.

6.5.5. Annealing buffer for mutagenesis

Tris-HCl pH 7.4 _____ 200 mM

MgCl₂ _____ 20 mM

NaCl _____ 50 mM

6.5.5. Double strand DNA Synthesis buffer

Tris pH 7.4 _____ 175 mM
MgCl₂ _____ 37.5 mM
Dithiothreitol (DTT) _____ 50 mM
ATP _____ 7.5 mM
dNTP (ATP,CTP,GTP,TTP) _____ 4 mM each

6.5.6. T4 DNA polymerase dilution buffer

Potassium phosphate buffer _____ 0.1 M
DTT _____ 5 mM
Glycerol _____ 50%

REFERENCES

1. Avers, C.J., (1986): Molecular cell biology. Adison-Wesley Publishing Co.
2. Baldari and Cessarani, (1985): Gene **35** 27-32
3. Beggs, J.D., (1978): Transformation of yeast by a replicating hybrid plasmid. Nature **275** 104-109.
4. Berghuis, A.M. and Brayer, G.D., (1992): Oxidation state-dependent conformational changes on cytochrome *c*. Journal of Molecular Biology **223** 959-976
5. Berghuis, A.M., Guillemette, J.G., Smith, M. and Brayer, G.D., (1994a): Mutation of tyrosine-67 in cytochrome *c* significantly alters the local heme environment. Journal of Molecular Biology **235** 1326-1341.
6. Berghuis, A.M., Guillemette, J.G., McLendon, G., Sherman, F., Smith, M. and Brayer, G.D., (1994b): The role of a conserved internal water molecule and its associated hydrogen bond network in cytochrome *c*. Journal of Molecular Biology **236** 786-799.

7. Bortstein, D. and Shortle, D., (1985): Strategies and applications of *in vitro* mutagenesis. *Science* **229** 1193-1201.
8. Bosshard, H.R., Zurrer, M., Schagger, H. and von Jagow, G., (1979): Binding of cytochrome *c* to the cytochrome *bc₁* complex (complex III) and subunits cytochrome *c₁* and *b₁*. *Biochemical and Biophysical Research Communications* **89** 250-258.
9. Bushnell, G.W., Louie, G.V. and Brayer, G.D., (1990): High-resolution three dimensional structure of horse heart cytochrome *c*. *Journal of Molecular Biology* **214** 585-595.
10. Capaldi, R.A., (1990): Structure and assembly of cytochrome *c* oxidase. *Archives of Biochemistry and Biophysics* **280** 252-262.
11. Clark-Walker, G.D. and Linnane, A.W., (1967): The biogenesis of mitochondria in *Saccharomyces cerevisiae*. *Journal of Cell Biology* **34** 1-14.
12. Cooper, C.E., (1990): The steady-state kinetics of cytochrome *c* oxidation by cytochrome oxidase. *Biochemica et Biophysica Acta* **1017** 187-203.

13. Cutler, R.L., Pielak, G.J., Mauk, A.G. and Smith, M., (1987): Replacement of cystein-107 of *Saccharomyces cerevisiae* iso-1-cytochrome *c* with threonine: improved stability of the mutant protein. *Protein Engineering* **1** 95-99.
14. Das, G., Hickey, D.R., McLendon, D., McLendon, G. and Sherman, F. (1989): Dramatic thermostabilization of yeast iso-1-cytochrome *c* by an asparagine to isoleucine replacement at position 57. *Proc. Natl. Acad. Sci. USA.* **86** 496-499.
15. Delange, R.J., Glazer, A.N. and Smith, E.L. (1970): Identification and location of α -N-trimethyllysine in yeast cytochrome *c*. *Journal of Biological Chemistry* **245** 3325-3327.
16. Dickerson, R.E., and Timkovich, R., (1975): Cytochromes *c*. In: *The Enzymes*, vol.iii; 3rd ed. p397-547. Ed. Boyer, P.D. Academic Press, New York.
17. Dickerson, R.E., Takano, T., Eisenberg, D., Kallai, O.B., Samson, L., Cooper, A. and Margoliash, E. (1971): Ferricytochrome *c*: General features of the horse and bonito proteins at 2.8 Å resolution. *The Journal of Biological Chemistry* **246** 1511-1535.

