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THE ROLE OF HEME IN THE BIOGENESIS AND ASSEMBLY OF MITOCHONDRIAL COMPLEX III IN YEAST CELLS

by

Ching-I Patsy Lin

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Mount Sinai School of Medicine of the City University of New York.

-1985-

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in Satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

THE ROLE OF HEME IN THE BIOGENESIS AND ASSEMBLY OF MITOCHONDRIAL COMPLEX III IN YEAST CELLS

by

CHING-I PATSY LIN

Advisor: Professor Diana S. Beattie

The purpose of my thesis was to investigate the role of heme in the biogenesis and assembly of mitochondrial complex III in yeast cells using a mutant strain yeast that lacks ALA-synthase. The purified complex III from yeast contains 8-10 protein subunits including two core proteins, apocytochromes b and c_1 and the Rieske's iron-sulfur protein. The results obtained from this study indicated that heme exert diverse effects on the biogenesis of different hemoproteins.

Using the 'Western' immunotransfer and the immunoprecipitation technique, the two core proteins, apocytochrome b and the Rieske's iron-sulfur protein of complex III were found to be present in the mitochondria of heme-deficient yeast cells; however, the apocytochrome c_1 was found to be present as the intermediate form in these heme-deficient yeast cells. Electron paramagnetic resonance signals due to all mitochondrial iron-sulfur clusters were found to be absent in these heme-deficient cells. Hence, heme governs the formation of yeast mitochondrial iron-sulfur cluster in yeast cells.

The orientation of complex III subunits in the mitochondrial membrane of wild-type yeast cells was studied by a protein labeling technique. Results from such labeling experiments revealed that the two core proteins and the apocytochrome b span the membrane while apocytochrome c_1 and the Rieske's protein are exposed on the cytosolic surface of the inner mitochondrial membrane. A model of the topography of complex III was deduced based on results of this study and from other complex III enzymatic studies.

In order to study the role of heme in the orientation of the complex III subunits in the membrane, parallel studies of protease digestion and immunoprecipitation followed by polyacrylamide gel electrophoresis were performed on mitoplasts from wild-type and heme-deficient mutant cells. Results from this study indicated that the two core proteins, apocytochrome b and the intermediate form of apocytochrome c_1 have similar orientations in the membrane in wild-type and heme-deficient mutant yeast cells. However, the Rieske's iron-sulfur protein is more imbedded in the membrane in heme-deficient cells. Therefore, heme also governs the assembly of the mitochondrial iron-sulfur clusters in yeast cells.

DEDICATION

To my dear parents; my husband, Kou-Kwang;
and my two wonderful children, Rony and Allen.

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FORWORD

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(4). Lin, C.I.P. and Beattie, D.S. (1984) "Chymotrypsin Digestion of the Subunits of Complex III in Mitoplasts Obtained from Heme-deficient Yeast Cells. (Manuscript in Preparation).

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CHAPTER I. INTRODUCTION

The mitochondria of eukaryotic cells occupy a substantial fraction of the cytoplasm and engage in several metabolic pathways such as the citric acid cycle, fatty acid oxidation, the urea cycle and the oxidation of NADH through the respiratory electron transfer chain with the production of the ATP. Besides metabolizing fuels and generating energy for the cell, the mitochondrion is also unique in that it posesses a circular mitochondrial genome which encodes some polypeptides of the mitochondrial proteins and RNAs. Only about 12 of the mitochondrial proteins, however, are synthesized on mitochondrial ribosomes, the rest of mitochondrial proteins are encoded by the nuclear genome, synthesized on the cytosolic ribosomes and subsequently transported into the mitochondria. The biogenesis of a functional mitochondrial respiratory chain requires the synthesis and the coordination between proteins synthesized from both mitochondrial systems and cytosolic systems. Under normal growth conditions, assembly of the mitochondrial membrane proceeds in an orderly manner such that a constant level of each respiratory chain complex is maintained.

The yeast Saccharomyces cerevisiae is an organism very suitable for the study of biogenesis and regulation of respiratory chain proteins. This strain of yeast is eukaryotic and facultative and can be genetically manipulated for biogenesis studies. The level of mitochondrial proteins in this strain of yeast cells can be regulated by different metabolites and different growth conditions. Various factors such as oxygen, GTP, cytoplasmic proteins and cyclic AMP have been investigated as possible regulators of the coordination process of biogenesis of mitochondrial proteins and complexes.

The major focus of this thesis project is on the role of heme in the biogenesis and assembly of mitochondrial complex III proteins and iron-sulfur centers in the yeast S. cerevisiae. For the studies on the role of heme in the biogenesis of complex III of yeast cells in this thesis, a yeast mutant strain (GL1) which is defective in ALA-synthase previously isolated is used. Different immuno techniques with an antiserum against complex III and antisera against specific subunits of complex III were used in the detection of complex III subunits in heme-deficient mutant yeast cells. The presence and the integration of the iron-sulfur clusters was studied by electron paramagnetic resonance technique. The topography of complex III in mitochondria of wild-type cells was studied by protein labeling technique using a membrane impermeant hydrophilic labeling reagent, DABS. The effects of heme on the assembly of complex III, however, were determined by the analysis of SDS-polyacrylamide gel analysis of immuno precipitates of complex III subunits from proteolytically digested mitoplasts of wild type and heme-deficient yeast mutant cells. A thorough discussion will be presented in Chapter IV on studies of the effects of heme on the biogenesis of many hemoproteins and respiratory chain proteins in yeast cells.

(1). The Mitochondrial Respiratory Chain

Hatefi et al. first suceeded in isolating five protein-lipid complexes from the inner mitochondrial membrane of beef heart mitochondria. Four of these complexes are involved in electron transfer (1) and the fifth in the respiration coupled ATP synthesis(2,3). These complexes are catalytically active within particular segments of the overall oxidation reaction of NADH and succinate. Mitchell (4) first proposed that the electron transfer complexes convert the free energy of oxidation into an electrochemical gradient, uH^+, which is used by complex V for the synthesis of ATP.

Various electron carriers, such as flavins, quinones, iron-sulfur clusters and hemes are involved. Besides transfering electrons, these carriers also serve as prosthetic groups for different protein subunits in the respiratory chain. Fig. (1) depicts the redox components of the mitochondrial respiratory chain complex I through IV from different substrate to oxygen (5). Much of the research on mitochondria has been focussed on the understanding of the biogenesis of the respiratory chain components and mechanism of oxidative phosphorylation.

Complex I, NADH-Coenzyme Q reductase, catalyzes the reduction of ubiquinones and ferricyanide by NADH. This complex is the first site of oxidative phosphorylation, and is comprised of 16-17 subunits (6). Resolution of this complex with chaotropic reagent and ammonium sulfate revealed that complex I contained a flavo Fe-S protein (FP), an insoluble iron-sulfide protein (IP) and a hydrophobic fraction. The FP fraction corresponds to soluble NADH dehydrogenase and contains three polypeptides and six nonheme iron atoms and acid labile sulfides (7,8). The hydrophilic Fe-S subunit (IP) contains four polypeptides and nonheme

Fe and acid labile sulfides in equivalent concentrations (9). However, the iron-sulfur clusters associated with NADH dehydrogenase in beef heart and other organisms are absent in S. cerevisiae mitochondria. The site I phosphorylation from complex I does not exist in this strain as suggested from several lines of evidence such as EPR studies, inhibitors studies and enzyme activity studies (13,14). Rotenone and Piercidine are known to be inhibitors of the electon transfer reaction in this segment of the chain (14). Detailed resolution of the complex I still remains to be elucidated.

Complex II, succinate-coenzyme Q reductase, catalyzes the reduction of coenzyme Q by succinate. Complex II contains succinate dehydrogenase (SDH), two small protein subunits of 15 kDa and 13.5 kDa associated with two hemes of b type (15,16). Pure and reconstitutively active preparations of SDH from beef heart mitochondria all contain 1 covalently bound FAD and 8 nonheme Fe atoms and 8 acid labile sulfides per molecule (17). SDH preparation preparations isolated from complex II using chaotropic agents revealed that the SDH is composed of two different subunits; the flavo Fe-S protein (FP) of 70 kDa. and the iron-sulfur protein (IP) of 27 kDa. From the EPR characteristics of paramagnetic redox centers of complex II preparations, the composition of Fe-S clusters was composed of S-1, S-2 and S-3 (18).

Complex III, coenzyme QH_2-cytochrome c reductase or the bc_1 complex, catalyzes the transfer of electrons coupled to ATP synthesis and ion transport from reduced ubiquinone to ferricytochrome c. The complex III preparations purified from beef heart (19,20), Neurospora crassa (21) or yeast (22-24) were found to be dimers with molecular weights ranging from 500 kDa to 560 kDa. The monomer of yeast complex

III purified previously from this laboratory contains two core proteins (50 and 40 kDa respectively), cytochrome b (30kDa), cytochrome c_1 (31 kDa), an iron-sulfur protein (24.5 kDa) and 3-5 smaller molecular weights subunits (23, 24). The composition, mechanism, topography and biogenesis of yeast mitochondrial complex III will be discussed in the following sections of this chapter.

Complex IV, cytochrome oxidase, catalyzes the terminal oxidative step in oxidative metabolism and is also one of the sites in ATP synthesis. This enzyme complex is ubiquitous in all aerobic cells. The enzymatic reacion catalyzed by complex IV results in the four electron reduction of oxygen to water. Complex IV contains four electron acceptors, two hemes (a and a_3) and two copper atoms, in a complicated complex (25). Cytochrome oxidase purified from eukaryotic systems is a dimeric protein and contains at least 7 subunits with molecular weights ranging from 36 kDa to 5 kDa (26,27). The three larger subunits are synthesized on the mitochondrial ribosomes while the other subunits of this complex are synthesized on cytoplasmic ribosomes and subsequently transported and integrated in the inner mitochondrial membrane. Recently, the biogenesis(28-29), topography (30-32) and mechanism (33) of this complex has been studied extensively in several laboratories.

Complex V, the mitochondrial ATPase is the terminal enzyme in oxidative phosphorylation and functions in the interconversion of the electrochemical potential energy of proton gradients and the chemical energy of the terminal phosphoryl bond in ATP (34). This complex consists of two parts, F_o and F_1. The F_1 portion fuctions in ATP synthesis and hydrolysis while F_o is a membrane sector and functions in the transport of protons (35-37). Purified F_1 of complex V contains five

different protein subunits, bound Mg and nucleotides of ATP and ADP (38). The F_0 portion, however, contains three protein subunits. Among these three subunits, the subunit c is found to bind DCCD covalently resulting in inhibition of proton translocation (39). Subunit a of F_0 is found to bind inhibitor oligomycin (40). Genes of all the complex V subunits are found to be linked in bacteria as an 'unc' operon (41,42).

(2). Yeast Mitochondrial Complex III

(a). Composition of Yeast Mitochondrial Complex III

Hatefi et al. first isolated an enzymatically active and pure complex III from beef heart mitochondria based on cholate and ammonium sulfate fractionation, and deoxycholate solubilization (43,44). Starting from succinate-cytochrome c oxidoreductase, Yu et al. isolated complex III from beef heart mitochondria using bile salts (45,46). Mitochondrial complex III from N. crassa, however, was first isolated by Weiss and Kolb using a Triton X-100 extract and affinity chromatography on immobolized cytochrome c (21). Purification of an enzymatic inactive complex III from yeast was reported by Katan et al. using submitochondrial particles solubilized by cholate followed by ammonium sulfate precipitation in the presence of antimycin (47). Palmer et al. (22) first isolated an enzymatically active complex III from submitochondrial particles of the baker's yeast using cholate as a solubilizing agent.

Recently, Sidhu et al. in this laboratory have modified and improved the purification method reported by Palmer et al. using ammonium sulfate fractionation and column chromatography on ultrogel AcA 34 (24). Preparation of the yeast complex III from several organisms and different laboratories are very similar in composition. The purified complex from beef heart, N.crassa, and yeast all contain 7-10 polypeptide subunits, a binuclear iron-sulfur cluster, quinones and phospholipids including phosphotidyl ethanolamine, cardiolipin, and phosphotidylcholine (48).

Isolated bc_1 complexes from beef heart, N.crassa and yeast all contained 2 moles of b heme per mole of c heme. The recently purified enzymatically active complex III from this laboratory (24) contained

7.03 nmol of cytochrome b and 4.24 nmol of cytochrome c_1 per mg of protein, respectively. The specific activity of this purified complex was 17.1 umol/min/mg of protein using the decyl analog of coenzyme Q as a substrate. This purified complex contains 7 subunits of molecular weights ranging from 50 kDa to 11 kDa. Table (1) lists the names of various subunits of recently purified preparation of complex III by Sidhu et al. (24) along with the molecular weights, stoichiometry and isoelectric points of these subunits. Antiserum against complex III and specific subunits of complex III were raised in rabbit and subsquently immuno tested and characterized in a series of studies (49,50). Throughout the experimental section of this thesis, these antisera were used for studies of both biogenesis and assembly of the complex.

Among complex III subunits, cytochromes b, c_1 and the Rieske's iron-sulfur protein have been extensively characterized (48,51,52). The functions of the larger molecular weight subunits, core proteins 1 and 2, and the low molecular weight subunits are not known. These subunits, however, are present in stoichiometric amounts in preparations from N. crassa, beef heart and yeast. In yeast and two other organisms studied so far, only apocytochrome b is encoded by the mitochondrial genome (53,54) whereas all the other subunits of complex III are encoded by the nuclear genome and synthesized on cytoplasmic ribosomes (55-57). The fact that two genetic systems are involved in the synthesis and regulation of complex III make this complex very suitable for studies on the biogenesis of respiratory chain proteins.

(b). Biogenesis of Yeast Mitochondrial Complex III

(i). Biogenesis of Apocytochrome b

Several lines of evidence suggest that apocytochrome b is a translational product of the mitochondrial genome. Results from this laboratory first demonstrated that the formation of complex III in the yeast S. cerevisiae required mitochondrial protein synthesis (54). Schatz and Mason also reported that in cytoplasmic petite mutants lacking mitochondrial DNA or in yeast cells grown in the presence of inhibitors of mitochondrial protein synthesis, the characteristic absorption bands of cytochrome b were not present (58). It is now known that the mitochondrial gene coding for cytochrome b (the box or cob gene) displays a mosaic organization characteristic of many nuclear genes in lower organisms such as yeast and N. crassa (59,60). In yeast, the box gene has 2 or 5 introns depending on the strain examined. In mammalian systems, however, the gene coding for apocytochrome b is colinear with that of the yeast but is continuous and without introns (61,62).

The box gene is located between the oli-1 and oli-2 loci of the mitochondrial genome (60). Recently, the deoxynucleotide sequences of the box genes from several organisms have been studied and fully elucidated. The box gene is highly conserved in many organisms. The apocytochrome b protein is a very hydrophobic protein and associates tightly with the inner mitochondrial membrane. Apocytochrome b has been proposed to traverse the membrane several times which could account for the high hydrophobicity of the protein and its close association with the membrane (64). The apocytochrome b has been shown to behave anomalously on SDS polyacrylamide gels (65). The molecular weight of apocytochrome b derived from the DNA sequence is higher than experimentally

obtained from SDS polyacrylamide gel electrophoresis (66).

Throughout the literature, the existence of two functionally different forms of apocytochrome b in purified complex III and in the mitochondrial membrane has been reported (44,66). Two midpoint potentials and two different wavelengths in the mitochondrial difference spectrum were found for the cytochrome b indicating the holoprotein is heterogeneous (67). Two forms of apocytochrome b with molecular weights of 31 kDa and 29 kDa have been purified from yeast in this laboratory (68). Controversies still exist as to the nature of dimerism of the apoprotein b in the membrane and the binding of heme to the apoprotein.

(ii). Biogenesis of Nuclear Gene Coded Subunits of Complex III

The synthesis, import and processing of cytoplasmically made precursors of mitochondrial proteins has been the focus of much attention in the field of mitochondrial research recently (69,70). It is now known that most respiratory chain protein subunits coded by the nuclear genes were initially synthesized as larger precursors on the free ribosomes before translocation into the mitochondria and integration to a fuctional complex. Pools of precursor proteins exist in the cytosol prior to their binding to the mitochondrial membrane (71). Most of these precursors studied contain amino-terminal peptide extensions which are proteolytically cleaved after internalization of the precursors into the mitochondrial inner membrane (72-74). These amino-terminal sequences are known to have a higher content of basic amino acids which could be helpful in membrane recognition, precursor translocation and proteolytic processing of precursor proteins (75-77).

The processing proteases are reported to be sensitive to metal chelators such as EDTA and o-phenanthroline and require divalent

cations, magnesium or zinc for full activity (78,79). Yeast or N. crassa mitochondria has been shown to incorporate precursors, synthesized in a cell free system, in the absence of protein synthesis. The transfer of mitochondrial protein precursors is post-translational (74) in contrast to the co-translational processing that takes place in the endoplasmic reticulum (80). The translocation of the precursor across the inner mitochondrial membrane has been shown to be dependent on a transmembrane electrical potential (81,82). The final stage in the import process involves the assembly of the mature protein with other protein subunits and prosthetic groups such as hemes and iron-sulfur clusters into a functional enzyme complex.

Many precursors to nuclear gene coded mitochondrial proteins have been studied and reported now. With pulse-chase experiments, the precursors to the Rieske's iron-sulfur protein and the apocytochrmoe c_1 were found to be cleaved proteolytically to an intermediate form first before the mature form (49,83). Ohashi et al. (84) reported that the precursor to apocytochrome c_1 is matured in two proteolytic steps and that the second step is heme-dependent. So far, all of the complex III subunits have been studied in yeast and N. crassa in several laboratories. Grivell (56,57) and his collaborators have studied and reported the molecular weights of precursors to all nuclear gene encoded mitochondrial complex III subunits as listed in Table (2). Five of these precursors (core 1, core 2, apo cytochrome c_1, iron-sulfur protein and 17 kDa subunit) contain additional amino-terminal peptides with molecular weights ranging from 0.5 kDa to 9 kDa.

In addition to studies on the precursors to complex III, the nuclear genes coding 5 of the complex III subunits; core protein 2, the

Rieske's iron-sulfur protein and the three smaller subunits of molecular weights of 17 kDa, 14 kDa and 11 kDa, were cloned, isolated and characterized by Grivell and his coworkers (57). These genes were found to be present as only single copies in the cell and are located at least a thousand base pairs apart in the yeast nuclear genome.

(c). Topography of Yeast Mitochondrial Complex III

(i). Mechanism of Mitochondrial Complex III

Respiratory chain complex III and the b_6-f complex of photosynthetic electron-transport chains all contain two forms of cytochrome b, one c-type cytochrome (c_1 of f), one Fe_2S_2 cluster and up to one bound ubiquinone or plastoquinone (48,87,88). Initially, a linear chain such as shown in Fig. (1) was proposed to be the sequence by which electrons flow through from substrate to oxygen (89,90). This linear scheme, however, failed to explain the observed stoichiometry of protons translocated to electons transferred in the complex III segment of the chain (91,92) and several observed phenomenon of complex III such as the oxidant induced extra reduction of cytochome b (93).

In 1972, Wilkstrom and Berden (94) first proposed the existence of a nonlinear pathway in this segment of the respiratory chain involving the oxidation and the reduction of ubiquinone through ubisemiquinone. Mitchell first proposed that ubiquinones possess the prerequisite intrinsic properties to function as transmembrane hydrogen and electron carriers (95). Ubiquinone is soluble in hydrocarbon solvents and can be oxidized or reduced through reactions which involve net release and uptake of protons.

The protonmotive Q cycle proposed by Mitchell in 1975 (96-99) is shown in Fig. (2). The oxidation factor (OxF) in the Q cycle has been isolated and identified as the Rieske's iron-sulfur protein by Trumpower (100). The mechanism as depicted in the Q cycle does illustrate how the transfer of two electrons from succinate dehydrogenase to cytochrome c covalently translocates 4 protons outward across the inner mitochondrial membrane (85) and the phenomenon of oxidant induced cytochrome b via

the oxidation and reduction of semiquinones and the Rieske's iron-sulfur protein (100-102).

In addition to the 'Q cycle mechanism', a similar but different 'b cycle' mechanism proposed by Wilkstrom(94) and other researchers (103, 104) also serves to explain the stoichiometry and redox behavior of complex III. The essence of this b cycle mechanism is that the apocytochrome b in complex III can serve as a proton pump in addition to ubisemiquinone. Papa et al. (104) proposed that cytochrome b_{566} may function as the proton pump in the respiratory chain complex. The proposed scheme of electron transport chain of complex III in the b cycle proposed by Wilkstrom (105) is shown on Fig. (3). Von Jagow et al. reported that the redox potential of cytochrome b displayed a pH dependence in the physiological range(103). Recently, work in this laboratory demonstrated that DCCD blocked the function of the electrogenic proton ejection but not electron transport in the reconstituted bc_1 complex (106,107). DCCD, a carboxyl group modifing agent, was found to bind to apocytochrome b selectively in these reconstitution experiments (108) and hence lent support to the hypothesis of the 'proton motive b cycle'. Although much research work in the past has been devoted to the understanding of the detailed mechanism of mitochondrial complex III in the membrane, the topic is far from being clear and still remains to be understood.

