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City University of New York PH.D. 1986

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THE ROLE OF CYTOCHROME B IN THE PROCESSING OF THE SUBUNITS OF COMPLEX III IN THE YEAST MITOCHONDRIA

by

Keya Gupta Sen

A dissertation submitted to the graduate faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University New York.

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ABSTRACT

THE ROLE OF CYTOCHROME B IN THE PROCESSING OF THE SUBUNITS OF COMPLEX III IN THE YEAST MITOCHONDRIA

Keya G. Sen

Adviser: Professor Diana S. Beattie

The work described in this dissertation deals with the effect of cytochrome b on the biogenesis and assembly of the subunits of complex III in the mitochondrial membrane of the yeast *Saccharomyces cerevisiae*. The cytochrome b $^{-}$ mutants (Box mutants) of *S.cerevisiae* form an excellent system to study such a role of cytochrome b. The steady state levels, the kinetics of synthesis and the assembly of the nuclear encoded subuinits of complex III have been investigated and compared in both the Box mutants and the wild type parental cells. Thus when mitochondria from wild type and four mutant strains were analyzed by immunoblotting and immunoprecipitation techniques, using specific antisera, the two core proteins and the iron-sulfur protein were decreased 50 % or more in the mitochondria from the mutants as compared to the wild type. By contrast, the amounts of cytochrome c_1 in the mitochondria, as determined both spectroscopically and immunologically, were not affected by the absence of cytochrome b. Pulse labeling of the cells with (^{35}S) methionine in the presence of CCCP showed the accumulation of the precursors to the core protein I and the iron-sulfur protein in similar amounts in the mutant Box 6-2 and the wild type cells. Synthesis of the

iron-sulfur protein and the cytochrome c_1 by *in vitro* translation of mRNA isolated from wild type and mutant Box 6-2 in a rabbit reticulocyte lysate system, also confirmed that the synthesis of the nuclear encoded subunits was not affected in the mutants. Pulse labeling of th e cells in the absence of CCCP and subsequent chase with cold methionine, however, showed much less of the mature subunits of core protein I and the iron sulfur protein in the mitochondria of the mutant cells relative to the wild type. Import of radiolabeled precursors, synthesized *in vitro*, into isolated mitochondria from the parental and the mutant strains indicated that less amounts of the iron-sulfur protein precursor was processed to the mature form in the mutant mitochondria of Box 6-2. Processing of the precursor of cytochrome c_1 into isolated mitochondria, remained unaffected in the mutant cells and similar amounts of the intermediate and the mature form of the subunit was found in the mitochondria of both strains. These results indicate that cytochrome b is necessary for the proper processing of certain subunits of complex III.

To my father

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Chapter I

INTRODUCTION

The mitochondrion is the organelle of the eucaryotic cell whose primary function is the conservation of oxidatively derived energy and its utilization for ATP synthesis. It has been extensively studied to elucidate the mechanism of ATP synthesis and the enzyme complexes involved in this process have been well characterized in the literature. Another line of research has focussed on the biosynthesis of the organelle itself. The work described in this dissertation represents a small facet of the large and complex study of mitochondrial biogenesis in the yeast *Saccharomyces cerevisiae*.

The generation of new mitochondria is not achieved by *de novo* synthesis as was once thought, but rather must occur by growth and division of existing organelles (1). The discovery of the existence of mitochondrial DNA and of a separate mitochondrial protein synthesizing system has revealed that the assembly of the newly synthesized mitochondrial material is highly complex. Most mitochondrial proteins are encoded by nuclear DNA and synthesized in the cytoplasam before their incorporation into the organelle (2). Mitochondrial biogenesis therefore requires an extensive coordinated effort of two physically separate protein synthesizing systems which are directed by two separate genomes. The regulation of the expression of the mitochondrial genome, the coordination of expression of nuclear and mitochondrial genes, the transport of cytoplasmically synthesized proteins into the mitochondria and their subsequent assembly with mitochondrially encoded proteins are topics of current interest. A few aspects of mitochondrial biogenesis

relevant to the subject of this thesis are reviewed briefly to provide some background information.

A. USE OF YEAST AS A STUDY SPECIES

Saccharomyces cerevisiae is an attractive eucaryotic organism for the study of the biosynthesis of mitochondria. First, it is a facultative anaerobe capable of both aerobic and anaerobic growth. Under anaerobic conditions the production of ATP by fermentation is sufficient for growth. Thus the cell can live indefinitely in circumstances in which mitochondrial function is impaired or impossible using a fermentative carbon source such as glucose. Second, it is well suited for genetic manipulation. Its life cycle lends itself to the relatively easy construction of both haploid and diploid strains with specific genetic markers. In addition being unicellular it can be handled using standard bacterial techniques.

B. MITOCHONDRIAL PROTEIN SYNTHESIS, ITS PRODUCTS, ITS GENOME

1. Mitochondrial Protein synthesis and its products

The possibe informational role of mitochondrial DNA was implied by reports from three groups in 1964, when they showed that isolated mitochondria could incorporate radiolabeled ribonucleotides into an acid insoluble product (3-5). Mitochondrial RNA polymerase has since been isolated by many groups (6-10). Reports of protein synthesis in isolated mitochondria first appeared over twenty years ago (11), however, it was not till the 1960s that several groups confirmed mitochondrial protein synthesis in isolated rat liver (12), rat brain (13), beef heart (14), yeast (15) or *Neurospora* mitochondria (16). These reports conflicted on the

amino acid and energy requirements for optimal protein synthesis *in vitro*. Wheeldon and Lehninger (17) showed that ATP itself, not some high energy intermediate generated by electron transport, provided the energy for maximal rates of mitochondrial protein synthesis; they observed amino acid incorporation by isolated mitochondria under conditions where bacterial contamination was minimized. Bacterial contamination as the cause of the observed *in vitro* incorporation of amino acids was definitively excluded by Beattie et al (18). They also demonstrated that mitochondrial translation products are predominantly incorporated into the inner mitochondrial membrane (19).