18. Faye, G., Leung, D.W., Tatchel, K., Hall, B.D. and Smith, M., (1981): Deletion mapping of sequences essential for *in vivo* transcription of the iso-1-cytochrome *c* gene. *Proc. Natl. Acad.Sci. USA.* **78** 2258-2262.
19. Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E., (1976): Correlation of the kinetics of electron transfer activity of various eukaryotic cytochromes *c* with binding to mitochondrial cytochrome *c* oxidase. *The Journal of Biological Chemistry* **251** 1104-1115.
20. Frauenhoff, M.M. and Scott, R.A., (1992): The role of tyrosine 67 in the cytochrome *c* heme crevice structure studied by semisynthesis. *Proteins: Structure, Function and Genetics* **14** 202-212.
21. Gao, Y., Boyd, J., Pielak, G.J. and Williams, R.J.P., (1991): Comparison of reduced and oxidized yeast iso-1-cytochrome *c* using proton paramagnetic shifts. *Biochemistry* **30** 1928-1934.
22. Garber, E.A.E., Luntz, T.L. and Margoliash, E., (1988): The interaction of cytochrome *c* with cytochrome oxidase In: King, T.E., Mason, H.S. and Morrison, M., eds *Oxidases and Related Redox Systems*, Alan R. Liss, Inc. pp 749-769.

23. Hampsey, D.M., Das G. and Sherman, F., (1986): Amino acid replacements in yeast iso-1-cytochrome *c*: comparison with the phylogenetic series and the tertiary structure of related cytochromes *c*. *Journal of Biological Chemistry* **261** 3259-3271.
24. Hanahan, D., (1983): Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166** 557-580.
25. Hennig, B., Koehler, H. and Neupert, W., (1983): *Proc. Natl. Acad. Sci. USA.* **80** 4963-4967.
26. Hickey, D.R., McLendon, G., and Sherman, F., (1988): Thermodynamic stabilities of yeast iso-1-cytochromes *c* having amino acid substitutions for lysine 32. *The Journal of Biological Chemistry* **263** 18298-18305.
27. Hickey, D.R., Berghuis, A.M., Lafond, G., Jaeger, J.A., Cardillo, T.S., McLendon, D., Das, G., Sherman, F., Brayer, G.D. and McLendon, G., (1991): Enhanced thermodynamic stabilities of yeast iso-1-cytochrome *c* with amino acid replacements at positions 52 and 102. *Journal of Biological Chemistry* **266** 11686-11694.
28. Inglis, S.C., Guillemette, J.G., Johnson, J.A. and Smith, M. (1991): Analysis of the invariant Phe82 residue of yeast iso-1-cytochrome *c* by site-directed mutagenesis using a phagemid yeast shuttle vector. *Protein Engineering* **4** 569-574.

29. Ito, H., Fukuda, Y., Murata, K., and Kimura, A., (1983): Transformation of intact yeast cells treated with alkali cations. *Journal of Bacteriology* **153** 163-168.
30. Kornblatt, J.A. and Luu, H.A., (1986): The interactions of cytochrome *c* and porphyrin cytochrome *c* with cytochrome *c* oxidase: the resting, reduced and pulsed enzymes. *European Journal of Biochemistry* **159** 407-413.
31. Kornblatt, J.A. and Laberge, M. (1988): Porphyrin cytochrome *c* pH effects and interaction with cytochrome *c* oxidase. *European Journal of Biochemistry* **175** 475-479.
32. Kornblatt, J.A. and Hui Bon Hoa, (1990): A non-traditional role of water in the cytochrome *c* oxidase reaction. *Biochemistry* **29** 9370-9376.
33. Kunkel, T.A., Roberts, J.D. and Zakour, R.A., 1987: Rapid and efficient site-specific mutagenesis without phenotype selection. *Methods in Enzymology* **154** 367-382.
34. Laycock, M.V., (1972): The amino acid sequence of cytochrome *c*. 553 from the chrysophycean alga *Monochrysis lutheri*. *Canadian Journal of Biochemistry* **50** 1311-1325.