(ii). Topography Studies of Mitochondrial Complex III

The 'Q cycle' and the 'b cycle' as shown in Fig. (2) and Fig. (3) requires that there are two topographically different sites at the cytosolic side and the mitochondrial side of the inner mitochondrial membrane. Intensive investigations on the topography and structure of respiratory chain complexes have been carried out in many laboratories to explain the unidirectional proton translocation at different sites of the respiratory chain.

Studies using proteolytic digestion, electron microscopy and neutron diffraction patterns suggested that 70 % of the complex III of N. crassa mitochondria extends into hydrophilic regions; cytochrome c_1 on one side and the core proteins on the other side of the membrane while apocytochrome b is integrated in the hydrophobic membrane (119, 110). Di Jeso et al. reported that antibodies against apocytochrome b inhibited the electon tranport from the cytosolic side of the mitochondrial membrane indicating the location of apocytochrome b on the cytosolic side of the membrane (111). Case and Leigh (112) demonstrated that both the hemes of cytochrome b are very close to the cytosolic side of the membrane, as determined by the EPR studies.

Labeling studies with the membrane non-permeant reagent DABS in beef heart mitochondria have indicated that cytochrome c_1 and the iron-sulfur protein are on the cytoplasmic side of the membrane while cytochrome b appears to span the membrane (113). Cross-linking experiments done by Capaldi et al. to elucidate nearest-neighbor relationships of the beef heart complex III subunits (114,115) also yielded similar results. Nelson et al. (116,117) using the same techniques found that the two core proteins of of the N. crassa mitochondria spanned the

inner mitochondrial membrane yet were exposed on the cytosolic surface. The topography of yeast mitochondrial complex III was investigated by DABS labeling method and proteolytic digestion method in this thesis.

(3). The Role of Heme in the Biogenesis of Yeast Mitochondrial Respiratory Chain Proteins

(a). Regulation of the Biogenesis of Mitochondrial Respiratory Chain Proteins in the Yeast Saccharomyces Cerevisiae-Anaerobiosis and Glucose Repression

The mitochondrial respiratory chain of the yeast S. cerevisiae can be modulated by oxygen and by carbon source in the growth medium. Fermentable carbon sources such as glucose (118) and low oxygen pressure (119) in the growth medium are known to repress the activity of mitochondrial respiratory chain proteins and other cellular proteins. Since the formation of the respiratory chain involves the synthesis and coordination of proteins encoded by both mitochondrial gene and nuclear gene, the phenomenon of catabolite repression and modulation by oxygen provides a convenient and useful system for studies on the regulation of the biogenesis of mitochondrial respiratory chain proteins.

Earlier work from this laboratory has shown that during glucose derepression, the activities of the various respiratory chain complexes appeared in the mitochondrial membrane at different times (120). Furthermore, the rate of cycloheximide insensitive protein synthesis into mitochondrial membranes in vivo was lowered over 90 % in glucose repressed cells. Although reduced, the mitochondrial gene synthesized products were qualitatively the same in SDS polyacrylamide gels (121).

Recently, the yeast CYC1 gene which codes for iso-1-cytochrome c has been cloned and studied extensively in several laboratories. This protein comprises 95 % of the cytochrome c in mitochondria of the yeast cells (122). Zitomer et al. (122) reported recently that the expression of the CYC1 gene is under transcriptional regulation by glucose(123).

Cells grown under derepressed conditions synthesized CYC1 RNA at a 6-fold greater rate than did repressed cells grown in medium containing glucose. In the case of complex III proteins, the effect of glucose repression has also been shown at the level of transcription. Van Loon et al. recently reported that glucose regulates the transcription of most of the complex III subunits; mRNAs for 4 subunits of this complex were reduced 2.5 to 25-fold with glucose concentrations ranging from 1 to 15 % (124) in the growth medium.

When S. cerevisiae cells were grown anaerobically on a fermentable carbon source, the respiratory deficient and less differentiated 'promitochondria' instead of the normal mitochondria are contained in these yeast cells (125). Oleic acid and ergosterol which are essential for yeast cells must be added to the growth medium. Anaerobically grown yeast cells lack cytochrome c peroxidase (127) and catalase (128) as well as respiratory chain complexes (129). However, upon aeration, activities of all these heme containing enzymes were rapidly regained and promitochondria increased differentiation.

The oxygen induced formation of cytochrome c peroxidase was shown to be insensitive to protein synthesis inhibitors and could be mimicked by the addition of heme to the extracts of the anaerobically grown cells (130). Apocytochrome c peroxidase is made in anaerobically grown yeast cells. Apoproteins of cytochrome c (131), c_1 (132) and catalase A and T (133), however, were not detected in the anaerobically grown yeast cells. Schatz et al. (134) reported that anaerobiosis did not have an effect on the synthesis of the three mitochondrially made subunits of yeast mitochondrial complex IV; however, only two of the cytosolically synthesized subunits were detected and they were not correctly assembled

with the three mitochondrially made subunits. Van Loon et al. (124) recently reported that anaerobiosis produced an approximately 12-fold decrease in mRNA levels for several of the complex III subunits (124).

Oxygen is known to function as an obligatory electron acceptor in the major pathway to protoheme IX in most eukaryotic cells (135). Since the heme biosynthetic pathway is impaired in anaerobically grown cells, the effects of anaerobiosis on biogenesis and assembly of respiratory chain proteins and hemoproteins could be a result of heme deficiency. Jayaraman et al. (136) had reported that the activities of yeast ALA-synthase and ALA-dehydratase of the heme biosynthetic pathway are lowered if the cells were grown in growth medium with higher glucose concentration (136). Thus it is possible that the heme synthesis is a limiting factor in the biogenesis of all hemoproteins under conditions of anaerobiosis and glucose repression.

Hemes in different hemoproteins serve as structural ligands and electron transfer moieties in virtually all cell types. The amino-acid sequences around the heme group in hemoproteins such as cytochrome c are knowned to be highly conserved throughout evolution (137). In mammalian systems, heme is known to be a physiological regulator of globin synthesis (138). Many studies have indicated that heme stimulates protein synthesis at the translation level by the specific interaction with eIF-2 and enhancing the rate of polypeptide initiation in lysates from reticulocytes (139) or other eukaryotic cells (140). Besides promotion of synthesis, heme has also been shown to inhibit the proteolytic degradation of globin in erythrocytes (141).

Work in this laboratory first suggested a possible relationship between heme synthesis and mitochondria biogenesis (142). Woods et al.

(143) reported that heme synthesis also regulate complex IV in fetal rat liver. In the mold N. crassa, iron deficiency leads to lower levels of ALA-dehydratase and cytochrome c oxidase (144). The recent isolation of ALA-synthase defective yeast mutant cells (145) has allowed and greatly accelerated the research on the role of heme on the biogenesis of almost all hemoproteins in cells.

(b). The Isolation and Characterization of 5-Aminolevulinic Acid Synthase Defective Yeast Mutant Cells

In 1974, Gollub at al. first isolated several strains of nyastatin-resistant and ergoserol requiring mutants of S. Cerevisiae strain D-587-4B (designated as the SG series) which also required Tween 80 and methionine for cell growth yet unable to grow on ethanol and glycerol. These mutant cells were petite and showed a complete absence of respiratory chain cytochromes and catalase (145). Supplementation with heme abolished these phenotypic properties of the mutant cells. A second group of mutants derived from wild type strain X2180 (designated as the GL series) exhibiting similar phenotypic properties have been genetically and biochemically characterized (146). Complementation tests revealed that different enzymes of the heme synthetic pathway were defective in some of these yeast mutants.

Strain GL1 of the SG series has been shown to be defective in the HEM1 loci of the gene that codes for ALA-synthetase due to a point mutation (145). Growth of GL1 mutants with ALA, or with heme and methionine, restored cytochromes and hemoproteins (145). Saltzgaber-Muller and Schatz (134) reported that when GL1 strain is grown in the absence of heme or a heme precursor, the mitochondria were devoid of all absorption bands and all enzyme activities due to cytochromes. The mitochondria were reported to be more fragile due to the different lipid composition in the mitochondrial membranes of these heme-deficient mutant cells. However, the level of mitochondrial ATPase and the oligomycin sensitivity of this enzyme were not appreciably affected (134). The GL1 mutant strain used in this thesis has also been used as a tool for biogenesis studies in several other laboratories.

(c). Initial Studies on the Role of Heme in the Biogenesis of Hemoproteins and Mitochondrial Respiratory Chain Proteins Using GL1 Yeast Mutant Cells

Schatz first reported that the apocytochrome c_1 (132) and several cytosolically made subunits of complex IV were not detected in the heme-deficient GL1 cells (134). All three of the mitochondrially made subunits of complex IV were present in the heme-deficient mutant cells as in the wild type cells although these subunits were not correctly assembled in the heme-deficient mutant cells. Woloszczuk et al. (133) reported the absence of apocatalase A and catalase T in these heme-deficient yeast mutant cells. In addition, the effect of heme on both catalases is suggested to be on the biosynthesis rather than degradation of the apocatalases (147).

Clejan et al. (148) from this laboratory recently demonstrated that apocytochrome b was present in the absence of heme in GL1 cells. The apocytochrome b synthesized in the presence or in the absence of of heme had the same molecular weights and an identical pattern after proteolytic digestion. The total mitochondrial translation products were also not affected appreciably in the absence of heme.

The role of heme on the biosynthesis and assembly of mitochondrial complex III subunits and the integration of Fe-S clusters has not been investigated using heme-deficient mutant cells in other laboratories. Experiments in this thesis were designed to answer these specific questions. Results on GL1 from this thesis and from other work in the current literature helps to reveal the many different and intriguing roles that heme plays in the biogenesis and assembly of cytosolic hemoproteins and mitochondrial respiratory chains protein complexes.

(4). EPR Studies on Iron-Sulfur Clusters of Mitochondrial Respiratory Chain of Yeast Saccharomyces cerevisiae

Transition metals such as Fe, Mn, Cu and Zn form an integral part of enzymes and are often associated with the functional properties of the enzyme (149). These metal enzyme systems can be examined by several probes that are characteristic of the unfilled 3d and 4d shells of the electron orbitals. EPR spectroscopy is one such probe used to obtain information regarding the elctronic structure of the bound metal ions or the atomic structure of their immediate environments.

The study of iron-sulfur clusters was pioneered in the laboratory of Beinert who first established their role in electron transfer chain by EPR techniques (151). Many different Fe-S clusters function in the respiratory chain of eukayotic cells as shown in Fig. (1). The Fe-S clusters associated with NADH dehydrogenase in other organisms such as beef heart are absent in S. cerevisiae (11,13). The EPR identification of various Fe-S clusters in complex II and complex III of the respiratory chain in Yeast S. cerevisiae have been completed. Epr field positions (g values), the number of irons and sulfurs per center, and midpoint redox potentials of these Fe-S clusters are listed in Table (3).

Ohnishi (152) and King (17) first reported that the flavoenzyme of succinate dehydrogenase contains two Fe-S centers, S-1 and S-2 associated with the the 70 kDa. flavoprotein. Each Fe-S center is of the plant ferrodoxin type which contain two sulfurs and two irons binding to cysteine of proteins. The structure of reduced binuclear clusters S-1 and S-2 displays rhombic symmetry which yield three absoption bands in EPR spectrum. A dynamic spin-spin interaction was observed between clusters S-1 and S-2 in all SDH preparations resulting in an enhancement

of EPR signals at their respective field positions (5).

Davis and Hatefi first reported that purified complex II also contains Fe-S centers associated with the 27 kDa protein subunit (155). Fe-S center S-3 of the complex II is now known to be tetranuclear like the ferredoxins of the bacterial type (156). This Fe-S cluster is paramagnetic only in the oxidized state (HIPIP type cluster), and shows relatively isotropic and highly temperature-sensitive EPR signals. Center S-3 is more stable in the membrane bound state. In the solubilized enzyme preparations, this center becomes extremely labile toward oxidant.

Rieske first isolated an Fe-S protein associated with a binuclear Fe-S centers from complex III of beef heart mitochondria (51). Both in isolated complex III and in whole mitochondria, the ascorbate reduced Rieske's Fe-S center exhibit a main signal at g = 1.90 which is different that the signal at g = 1.94 due to Fe-S centers of complex II.

The Fe-S centers, like heme, are not only structural elements but also electron transfer moieties of the respiratory chain. Although the EPR properties of various Fe-S centers in the respiratory chain have been studied extensively, little information is known in these areas such as the biogenesis of the Fe-S center, import of the Fe and S into the mitochondria and the association between these Fe-S centers with other respiratory chain components. An iron-transporter comparable to ferritin in mammalian cells has not been identified (157) in yeast cells. In plant ferredoxins and bacteria ferredoxins, the iron atoms are known to bind to cysteine residues of the apoproteins (154). The factors that control the integration of Fe-S clusters into the mitochon-

drial respiratory chain have just started to be investigated in several laboratories (158). The role of heme in the integration of S-1, S-2 and S-3 of complex II and Rieske's Fe-S center of complex III are investigated EPR spectroscopy in this thesis.

Chapter II. EXPERIMENTAL PROCEDURES

(1). Yeast Strains and Cell Growth

The haploid yeast strain KL14-4A was used in DABS labeling studies. This strain of yeast cells was grown to late logarithmic phase at 30 C in a semi-synthetic medium containing 0.2 % yeast extract and 2 % galactose (65,159). The yeast mutant strain lacking 5-aminolevulinic acid synthase (strain GL1, a gal 2, hem1) (146) and the wild-type parent strain (x2180, a gal 2) of S. cerevisiae were maintained on a medium containing 0.2 % yeast extract, 1 % dextrose and 0.6 % yeast nitrogen base (without amino acids) solidified with 2 % agar. The medium for the heme-deficient mutants was supplemented with 0.25 % Tween 80 as a source of oleic acid and 20 mg/l of ergosterol. To obtain heme-sufficient (GL1) mutant cells, 30 mg/l of 5-aminolevulinic acid was added to the growth medium (146,160). For biogenesis studies, wild-type cells and heme-sufficient and heme-deficient yeast mutant cells were grown to mid-log phase in Erlenmeyer flasks at 30 C with shaking in water baths or metabolic shakers. Samples of cell cultures were checked under the microscope for bacterial contamination.

(2). Labeling of Yeast Cells with [^{3}H]leucine and [^{35}S]methionine

For pulse labeling, wild-type cells (2180) and heme deficient or sufficient yeast mutant cells (GL1) were grown to mid-log phase and harvested by centrifugation at 1500 x g for 5 min at room temperature (84). The labeled cells were then resuspended at 200-300 mg/ml in growth medium in which the yeast extract had been omitted, and the concentration of glucose had been lowered to 0.3 %. The cells were labeled at 30 C under gentle shaking with either 300-400 uCi/ml of [^{3}H]leucine or 300-400 uCi/ml of [^{35}S]methionine. The optimal

labeling time was 55 min for wild-type yeast cells and 80 min for heme-deficient yeast mutant cells. Labeling was stopped by adding either 1 mM cold leucine or 1 mM methionine and cysteine to the reaction mixture followed by 5 min incubation at 30 C with gentle shaking.

(3). Preparation of Mitochondria, Mitoplasts and Submitochondrial Particles

To obtain mitochondria from wild-type or heme-mutant cells, two previously reported methods were used. For DABS labeling studies, wild-type yeast strain KL14-4A cells were broken with glassbeads (diameter of 0.45-0.5 mm) in a medium containing 0.25 M sucrose, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and 1 mM PMSF (65). For spectral analysis, cells were broken with the same size of glassbeads but in a medium containing 0.65 M sorbitol, 10 mM potassium phosphate buffer, pH 6.3, 2 mM EDTA, 0.1 mM $MgCl_2$, 20 mM KCl and 0.1% bovine serum albumin (161).

For chymotrypsin digestion experiments and DABS labeling experiments, the mitochondria of the wild-type cells and heme-mutant cells were obtained by using the previously described zymolyase digestion procedures (162). To obtain spheroplasts, yeast cells were preincubated for 20 min at 30 C in a medium containing 20 mM Tris-HCl, 20 mM EDTA, pH 9.0 containing 2-mercaptoethanol of 35 ul/100 ml buffer. Zymolyse 5000, suspended in the same buffer was added to the cell suspension at a final concentration of 3-4 mg zymolyase/g cells. An initial optical absorbance reading at 800 nm of a 1:100 water diluted cell suspension was recorded before incubation at 30 C with gentle shaking. The incubation was carried out until 10 % of the original absorbancy was reached.

The spheroplasts obtained after zymolyase digestion were isolated

and washed by centrifugation at 1500 g for 10 min using the same buffer from which 2-mercaptoethanol was omitted. The washed spheroplasts were then resuspended in a medium containing 0.6 M mannitol, 1 mM EDTA, 1 mg/ml bovine serum albumin, and 10 mM KH_2PO_4, pH 6.8, and shaken in 10-20 ml aliquots in a small waring blender for 25 sec at high speed. Mitochondria were then isolated by centrifugation at 9,000 x g for 20 min and washed twice by centrifugation with the above mannitol buffer at the same speed (162).

Mitoplasts were prepared by the digitonin fractionation procedure (163) from mitochondria of wild-type cells and heme-deficient yeast mutant cells. An osmotic shock procedure recently reported by Schatz et al. (164) was also used in the preparation of mitoplasts from mitochondria of wild-type cells in the chymotrypsin digestion studies.

For digitonin treatment, mitochondria of wild-type cells or heme mutant cells were suspended in a medium containing 0.6 M mannitol, and 10 mM sodium phosphate, pH 7.0 at a protein concentration of 10 mg/ml. A stock solution of freshly prepared 35 mg digitonin per ml buffer was added to the mitochondrial suspension to a final concentration of 3 mg digitonin/10 mg protein. The mixture was incubated for 1 min at 4 C with occasional vortexing and subsequently diluted 1 to 5 with the same buffer and sedimented by a 10 min centrifuation at 12,000 g to obtain the pellet of mitoplasts (50).

For the osmotic shock treatment, the mitochondria from wild-type (2180) cells were resuspended at a concentration of 6 mg protein/ml in a buffer containing 0.6 M mannitol, 20 mM Hepes, pH 7.4. This suspension was then diluted to a final concentration of 0.1 M mannitol by adding dropwise 5 volumes of hypotonic buffer of 10 mM Tris-HCl, pH 7.4

containing 1 mM PMSF in 20 min. The dilution process was carried out at 0-4 C with gentle stirring. Mitoplasts were sedimented at 12,000 g for 10 min by centrifugation (164).

Submitochondrial particles were prepared by suspending the mitochondrial pellet in 0.1 M sodium phosphate buffer, 0.5 mM EDTA, pH 7.5) and sonicating in three 20 seconds bursts in a Branson sonifier at a power setting of 4.5 (65). The suspension was centrifuged for 10 min at 8000 g. The pellet was discarded and the supernatant was centrifuged for 30 min at 100,000 g.

Amounts of proteins of mitochondria, mitoplasts and submitochondrial particles were determined by Lowry procedure (165).

(4). Spectral Studies

Mitochondrial difference spectrum were obtained from wild-type and heme mutant cells. Washed mitochondrial pellets of wild-type cells or heme-mutant cells were suspended in a medium containing 0.65 M sorbitol, 10 mM potassium phosphate buffer, pH 6.3, 2 mM EDTA, 0.1 mM $MgCl_2$, 20 mM KCl and 0.1% bovine serum albumin. Difference spectra were obtained from dithionite reduced versus ferricyanide oxidized mitochondrial samples at room temperature in a Perkin-Elmer Model 557 spectrophotometer. A 2-nm slit width and a scanning speed of 120 nm/min were used (159).

(5). Electrophoresis and Autoradiography

Slab gels (approximately 12 cm to 15 cm long, 1.2 mm thick) were prepared according to Studier (166) with minor modifications. The discontinuous sodium dodecyl sulfate-Tris buffer system, described by Laemmli (167), was used for electrophoresis. The stacking gels were 5 % acrylamide and the running gels were 10 % or 12.5 % acrylamide, respectively. The dissociation buffer for solubilizing proteins for electrophoresis contains 0.05 M Tris-HCl, pH 6.8, 2 % sodium dodecyl sulfate, 2 mM EDTA, 150 mM 2-mercaptoethanol and 10 % glycerol (159). Membrane proteins or proteins in immunoprecipitates were solubilized in the dissociation buffer overnight before electrophoresis. All slab gel electrophoresis procedures were performed at room temperature for approximately 4 h. After electrophoresis, slab gels were stained for 1 h with 1.25 % Coomassie Blue in 45 % methanol- 7.5 % acetic acid (v:v) and destained overnight in 5.5 % methanol- 7.5 % acetic acid (v:v).