What are the products of the mitochondrial protein synthesis machinery? The number of proteins made on the yeast mitochondrial genome and their relative contribution to the overall mass of the organelle have been assesed by two main approaches :

1. One has been with the help of inhibitors of the protein synthesis machinery. The antibiotics inhibiting mitochondrial translation, such as chloramphenicol or erythromycin, have been highly instrumental in the identification of the products of mitochondrial protein synthesis, because these bacterial inhibitors have no effect on yeast cytoplasmic protein synthesis. Conversely, yeast cytoplasmic translation inhibitors, such as cycloheximide, does not affect mitochondrial synthesis. Proteins synthesized in the mitochondria are thus selectively labeled (*in vivo* by intact cells in the presence of cycloheximide or *in vitro* by isolated mitochondria) and their functional role assigned by reacting with specific antibodies raised against purified mitochondrial enzyme complexes, either before or after electrophoretic separation.
2. The isolation in 1975 (20, 21) of several respiratory deficient mutants

of yeast having lesions in the mitochondrial genome (reviewed in the next section) gave rise to the other main approach. Respiratory deficient ρ^- mutants display a non-mendelian inheritance pattern. Thus when the protein composition of mitochondria from wild type and ρ^- mutants is compared proteins present in wild type but not in mutant mitochondria correspond to mitochondrial translational products. Examination of the products with specific antibodies indicates the defective subunit or subunits in the mutant and also identifies them as mitochondrial gene products.

Thus Tzagoloff et al. pulse labeled yeast cells in the presence of cycloheximide and rapidly isolated the mitochondrial rutamycin sensitive ATPase by immunoprecipitation, revealing that four of the subunits, 5, 6, 7 and 9, were mitochondrial in origin (22). Mason and Schatz (23) and Rubin and Tzagoloff (22) used similar techniques to demonstrate that the three large subunits of cytochrome oxidase were also of mitochondrial origin. Weiss (24) and Lin and Beattie (25) showed in *Neurospora* and yeast respectively that cytochrome b was also synthesized in the mitochondria. Similarly one protein component associated with the small mitochondrial ribosome was shown to be of mitochondrial origin by Lambowitz et al. in *Neurspora* (26) and Groot et al. (27) in *S. cerevisiae*. This protein is designated as var. I and appears to play a role in the assembly of the mitochondrial small ribosome. Mitochondrially inherited mutants with specific lesions in cytochrome c oxidase, oligomycin sensitive ATPase or coenzyme QH_2- cytochrome c reductase have been isolated and mapped into six different complementation regions on the mitochondrial genome both by genetic mapping and by the use of restriction enzymes (reviewed in section B.2).

2. The Mitochondrial genome

A big step forward in our understanding the mitochondrial genome and its subsequent role in mitochondrial biogenesis was made possible by the isolation of several classes of mitochondrially inherited mutations in the yeast. Mitochondrial mutants in yeast are separated into four categories : ρ^-, ant^R, mit^-, syn^-.

One type is the cytoplasmic "petite " or ρ^- mutant. These clones of *S.cerevisiae* with large deletions in mtDNA (28) can occur spontaneously (29) or can be induced with a number of chemical agents, such as ethidium bromide (30). The retained fragment is repeated and amplified to the size of wild type mt DNA. These mutants are invariably deficient in mitochondrial protein synthesis (31). The ρ^- genome is capable of undergoing recombinational events with genomes of other ρ^- mutants (provided there is sequence overlap) or with wild type mt DNA (32). These circumstances have made ρ^- mutants very useful tools for genetic and physical mapping of mt DNA. The ρ^-clone can be localized to a specific region of the wild type genome by genetic means. The extreme case of this type of mutation, the complete loss of all mt DNA is referred to as ρ^0. These mutants are very useful for ascertaining the location of mutations, either nuclear or mitochondrial.

A second type of mitochondrial mutant is recognized by its resistance to inhibitors (antibiotics and drugs) of several mitochondrial functions such as protein synthesis, electron transport and oxidative phosphorylation. Mutants resistant to these agents represent the ant^R category. Thus, chloramphenicol, erythromycin and paromomycin are inhibitors of mitochondrial protein synthesis (33, 34); antimycin, diuron, and mucidin of electron transport, inhibiting cytochrome b (35-37) and

oligomycin of oxidative phosphorylation (38, 39). The ant^R mutants greatly expanded the field of mitochondrial genetics because the resistance phenotypes were caused by point mutations, not large deletions as in the ρ^- mutants and these point mutation served as specific genetic markers.

Mutants of the remaining two categories, mit^- and syn^-, arise from point mutations or small intragenic deletions, making them very useful in gene mapping. The mit^- mutants have defects in the respiratory chain and oxidative phosphorylation but are capable of mitochondrial protein synthesis (20, 21, 40). The mutation is presumably located in the structural gene for one or at most two translation products. Mit^- mutants have been shown to be associated with subunits of cytochrome oxidase, ATPase and the respiratory carrier cytochrome b (41). In a number of cases, mit^- mutations generate premature termination codons resulting in novel peptides with altered electrophoretic mobilities (42). Such mutations have been instrumental in some of the earlier identification of mitochondrial gene products. The syn^- mutants on the other hand have lesions in components of the protein synthesis machinery and as a result are pleiotropically deficient for respiratory functions, as are the ρ^- mutants, because of the lack of mitochondrial protein synthesis (43). The syn^- mutants have mutation in mtDNA genes coding for tRNA's and rRNA's.