35. Lederer, F., Simon, A.M. and Verdiere, J. (1972): *Saccharomyces cerevisiae* iso-1-cytochrome *c*: revision of the amino acid sequence between the cysteine residues. *Biochem. Biophys. Res. Commun.* **47** 55-58.
36. Leung, C.J., Nall, B.T. and Brayer, G.D. (1989): Crystalization of yeast iso-2-cytochrome *c* using a novel technique. *Journal of Molecular Biology* **206** 783-785.
37. Louie, G., Pielak, G.J., Smith, M. and Brayer, G.D., (1988): Role of phenylalanine-82 in yeast iso-1-cytochrome *c* and remote conformational changes induced by a serine residue at this position. *Biochemistry* **27** 7870-7876.
38. Louie, G. and Brayer, G.D., (1990): High-resolution refinement of yeast iso-1-cytochrome *c* and comparisons with other eukaryotic cytochromes *c*. *Journal of Molecular Biology* **214** 527-555.
39. Luntz, T.L., Schejter, A., Garber, E.A.E. and Margoliash, E., (1989): Structural significance of an internal water molecule studied by site-directed mutagenesis of tyrosine-67 in rat cytochrome *c*. *Proc. Natl. Acad. Sci. USA* **86** 3524-3528.
40. McGowan, E.B. and Stellwagen E. (1970): Reactivity of tyrosyl residues of horse heart ferricytochrome *c* towards iodination. *Biochemistry* **9** 3047-3052.

41. **Messing, J., (1987): New M13 vectors for cloning. *Methods in Enzymology* **101** 20-79.**
42. **Moore and Petigrew, (1990): *Cytochrome c: Evolutionary, Structural and Physicochemical Aspects*. Springer Verlag, Berlin**
43. **Nicholls, P., (1965): *Cytochrome oxidase in situ: Concepts and questions*. In: King, T.E., Mason, H.S. and Morrison, M. eds. *Oxidases and Related Redox Systems*. New York: Wiley, pp 764-777.**
44. **Ochi, H., Hata, Y., Tanaka, N., Kakudo, M., Sakurai, T., Aihara, S. and Morita, Y., (1983): Structure of rice ferricytochrome *c* at 2 Å resolution. *Journal of Molecular Biology* **166** 407-418.**
45. **Pielak, G.J., Mauk, G.A. and Smith, M., (1985): Site-directed mutagenesis of cytochrome *c* shows that an invariant Phe is not essential for function. *Nature* **313** 152-154.**
46. **Rashin, A.A., Iofin, M., and Honig, B., (1986): Internal cavities and buried water in globular proteins. *Biochemistry* **25** 3619-3625.**
47. **Rickwood, D.; Wilson, M.T. and Darley-USmar, V.M., (1987): Isolation and characteristics of intact mitochondria - a practical approach. In *Practical Approaches* IRL Press, Oxford Washington DC. Ed. Darley-USmar and Wilson. p 46-49.**

48. Rieder, R. and Bosshard H.R., (1978): The cytochrome *c* oxidase binding site on cytochrome *c*: Differential chemical modification of lysine residues in free and oxidase-bound cytochrome *c*. *Journal of Biological Chemistry* **253** 6045-6053.
49. Sambrook, J.; Fritsch, E.F. and Maniatis, T., (1989): *Molecular cloning: A laboratory manual* Cold Spring Harbour Laboratory Press.
50. Sanger, F., Nicklen, S. and Coulson, A.R., (1977): DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74** 5463-5467.
51. Schejter, A., Aviram, I. and Sokolovsky, M. (1970): Nitrocytochrome *c*. II. Spectroscopic properties and chemical reactivity. *Biochemistry* **9** 5118-5123
52. Schejter, A., Luntz, T.L., Koshy, T.I. and Margoliash, E., (1992): *Biochemistry* **31** 8336-8341
53. Schroeder, H. and McOdimba, F.A., (1994): Yeast Iso-1-cytochrome *c*: Tyrosine 67 becomes more polar on going from oxidized to reduced state. 37th Annual Meeting of the Canadian Biological Societies.
54. Schroeder, H., (1994): Thesis in preparation