The proteins in the low molecular weight standards kit from Pharmacia with the molecular weight range between 14 kDa and 94 kDa was used as standards. Slab gels were dried for autoradiography on Whatman 3 MM filter paper by using a Biorad slab gel drier (Model 224) and exposed at -70 C to Kodak NS-5T X-ray film for several days. In some experiments, the autoradiogram were scanned in a Canalco densitometer (159).

(6). Labeling of Mitochondria and Submitochondrial Particle with DABS and Immunoprecipitation of Complex III from DABS Labeled Membranes

[^{125}I]diazotized iodosulfanilic acid was prepared from [^{125}I]iodosulfanilic acid according to New England Nuclear product information (159). After addition of 45 nmol of cold sulfanilic acid as a carrier, 1 mCi of [^{125}I]iodosulfanilic acid (2200 Ci/mmol) in 10 ul of water was diazotized by adding 5 ul of sodium nitrite and 5 ul of 0.1 N HCl at 4 C, followed by neutralization with 170 ul of 100mM phosphate buffer, pH 8.3. An aloquot of 100 ul of mitochondria was suspended at 2 mg protein/ml in 0.25 M sucrose and 10 mM sodium phosphate, pH 7.5 and an aliquot of 100 ul of submitochondrial particles was suspended at 2 mg protein/ml in 0.1 M sodium phosphate, pH 7.5 containing 0.5 mM EDTA. Both aliquots were incubated with 400 uCi of [^{125}I]DABS at 4 C for 30 min with several times vortexing.

Labeling was terminated by addition of 5 mM histidine followed by recentrifugation. Iodinated mitochondria and submitochondrial particles were washed twice with 4 ml of 100 mM Tris-HCl, pH 7.5 with 5 mM histidine and twice with 100 mM Tris-HCl containing 2.5 % bovine serum albumin and then solubilized with 1 % lubrol and 1 % sodium deoxycholate at 4 C for 1 h.

After centrifugation at 12,000 g to remove unsolubilized membranes, 20 ul of labeled mitochondria and submitochondrial particles were immuno incubated with an optimal amount of antiserum against complex III. The iodinated immuno precipitates were solubilized in 5% SDS and glycerol overnight at room temperature before SDS polyacrylamide gel electrophoresis. The final gels were dried and autoradiogrphed using Kodak film (159).

(7). Chymotrypsin Digestion of Mitoplasts

Mitoplast pellets prepared from wild-type (2180) cells or heme-deficient mutant cells were resuspended in a solution containing 0.67 M sucrose, 0.05 M Tris-HCl, and 1 mM histidine, pH 8.0, to a final concentration of 2 mg protein/ml buffer (50). Various amounts of chymotrypsin were added to the mitoplasts suspensions followed by gentle shaking at 30 C for 30 min. Proteolytic digestion was stopped by adding 2 mM PMSF to the mixture followed by centrifugation at 12,000 g for 10 min. Pellets of mitoplasts obtained were washed by centrifugation at the same speed with the sucrose buffer containing 2 mM PMSF before immunoprecipitation and SDS-polyacrylamide gel electrophoresis.

(8). Solubilization of Proteins from Control and Chymotrypsin Digested Mitoplasts for Immunoprecipitation

Pellets of control or chymotrypsin digested mitoplasts were resuspended in 0.1 M sodium phosphate buffer, pH 8.0, at a protein concentration of 1.5 mg/ml. Triton X-100 (20% solution) was added to the mitoplast suspensions to a final concentration of 1 % Triton (50). After incubation at 0-4 C for 1 h with occasional vortexing, the mixture were centrifuged at 100,000 g for 45 min to remove any unsolubilized material. The supernatants were then used for immunoprecipitation.

(9). Immuno Precipitation of Complex III from Control and Chymotrypsin Digested Mitoplasts of Wild-type and Heme Mutant Cells

Antiserum against purified complex III and antiserum against purified Rieske's iron-sulfur protein were prepared and characterized by several investigators in this laboratory (23,24,49,50). For assembly of complex III in heme-mutant cells studies, 1% Triton x-100 extracted labelled proteins from control or digested mitoplasts were mixed with an optimal amount of antiserum, and incubated for 16 hr at 4 C with shaking. Sepharose-bound protein A (10 mg dry weight preswollen in 50 ul of 10 mM sodium phosphate, pH 7.4 and 150 mM NaCl) was added to precipitate the antigen-antibody complex and the incubation continued for 1 h. The Separose beads were then cellected by centrifugation at 8,000 g for 5 min and washed three times by centrifugation with 10 mM sodium phosphate, pH 7.4, 150 mM Nacl and 0.15 % Triton X-100 and two times with the same buffer without Triton. The Sapharose bound immunoprecipitates were suspended in 40 ul of gel dissociation buffer as previously described with 5 % 2-mercaptoethanol and 1 mM PMSF. The mixtures were then boiled in 95 C hot water bath for 5 min in order to solubilize the proteins from beads. The supernatant containing proteins of complex III were stored overnight at room temperature. The entire supernatants were analyzed by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained and destained, sliced into 2-mm slices, digested with hydrogen peroxide, mixed with liquiscint and counted for their radioactivity.

(10). 'Western Blotting' Immuno Transfer Technique

This relatively new immuno transfer technique (168,169) involves the transfer of proteins electrophoretically from SDS-polyacrylamide gels to nitrocellulose filters. The filters are then treated with specific antibodies, followed by the binding of radioiodinated Staphylococcus aureus protein A to the immume complexes, and visualization of the labeled proteins in the immune complexes by autoradiography.

(a). Electrophoretic Transfer of Proteins from SDS-polyacrylamide Gels to Nitrocellulose Papers for Immuno Reaction

Slab gels were prepared by the method of Laemmli (167) as previously described in this chapter. Mitochondrial samples of wild-type cells, heme-sufficient and heme-deficient yeast mutant cells were suspended at 5 mg protein/ml in 0.25 M sucrose and 10 mM sodium phosphate, pH 7.5 followed by solubilization overnight in a dissociation medium containing 5 % sodium dodecyl sulfate, 5 % 2-mercaptoethanol, 2 mM EDTA, 50 mM Tris-HCl, pH 6.8 and 10% glycerol. Approximately 100 ug of mitochondrial proteins was loaded into each well of the slab gel.

After gel electrophoresis, the portions of the gel with molecular weight standards were removed, stained with Coomassie Blue and destained for visualization. The remainder of the gel containing samples were than transferred electrophoretically to nitrocellulose filters according to the method of Towbin et al. (170). The transfer buffer contained 120 mM glycine, 24.8 mM Tris-HCl, pH 8.4 and 10 % ethanol. The total transfer time is 1.5 to 2 h. After the transfer was complete, the filters were trimmed to the exact size as the gel slice then incubated in phosphate buffer saline (10 mM sodium phosphate, 150 mM NaCl, pH 6.8)

with 5 % new born calf serum for 60 min at room temperature with gentle shaking to saturate the remaining protein-binding sites. After washing, the nitrocellulose filters were blotted dry between two sheets of Whatman filter papers and immunoincubated immediately with antiserum or stored in the cold room for future use.

(b). Iodination of Protein A of Staphylococcus Aureus

Staphylococcus aureus protein A was iodinated using $Na^{125}I$ according to the instructions supplied by Bio-Rad. The protein A was radiolabeled with a gentle enzymatic procedure using a new solid phase radioiodination reagent involving enzymobeads. The enzymobead reagent consists of immobilized preparations of lactoperoxidase and glucose oxidase on hydrophilic spheres several microns in diameter. On addition of glucose to a suspension of enzymobeads, $Na^{125}I$ and protein A, the immobilized glucose oxidase continuously generates a small, steady amount of H_2O_2. The lactoperoxidase, in turn, catalyzes the peroxide oxidation of labeled iodide to iodine. Then the radiolabeled iodine reacts with the protein A to produce radioiodinated protein A (171,172).

The dry enzymobead reagent obtained from Bio-Rad was first rehydrated with distilled water at least 1 h before use. For radioiodination, 1 mCi $Na^{125}I$ (25 ul), was added to a small Eppendorf vial containing 50 ug protein in 75 ul 0.2 M phosphate buffer, pH7.2, 50 ul enzymobeads suspension and 25 ul of 1 % beta-D-glucose. The incubation was carried out at room temperature for 25 min followed by the addition of 200 ul of 0.05 M sodium phosphate buffer to terminate the iodination. The Eppendorf vial containing the mixture was then centrifuged at 17,000 g for 5 min to remove the pellet containing the

enzymobeads. The supernate was applied to a G25 Sephadex gel filtration column pre-equilibrated with 0.05 M phosphate buffer saline containing 1 mg/ml bovine serum albumin. This same buffer without bovine serum albumin was used in eluting the iodinated protein A. A Geiger counter was used in monitoring the fractions containing the highest iodinated protein A (168).

Eluate fractions containing the iodinated protein A were then pooled and dialyazed in 500 ml of 0.05 M phosphate buffer saline overnight to get rid of the unbound iodide. The final product of radioactive iodinated protein A contained 7.8×10^6 counts/min, ug of protein A and was stored in aliquots at -20 C prior to use. Samples were used less than 1 month after preparation.

(c). Immunoincubation and Radioactive Protein A Reaction of the Nitrocellulose Paper for Autoradiography

The immunoincubation was performed overnight at room temperature with shaking in 20-30 ml of phosphate buffered saline containing 10 % new born calf serum with 10 ul of antiserum against complex III. The filters were then washed three times,for 30 min each, in 50 ml phosphate buffered saline containing 3 mg of bovine serum albumin/ml.

The immunoincubated filters were then incubated in 30 ml phosphate buffered saline with 10% new born calf serum containing 1×10^7 counts/min of radiolabeled protein A for 1 h at room temperature with gentle shaking. After incubation, the filters were washed with the same buffer containing 3 mg/ml bovine serum albumin for three times. Blotted dried filters were then wrapped with Saran Wrap and autoradiographed on a Kodak film using a Kodak enhancer screen (160).

(11) Electron Paramagnetic Resonance Studies on the Mitochondrial Respiratory Chain Iron-Sulfur Clusters of Wild-Type Cells and Heme-Deficient Mutant Yeast Cells

EPR spectra of the iron-sulfur clusters of the mitochonrial respiratory chain of wild-type and heme-deficient yeast mutant cells were obtained using a Varian E109 spectrophotometer with the help of Dr. Tomoko Ohnishi at the University of Pennsylvania. The sample temperature was controlled within 15-50 K by using a liquid helium variable temperature cryostat system (Air Product Ltd 3-110) and monitored with an Allen-Bradley carbon resistor placed directly below the sample. Double integration of the spectra was performed on a Nicolet signal averager (NIC-1024) using Cu(II)-EDTA complex as a standard (158,182).

Mitochondrial pellets from the heme-deficient mutant yeast cells and wild-type yeast cells were resuspended in 0.6 M sorbitol, 0.25 mM EDTA, 50 mM Tris-HCl and 40 mM 4-morpholinopropanesulfonate (MOPS), pH 7.4 at 42.9 and 46.8 mg of protein per ml respectively. The mitochondrial suspensions were then reduced with either a few crystals of dithionite or with ascorbate. The samples were then frozen in the EPR tube by immersion into a 1:5 mixture of methyl cyclohexane/isopentane chilled to approximately 81 K. The frozen samples were stored in liquid nitrogen until the EPR experiments were performed (173).

(12). Materials

Uniformly labeled [^{3}H]leucine and [^{35}S]methionine were purchased from Amersham. [^{125}I]iodosulfanilic acid and Na[^{125}I] were purchased from New England Nuclear. Zymolyase 5000 was from Miles Lab., Inc. Yeast extract and yeast nitrogen base (without amino acids) and new born calf serum were from Difco. N,N'-methylene-bis-acrylamide was from Biorad. Acrylamide was from Serva. Sepharose bound protein A was from Pharmacia. Nitrocellulose filters (0.45 um) was from Millipore. Enzymobead reagent was from Bio-rad. Glassbeads were from VWR. Ergosterol, Tween 80, 5-aminolevulinic acid, Triton X-100, chymotrypsin, Digitonin, Coomassie Brilliant Blue, Phenylmethanesulfonyl floride and sodium dodecyl sulfate were from Sigma. All other reagents were of the highest purity commercially available.

The wild type yeast stain KL14-4A used in this study was generous gift of Dr. A. Goffeau (University of Louvain, Belgium). The ALA-synthase defective mutant strain GL1 and its parent strain 2180 were a generous gift of Dr. E. Gollub at the Rockefeller University (New York). Dr. T. Ohnishi kindly assisted in performing the electron paramagnetic resonance experiments at the University of Pennsylvania at Philadelphia. The antiserum against purified complex III and purified Rieske's iron-sulfur protein were a gift from Dr. C. Battie and Dr. A. Sidhu at Mount Sinai School of Medicine. Dr. L. Clejan kindly contributed purified complex III preparation for some experiments in this thesis.

Chapter III. Experimental Results

(1). Growth Properties and Mitochondrial Properties of Heme-deficient Yeast Mutant Cells

(a). Growth Curves of Wild-type and Heme Mutant Yeast Cells

Initially, growth curves of the ALA-synthase defective (GL1) mutants in the presence of ergosterol or Tween 80 (heme-deficient) or 5-aminolevulinic acid (heme-sufficient) and its parent strain (2180) were determined and compared to standardize conditions for biochemical studies. As shown on Fig. (4), the heme-deficient yeast mutant cells have a lag phase of approximately 10 h before cell division begins in the logrithmic phase of cell growth. In contrast, the wild-type yeast cells and heme-sufficient mutant cells have a lag phase of only about 2 h. Both the wild-type and heme-sufficient mutant cells reach the same cell density (by optical density of the cell culture) at stationary phase of the cell growth; however, heme-deficient mutant cells never attain this cell density. Therefore, the absorbancy of the cell cultures can be used as a convenient tool for monitoring the growth of the wild-type and heme-deficient yeast mutant cells.

For experiments in this thesis, it was important that the initial inoculum were from a freshly grown preculture in the late-log phase of the growth, or reproducible growth curves could not be attained. In addition, it was important to use cultures harvested at the mid-log phase of the growth since a higher reversion rate was observed when cells reached late-log and stationary phase.

(b). Mitochondrial Cytochrome Levels of Heme-deficient and Heme-sufficient Yeast Cells

Mitochondrial cytochrome content was obtained from dithionite

reduced versus ferricyanide oxidized mitochondrial samples from wild-type, heme-sufficient and heme-deficient yeast mutant cells. As shown in Fig. (5), Wild-type and heme-sufficient mutant cells all contain cytochromes b, c, c_1, a and a_3, while the heme-deficient mutant cells have no spectrally detectable mitochondrial cytochromes.

The mitochondria of the heme-sufficient mutant yeast cells were observed to contain approximately 60 % to 90 % of the cytochrome level observed in the wild-type cells (data not shown). The levels of mitochondrial cytochromes are known to vary in different experiments depending on the uptake of the added 5-aminolevulinate and the different growth phase of the cell (146).

Mitochondrial cytochrome levels were determined in different experiments to ensure that the mutant had not reverted to wild-type cells. The colors of the mitochondrial pellets of wild-type and heme-deficient cells were also visibly different. Mitochondria isolated from wild-type cells and heme-sufficient mutant cells are brown in color while those from the heme-deficient mutant cells are white due to the absence of the cytochromes. Similarly, microsomes of heme-deficient mutant cells are also white in color instead of cherry red as those in wild-type yeast cells.

(c). Separation of Mitochondrial Proteins of Wild-Type and Heme-deficient Mutant Cells on SDS-polyacrylamide Gels

Mitochondrial proteins of wild-type and heme-deficient yeast mutant cells were separated on SDS polyacrylamide gel for comparison. As shown in Fig. (6), mitochondria from wild-type yeast cells and heme-deficient mutant cells contain similar patterns of protein bands. However, several different band patterns in these two mitochondria do exist.

These results indicate that only a relatively small portion of the mitochondrial proteins are affected in the heme-deficient yeast mutant cells, the majority of the mitochondrial proteins are probably not affected by the absence of intracellular heme. Previously, Saltagaber-Muller et al. (134) reported that the mitochondrial ATPase level of the heme-deficient GL1 mutant cells is in the same order as those in the wild-type yeast cells, while the activities of cytochrome oxidase and succinate-cytochrome c reductase were extremely low or near zero in the heme-deficient GL1 cells. In addition, Gollub et al. (146) also reported the similar activities of several cytosolic enzymes such as glucose-6-phosphate dehydrogenase and HMGCoA reductase in heme-deficient yeast cells and in wild-type.

(2). Immunological Detection of Complex III Subunits in Mitochondria of Heme-deficient Yeast Cells

Previous results from this (148) and other laboratories (134) have demonstrated the existence of normal levels of mitochondrial translational products including the apocytochrome b of complex III in heme-deficient GL1 cells. My initial experimental approach to the detection of other complex III subunits in heme-deficient yeast mutant cells involved immunoprecipitation of the complex from detergent solubilized mitochondrial membranes with antiserum against purified complex III from bakers yeast (23, 24).

An immuno titration curve was obtained first using 1 % lubrol WX and 1 % deoxycholate solubilized mitochondria isolated from heme-deficient and heme-sufficient mutant yeast cells grown in [^{3}H]leucine (Fig. 7). The antiserum against complex III immunoprecipitated almost 10 % of the total radioactivity from mitochondria of the heme-sufficient mutant cells, a value identical to that obtained with wild-type cells. By contrast, the antiserum against complex III did not immunoprecipitate from the heme-deficient mitochondria radioactivity at a level significantly above that precipitated by the pre-immune serum. The incapability of immunoprecipitating substantial counts in these experiments could be due to reasons such as detergent extraction that was involved in the immunoprecipitation affected the antigen-antibody reaction in the heme-deficient mutant cells.

The immunoprecipitates obtained at the optimal antibody-antigen ratio were examined by SDS-polyacrylamide gel electrophoresis to determine which subunits of complex III were present. As seen in Fig. (8), the lubrol and deoxycholate solubilized mitochondrial suspension of

heme-sufficient mutant cells contained labeled bands corresponding in molecular weight to six of the subunits of complex III. By contrast, the gels of the immunoprecipitates formed with the mitochondrial membranes of the heme-deficient yeast cells and the antiserum against complex III revealed only two major protein bands. The major band in the immunoprecipitates has a molecular weight corresponded to the core protein 1 of complex III. Interestingly, no labeled band with a mobility corresponding to apocytochrome b was observed in Fig. (8), in contrast to previous studies done in this laboratory (148).

The second approach to determining the presence of the subunits of complex III in the heme-deficient yeast mutant cells involved a new immunotransfer technique perfected by Groot and his coworkers (168). One advantage of this technique is that problems arising from detergent extraction and immunoprecipitation are avoided. The results of Fig. (9) reveal that mitochondria of heme-sufficient (lanes 2-4) mutant cells contain all of the subunits of complex III with the exception of subunit VI and VII which are not easily detected with this technique even in an identical transfer of purified complex III (lanes 5,6) in Fig. (9). Similarly, subunit II is only faintly detected by this antibody against complex III with this immunotransfer technique.

Contrary to the results from immunoprecipitation, results from the immunotransfer experiment (Fig. (9)) demonstrated that the mitochondria from heme-deficient yeast cells contain most of the subunits of complex III. Proteins with molecular weights of core proteins 1, core protein 2, apocytochrome b and the Rieske's iron-sulfur protein of complex III are present in mitochondria of wild-type, heme-sufficient and heme-deficient yeast mutant cells, In addition, three new proteins with

apparent molecular weights of 34.5 kDa, 29 kDa and 28 kDa were observed in the mitochondria of the heme-deficient yeast cells using antiserum against complex III. These proteins were not detectable in the mitochondria of heme-sufficient mutant cells or wild-type cells. No labeled bands were present in either heme-deficient or heme-sufficient yeast cells, when preimmune serum was used in place of antiserum.

Previous studies by Van Loon et al. (174) and Sidhu (49) of this laboratory has demonstrated that the processing of the iron-sulfur protein of complex III involves a precursor form (28 kDa) and an intermediate form (26 kDa) of the precursor protein. In order to establish the possible identity of the polypeptides of 28 kDa and 29 kDa in Fig. (9) as these values correspond to the reported molecular weight for the precursor form of the iron-sulfur protein in yeast, an immunotransfer experiment was performed with the antiserum against purified iron-sulfur protein.

As shown in Fig. (10), the iron-sulfur protein antiserum immunoprecipitated only a major protein of 25 kDa from a 3 % SDS extracts of the mitochondria from [^{35}S]methionine labeled heme-deficient mutant cells and wild-type cells. No bands correspond to the precursor and intermediate form of the iron-sulfur protein were detected. With the immunotransfer technique, the antiserum against iron-sulfur protein precipitated one major band of 25 kDa (Fig. (11)) from mitochondria of wild-type and heme-deficient yeast cells. In addition, faint bands of 25.5 kDa were also present in both wild-type and heme-deficient mutant yeast cells. Hence, the precursor of the Rieske's protein can be processed to the mature form in the absence of heme in yeast mutant cells.