Mitochondrial genes have been mapped by three independent methods. The earliest mapping studies employed classical recombinational analysis of mitochondrial markers, while subsequent studies used deletion mapping with ρ^- clones. Recombinational analysis provided a wealth of information on the fine structure of individual genes, but have not been very useful in establishing linkage relationships.

Subsequently, several laboratories published restriction maps of wild type mt DNA for several laboratory strains of *S. cerevesiae* (44, 45). These maps depicted the locations of restriction sites for endonucleases with recognition sequences of 6 nucleotides. These physical maps allowed the mt DNA segments of different ρ^- mutants to be readily placed and oriented on the wild type genomes. Genetic markers retained by ρ^- mutants could therefore be mapped on wild type mt DNA within the physical limits defined by the deletion end points of the comminuted ρ^- genome.

The most accurate maps of the yeast mitochondrial genome have been obtained by DNA sequencing. DNA sequence analysis by Tzagoloff, his collaborators and others completed the map of the mitochondrial genome. The genetic and physical map of the mitochondrial genome showing the physical location of the mitochondrial genes has been shown in Fig 1a (44) and 1b (46). The 25 µm circular genome is 70 Kb long. The GC content is 18 %, their being large AT-rich regions between and sometimes within genes (47). Examination of the genetic and physical maps reveal that there is a scattering of functionally related genes. Thus the three cytochrome oxidase loci, *oxi* 1, *oxi* 2 and *oxi* 3 are separated by either tRNA genes or genes coding for other mitochondrial translation products. Similarly the ATPase loci *pho* 1 and *pho* 2 and the two ribosomal RNA genes are scattered. Furthermore, some 40 % of the genome consists of noninformational DNA since they lack any genetic markers. These are the regions very rich in AT sequences. Therefore the yeast mitochondrial genome is extremely specialized.

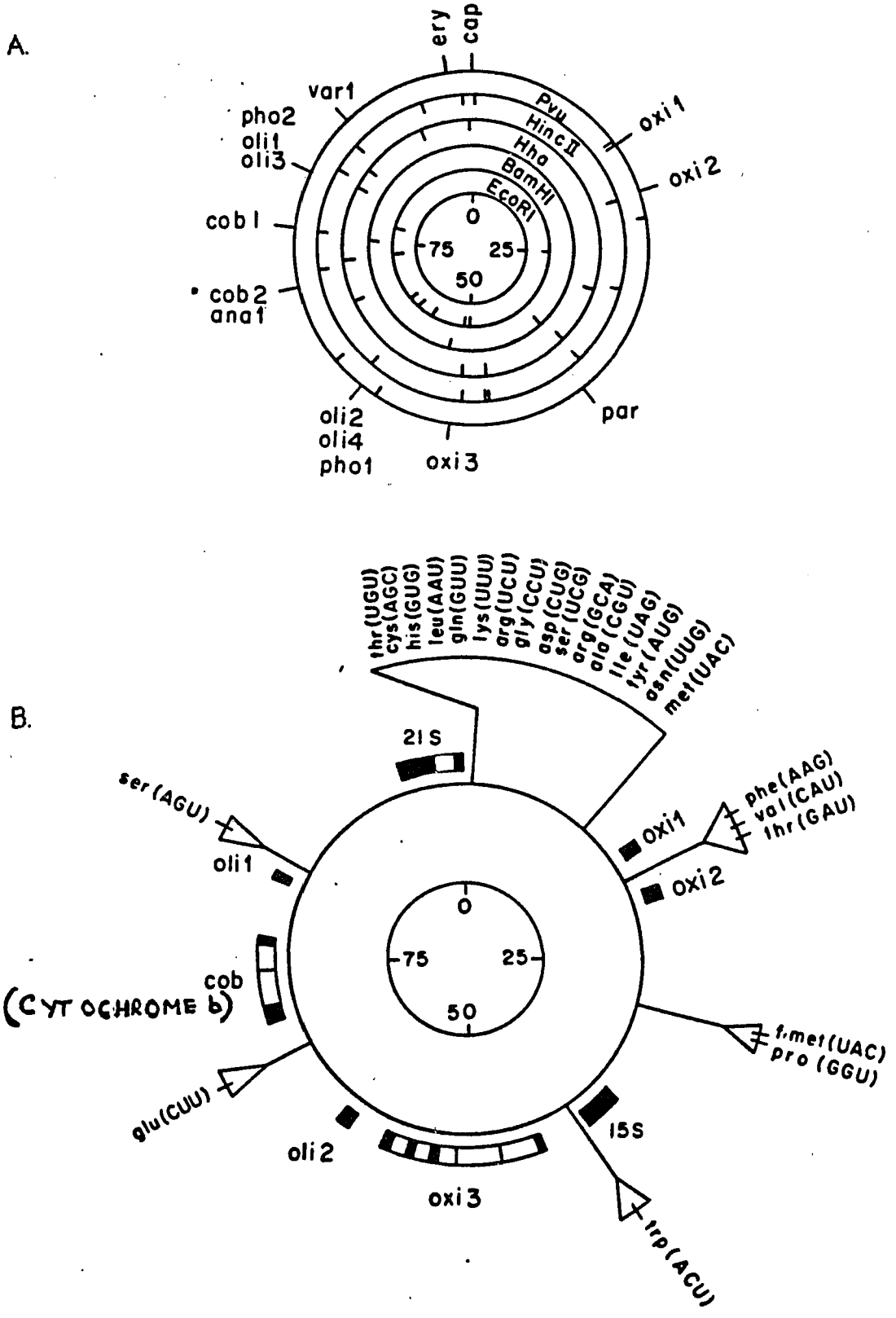
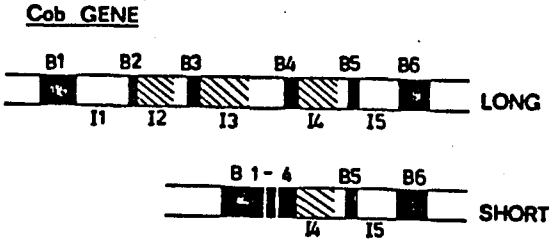


Figure 1. Physical and Genetic Map of mt DNA in *S. cerevisiae* D273-10B. A. The location of the antibiotic resistance and mit loci are indicated on the outer circle (44). B. Physical location of the mitochondrial genes that have been sequenced (46).