55. Schweingruber, M.E., Stewart, J.W. and Sherman, F., (1978): Amino acid replacements of the evolutionary invariant tryptophan at position 64 in the mutant forms of iso-1-cytochrome *c* from *Saccharomyces cerevisiae*. *Journal of Molecular Biology* **118** 481-496.
56. Schweingruber, M.E., Stewart, J.W. and Sherman, F., (1979): Primary site and second site revertants of missense mutants of the evolutionarily invariant tryptophan 64 in iso-1-cytochrome *c* from yeast. *The Journal of Biological Chemistry* **254** 4132-4143
57. Sherman, F. Stewart, J.W., Margoliash, E., Parker, J. and Campbell, W. (1966): The structural gene of yeast cytochrome *c*. *Proc. Natl Acad. Sci. USA.* **55** 1498-1504.
58. Sherman, F. Stewart, J.W., Parker, J., Inhaber, E. and Shipman, N.A. (1968): The mutational alteration of the primary structure of yeast iso-1-cytochrome *c*. *The Journal of Biological Chemistry* **243** 5446-5456.
59. Sherman, F. and Stewart J.W., (1971): Genetics and biosynthesis of cytochrome *c*. *Annual Review of Genetics* **5** 257-296.
60. Sinjorgo, K.M.C., Meijling, J.H. and Muijsers, O., (1984): The concept of high and low-affinity reactions in bovine cytochrome *c* oxidase steady-state kinetics. *Biochimica et Biophysica Acta* **767** 48-56.

61. Sinjorgo, K.M.C., Steinebach, O.M., Dekker, H.L. and Muijsers, A.O., (1986): The effects of pH and ionic strength on cytochrome c oxidase steady-state kinetics. reveal a catalytic and non-catalytic interaction domain for cytochrome c. *Biochimica et Biophysica Acta* **850** 108-115.
62. Smith, M., Leung, D.W., Gillam, S. and Astell, C.R., (1979): Sequence of the gene for iso-1-cytochrome c in *Saccharomyces cerevisiae*. *Cell* **16** 753-761.
63. Speck, S.H., Ferguson-Miller, S., Osheroff, N. and Margoliash, E., 1979: Definition of cytochrome c binding domains by chemical modification: Kinetics of reaction with beef mitochondrial reductase and functional organization of the respiratory chain. *Proc. Natl. Acad. Sci. USA* **76** 155-159.
64. Tabor, S. and Richardson, C.C., 1987: DNA sequencing analysis with a modified bacteriophage T7 polymerase. *Proc. Natl. Acad. Sci. USA*. **84** 4767-4771.
65. Takano, T. and Dickerson, R.E., 1981a: Conformation change of cytochrome c: I. Ferricytochrome c refinement at 1.8 Å and comparison with the ferrocytochrome structure. *Journal of Molecular Biology* **153** 79-94.

66. Takano, T. and Dickerson, R.E., 1981b: Conformation change of cytochrome *c*: II. Ferricytochrome *c* refinement at 1.8 Å and comparison with the ferrocyclochrome structure. *Journal of Molecular Biology* **153** 95-115.
67. ten Kortenaar, P.B.W., Adams, P.S.H.M. and Tesser, G.I., (1985): Synthesis of horse heart cytochrome *c* from two or three fragments. *Proc.Natl. Acad. Sci. USA* **82** 8279-8283.
68. Vanneste, W.H., (1966): Molecular proportion of the fixed components of the respiratory chain of the Keilin-Hartree particles and beef heart mitochondria. *Biochim et Biophysica Acta* **113** 175-178.
69. Vieira, J. and Messing, J., (1987): Production of single-stranded plasmid DNA. *Methods in Enzymology* **153** 3-11.
70. Wallace, C.J.A., Mascagni, P. Chait, B.T., Collawn, J.F., Paterson, Y., Proudfoot, A.E.I. and Kent, S.B.H., (1989): Substitutions engineered by chemical synthesis at three conserved sites in mitochondrial cytochrome *c*: Thermodynamics and functional consequences. *Journal of Biological Chemistry* **264** 15199-15209.
71. Wikstrom et al (1981): Cytochrome oxidase: A synthesis. Academic Press, London

72. Zitomer, R.S. and Hall, B.D., (1976): Yeast cytochrome *c* messenger RNA. *Journal of Biological Chemistry* **251** 6320-6326.
73. Zitomer, R.S. and Nichols, D.L., (1978): Kinetics and glucose repression of yeast cytochrome *c*. *Journal of Bacteriology* **135** 39-44.
74. Zoller M.J. and Smith M., 1987: Oligonucleotide-directed mutagenesis: A simple method using two oligonucleotide primers and a single stranded DNA template. *Methods in Enzymology* **154** 329-367.