Recently, Schatz and his collaborators have modified and improved

the techniques of immunoprecipitation of mitochondria and mitoplasts proteins using protein A bound to Sepharose beads (175). In order to study the synthesis and assembly of complex III in heme-deficient mutant cells, mitoplasts of the wild-type and heme-deficient yeast cells were extracted with 1 % Triton before immunoprecipitaton with antiserum against complex III. As can be seen in the gel lane containing the undigested mitoplasts of the heme-deficient yeast cells in Fig. (20), the complex III antiserum immunoprecipitated proteins with the same apparent molecular weights as core protein 1, core protein 2, apocytochrome b, and three small proteins of molecular weights 18, 14 and 11 kDa and a 34.5 kDa protein from mitoplasts of heme-deficient mutant cells. The antiserum aginast complex III also immunoprecipitated the same proteins from wild-type mitoplasts with the exception of a 31.5 kDa protein instead of the 34.5 kDa as observed in the heme-deficient mitoplasts in these experiments.

Results obtained from several different immuno experiments in this study therefore demonstrated that core protein 1, core protein 2, apocytochrome b and the iron-sulfur protein of complex III are synthesized, processed and integrated into the inner mitochondrial membrane in the absence of heme in heme-mutant yeast cells. The 34.5 kDa protein bands in gel lanes containing heme-deficient mitochondrial or mitoplasts proteins in Fig. (9) and (20) are tentitively identified as the intermediate form of apocytochrome c_1 since the same molecular weight of the intermediate form of this protein has recently been reported by Ohashi et al. (84) in the same yeast strain GL1.

Interestingly, the two unidentified proteins of 28 and 29 kDa molecular weights that were present in the autoradiogram of the

of heme-deficient mitochondria with antiserum aginst complex III in Fig. (9) were both absent in the immunoprecipitates obtained from Triton solubilized heme-deficient yeast mitoplasts as shown on Fig. (20). Therefore, these two protein bands might be due to contaminating antigenic cross reacting proteins existing in the mitochondria of heme-deficient yeast mutant cells (176).

(3). Detection of Iron-Sulfur Clusters in Mitochondria of Heme-deficient Yeast Mutant Cells

Since most of the subunits of complex III including the Rieske's iron-sulfur protein were found to be synthesized and integrated into the inner mitochondrial membrane of yeast cells in the absence of heme, the next logical step was to detect the presence of iron-sulfur clusters of the respiratory chain in heme-deficient yeast mutant cells. EPR spectra of dithionite-reduced mitochondria prepared from either wild-type or heme-deficient cells were compared to investigate the presence or absence of the yeast mitochondrial respiratory chain iron-sulfur clusters in yeast cells in the absence of heme.

As shown in Fig. (12), the EPR signals at g=2.03, g=1.93 and g=1.91 due to clusters S-1 and S-2 of succinate dehydrogenase of the wild type cells were clearly observed. The EPR signal arising from Rieske's iron-sulfur cluster was also discernible with a negative peak at g=1.89 in the dithionite reduced mitochondria. By contrast, no EPR signals due to S-1, S-2 and the Rieske's iron-sulfur protein were observed in mitochondria from the heme-deficient yeast mutant cells.

EPR spectra of iron-sulfur cluster S-3 of succinate dehydrogenase were examined at a lower temperature of 9 K in mitochondria reduced with ascorbate. As shown in Fig. (13), a pronounced S-3 signal centered at g=2.01 was observed in the mitochondria of wild-type cells. The peak at g=2.02 and the trough at g=1.99 with a half width of approximately 24 g (2.4mT) is characteristic of the Hipip-type iron-sulfur cluster S-3 reported for complex II from beef heart mitochondria (177). However, the S-3 signals were not detected in the mitochondria from heme-deficient mutant cells. The broad peak at g=2.07 observed in Fig. (13)

probably reflects unidentifeid metal ions such as Cu^{+2} in the preparation.

The EPR spectra due to Rieske's iron-sulfur cluster were clearly shown when ascorbate was used as a reductant for wild-type mitochondria. The spectrum at a lower gain as shown in Fig. (14) shows the overlapping signals from the partially reduced clusters S-1 and S-2 described previously. The g=2.03 signal of the Rieske's iron-sulfur cluster overlapped with the signals of S-1 and S-2 in Fig. (12) and that of S-3 in Fig. (13). The g_x signal around g=1.8 in Fig. (14) was not clearly revealed because of the low signal-to-noise ratio (178,179). A comparable sample of mitochondria from the heme-deficient mutant is not presented since the data of Fig. (12) and (13) had demonstrated the lack of any iron-sulfur clusters in mitochondria from the heme-deficient mutant cells.

The lack of EPR signals due to the iron-sulfur clusters of complex II and complex III in this study suggested that, in the absence of heme, iron-sulfur clusters of yeast mitochondrial respiratory chain are either not formed or not properly integrated with iron-sulfur proteins and other components of the respiratory chain.

(4). Localization of Subunits of complex III in Wild-type Cells by DABS Labeling Techniques

The initial approach to determining the localization of complex III subunits in the inner mitochondrial membrane involved protein labeling with the membrane-nonpermeant reagent [^{125}I]DABS. This reagent, like other diazotized sulfanilic acid derivatives, selectively reacts with amino acid residues of proteins extending from or exposed on the surface of the membrane. Subunits of complex III were immunoprecipitated from mitochondria and submitochondrial particles labeled with [^{125}I]DABS prior to SDS-polyacrylamide gel electrophoresis and autoradiography. It should be noted that both mitochondria and submitochondrial particles were labeled to approximately the same extent under the labeling conditions, such that similar numbers of counts were applied to the gel.

The proper inversion of the inner mitochondrial membrane in the submitochondrial particles had previously been studied and ascertained by enzyme studies such as succinate dehydrogenase and coenzyme QH_2-cytochrome c reductase assays (159). It was observed that the addition of the coenzyme Q analogue to mitochondria caused reduction of 15-30 % of the total ditionite reducible b and 100 % of cytochrome c_1; while the addition of the coenzyme Q analogue to the submitochondrial particles led to the reduction of 70 % of the total dithionite reducible cytochrome b but insignificant amount of cytochrome c_1. In addition, the autoradiogram profiles of gels containing [^{125}I]DABS labeled mitochondria differed completely from that of the submitochondrial particles suggesting an inversion of the inner mitochondrial membrane occurred during sonication (159,180).

The final autoradiogram of a 10 % SDS-polyacrylamide gel containing the immunoprecipitates obtained from [^{125}I]DABS labeled praparations of mitochondria and submitochondrial particles using the antiserum against complex III are shown on Fig. (15). As indicated in this figure, a completely different labeling pattern in the mitochondria and submitochondrial particles again indicates that different proteins are exposed on the surface of the inner mitochondrial membrane. Three protein bands with molecular weights of 29 kDa (apocytochrome c_1), 25 kDa (the Rieske's iron-sulfur protein) and 17 kDa (subunit VI) are labeled with [^{125}I]DABS in mitochondria but not in submitochondrial particles, indicating that these proteins are localized on the outer surface of the membrane. By contrast, two proteins with molecular weights of 50 kDa (core protein 1) and 31 kDa (apocytochrome b) are heavily labeled with [^{125}I]DABS while two other proteins with molecular weights of 47 kDa and 39 kDa (core protein 2) are lightly labeled in both mitochondria and submitochondrial particals, suggesting that all these proteins span the inner mitochondrial membrane.

The [^{125}I]DABS labeled band with an apparent molecular weight of 56 kDa observed in Fig (15) in the immunoprecipitates obtained with antibodies against complex III or apocytochrome b (data not included) may represent a dimer of cytochrome b present in the membrane as suggested by Von Jagow and Engel (103). Alternatively, this band may represent a nonspecific aggregate of cytochrome b or other proteins as previously reported by Clejan et al. from this laboratory (54,181).

In a separate experiment, the labeling pattern of immunoprecipitates obtained from [^{125}I]DABS-labeled mitoplasts were compared to that of the mitochondria. As indicated in Fig. (16), a similar pattern

of labeled proteins were observed, although quantitative differences in labeling of the peaks were observed. This similarity suggested that the outer membrane does not prevent the reaction of [^{125}I]DABS on the outer surface of the inner membrane. The gel profile in Fig. (16) for mitoplasts also shows fewer labeled bands than those in mitochondria as shown in Fig. (15) suggesting that fewer contaminating or cross reacting proteins exist in mitoplasts compared to mitochondria. The data in Fig. (16) show that both core protein I (39 kDa) and the iron-sulfur protein (25 kDa) were more heavily labeled relative to apocytochrome b (31 kDa) and apocytochrome c_1 (29 kDa) in the mitoplasts compared to mitochondria, while that of core protein 1 (49 kDa) was less heavily labeled. The quantitative differences in labeling of the mitoplasts and mitochondrial proteins of complex III could be due to the digitonin treatment that is necessary for the preparation of mitoplasts.

Simultanous [^{125}I]DABS labeling experiments with the same membrane preparations as in this study using the antiserum against apocytochrome b were performed by Chen (159,180) in this laboratory. Apocytochrome b was found to be labeled with [^{125}I]DABS more in mitochondria than that in the submitochondrial particles indicating further that apocytochrome b spans the inner mitochondrial membrane.

A model for the topography of complex III in the inner mitochondrial membrane consistent with the current data of the [^{125}I]DABS labeling studies and other complex III enzymatical studies done in this laboratory (159) is shown in Fig. (17).

(5). Chymotrypsin Digestion of the Subunits of Complex III in Mitoplasts of Wild-type and Heme-deficient Yeast Cells

In this study, the sensitivity of the various subunits of complex III to chymotrypsin digestion was compared by analyzing immunoprecipitates obtained with antiserum against complex III and mitoplasts prepared from wild type (2180) and heme-deficient yeast mutant (GL1) cells grown in [^{3}H]leucine to label all cellular proteins. The proteins present in the immunoprecipitates were separated on SDS polyacrylamide gels for analysis. The molecular weights of the different subunits of complex III in the 12.5 % SDS polyacrylamide gels are: 49 kDa (core protein 1, subunit I), 39 kDa (core protein 2, subunit II), 34 kDa (the intermediate form of apocytochrome c_1, subunit IV), 31.5 kDa (the mature form of apocytochrome c_1, subunit IV), 30 kDa (apocytochrome b, subunit III) and 24.5 kDa (The Rieske's iron-sulfur protein, subunit V), plus three smaller subunits of appropriately 18, 14, and 11 kDa as observed in Fig. (18), (19) and (20). The assignments of the different molecular weights to their corresponding subunits were the same as those reported in this thesis.

The ^{3}H radioactivity profiles of the SDS polyacrylamide gels of several chymotrypsin digestion experiments are presented in the figures of this section. The amount of digestion by chymotrypsin was calculated, by obtaining for each individual subunit of complex III, the ratio of the radioactivitiy remaining in the immunoprecipitates of the protease-digested mitoplasts to that of the radioactivity in the immunoprecipitates of the control mitoplasts. In table (IV) such percentages are listed for comparison for different subunits of complex III at different chymotrypsin concentrations.

(a). Accessibility of Core Protein 1 and Core Protein 2 to Chymotrypsin in Wild-type and Heme-deficient Yeast Mutant Cells

In the experiments of Fig. (18), the wild-type mitoplasts that were used for digestion were prepared by the digitonin method. The [^{3}H] radioactivity profiles in Fig. (18) show that the two largest proteins of the complex, core protein 1 and core protein 2 are not digested by either 400 or 800 ug of chymotrypsin per mg of mitoplast protein. When mitoplasts were prepared from wild type cells by the osmotic shock method, the two core proteins displayed somewhat different sensitivities to chymotrypsin. As shown in Fig. (19), core protein 1 was gradually digested away up to 57.1% of the control value with increasing amounts of chymotrypsin ranging from 275 to 822 ug of chymotrypsin per mg mitoplast protein. With the same concentrations of chymotrypsin, core protein 2 was found to be digested only up to 22.6 % as shown in Fig. (19) and Table (1). These results suggest that in mitoplasts obtained from the osmotic shock method the two core proteins are more exposed to the cytosolic side of the membrane than in mitoplasts prepared by the digitonin method and hence are more sensitive to chymotrypsin.

In the case of heme-deficient mutant cells, the results of Fig. (20) (bottom panel) demonstrated that core protein 1 and core protein 2 were completely resistant to proteolysis by chymotrypsin concentrations of 780 or 1560 ug per mg of mitoplast protein. In the experiment reported in Fig. (20) (top panel), the core protein 2 was not labeled well with [^{3}H]leucine and was not well resolved very well from core protein 1 in this gel. Core protein 1, however, did not seem to be very sensitive to chymotrypsin concentrations of 400 and 800 ug per mg mitoplast protein. The digitonin method instead of the osmotic shock method was chosen for mitoplasts preparation from heme-deficient mutant

cells, since the amount of immunoprecipitable material obtained from the mitoplasts was decreased considerably after osmotic shock treatment.

(b). Accessibility of Iron-Sulfur Protein to Chymotrypsin in Wild-type and Heme-deficient Yeast Mutant Cells

The iron-sulfur proteins present in mitoplasts obtained from wild-type and heme-deficient mutant cells were noted to have different sensitivities to chymotrypsin. The result in Fig. (19) shows that the iron-sulfur protein of the wild type cells was progressively more sensitive to chymotrypsin concentrations ranging from 275 to 685 ug per mg of mitoplast protein; however, this protein was completely digested by 800 ug of chymotrypsin per mg of mitoplast protein in the heme-deficient mutant cells (Fig. (20) and Table (IV)). These results agree with those obtained previously from protease digestion studies, immunoinhition studies and DABS labeling studies, suggesting that this protein is exposed on the outer surface of the inner mitochondrial membrane.

In contrast to the iron-sulfur protein present in wild-type cells, the iron-sulfur protein of the heme-deficient mutant mitoplasts was found to be resistant to digestion by chymotrypsin concentrations ranging from 450 to 1560 ug per mg of mitoplast protein (Fig. (20) and Table (IV)). These results suggest that the iron-sulfur protein is not exposed on the cytosolic side of the inner mitochondrial membrane of heme-deficient mutant cells such that it is digested with chymotrypsin.

(c). Accessibility of Apocytochromes b and c_1 to Chymotrypsin in Wild-type and Heme-deficient Mutant Yeast Cells

For apocytochrome b, similar patterns of chymotrypsin sensitivity was observed in mitoplasts obtained from wild-type and heme-deficient

mutant yeast cells. The apocytochrome b was progressively more degraded with increasing concentrations of chymotrypsin ranging from 275-822 ug per mg of mitoplast protein in wild-type cells (Fig. (18)) and 450 ug and 900 ug per mg of mitoplast protein in heme-deficient mutant cells (Fig. (20) and Table (IV)).

The intermediate form of apocytochrome c_1 in these gels has a molecular weight of 34 kDa (Fig. (20)), which is clearly different from the mature form of mature apocytochrome c_1 (31.5 kDa) of wild type cells observed in Fig. (18) and (19). The results of Fig. (18) and Table (IV) indicate that the mature form of apocytochrome c_1 was progressively more digested with increasing concentrations of chymotrypsin (275-822 ug per mg of mitoplasts proteins) in wild type cells. The results of the top pennel of Fig.(20) also indicate that the intermediate form of apocytochrome c_1 in the heme-deficient mutant cells was also sensitive over approximately the same range of chymotrypsin concentrations (Table (IV)).

CHAPTER IV. DISCUSSION

In the past two decades, extensive studies on the biogenesis, mechanism and topography of complex III in yeast and other organisms has led to our greater understanding on this respiratory chain complex. However, the regulations on the biogenesis of this complex by physiological regulators such as heme, oxygen and glucose is still not clearly characterized. The recent isolation of several yeast mutant strains defective in heme-biosynthetic pathway enzymes has provided researchers a good system for studing the effects of intracellular heme on the biosynthesis and assembly of hemoprotein complexes and respiratory chain protein chain complexes. In this thesis project, studies on the role of heme in the biogenesis and assembly of yeast mitochondrial complex III was investigated in a heme-deficient yeast mutant strain of GL1.

(1). The Role of Heme in the Biosynthesis and Precursor Processing of Complex III Subunits in Yeast S. cerevisiae

The majority of the complex III subunits were detected by two different immuno techniques in this thesis work. The roles of heme in the biosynthesis of various subunits of complex III are discussed in following sections.

(a). The Role of Heme in the Biosynthesis and Processing of Core Protein 1, Core Protein 2 and the Rieske's Protein of Complex III

Results from the immunotransfer experiment with antiserum against complex III in this study demonstrated that mature forms of core protein 1, core protein 2 and the Rieske's iron-sulfur protein were present in roughly equal amounts in mitochondria of heme-deficient yeast mutant cells and heme-sufficient yeast mutant cells. With the antiserum against purified Rieske's iron-sulfur protein, the presence of mature

iron-sulfur protein, but not the precursor or the intermediate form of the iron-sulfur protein of complex III in the mitochondria of heme-deficient yeast mutant cells was demonstrated in this study. Immuno precipitation experiments performed with mitoplasts isolated from heme-deficient cells and wild-type yeast cells also indicated that core protein 1, core protein 2 and the iron-sulfur protein of complex III were present in the inner mitochondrial membrane of the heme-deficient yeast cells. Hence, the absence of heme does not appear to affect the biosythesis, translocation and processing of the precursors to core protein 1, core protein 2 and the Rieske's iron-sulfur protein of complex III in mitochondria of yeast cells.

Van Loon et al. (56) recently reported that, relative to the mRNA levels measured in wild type yeast cells grown in 2.5 % lactate, the translatable mRNA levels coding for both core proteins 1 and 2 were reduced roughly 4-6 fold in cells grown in 1 % glucose and reduced 6-7 fold in cells grown in 2 % galactose or under anaerobiosis. Hence, they concluded that the effect of anaerobiosis and glucose repression was not significantly more effective than either condition alone in reducing the transcription of core protein 1 and core protein 2 of yeast mitochondrial complex III. Anaerobically grown cells and heme mutant cells are both devoid of intracelluar heme (135). Wild-type cells and heme mutant cells in this thesis were grown in growth media containing 2 % glucose as carbon source. As discussed in chapter 1 of this thesis, it is known that the biosythetic pathway of heme is affected in cells grown in glucose (136). Therefore, the present result of the similar levels of core protein 1 and core protein 2 in heme-sufficient and heme-deficient yeast mutant cells agrees with Van Loon's observation on the effects of

anaerobiosis and glucose repression on the mRNA levels of these two proteins in the wild-type yeast cells.

(b). The Role of Heme in the Biosynthesis of Apocytochrome b of Complex III in Yeast Cells

The presence of apocytochrome b in the mitochondrially synthesized products of heme-deficient mutant cells had been previously demonstrated by Clejan et al. (148) from this laboratory using antiserum against apocytochrome b. The SDS-polyacrylamide gel pattern of protease digested apocytochrome b of heme-deficient yeast mutant (GL1) cells and wild-type (2180) cells were reported to be identical. In the present study, roughly equal amounts of apocytochrome b was also detected in the mitochondria from heme-sufficient and heme-deficient yeast mutant cells.

Previous studies on GL1 heme-deficient mutant cells have demonstrated that the synthesis of most of the mitochondrial translational products of yeast cells are not affected appreciably by the absence of heme (134,148). However, the assembly of these proteins such as subunits I, II and III of the cytochrome oxidase in this yeast strain was found to be different than that in the wild type cells (134).

(c). The Role of Heme in the Biosynthesis and Processing of Apocytochrome c_1 of Complex III in Yeast Cells

Ohashi (84) et al. recently demonstrated that the precursor to apocytochrome c_1 is synthesized and processed to an intermediate form in the heme-deficient GL1 cells. With the antiserum agaisnt complex III and both the immuno transfer and immuno precipitation technique in this study, proteins with the apparent molecular weights corresponding the the putative intermediate form of apocytochrome c_1 in heme-defi-

cient mutant cells and the mature form of apocytochrome c_1 in wild-type cells and heme-sufficient yeast mutant cells were also observed. Therefore, the present result support the previous observation that heme is necessary for the second proteolytic processing step of this protein. Since the second proteolytic processing step of the Rieske's iron-sulfur protein did not depend on the presence of heme in the present study, a different interaction of heme could be involved with the precursor to apocytochrome c_1 in the processing pathway of this protein than that of the precursor of iron-sulfur protein. More studies on the gene level and molecular level are necessary to investigate the precise role of heme on the precursor processing and assembly pathways of these complex III subunits.

(d). The Role of Heme in the Biosynthesis of 17 kDa, 14 kDa and 11 kDa Subunits of Complex III in Yeast cells

With the the antiserum against complex III, visible amounts of the three small subunits of complex III in the mitochondria of heme-deficient mutant cells were not detected with the immuno transfer technique used in this study. However, with the immuno precipitation technique, this antiserum was able to immuno precipitate subunits of apparent molecular weights of 17, 14 and 11 kDa from 1 % Triton solubilized mitoplasts of wild-type yeast cells and heme-deficient yeast mutant cells. Differences in these results could be explained by the loss of these small subunits due to their faster migration rates in the electrophoretic transfer process of the immuno transfer experiments (168,176).