Locus	Span
box 4/5	B1
box 3	I2
box 8	B3
box 10	I3
box 1/9	B4
box 7	I4
box 2	B5
box 6	B6
cob 2 (short gene)	B5
cob 1 (short gene)	B5+B6

Figure 2. Diagrammatic Representation of the Long and Short Form of Cob-Box gene of *S. cerevisiae* B, exon *Black areas* ; I, intron, *open and cross hatched areas*. The cross hatched sections correspond to open reading frames in phase with preceding exons; open areas are closed reading frames (80). The table on the right is adapted from Tzagoloff, A., (1982) 'Mitochondria' , p. 306.

3. Cytochrome b gene and cob- box mutants of yeast

Since the box mutants of *S. cerevisiae* are the subject of this thesis they will be discussed in some detail. The apo-cytochrome b gene is located between the *oli*-1 and the *oli*-2 loci on the yeast mitohondrial genome (Fig1b). The gene has a mosaic structure and maps between 71.5 and 76.2 map units (48-52). Both genetic and DNA-RNA hybridization studies indicate that there are two forms of the gene. In *S. cerevisiae* strain D273-10B, the apo- cytochrome b gene is a 'short gene 'and contains three exons and two introns (53, 54). The first 252 residues from the amino terminal end of the protein are specified by the first exon (B1-4 in Fig. 2). This exon has a reading frame which is continuous and in frame with the intron which separates it from the next exon (B5). The second intron, which separates B5 and B6 (Fig. 2), has no reading frame of any significant length. In other strains of *S. cerevisiae* such as KL14 and 777-3A a longer version of the gene exists due to the presence of additional introns (52 , 55 , 56). As shown in Fig. 2, the most substantial difference occurs in the structural sequence coding for the first 252 residues of the protein; in the long gene this part of the protein is encoded by 4 short exons. The only two introns of the short form are equivalent to the I4 and I5 of the long form. Among the five introns of the *cob* long form three; I2, I3, and I4 contain open reading frames in phase with the preceding exon sequences (53, 55). The molecular weightof the protein encoded by the cytochrome b or *cob-box* gene is 44,000 a value considerable in excess of the molecular weight estimated by SDS-polyacrylamide gels.

Point mutations mapping in any region of the cytochrome b gene

result in what is termed as cob-box mutants. These mutants were first isolated by Tzagoloff et al. (20 , 21) ; approximately 200 cob-box mutants are now available. These mutants are incapable of forming the wild type polypeptide and lack a spectrally or immunologically detectable cytochrome b but instead form polypeptide fragments immunologogically related to cytochrome b (mutations in clusters Box 4/5, Box 1, Box 2 and Box6) (49, 51, 58, 59) or accumulate novel mitochondrially made polypeptides (mutations in Box 3, Box 7 and Box 10) (49 , 51, 58, 60-62). Mutations in Box 3 also result in lack of cytochrome oxidase (48, 57, 62). All of these mutations result in a lack of functional ubiquinol-cytochrome reductase; they, however, have a spectally detectable cytochrome c_1 (62, 63).

What is the function of the open reading frame of some of the introns? Slonimski and co-workers have proposed that after the initial step in splicing of the first intron from the cytochrome b precursor mRNA, there is translation of an exon-intron 2 coded 'maturase' protein (55). The 'maturase' protein acts in trans for the subsequent splicing of the pre-mRNA for cytochrome b to yield the final mRNA. In support of this model, these workers have shown that mutations in the intron reading frames cause the appearance of higher molecular weight translation products not seen in wild type cells. Since the defective maturases cannot catalyze the splicing reaction, the mutants accumulate high levels of the maturase message and hence of the translation product. Recently they have also identified immonologically the mRNA maturase encoded by the second intron and shown it to be a polypeptide of molecular weight 42 KDa. (64). Similarly, intron 4 which also contains an open reading frame in

phase with the preceding sequence and possibly codes for a 'maturase' protein, is not only involved in the processing of the mRNA for cytochrome b but also subunit I of cytochrome oxidase (65, 66). This may explain the pleiotropic nature of some of the box mutants.

C. NUCLEAR GENE PRODUCTS CONTRIBUTING TO THE BIOGENESIS OF MITOCHONDRIA

Clearly the products of the mitochondrial genome are not sufficient for the formation of a respiratory competent mitochondrion. In yeast, the mitochondrially synthesized proteins have been estimated to represent 5-10% of the total 400-500 mitochondrial proteins needed for a functional mitochondria, the remaining being made on the cytoplasmic ribosomes (1). Identification and characterization of the nuclear gene products are being approached by methods similar to those used for the identification of the mitochondrial gene products. The situation however is more complex. Nuclear mutants conferring a respiratory defective phenotype, called '*pet*' mutants, were first isolated in 1963 (67). Until recently however, only a small fraction of the required nuclear genes had been identified in this manner and, in most cases the exact nature of the defect was not known. In the past several years Tzagoloff and co-workers have isolated over 2000 nuclear recessive respiratory deficient *pet* mutants and they have been used to identify 207 nuclear genes involved in mitochondrial function (68, 69).