Among the three small subunits of complex III, only the 17 kDa subunit is synthesized as a larger precursor of 26 kDa in the yeast cells. The 14 kDa and 11 kDa subunits are synthesized without additional amino-

terminal extensions (55). Recently, the nuclear genes coding for these three small subunits of complex III were cloned and studied in Grivell's laboratory (57). The most notable feature in the amino acid sequence derived from the cloned gene of the 17 kDa subunit is in its high content of acidic amino acid residues (52 %). De Haan et al. (182) recently reported that the predicted protein from the nuclear gene coding for the 14 kDa subunit of complex III is hydrophilic. The cloned gene codes for a polypeptide of 14,561 Da. The membrane targetting element of this protein has not been revealed in this precursor protein. The fact that all three small subunits of the complex III are made in the absence of heme in yeast cells is a good example of coordinated gene regulation of eukaryotic cells.

The presence of all three small subunits in the mitoplasts of yeast cells in the absence of heme in this study suggest that heme does not affect the integration and processing of the precursors to these three small subunits of complex III. However, the amounts of these proteins in the heme-deficient mutant cells could not be determined from results of this study since the mitoplasts were solubilized with detergent before immunoprecipitation.

Steady state levels of the 14 kDa and 11 kDa subunits of complex III were reported to be severely reduced in box mutant yeast cells (57). The biosynthesis and association of these two subunits with the complex was hence suggested to be mediated by the interaction of cytochrome b. In contrast to box mutant cells, heme mutant cells are able to synthesize apocytochrome b (148). However, heme mutant cells and box mutant cells are both incapable of forming the electron transfer chain. Studies on the gene level will be helpful in establishing the effects of

heme in the transcription and translation of all complex III protein subunits.

(2). The Role of Heme in the Formation and Integration of Iron-Sulfur Clusters in the Respiratory Chain in Yeast Cells

The lack of detectable EPR signals due to S-1, S-2, S-3 and the Rieske's iron-sulfur clusters of the respiratory chain in the mitochondria of heme-deficient GL1 yeast mutant cells in this study raises interesting questions on the biogenesis of these iron-sulfur clusters and on their integration in the respiratory chain. The absence of EPR signals due to the respiratory chain iron-sulfur clusters could result from either their incorrect assembly with other components of the respiratory chain or their incapability of forming the iron-sulfur clusters in heme-deficient yeast mutant cells. Possible roles of heme on the biogenesis and integration of mitochondrial iron-sulfur proteins and iron-sulfur clusters of the yeast are discussed below:

(a). The Effects of Heme on the Assembly of the Iron-sulfur Clusters in the Mitochondria of Heme-deficient Yeast Mutant Cells

The apoproteins of the complex II and most subunits of complex III including the Rieske's iron-sulfur protein were immunodetected in heme-deficient yeast cells in this study. The chymotrypsin sensitivities of most of the complex III subunits were also demonstrated to be similar in wild type and in heme-deficient mutant yeast cells. The chymotrypsin sensitivity of the Rieske's iron-sulfur protein, however, differs from that of the wild type cells. This difference indicates that the Rieske's protein may be integrated to the membrane in a different manner in the heme-deficient mutant cells compared to wild-type cells. When reducing agents such as ascorbate or dithionite were added to the mitochondria of the heme-deficient cells, the iron-sulfur clusters might not be reduced due to the improper assembly in mutant cells.

(b). Possible Effects of Heme in the Transport and Reduction of Iron in Yeast Cells

Ferric iron atoms have to be transported and reduced to ferrous irons before integration in heme-containing enzymes and iron-sulfur clusters of all cells (183). Therefore, the absence of the iron-sulfur clusters of the respiratory chain in heme-deficient mutant yeast cells could also have resulted from a deficiency in iron transport or reduction of the ferric irons in these heme-deficient mutant yeast cells.

Several hundreds of different iron binding proteins, or 'siderophores' have been reported in the literature (184). The general feature of these proteins are their cage-like structures for chelating ferric irons. Recently, Bindereif and Nieland (185) were able to analyze the molecular biology of one elaborate iron transport system in E. coli through studies with cloned genes and plasmids. The structural genes coding for various subunits of one siderophore and its protein receptor were found to be linked in an operon and were preceded by genes coding for repressor, promotor and operator genes of the operon. The transport of iron has not been investigated in yeast cells as in liver cells or in bacteria. It is conceivable that the absence of heme in yeast cells can lead to an impaired iron transport system for the formation of all iron-containing enzymes of the cell.

Subsequent to import of the ferric iron, a substantial membrane potential is necessary to reduce the ferric iron to the ferrous state. The membrane potential of the mitochondria of heme-deficient yeast mutant cells should be different from that of the wild-type cells since a fuctional mitochondrial respiratory chain does not exist in the absence of heme in yeast cells. The observation by Ohnishi et al. (158) that

S-1, S-2 and S-3 of the complex II were reduced in amount and the Rieske's iron-sulfur protein of complex III was not present in box mutant cells also is indicative that a functional electron transport chain in yeast cells is necessary for the formation and integration of various iron-sulfur clusters into the inner mitochondrial membrane of yeast cells.

(c). Possible Effects of Heme in the Generation of Sulfides in Yeast Cells.

Sulfates and sulfites transported into the cell have to be reduced into sulfides before their integration into the iron-sulfur clusters. The last step in this enzymatic pathway of sulfide formation involves a heme-containing enzyme sulfite reductase (186). The GL1 heme mutants have been reported to be deficient in the formation of sulfides containing amino acids such as methionine (145). Hence, the absence of iron-sulfur clusters in the heme-deficient mutant cells could also have resulted from the deficiency in the formation of sulfides.

(3). A Summary of the Effects of Heme in the Biosynthesis of Hemoproteins and Mitochondrial Respiratory Chain Proteins Using Heme-Deficient Yeast Mutant Cells

Since the isolation of the heme-deficient yeast mutant strains ten years ago researchers in several laboratories around the world have devoted their research efforts to studies of hemoproteins and the biogenesis of mitochondrial respiratory chain proteins using these yeast mutant cells. From such studies, several different and intriguing roles of heme on the biosynthesis and assembly of several hemoproteins and respiratory chain proteins have recently been revealed. Fascinating information on the role of heme on the regulation of several genes of S. cerevisiae has been resulted from these studies.

(a). The Role of Heme in the Biosynthesis of Yeast Cytochrome c

The yeast S. cerevisiae contains two distinct mitochondrial c-type cytochromes; a major form of iso-1-cytochrome c and a minor form of iso-2-cytochrome c. The CYC1 and CYC7 genes encode, respectively, iso-1-cytochrome c and iso-2-cytochrome c (187,188). In yeast cells, the CYC1 gene has been previously shown to be regulated at the transcriptional level by catabolite repression, oxygen and heme, while the CYC7 gene does not appear to be regulated by these physiological regulators (189).

Guarante and Mason reported that the expression of the CYC1 gene is tightly regulated by the levels of intracellular heme (190). In their studies, the expression of the CYC1 gene was examined by the effects of anaerobiosis and HEM1 mutation on β-galactosidase activity in yeast cells harboring the CYC1-lacZ fused gene (191). The fused gene contained only the first codon (ATG) of CYC1 coding DNA and the bacterial lac z gene

coding for the -galactosidase. A change in β-galactosidase activity in response to heme deficiency hence suggests a role for heme in the control of CYC1 expression at the level of transcription since the host yeast cells do not have endogenous β-galactosidase (192). Guarante and Mason (190) reported that the β-galactosidase levels directed by such plasmids were reduced at least 100-fold to 500-fold by anaerobic growth in different wild type cells and reduced more than 800-fold by HEM1 mutation in yeast cells. Furthermore, the heme regulatory sites in the CYC1 promoter region are found to be in two upstream activation sites, at about -275 (UAS_1) and -225 (UAS_2) base pairs upstream from the region of transcriptional initiation.

Guarante et al. further characterized the UAS sites of the CYC1 gene and investigated the relation of catabolite repression on these sites (193,194). Each of the UAS site, when placed upstream of the transcriptional initiation region of the yeast LEU2 gene, also activates LEU2 trancription which is regulated by catabolite repression. UAS1 but not UAS2 are found to be regulated distinctly at the molecular level. UAS1 is responsible for most of the transcription under glucose repressed condition, While UAS1 and UAS2 contribute equally to glucose derepressed conditions. UAS1 but not UAS2 is fully derepressed in glucose by increasing the levels of intracellular heme. Transacting regulatory mutations, hap-1 (heme-activation protein) and hap-2, selectively abolish the activity of UAS1 and UAS2. Furthermore, HAP1 of the UAS region appeared to encode a protein that mediates catabolite repression of UAS1 by responding to intracellular heme levels. A mutation in the locus HAP1 abolished UAS1 activity and renders the site uninducible by the heme analogues.

(b). The Role of Heme in the Biosynthesis of Cytosolic Catalase A and Catalase T in Yeast Cells

Catalase is present in almost all prokaryotic and eukaryotic cells. The yeast S. cerevisiae contains three catalase proteins. Two of these catalases, catalase A and catalase T (194,195) are tetrameric hemoproteins that differ in molecular weight and spectral properties. The prosthetic group, protoheme, is noncovalently bound to each of the subunit of catalase A and catalase T. The catalase T was reported to be a cytosolic enzyme while the catalse A is a vacuolar enzyme in yeast cells. The third minor form of catalase is a protein containing nonheme iron and RNA.

The activities of these catalases are not detectable in anaerobically grown yeast cells (130). During reoxygenation of the glucose grown yeast cells, the catalase T is reported to form via a heme-less and heme-containing precursor (196). Woloszczuk et al. (197) reported that both apoproteins of catalases A and catalase T could not be immuno detected in heme-deficient GL1 yeast mutant cells. Control by heme of the biosynthesis of catalase A and T was reported to be at the level of apoprotein synthesis rather than increased proteolytic degradation of the apoproteins.

The regulation by heme was reported to be mainly at the transcriptional level, since the mRNA for catalases A and T were not detected in the heme-deficient GL1 cells (198). In addition, the presence or absence of heme also affected the in vivo synthesis of these two hemoproteines (199). Recently, Hamilton et al. (200) reported that the effect of heme was also on the translational level since the mRNA coding for the catalases could not be effectively translated in the protein

synthesizing system prepared from heme-deficient yeast mutant cells. However, when total mRNA was added to such mRNA-dependent systems prepared from both heme-sufficient and heme-deficient yeast cells, both systems translate most mRNAs with comparable efficiencies. Addition of 10 uM hemin to the system from heme-deficient cells stimulates translation of catalase mRNAs significantly.

In conclusion, the gene expression of the catalases A and T has been shown to be regulated by control not only of the concentration of mRNAs but also by the rate of their their translation. Further studies are clearly necessary to clarify the mechanism of such dual control mechanism of catalase genes. In the case of Drosophila heat shock protein, a similar pattern of contol on gene expression has also been previously reported (201). Hence such modes of gene expression might be a fairly common phenomenon in providing more regulatory flexibility to different cells.

(c). The Role of Heme on the Biosynthesis of Mitochondrial Apocytochrome b_2 and Cytochrome c Peroxidase in Yeast Cells

The yeast intermitochondrial membrane space enzyme, Cytochrome b_2 and cytochrome c peroxidase are encoded by the nuclear genes and synthesized as larger precursors in the cytosol (202). The precursors to these two proteins, are processed in two steps. Similar to the maturation of apocytochrome c_1 (84), a matrix-localized soluble protease (203) first cleaved the precursors to apocytochrome b_2 to an intermediate form after their transfer across the inner mitochondrial membrane (204). In contrast to apocytochrome c_1, however, the second proteolytic processing step of apocytochrome b_2 and cytochrome peroxidase does not depend on the presence of heme in heme-deficient yeast mutant cells.

(4). The Orientation of Complex III in Wild-type Yeast Mitochondrial Membrane

The opposite orientation of mitoplasts and submitochondrial particles in DABS labeling experiments performed in this thesis was obtained from several kinds of studies; (i) Different labeling patterns of proteins by [^{125}I]DABS was observed in this study. (ii). Different steady state reduction of cytochrome b and c_1 by the addition of succinate or coenzyme Q analogues and different effects of trypsin and chymotrypsin digestion on succinate dehydrogenase activityies in mitoplasts and submitochondrial particles was observed by other colleagues in this laboratory (159).

The localization of cytochrome c_1 and the iron-sulfur protein on the cytosolic side of the inner mitochondrial membrane is based on the labeling patterns observed with the [^{125}I]DABS in mitochondria and the lack of labeling of these proteins in submitochondrial particles. By contrast, core protein 1, core protein 2 and the apocytochrome b were labeled with [^{125}I]DABS in both mitochondria and submitochondrial particles indicating that these proteins span the inner mitochondrial membrane from both surfaces. The results of the [^{125}I]DABS labeling experiment performed in this laboratory (159,205) using the antiserum against apocytochrome b also indicates that the apocytochrome b spans the inner mitochondrial membrane.

A model for the topography of complex III in the inner mitochondrial membrane consistent with the results obtained from [^{125}I]DABS labeling ,enzymatic and immuno studies performed in this laboratory is shown on Fig. (17). Previously, a similar localization of complex III subunits of beef heart mitochondria based on

cross linking studies, and surface labeling studies was reported by Bell et al. (205). In their study, however, core protein 1 appeared less accessible to the hydrophilic labeling reagent in submitochondrial particles than in this study. In addition, the intensive investigation of topography of complex III carried out with the complex from N. crassa by Weiss and his coworkers (109,206) using proteolysis and electron microscopy techniques also yielded a similar topography of complex III although the core proteins were concluded to be more exposed on the matrix side of the membane. Results from immuno studies on complex III from N. crassa reported by Nelson and his co-workers (116,117,207) supported these results on core proteins from this organism. Recently studies performed in this laboratory using protease digestion and immuno techniques further supported the topography of complex III as in Figure (21).

In 1983, Hauska et al. (48) compared and combined universal features of the different quinol cytochrome c oxidoreductase into one general, topographical scheme of respiratory and photosynthetic membranes. This scheme is reproduced in Fig. (21) of this thesis for comparison. Since the fuction and topography of the two core proteins are still not very clear, their scheme did not include the orientation of these two proteins. The model proposed by Beattie et al. as discussed in this thesis agrees generally with those obtained from other researchers from other organisms. It is interesting to note that the mitochondrial complex III and plant cytochrme b_6-f complex has such close resemblance in topography. The proposed model in Fig. (21) also supports the proposed 'Q cycle' and 'b cycle' mechanism, although not enough to differentiate either one from the other mechanism.

(5). The Role of Heme in the Integration and Assembly of Complex III Subunits in Yeast Cells

The absence of heme was found to have no effect on the biosynthesis of most of the complex III subunits in this thesis project. The topography of complex III in wild-type cells has been studied with various techniques as described and discussed in this thesis. Therefore, the last topic of this thesis project was the assembly of these complex III subunits in the inner mitochondrial membrane in heme-deficient mutant GL1 cells. Since the role of heme on the assembly of respiratory chain proteins or hemoproteins are still not understood at this stage, results from this study on the assembly of complex III in the absence of heme would increase our knowledge in this respect.

In this study, the assembly of the various complex III subunits in the inner mitochondrial membrane of the mitoplasts was studied by comparing the sensitivity of the individual subunit of complex III to proteolytic digestion with chymotrypsin in wild-type and in heme-deficient mutant cells. The results obtained from these comparisons suggest that with the possible exception of the iron-sulfur protein, the other subunits of complex III of mitoplasts of heme-deficient cells display similar patterns of chymotrypsin sensitivities as they do in the mitoplasts of wild-type cells.

The various subunits of complex III in mitoplasts of wild type cells and heme-deficient cells were sensitive to digestion by chymotrypsin as follows ; (a) Core protein 1 and core protein 2 were found to be rather chymotrypsin insensitive in mitoplasts of either wild-type or heme-deficient yeast mutant cells. (b) Apocytochrome b, plus the intermediate and mature forms of apocytochrome c_1 were found to be

be progressively more sensitive to digestion in the mitoplasts of wild-type cells, but were more resistant to digestion in the mitoplasts of heme-de- ficient mutant cells with increasing concentrations of chymotrypsin in the concentration range tested.

The results from the current study suggest that the absence of heme does not seem to affect the synthesis and integration of the two core proteins of complex III in heme-deficient yeast mutant cells. Since core proteins of complex III neither contain nor were known to bind prosthetic groups such as heme and iron-sulfur clusters, the integration and assem- bly of these two core proteins probably should be least affected among complex III subunits.

The similiar sensitivity of apocytochrome b to chymotrypsin in wild type cells and in heme-deficient cells in this study suggests that, in the absence of heme, this protein is integrated into the inner mitochondrial membrane with a portion of the protein protruding from the cytosolic side of the membrane. Recently, the amino acid sequence of apocytochrome b from several species was compared by Widger et al. (208) and a model for the insertion of this protein in the membrane was also proposed. The polypeptide chain of apocytochrome b was predicted to span the inner mitochondrial membrane several times with several segments of the polypeptide chain exposed on both sides of the membrane. Previous results from our laboratory also indicated that the heme b group of apocytochrome b in the wild type cells was buried in the inner mitochondrial membrane and hence chymotrypsin insensitive (50). The absence of the prosthetic group (or ligand) heme b, however, does not seem to affect the integration of apocytochrome b in the inner mitochondrial membrane.

Concluding Remarks

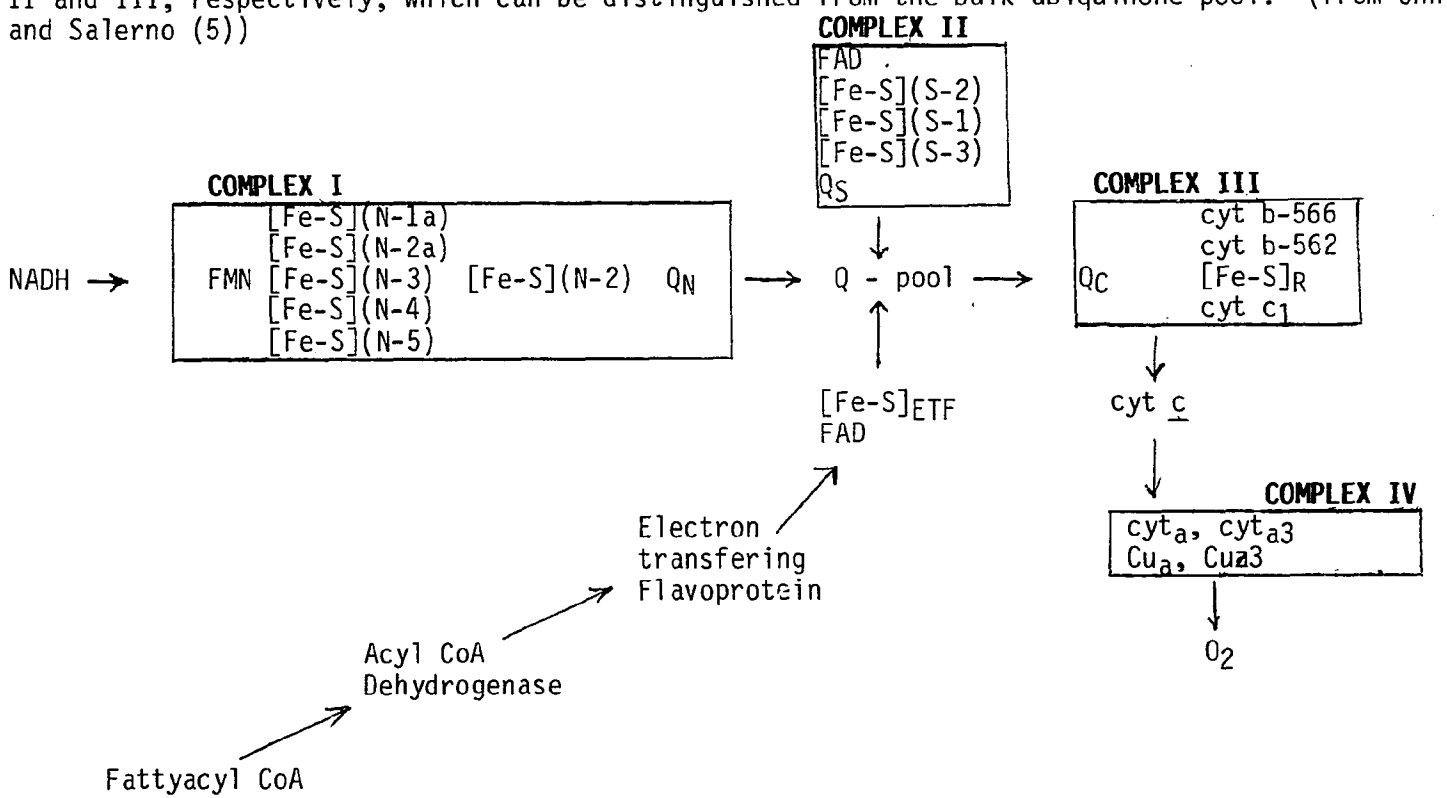
The role of heme in the biogenesis of hemoproteins and respiratory chain proteins has recently attracted much research interest in this and many other laboratories. Several ALA-synthase defective yeast mutant strains have been used in these biogenesis studies. Results from these studies indicate that the absence of heme has very diverse effects on the biosynthesis and assembly of different hemoproteins and respiratory chain proteins.

Results from my thesis work can be summarized as follows: (a) The biosynthesis and processing of all complex III subunits is not affected in the absence of heme except the apocytochrome C_1. The precursor of apocytochrome c_1 can only be processed to the intermediate form in the absence of heme. (b). The assembly of complex III in wild-type cells deduced from DABS labeling experiments indicate that core protein 1, core protein 2 and apocytochrome b have a transmembrane orientation; while apocytochrome c_1 and the Rieske's iron-sulfur protein are exposed to the cytosolic surface of the inner mitochondrial membrane. These results agree with data obtained by researchers from other laboratories using different experimental approaches. (3). The EPR signals due to S-1, S-2 and S-3 of the complex II and the Rieske's iron-sulfur cluster signal of complex III are missing in the mitochondria of heme-deficient yeast mutant cells. Hence, heme controls the integration or formation of the iron-sulfur clusters in the mitochondria of yeast cells. (4). The orientation of most of the complex III subunits of heme-deficient mutant cells in the inner mitochondrial membrane was investigated using chymotrypsin digestion reactions. Core protein 1, core protein 2, apocytochromes b and c_1 were found to have similar

orientation in the heme-deficient yeast cells as they are in the wild-type cells. However, the Rieske's iron-sulfur protein was found to be less exposed on the cytosolic surface of the inner mitochondrial membrane.

Recently, heme was found to govern the transcription of iso-1-cytochrome c, catalase A and catalase T and possibly two subunits of the mitochondrial complex IV in yeast cells. The results of this thesis on the role of heme on the biogenesis and assembly of mitochondrial complex III suggest that heme plays many different roles in the biogenesis of hemoproteins.

Fig (1) Respiratory Chain Redox Components Present in the Inner Mitochondrial Membrane. Iron sulfur clusters associated with the mitochondrial respiratory chain of eukaryotic cells are depicted in the scheme below. Fe-S clusters associated with the complex I and II are designated with suffixes N-x and S-x, respectively. Q_S, Q_N and Q_C are protein associated pools of ubiquinone in complex I, II and III, respectively, which can be distinguished from the bulk ubiquinone pool. (from Ohnishi and Salerno (5))



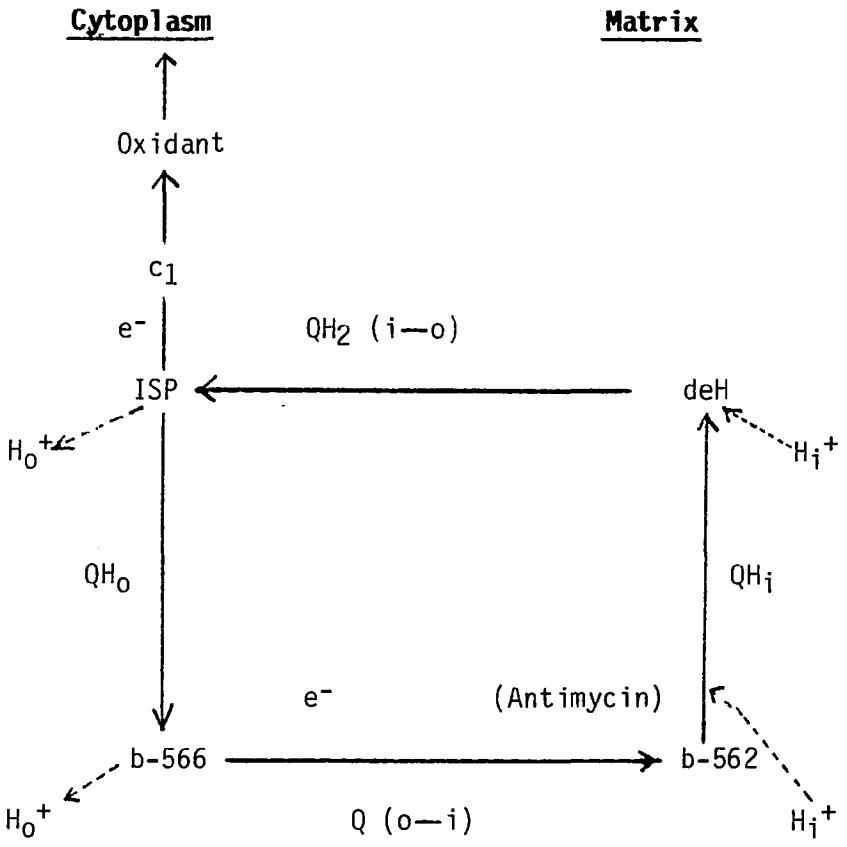


Fig. (2) A Protonmotive Q Cycle Mechanism for Electron Transfer Through the Complex III. Iron-sulfur protein (ISP) designates the iron-sulfur protin of the complex III and deH refers to an iron-sulfur cluster of complex II or complex I. (from Trumpower(87))

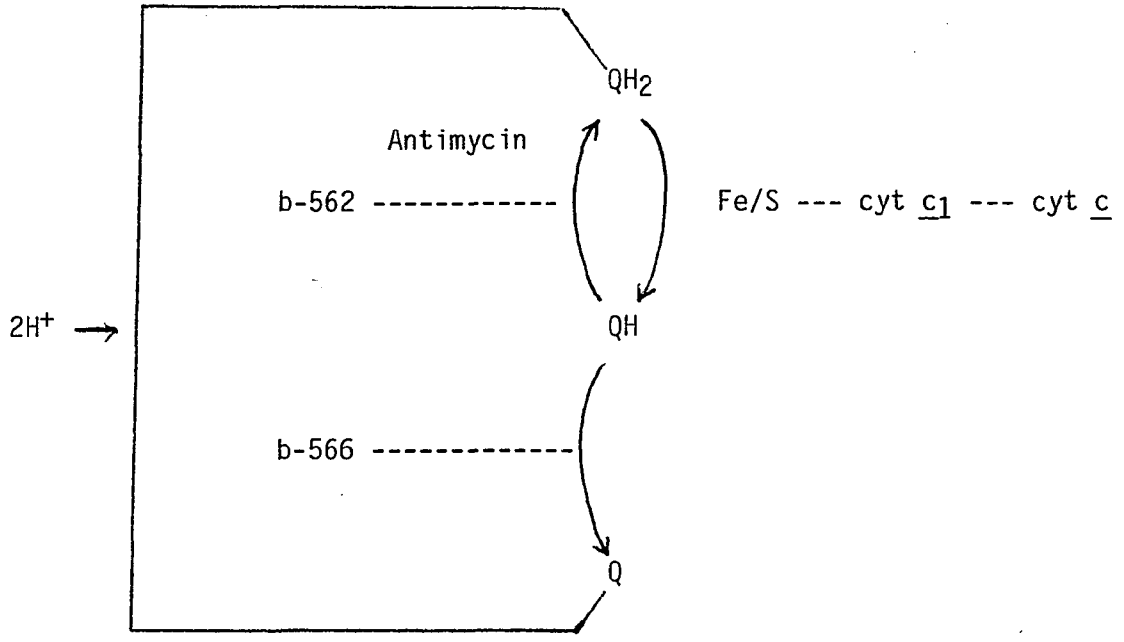


Fig. (3) Proposed Scheme for Electron Transport in Mitochondria Complex III. Q, QH and QH_2 represent the quinone, semiquinone and hydro-quinone states respectively, of a protein bound species of ubiquinone. Fe/S, Rieske's iron-sulfur cluster; $2H^+$ depicts input of hydrogen from the bulk pool of ubiquinone 10. (from Wilkstrom and Krab (105))

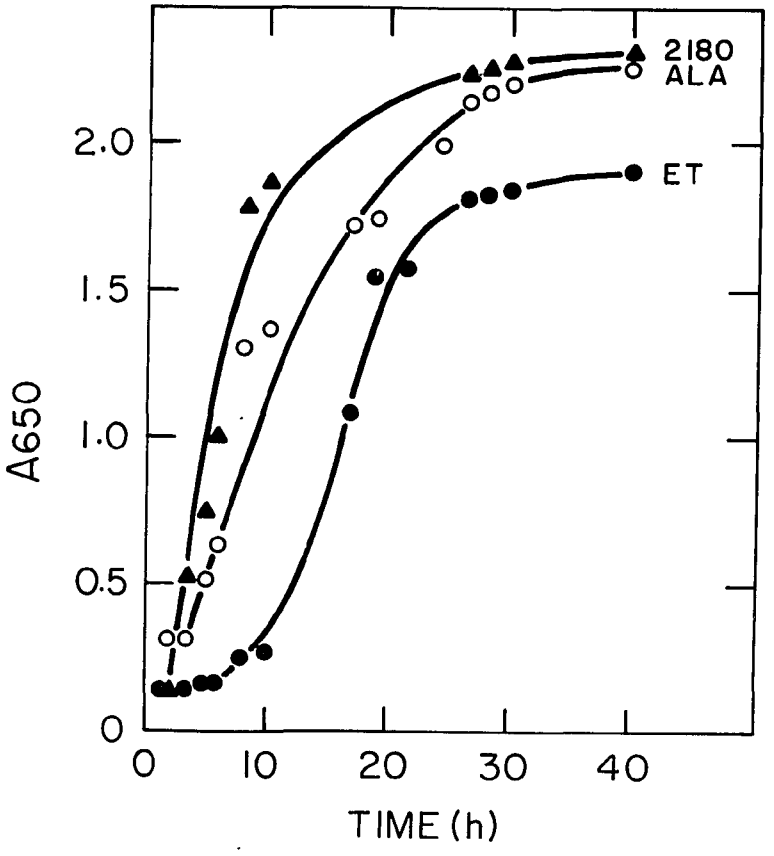


Fig. (4). Growth Curves of Wild-type Yeast cells, Heme-sufficient and Heme-deficient Yeast Mutant Cells. Yeast mutant strain GL1 was grown in a semisynthetic medium supplemented with 5-aminolevulinic acid (o---o) or with ergosterol and Tween 80 (●---●). Wild-type strain 2180 (▲---▲) was grown in semisynthetic medium without supplements. Cultures (250ml) were grown at 30 C on a metabolic shaker. Aliquots of 3 ml were withdrawn at various times and the absorbance determined at 650 nm on a Gilford spectrophotometer model 240.

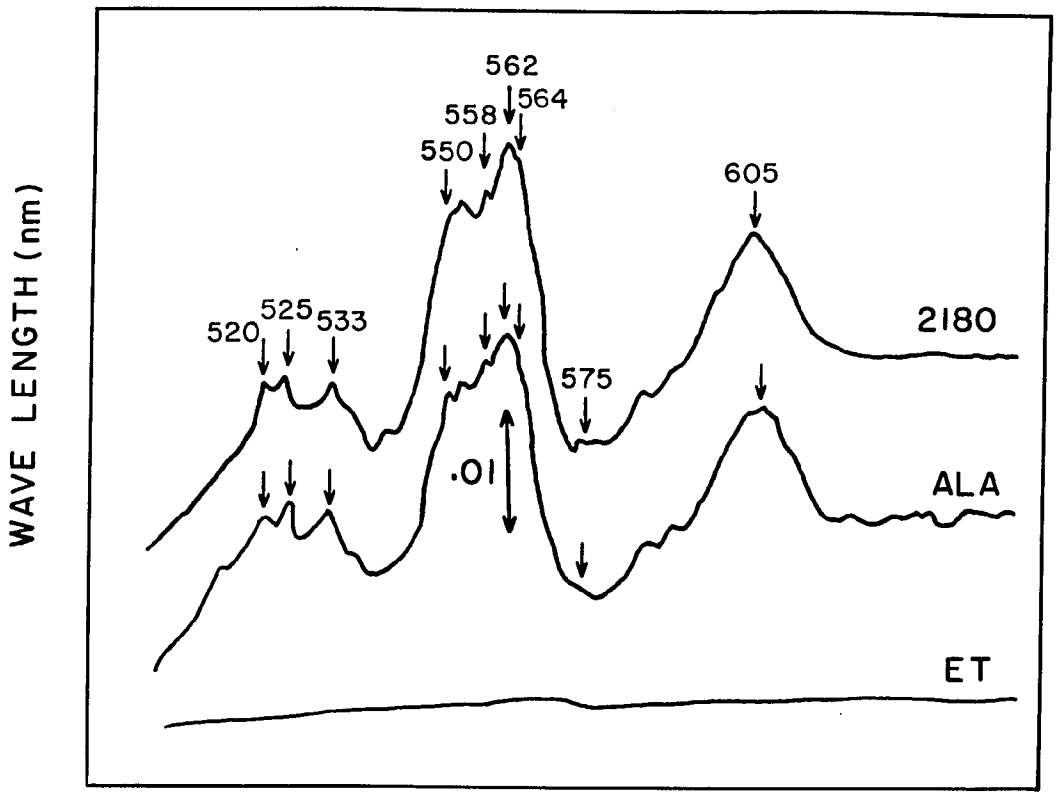


Fig. (5) Difference Spectra of Mitochondrial Cytochromes of Wild-type Yeast Cells, Heme-sufficient and Heme-deficient Yeast Mutant Cells. Difference spectra were obtained from dithionite reduced versus ferricyanide oxidized mitochondrial samples at room temperature in a Perkin-Elmer Model 557 spectrophotometer. Mitochondrial suspensions of wild-type cells (2180, 4.3 mg protein/ml), heme-sufficient GL1 mutant cells (ALA, 6.2 mg protein/ml) and heme-deficient GL1 mutant cells (ET, 4.0 mg protein/ml) were suspended in a sorbitol containing buffer described in Chapter II of this thesis. Numbers on top of the arrows in this figure denotes respective wavelengths of various peaks of absorbance.

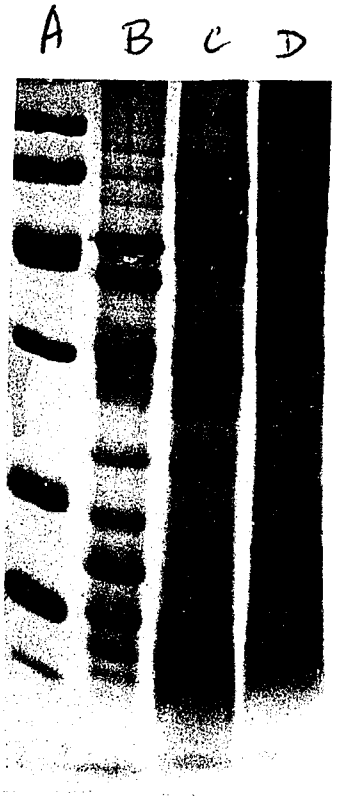


FIG. (6) SDS Polyacrylamide Gel Seperation of Total Mitochondrial Proteins of Wild-type and Heme-deficient Mutant Yeast Cells. Mitochondria were isolated from wild-type (2180) cells and heme-deficient yeast mutant (GL1) cells with the zymolyase procedure. Freshly prepared mitochondrial pellets were dissociated overnight in a buffer containing 5 % sodium dodecylsulfate, 2 mM EDTA, 5 % 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.8 and 10 % glycerol. Solubilized proteins were then seperated on a 12.5 % SDS polyacrylamide gel, stained and destained for visualization. Lane A: proteins of molecular weight standards. (94, 67, 43, 30, 20 and 14 KDa, respectively) Lane B; 10 ug protein of partially purified complex III of yeast isolated by Dr. Liviu Clejan. Lane C and D: 200 ug of mitochondrial proteins of heme-deficient mutant cells and wild-type cells respectively.

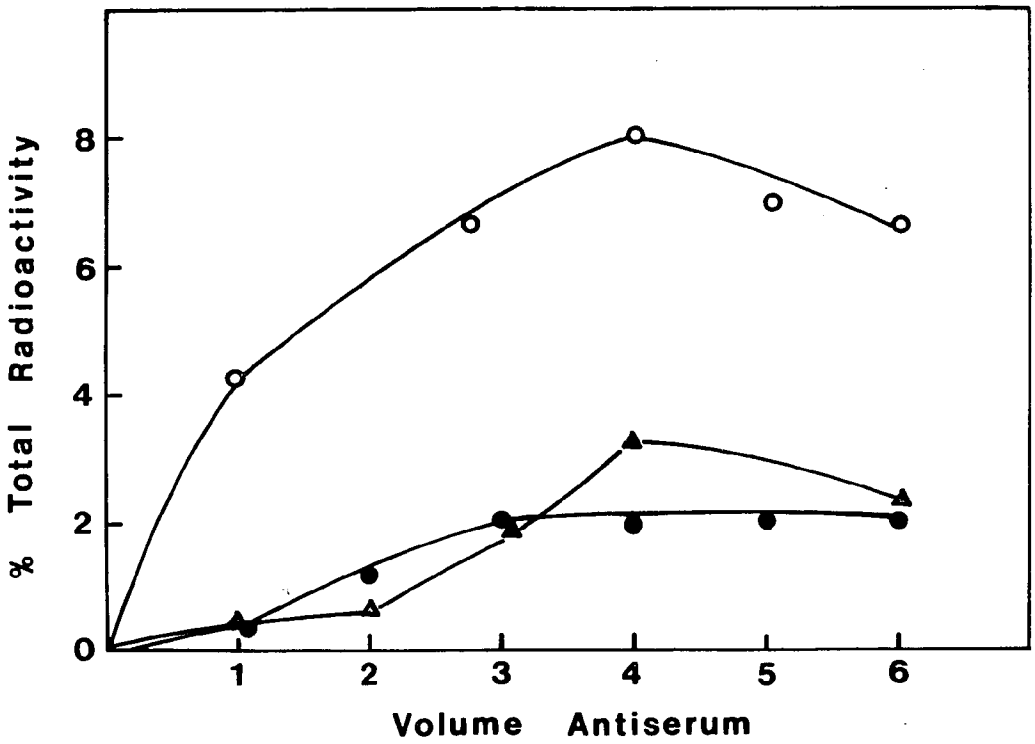


Fig. (7) Titration of Mitochondrial Extracts with Antiserum Against Purified Mitochondrial Complex III Mitochondrial pellets were isolated from heme-sufficient and heme-deficient yeast mutant (GL1) cells grown to mid log phase in appropriate medium containing 5 mCi/l [^{3}H]leucine and subsequently extracted with 1% Lubrol WX and 1% deoxycholate as described in Chapter II of this thesis. Increasing amounts of antiserum against complex III or preimmune serum were added to 10 ul aliquots of the mitochondrial extracts containing 10 mg protein/ml buffer. (o---o) and (▲---▲) denotes the radioactivity profiles of the immunoprecipitates of heme-sufficient and heme-deficient mitochondrial extracts with the antiserum against complex III. (●---●) denotes the radioactivity profile of the immunoprecipitates of the preimmune serum with the mitochondrial extracts of the heme-sufficient yeast mutant cells.

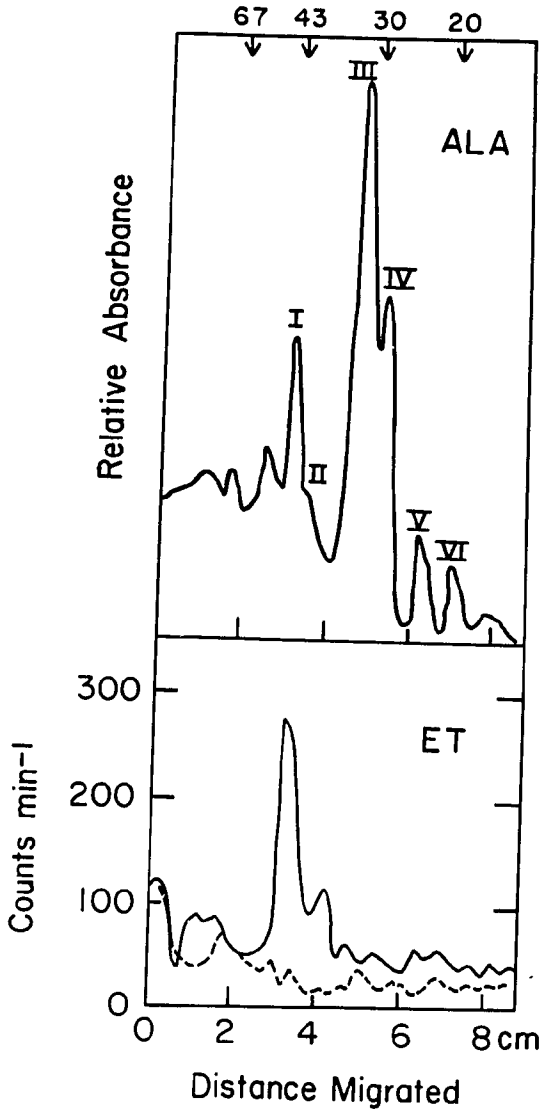


Fig. (8) Separation by SDS Polyacrylamide Gel Electrophoresis of Complex III Immunoprecipitated from Detergent Solubilized Mitochondria. Mitochondria were isolated from heme-sufficient (ALA) and heme-deficient (ET) mutant (GL1) yeast cells labeled with 1 uCi of [^{35}S]methionine per mg wet weight of cells under non-growing conditions and subsequently solubilized with 1 % Lubrol and 1 % deoxycholate. Immunoprecipitates of mitochondrial extracts with the antiserum against complex III were analyzed by seperation on a 12.5 % SDS-polyacrylamide gel. The gel lane containing immunoprecipitate was subjected to autoradiography and scanned in a Canalco microdensitometer as shown on penel A. The gel lane containing immunoprecipitate of heme-deficient mitochondrial extract was sliced into 1 mm gel slices and their radioactivities counted (panel B). (Dashed line is the immunoprecipitate of the preimmune serum).