Approximately 25% of the nuclear *pet* mutants exhibit same pleiotropic deficiencies as do mitochondrial ρ^- mutants (68) and show similar phenotypic characteristics. The absence in pleiotropic *pet* strains

of cytochrome a , a_3, b and oligomycin sensitive ATPase is most likely to be a secondary effect of a primary lesion in a component essential for DNA replication, transcription, RNA processing and mitochondrial protein synthesis. Relatively little work has been done to characterize the primary lesions of these pet mutants. Several groups have studied the single nuclear *pet* mutant 936 (70, 71) and shown that a single nuclear lesion in *pet* 936 hindered the translation of mRNAs coding for ATPase subunits as well as subunits of cytochrome oxidase. This indicates that common translational control elements exist at least for the ATPase and cytochrome oxidase complexes.

Pet mutants which affect the accumulation of a specific protein have been investigated more thoroughly. Sherman et al. have identified and characterized the structural genes for iso-1 and iso-2 cytochrome c and seven additional genes required for production of these proteins by studying a large number of cytochrome c deficient strains (72). Recent advances in yeast biotechnology have led to the rapid progress in this area. Genes encoding five subunits of complex III, (73) several subunits of cytochrome oxidase (complex IV) (74) and the ATPase ß subunit have been cloned (75).

Another interesting class of *pet* mutants have lesions that block the accumulation of a specific mitochondrially encoded protein. The genes defined by this group of mutants are probably involved in the expression of the mitochondrial gene coding for the missing product. Facilitated by the extensive information available on the target mitochondrial gene, some of the defects observed have been described at the moleclular level. Muller and coworkers have delineated the expression of subunit 3 of cytochrome

oxidase in some details (76). They have shown that the mitochondrial gene encoding subunit 3 of cytochrome oxidase (oxi 2) is transcribed but not translated in the mutant *pet* 494-1. In addition they isolated a revertant of *pet* 494 -1 in which the nuclear effect has been compensated by the presence of a grossly rearranged ρ^- genome maintained in a heteroplasmic state with a ρ^+ genome. Their results suggest that a nuclear encoded factor is needed for the translation of the *oxi* -2 gene. Similarly, Faye et al. have characterized a nuclear gene involved in the splicing of the transcript for subunit 1 of cytochrome oxidase (oxi -3) (77). Another set of *pet* genes that have been extensively analyzed affect the expression of the mitochondrial cytochrome b gene. Five of these genes, *CBP* 1 - *CBP* 5 mediate processing of the RNA. The *cbp* 1 mutants are defective in the processing step that is required to generate the 5' end of the cytochrome b mRNA (78). The *cbp* 3, *cbp* 4 and *cbp* 5 mutants cannot remove the first intron from the cytochrome b RNA. In addition they cannot complete splicing of the RNA for subunit 1 of cytochrome oxidase (*oxi3*) (69). The requirement of three nuclear gene products in addition to the maturase protein (55), suggests the existence of a complex that mediates the splicing of introns present in the cytochrome b and the *oxi* 3 genes. The *CBP* 2 gene has recently been characterized and it has been shown that *cbp* 2 mutants do not remove the second intron from the cytochrome b RNA (78). A sixth nuclear gene needed for the cytochrome b expression has been recently identified and falls in the nuclear complementation group G154. The mutant in this group produces the transcript of cytochrome b mRNA but fails to translate it (68), similar to the *pet* 494-1 phenotype for *oxi* 2 gene described earlier. Similarly Kaudewitz and co-workers

have worked with *pet* mutants having lesions in the 'long gene' of cytochrome b and reported that two nuclear elements are needed for the processing of the introns present only in the long form of the *cob* gene (80).

D. TRANSPORT OF PROTEINS INTO MITOCHONDRIA

The several hundred proteins made on the cytoplasmic ribosomes must be transported into the mitochondria. Some of these imported proteins are integrated firmly with the proteins translated within the organelle to act as structural proteins while others act as signals through which the nucleus regulates mitochondrial growth and division. The import process must, therefore, be highly specific as the structural proteins occupy a precise location within the organelle. *In vivo* studies with radio-isotopic tracers in *Neurospora* and yeast (81) have established that the import process is a post translational event. Thus pulse labeled mitochondrial proteins are initially found outside the mitochondria and these newly synthesized proteins can be chased into the mitochondria only after a lag. The labeled proteins fail to appear in the mitochondria if cytoplasmic protein synthesis is inhibited with cycloheximide (82, 83). *In vitro* studies with a cell free system have demonstrated clearly that the synthesis of mitochondrial proteins can be separated from its transport in space and in time.

What are the characteristics of the import process? Based on the variations in the process, proteins can be classified into two groups :

1. Proteins that have their final destination in the inner membrane, inter-membrane or the matrix of the organelle (cytochrome c and

adenylate kinase are exceptions).