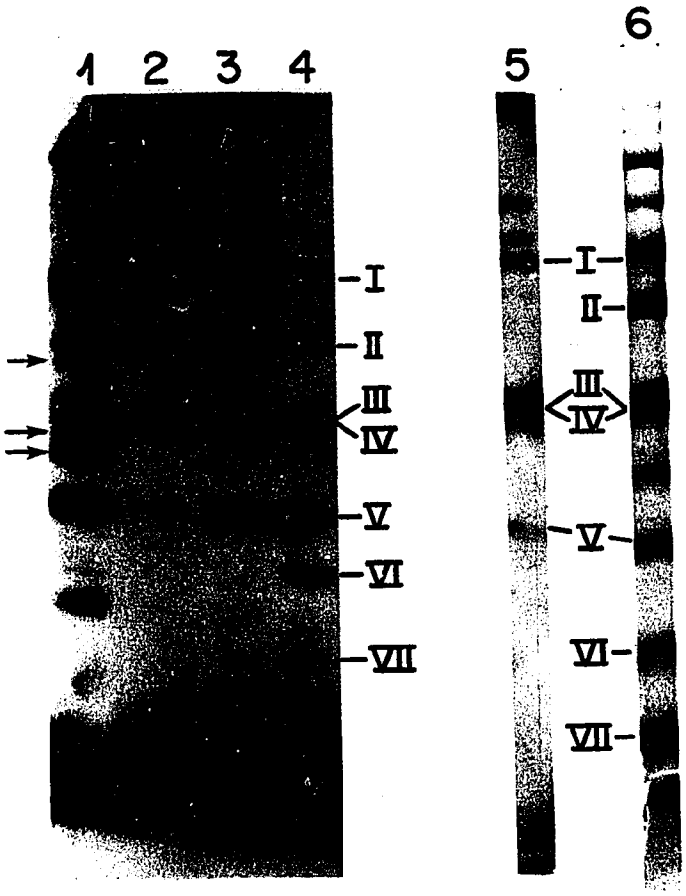


Fig. (9) Immunodetection of Complex III Subunits by the Radioimmunotransfer Technique. Mitochondria were prepared from wild-type (2180) yeast cells, heme-sufficient and heme-deficient yeast mutant cells grown to mid-log phase as described in Chapter II of this thesis. Mitochondrial proteins and complex III proteins (previously purified by Dr. Liviu Clejan in this laboratory) were seperated on 12.5% SDS polyacrylamide gels. Lanes 1 and 4 contain 100 ug of mitochondrial proteins from heme-deficient mutant cells and wild-type cells. Lanes 2 and 3 contain 50 ug and 100 ug of mitochondrial proteins from heme-sufficient yeast mutant cells. Lanes 5 and 6 contain 85 ug of purified complex III protins. The gel portion containing lane 6 was stained with Coomassie Blue and destained to serve as a standard. The gel portion containing lanes 1-4 and lane 5 were processed by the radio-immunotransfer technique with antiserum against complex III described on Chapter II of this thesis. The arrows on the left show the proteins with the apparent molecular weight of 34.5, 29 and 28 kDa, respectively. The numerals I-VII indicates the subunits in yeast mitochondrial complex III.

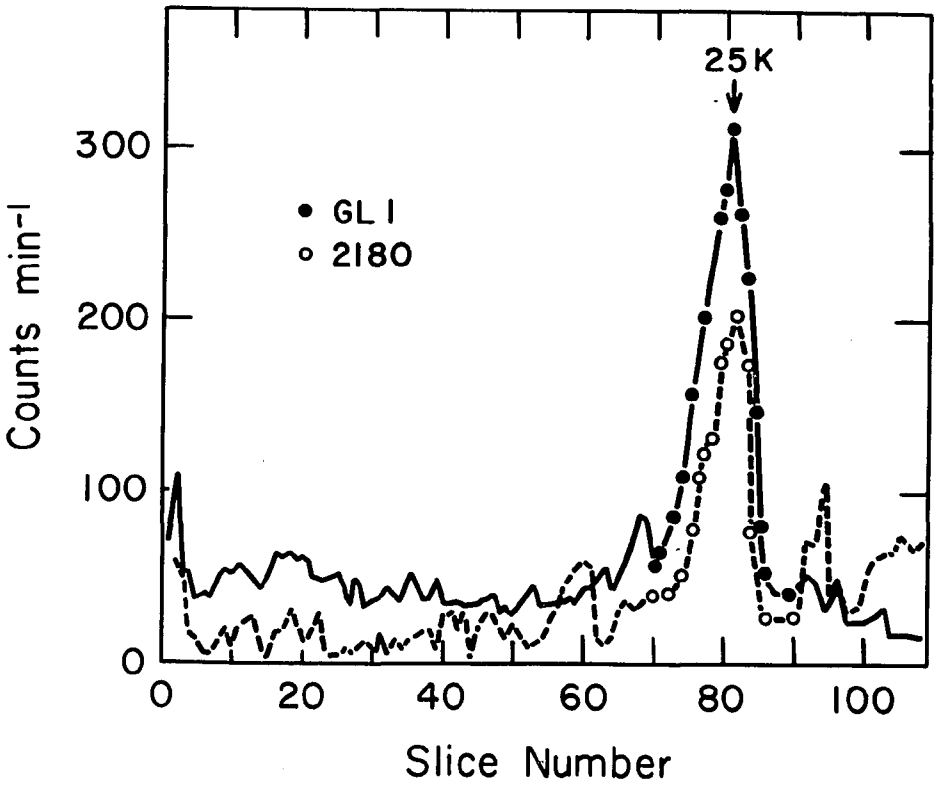


Fig. (10) Immunoprecipitation of the Rieske's Iron-sulfur Protein from Mitochondria of Wild-type and Heme-deficient Yeast Mutant Cells with Specific Antiserum Against Purified Iron-sulfur Protein. Wild-type (2180) and heme-deficient mutant (GL1) yeast cells were pulse labeled with [^{35}S]methionine. Mitochondria were prepared with the glassbeads breaking method and solubilized with 3% SDS and immunoprecipitated with specific antiserum against the Rieske's iron-sulfur protein previously prepared and characterized by Dr. Anita Sidhu of this laboratory. The immunoprecipitates were analyzed by electrophoresis on a 12.5% polyacrylamide gel. Gel lanes containing the immunoprecipitates were sliced into 1-mm slices and radioactivities counted. Immunoprecipitates obtained with mitochondria from wild type cells (o---o) and from heme-deficient mutant cells (•---•) were plotted on the same graph. The arrow indicates the position of the radioactivity peak at 25 kDa.

1 2

Fig. (11) Detection of the Rieske's Iron-sulfur Protein of Complex III in Mitochondria of Heme-deficient Yeast Mutant Cells by a Radioimmunotransfer Technique. Mitochondria of wild-type yeast (2180) cells and heme-deficient yeast mutant (GL1) cells were prepared and subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel as previously described in Chapter II of this thesis. Lane 1 and lane 2 each contains 100 ug of mitochondria from wild-type yeast cells and heme-deficient yeast mutant cells, respectively. 20 ul of antiserum against purified Rieske's iron-sulfur protein prepared previously by Dr. Anita Sidhu in this laboratory was used in each lane in the immunotransfer experiment. The numbers on the right refer to the molecular weight of the bands in kDa, calculated from molecular weight standards used in this experiment.

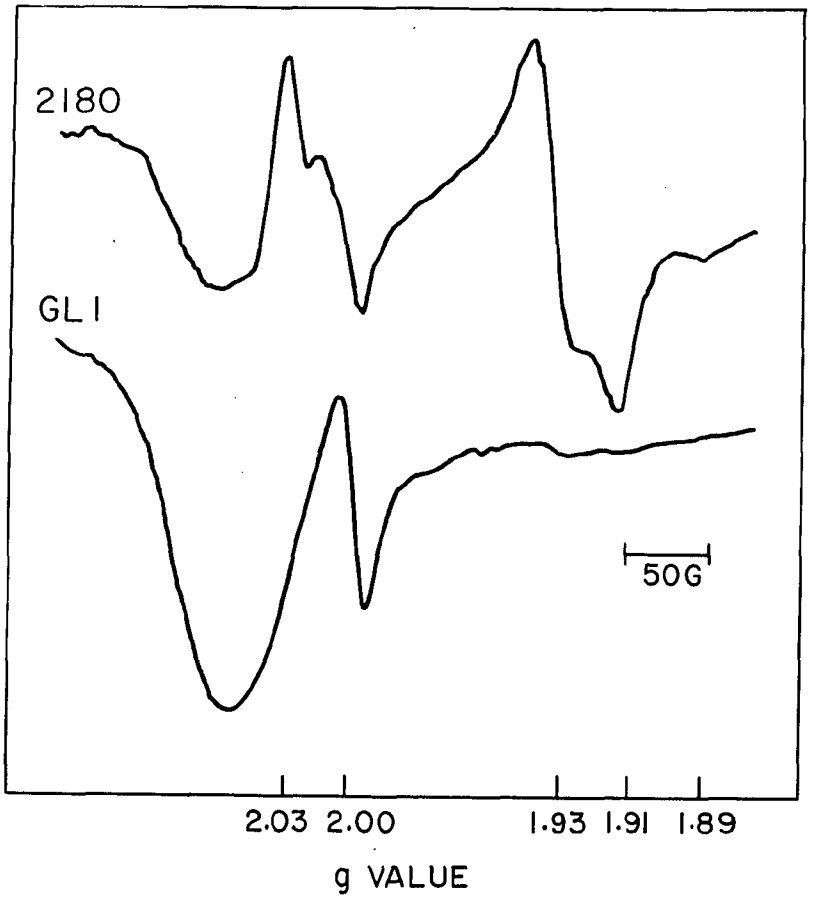


Fig. (12) Electron Paramagnetic Resonance Spectra of Iron-sulfur Clusters S-1 and S-2 of Mitochondrial Complex II at 15 K. Mitochondrial suspensions of wild-type yeast (2180) cells and heme-deficient mutant (GL1) yeast cells were treated with dithionite crystals before freezing in liquid helium for EPR measurement. Upper trace is of mitochondria (46.8 mg protein / ml) of wild-type cells and lower trace is of mitochondria (42.9 mg protein / ml) of heme-deficient yeast mutant cells.

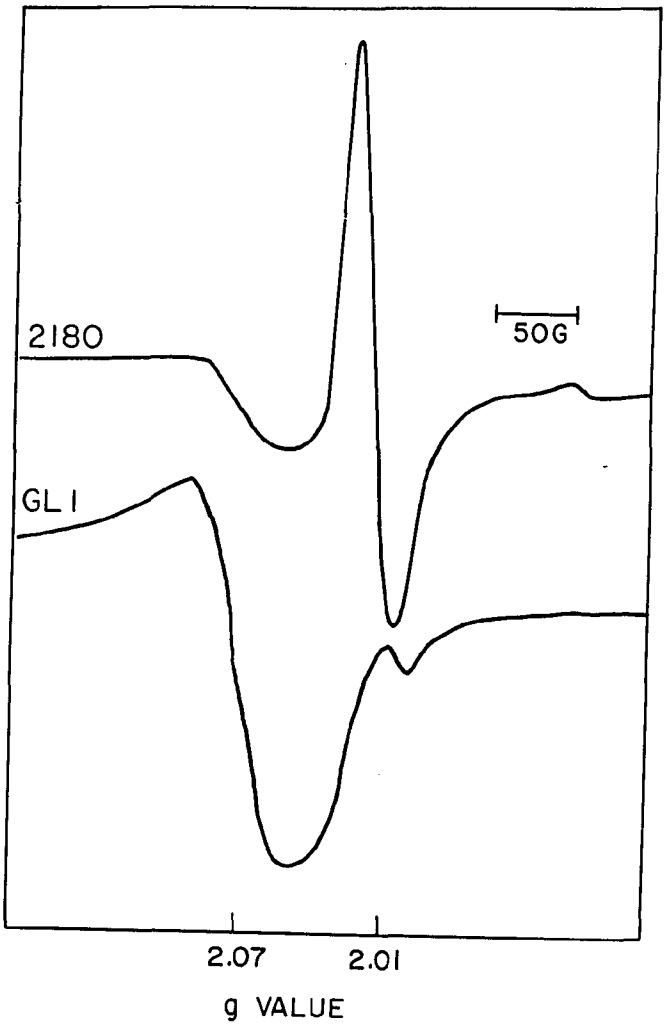


Fig. (13) Electron Paramagnetic Resonance Spectra of the Cluster S-3 of Mitochondrial Complex II at 9 K. Mitochondrial preparations of wild-type (2180) yeast cells and heme-deficient yeast mutant (GL1) cells were treated for 15 min at room temperature with 20 mM ascorbate before freezing in liquid helium for EPR measurement. Upper trace is of mitochondria (46.8 mg protein/ml) of wild-type yeast cells. Lower trace is of mitochondria (42.9 mg protein/ml) of heme-deficient yeast mutant cells.

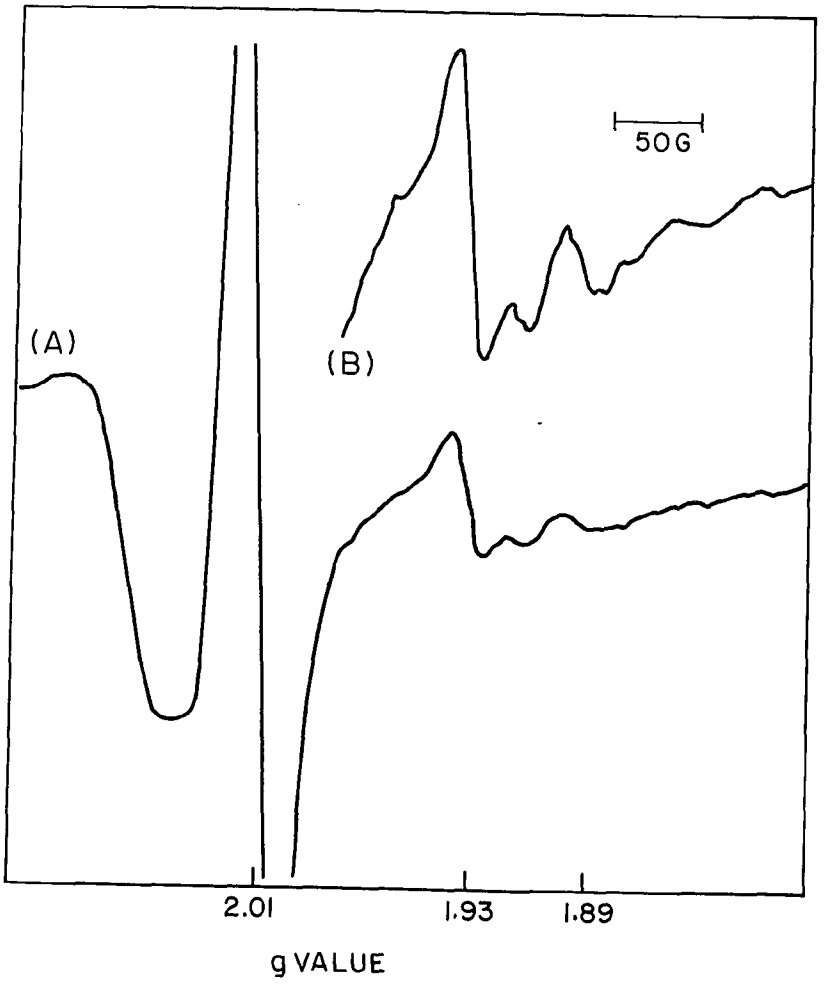


Fig. (14) Electron Paramagnetic Resonance Spectra of the Rieske's Iron-sulfur Cluster at 15K. Mitochondria of wild-type yeast (2180) and heme-deficient yeast mutant (GL1) cells were treated with several crystals of dithionite before they were frozen in liquid helium. Trace (A) is the complete EPR spectrum while trace (B) is the enlarged 2.5 times spectrum containing g_y and g_x of trace (A).

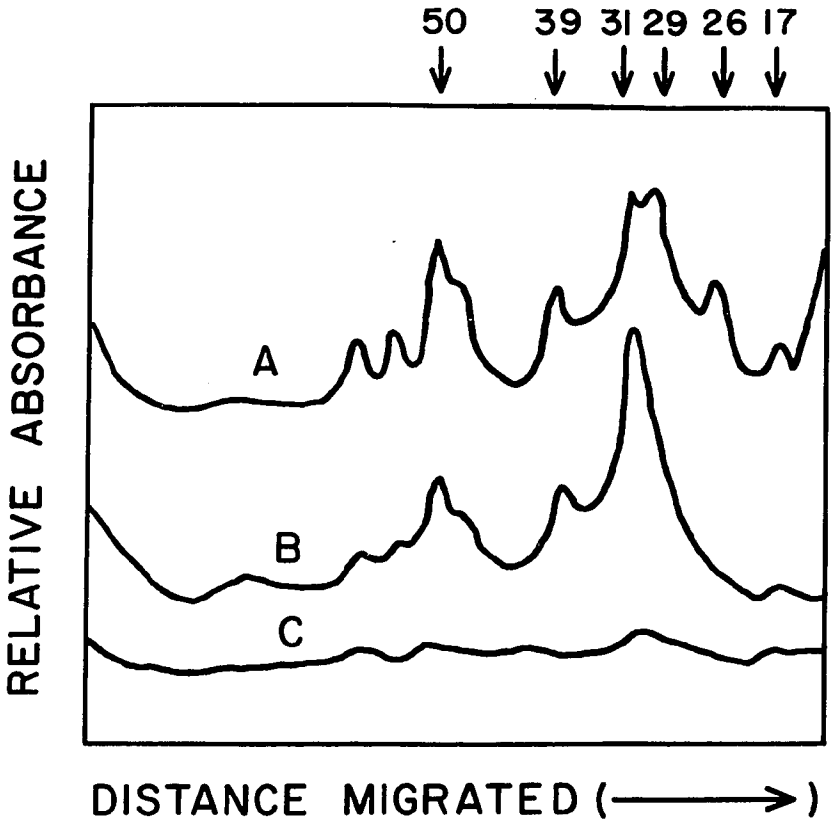


Fig. (15) Autoradiogram Scanning of SDS-polyacrylamide Gel Pattern of Immunoprecipitates Formed with Antibodies Against Complex III and Mitochondria or Submitochondrial Particles Labeled with [^{125}I]DABS
Mitochondria prepared from mid-log phase grown wild type yeast (KL14) cells were reacted with [^{125}I]DABS for 30 min at 4 C and solubilized with 1% deoxycholate and 1% Lubrol prior to immunoincubation. Submitochondrial particles of KL14 yeast cells labeled the same way with [^{125}I]DABS were solubilized with 1% Triton and 1M KCl. Immunoincubation and gel electrophoresis (12.5% SDS-polyacrylamide gel) were performed as described in Chapter II of this thesis. Lanes A and B: immunoprecipitates of antiserum against purified complex III with labeled mitochondria and submitochondrial particles, respectively. Lane C: immunoprecipitates of labeled mitochondria with preimmune serum.

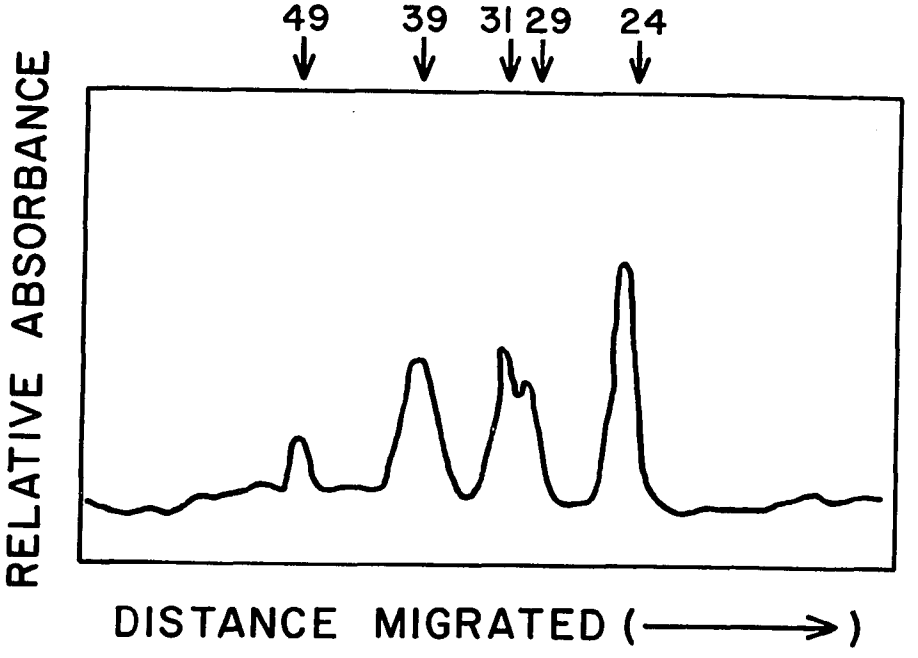


Fig. (16) Scan of Autoradiogram of SDS-polyacrylamide Gel Pattern of Immunoprecipitates Formed with Antiserm Against Complex III and Mitoplasts labeled with [^{125}I]DABS.Labeling and preparations for immunoprecipitation are as described in Chaper II and in the legend to Fig. (15) of this thesis. Numbers above the trace indicate the molecular weights of the labeled proteins calculated from the migration of standard molecular weight proteins on the same gel.

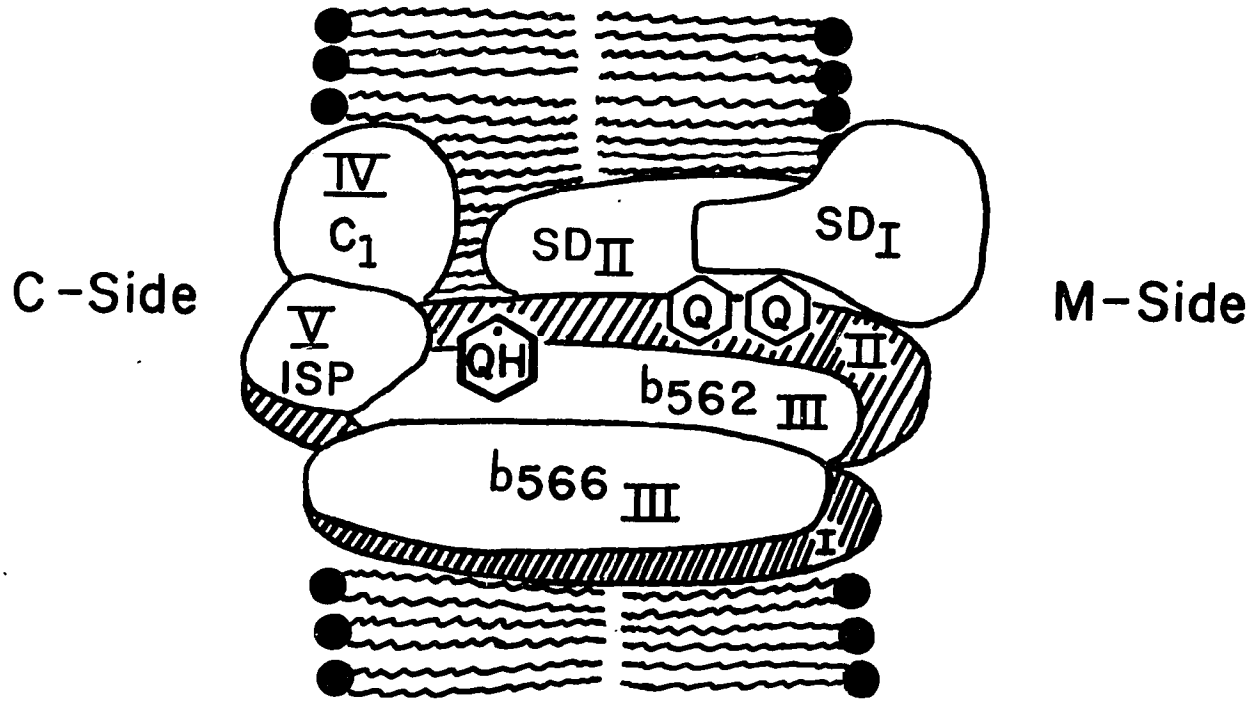


Fig. (17) The Topography of Complex III Subunits in the Inner Mitochondrial Membrane of Wild-Type Yeast Cells. Topography of complex III was investigated with the [^{125}I]DABS labeling techniques as described in this thesis and other enzyme activities studies of the complex III performed in this laboratory (159). A model of a possible arrangement of complex III subunits in the inner mitochondrial membrane of wild type yeast cells is presented here based on data obtained from these different studies.

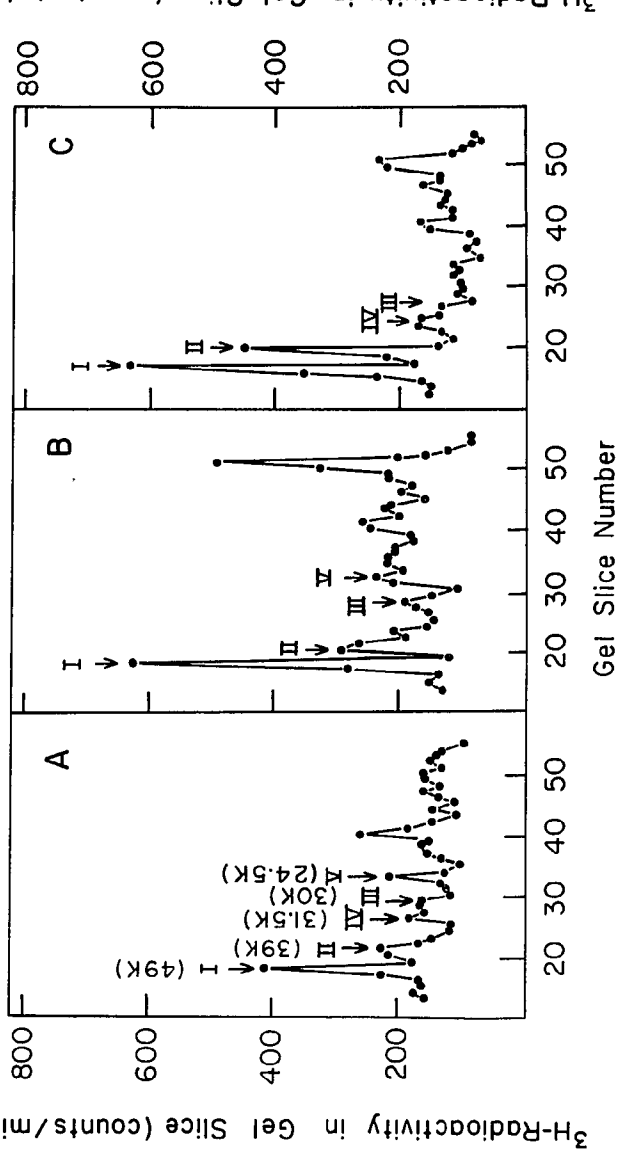


Fig. (18) Chymotrypsin Digestion of Wild-type Yeast Mitoplasts Prepared by the Digitonin Method. Mitochondria were prepared from [^{3}H]leucine labeled wild-type yeast cells by the zymolyase procedure. Mitoplasts were prepared from mitochondria by the digitonin method with 3 mg digitonin per mg of mitoplast protein. The washed mitoplasts were incubated at 30 C for 30 min in the absence of chymotrypsin (A) or in the presence of 400 ug (B) and 800 ug (C) of chymotrypsin per mg of mitoplast protein. After chymotrypsin digestion, the proteins were solubilized from the mitoplast membrane with 1% Triton X-100 for 1 h. Antiserum against complex III was immunoincubated with the Triton X-100 extracts of the control and chymotrypsin treated mitoplasts overnight at 4 C. The immunoprecipitates were absorbed on Sepharose bound protein A and washed by centrifugation. Proteins in immunoprecipitates were separated on a 10% SDS-polyacrylamide gel. Individual gel lanes were sliced into 2 mm slices and radioactivities counted and graphed.

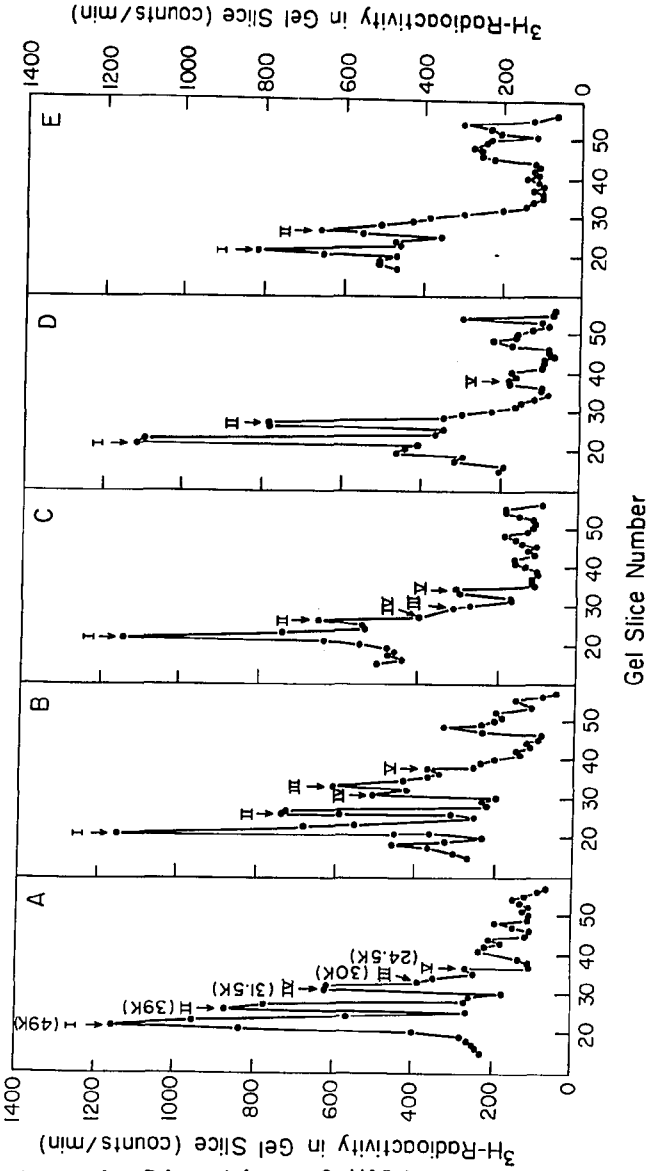


Fig. (19) Chymotrypsin Digestion of Wild-type Yeast Mitoplasts Prepared by the Osmotic Shock Method. Mitochondria were prepared from [^{3}H]leucine labeled wild type yeast (2180) cells by the the Zymolyase procedure. Mitoplasts were prepared by the osmotic shock method as described in Chapter II. The mitoplast suspension was incubated at 30 C for 30 min in the absence of chymotrypsin (A) or in the presence of 275 ug (B), 550 ug (C), 685 ug (D) and 822 ug (E) of chymotrypsin per mg of mitoplast protein. Immunoprecipitates of the antiserum against complex III and the Triton X-100 extracts of the control and the chymotrypsin digested mitoplasts were analyzed on a 10 % SDS polyacrylamide gel as described in the legend to Fig. (18).

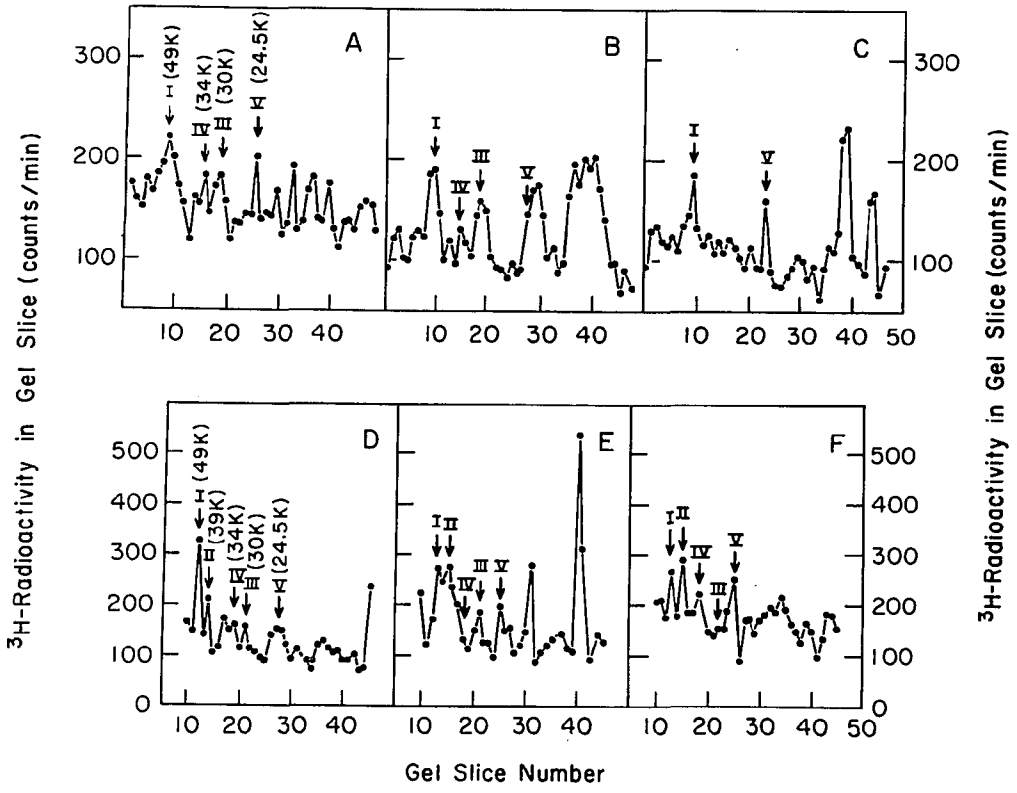


Fig. (20) Chymotrypsin Digestion of Heme-deficient Mutant Mitoplasts Prepared with the Digitonin Method. Mitochondria were prepared by the Zymolyase procedure from [^{3}H]leucine labeled heme-deficient yeast mutant (GL1) cells. Mitoplasts were prepared by the digitonin method with 2 mg of digitonin per mg of mitoplast protein, and resuspended in sucrose buffer as described Chapter II of this thesis. The mitoplast suspension was incubated at 30 C for 30 min in the absence of chymotrypsin (A) and (D), or in the presence of 450 ug (B), 900 ug (C), 780 ug (E), and 1560 ug (F) of chymotrypsin per mg of mitoplast protein. Immunoprecipitates of the antiserum against complex III and the Triton X-100 extracted proteins from the control and chymotrypsin digested mitoplasts were analyzed by SDS polyacrylamide gels as described in the legend to Fig. (18). The results in the top and the bottom panel were from two separate experiments.

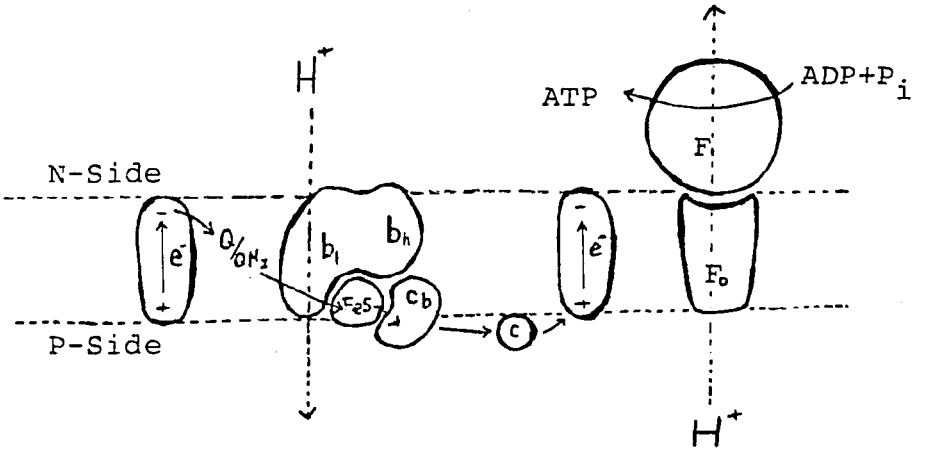


Fig. (21) Topography of Cytocrome b-c_1 or b_6-f Complexes in Electron Transporting Membranes. The small polypeptides of the complexes have been neglected in this figure (from Hauska et al. (48)). P- and N-side denote the aqueous surfaces which become positively and negatively charged during electron transport, respectively. F_1 and F_0 represent the peripheral and membrane-integrated portion of the coupling factor complex; c_b and c denote membrane-bound cytochrome c (c_1 or f) and water soluble cytochrome c (or plastocyanin), respectively. b_1 and b_h are the two forms of apocytochrome b, respectively.

Table (1) Subunit Composition of Complex III

Subunit number (names)	Stochiometry	Molecular Weights (kDa)	Isoelectric points	Prosthectic groups
I (core protein 1)	2	49.0	6.53	
II (core protein 2)	1	39.0	6.37	
III(apocytochrome b)	1	30.0	8.3	heme b
IV (apocytochrome c_1)	1	29.0	6.45	heme c
V (Rieske's Fe-S protein)	1	24.5	6.57	Fe_2S_2
VI (subunit 6)	1	17.7	6.2	
VII(subunit 7)	1	15.5	7.15	

Data presented on this table are based on those obtained by Sidhu et al. (23,24) from this laboratory.

TABLE (2). PROCESSING OF CYTOPLASMICALLY MADE PRECURSORS OF SUBUNITS OF MITOCHONDRIAL COMPLEX III

Subunits	Molecular weight (KDa) Mature	Inter-mediate	Pre-cursor	Organism
I (core protein 1)	44		44.5	yeast
II (core protein 2)	40		40.5	yeast
IV (apocytochrome c_1)	31.5	34	37	yeast, N. crassa
V (Rieske's protein)	24.5		28	yeast
VI	17		26	yeast
VII	14		14	yeast
VIII	11		11	yeast

Molecular weights listed in this table are from Van Loon et al (55,57).

TABLE (3)

PROPERTIES OF THE IRON SULFUR CENTERS OF THE MITOCHONDRIAL RESPIRATORY CHAIN OF YEAST S. CEREVISIAE

Center	(a) Complex	Number of Fe and S per center	Mid-point redox potentials(b)	Field positions (g values) g(z)	g(y)	g(x)
S-1	II	2 : 2	0 mV	2.03	1.93	1.91
S-2	II	2 : 2	400 mV	2.03	1.93	1.91
S-3	II	4 : 4	65 mV	2.02	2.01	1.99
Rieske	III	2 : 2	230 mV	2.025	1.89	1.81

(a) Designation of the Fe-S centers and their epr field positions listed in this table are based on data of Rieske (51), Orma-Johnson (154) and Ohnishi et al. (5,152).
(b) The mid-point redox potentials listed were measured in isolated complexes (5).

TABLE (4) CHYMOTRYPSIN SENSITIVITIES OF COMPLEX III SUBUNITS

Mitoplasts, labeled with [^{3}H]leucine, were digested for 30 min with the indicated amounts of chymotrypsin. Following digestion, the mitoplasts were solubilized and subunits to complex III were immunoprecipitated and analyzed on SDS polyacrylamide gels. The percentages of digestion were calculated from the ratio of areas under the peaks in radioactivity profiles of Figures (18), (19) and (20).

			Percentages of digestion	
Subunit	Fig.	Chymotrypsin concentration	Wild type cells	Heme mutant cells
Core protein 1	(19)	400 (*)	0	
		800	0	
	(18)	275	24.9	
		550	24.7	
		685	31.4	
		822	57.1	
	(20)(a)	450		33.0
		900		33.4
	(b)	780		0
		1560		0
Core protein 2	(19)	400	0	
		800	0	
	(18)	275	22.6	
		550	11.3	
		685	7.3	
		822	13.3	
	(20)(b)	780		0
		1560		0
Iron-sulfur protein	(19)	400	0	
		800	100	
	(18)	275	4.7	
		550	11.3	
		685	36.4	
		822	100	
	(20)(a)	400		0
		800		0
	(b)	780		0
		1560		0
Apo-cytochrome b	(18)	275	0	
		550	36.4	
		685	100	
		822	100	
	(20)(a)	450		33.4
		900		72.1
Apo-cytochrome c_1	(18)	275	21.4	
		550	38.6	
		685	0	
		822	0	
	(20)(a)	450		49.4
		900		71.4

(*) Micrograms of chymotrypsin per mg of mitoplast protein.
(a) and (b) are results from the top and the bottom panels of Fig. (20) respectively.

LIST OF ABBREVIATIONS

ALA	5-aminolevulinic acid
ATP	adenosine triphosphate
DABS	diazobenzene sulfonate
DCCD	dicyclohexylcarbodiimide
EDTA	ethylene diamine tetraacetic acid
ET	ergosterol and Tween 80
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIPIP	high potential iron-sulfur protein
ISP	iron-sulfur protein
KDa	kilodaltons
MOPS	4-morpholinopropanesulfonate
NADH	reduced nicotiamide adenine dinucleotide
PMSF	phenylmethylsulfonate
SDS	sodium dodecyl sulfate

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