2. Proteins destined for the outer mitochondrial membrane.

Proteins that belong to the first group are usually synthesized as longer precursors and differ from the corresponding mature polypeptides by an amino terminal extension (81, 84, 85). The first step in the import process is binding of the precursors to a protease sensitive receptor molecule on the cytoplasmic side of the mitochondrial outer membrane (86). This binding is not competed by a large excess of the corresponding mature proteins and is abolished by removing the amino terminal extension from the precursor (81). Import of the proteins into the organelle requires a membrane potential (87, 88) and the precursor forms of these proteins are accumulated in the presence of uncouplers of oxidative phosphorylation (89). However, the initial binding does not require an energized state and can be obtained with isolated outer membrane vesicles (90, 91). During translocation, the precursor sequence is cleaved off by a protease located in the matrix (92). This protease is chelator sensitive, and is specific for only mitochondrial precursor proteins. It does not act on mature proteins and interestingly enough it is itself imported from the cytoplasam (93). Inter-membrane polypeptides such as cytochrome c peroxidase and cytochrome b_2 undergo two processing steps, an intermediate form being released first before the final mature form. Unexpectedly, the matrix located protease acts on the first of the two processing steps (94, 95), indicating that the precursors to these intermembrane space polypeptides must at least in part pass through the inner membrane (via a transmembrane intermediate) before processing can occur. The mature form is then released into the intermembrane space and cleaved by a second protease, located probably

on the outer surface of the inner membrane (81).

Information on the nature of the pre-sequence has been obtained by analysis of the DNA sequence of the genes for some of the precursor proteins. Comparison of the predicted protein sequences with that of the known sequences for 7 proteins has revealed that the precursor sequences are located at the amino terminus (84, 85, 96-100). Furthermore, there are only basic and no acidic amino acid residues in the precursor region with a high concentration of serine and threonine residues. It is speculated that the absence of acidic residues and clustering of basic aminoacid residues renders the precursor more basic and hydrophilic than the mature protein, to facilitate its import into the mitochondria (81). A few polypeptides like the adenine nucleotide translocator though destined for the inner membrane have no presequences; however the adenine nucleotide translocator differs from the mature form in solubility and chromatographic properties and therefore achieves a new conformation on being inserted into the membrane (101, 102).

Import of proteins into the outer membrane differs from import into inner membrane in several ways. First no energized membrane is required for the import (103,104). Second no cleavable presequences have been observed in any of the polypeptides isolated from yeast or *Neurospora*. No trypsin sensitive component seems to be necessary either, for binding of the protein to the outer membrane. Indeed outer membrane proteins synthesized *in vitro* can be imported into purified outer membrane vesicles. Therefore, either there is a separate receptor species, which is not trypsin sensitive, for the outer membrane proteins or no receptor is required for the binding and integration of these proteins into the outer membrane. Cytochrome c, however, is an intermembrane space protein

which is not synthesized as a longer precursor. Zimmerman et al. have shown that only the apocytochrome c is imported and not the heme containing holoenzyme (105). The heme is inserted into the protein within the organelle to form the holoenzyme. Furthermore, the binding site for apocytochrome c on the mitochondrial membrane surface is different from the binding site of the other imported innermembrane or intermembrane proteins. Although this site is also trypsin sensitive, it cannot be competed by other innermembrane precursors but only by excess apocytochrome c (86,106). Similarly adenylate kinase appears to be imported into the intermembrane space without a proteolytic cleavage (107).

The import process is a subject of much research. Effort is being made by several laboratories to isolate the components of the import 'machinery'. The processing protease has been purified 100-fold from yeast mitochondria (93,108). Schatz et al. recently reported the isolation of microgram quantities of the precursor to ß subunit of ATPase from yeast; they have shown that the import of the isolated precursor by energized yeast mitochondra requires a 40 KDa protein fraction from the yeast cytoplasam (108). Gene fusion studies, using the isolated gene for this protein, by Douglas et al. have firmly established that the amino acid sequences targeting the precursor protein are located within the first 350 amino terminal residues (109). Using similar techniques Schatz et al. recently showed that the first 12 amino acids of cytochrome c oxidase subunit IV presequence from yeast were sufficient to direct dihydrofolate reductase into the mitochondrial matrix (110). Isolation of two yeast mutants (*mas* mutants) resulting from lesions in the nuclear gene and which are temperature sensitive for import of mitochondrial proteins has

recently been reported (112). One of these mutants (*mas1*) is deficient in the matrix located protease activity (113). These mutants should facilitate the molecular analysis of the mitochondrial import process in great details.

E. BIOGENESIS & TOPOGRAPHY OF COMPLEX III

1. Topography of complex III

Hatefi and Green first isolated four enzymatically active protein -lipid complexes from the inner mitochondrial membrane: Complex I, II, III, IV (114). These four complexes are involved in electron transfer across the respiratory chain, while a fifth complex, the ATP synthetase, couples the respiratory or electron transfer chain to ATP synthesis (115,116). These complexes, since then, have been isolated by several groups from different organisms and both their electron transport properties and their biosynthesis have been studied extensively (for a review see 46, 119).

Complex III or coenzyme QH_2-cytochrome c reductase catalyzes the the transfer of electrons from coenzyme QH_2 to cytochrome c causing at the same time an electrogenic proton translocation across the inner membrane (118, 119). The enzyme has been isolated in an enzymatically active and pure form from several species (120-127). The complex isolated from either beef heart or *Neurospora* has been shown to be present as a dimer both in solution (124, 125) and in the membrane bound form (128) with molecular weight ranging from 440 KDa to 560 KDa. Electron microscopy of membrane crystals of complex III from *Neurospora* has shown that the dimeric enzyme has the two fold axis running perpendicular to the membrane. The monomeric unit is elongated

and extends approximately 15 nm through the membrane; only about 30% of the total mass of the complex is buried in the lipid bilayer, while 70% protrudes on either side of the membrane (128). The localization in yeast and beef would probably prove to be similar since the composition of the complex in different organisms is highly conserved.

The purified complex from *Neurospora*, beef and yeast all contain 7-10 polypeptide subunits, a binuclear iron-sulfur cluster (the Rieske's iron sulfur cluster), quinones and phospolipids which include phosphatidyl ethanolamine, cardiolipin and phosphatidyl choline (129). The complex recently purified in this laboratory contained 7.03 nmol of cytochrome b and 4.24 nmol of cytochrome c_1 per mg of protein. This purified complex contained 7 subunits: cytochromes b and c_1, the Reiske's iron-sulfur protein (ISP), core proteins I and II and three smaller molecular weight subunits (130).

The topographical distribution of the individual subunits has been studied by several methods. Membrane surface labeling studies with the membrane permeant reageant diazobenzene sulfanilic acid (DABS) in beef heart by Bell et al. (120) and in yeast mitochondria by Beattie et al. (131, 132), indicated that cytochrome c_1 and the ISP are on the cytoplasmic side of the inner membrane, while cytochrome b and the two core proteins span the inner membrane, with core protein I being within the membrane, and core protein II being exposed on both sides (Fig. 3). Core protein II appeared to protrude into the matrix (132).

Lactoperoxidase-catalyzed iodination of mitochondria from

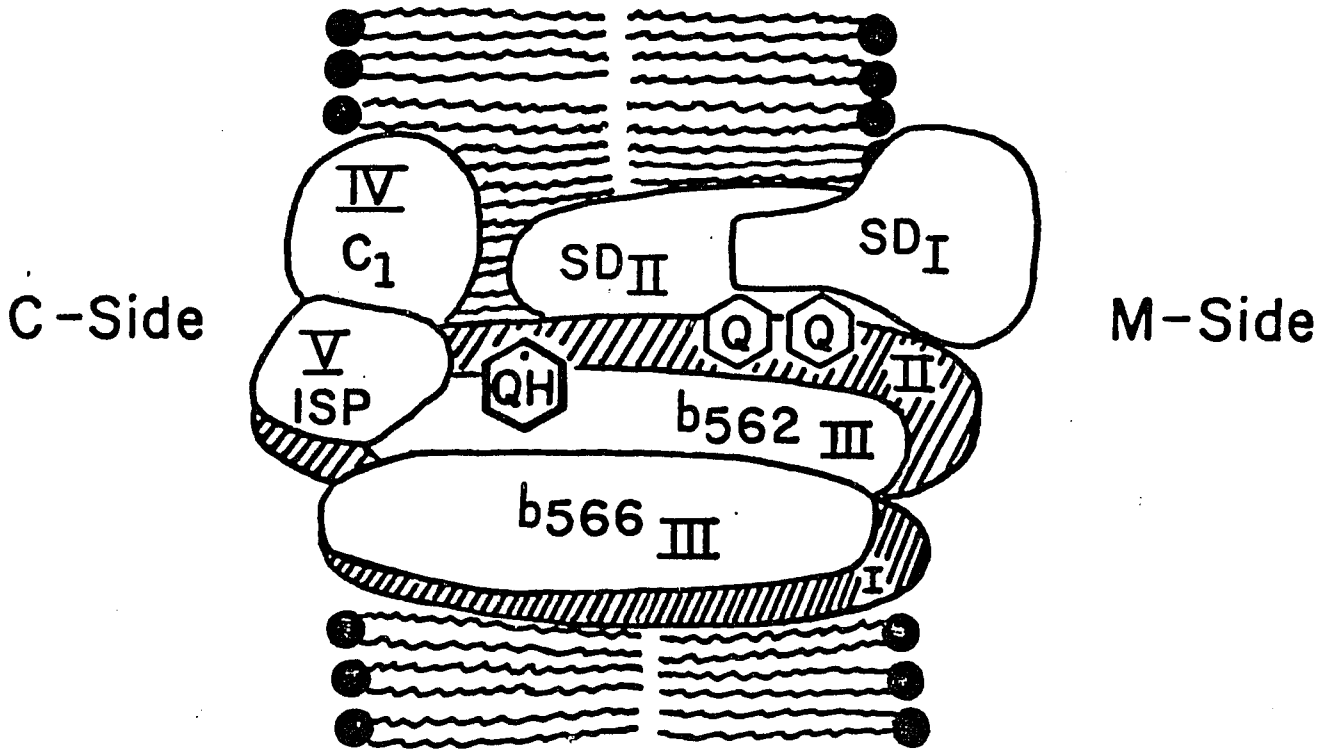


Figure 3. A Model for the Orientation of Complex III Subunits in the Inner Mitochondrial Membrane. *C-Side* refers to the cytoplasmic side and *m-side,* the matrix side of the inner membrane. I and II are core proteins I and II respectively; b-562 and b-566 are the two proposed forms of cytochrome b; IV is the cytochrome c_1 and V is the iron-sulfur protein. SD_I and SD_{II} are the subunits of succinate dehydrogenase; the two Q's represent the ubiquinones associated with complex III (131).

Neurospora indicated that the bulk of cytochrome b was localized in the interior of the membrane as this subunit was almost inaccessible to the probe (133) while Di jeso et al. (134a) and Chen et al. (134b) using cytochrome b specific antibodies to inhibit electron transport concluded that the subunit was located near the outside of the inner membrane. Electron spin resonance studies with piegon heart mitochondria indicated that the hemes of the dimeric b are localized on the cytoplasmic side of the membrane (135). Hydrophobic photo-labeling with lipid analogs demonstrated that cytochrome b was in contact with lipids; part of core protein I was also exposed to lipids while very little core protein II was present at the lipid -protein boundary of the enzyme complex (136). This suggests that core protein II is buried within the complex through protein-protein type of interactions. Smith et al. through cross linking studies found that the two core proteins lie next to each other while the ISP is adjacent and peripheral to cytochrome c_1 (137, 138). Cytochrome c_1 appears to be ampipathic, with its hydrophilic domain exposed on the cytoplasmic side of the membrane, since it is labeled by both hydrophilic and hydrophobic probes and it binds cytochrome c (139, 140). The topology of the smaller subunits is not very clear due to the large variation in the number and the molecular weight of the species. However, DABS labeling studies (120, 131) along with chemical cross linking data suggest (138) that two lower molecular weight species, 17 KDa and 15 KDa, are exposed on the cytoplasmic side of the membrane.

Recent immunoinhibition studies of cytochrome c reductase activity using antisera aginst complex III, the ISP protein and core proteins I and II by Sidhu et al.,1983 (132), suggest a transmembraneous orientation of the

complex with the antigenic site of ISP exposed on the cytoplasmic side. Immunoprecipitation studies of SDS and Triton-X solubilized mitochondria with the specific antisera indicated that cytochrome b, c_1 and core protein I are tightly associated in complex III while the iron-sulfur protein and core protein II are loosely associated with the other subunits of the complex such that they are dissociated by low concentrations of the detergent (132).

2. Biogenesis of complex III

Considerable interest in the study of the biogenesis of complex III has been generated as only one subunit, cytochrome b, is made by the mitochondrial protein synthesis machinery, the remaining 6-7 subunits being made on the cytoplasmic ribosomes (reviewed in section B). The complex thus serves as an ideal system to gain information on the nucleo-cytoplasmic interactions. It has been shown that 5 of the nuclear encoded subunits of complex III are encoded as longer precursors in the yeast, the extensions ranging from 0.5 to 9 KDa (Table IV) (141-147). The precursors are transported into the mitochondria in a post translational event and cleaved to the mature form by a protease located in the matrix (reviewed in section D). Cytochrome c_1 and the ISP are first cleaved into an intermediate form before the final mature form is reached (141, 142). The intermediate form of cytochrome c_1 is converted to the mature cytochrome c_1 by covalent linkage of heme to the polypeptide accompanied by cleavage by another unknown protease (145). Thus heme deficient mutants fail to show the mature form. Cytochrome b does not seem to be synthesized in a precursor form. Comparisons of the gene and amino acid sequence of cytochrome b indicated that the sequence of the mature

protein begins with the initiator methionine (53).

The nuclear genes for five of the subunits, core protein II the ISP and three smaller molecular weight subunits of mass 17 KDa, 14 KDa, 11KDa, have been cloned and characterized by Van Loon et al.,1983 (148, 149). They have shown that the genes are present in single copies in the yeast haploid genome. Furthermore, at least three of them are located on separate chromosomes (73). The rate of assembly of the various subunits into the holoenzyme was recently studied using double labeled yeast cells, followed by immunoprecipitation of complex III by Sidhu et al. (142). Such studies indicated that cytochromes b and c_1 and the 15 KDa subunit were the first polypeptides to be assembled into the mitochondria while core protein I and the ISP were inserted more slowly. Calculations of the precursor pool sizes of the subunits showed that both core protein I and the ISP had large precursor pools. Thus the subunits of the complex are assembled at very different rates. Studies by Van Loon et al. indicated that import of the subunits into the mitochondria is not coupled to the assembly of the complex. Thus overproduction of one subunit, e.g., the 11KDa or core protein II, by reintroduction into the cell the gene coding for either of these proteins on a free replicating plasmid, did not influence the synthesis or steady state concentrations of the other subunits; the overproduced subunit could be located in the mitochondria (150).

The control of expression of mitochondrial cytochrome b gene by nuclear gene products has been reviewed in section B.3. It was thought that it would be interesting to explore the role of the mitochondrially encoded subunit on the assembly of the nuclear encoded subunits in the complex III. The following section discusses the scope of the thesis in detail.

F.SCOPE OF THE THESIS

Mutants of yeast having a lesion either in the mitochondrial or the nuclear gene have proved to be very useful not only in delineating the contents of the two genomes and their products but also in studying different aspects of regulation and coordination of mitochondrial biosynthesis. Box mutants of yeast that lack cytochrome b or synthesize partial fragments of it have been used in this study. By studying the assembly of complex III into the mitochondrial membrane of these mutants the role of cytochrome b in the processing and assembly of the nuclear encoded subunits has been elucidated. Some of the goals of this dissertation were to :

1. Characterize complex III in these mutants; whether the subunits are present or absent, if present then in what amounts relative to the wild type.
2. Investigate whether the subunits are bound firmly to the mitochondrial membrane in the mutants.
3. Investigate the kinetics of subunit protein synthesis and their assembly into the inner membrane by *in vivo* pulse and chase studies with radioisotopic tracers in the mutants and the wild type and compare them.
4. Examine the mRNA levels and compare rates of protein synthesis in mutants and wild type.
5. Examine the turnover of the subunits in the mutants and the wild type by double label analysis.
6. Study import of *in vitro* synthesized subunits into isolated mitochondria from mutant and wild type cells in presence of energy to confirm observations obtained with *in vivo* experiments and conclude

what role cytochrome b plays in the processing of the subunits and their assembly into the complex.

Chapter II

EXPERIMENTAL PROCEDURES

A. YEAST STRAINS

Mutants lacking cytochrome b, viz., W-267 (Box6 -2), G-392 (Box5-1), G-1334 (Box 4-1), G-2286 (Box 1-2) and G-1988 (Box 1-1) were obtained from the Slonimski collection (63). The wild type parent was 777-3A. Spectral and protein analysis further established the nature of the mutants used in this work as cytochrome b^-.

B. GROWTH OF CELLS AND PREPARATION OF MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

The yeast cells were grown at 30^0C in a semisynthetic medium (151) containing 3% galactose supplemented with adenine at 80 mg/l. The semisynthetic medium contained 0.3 % yeast extract, 0.04% $CaCl_2$, 0.05% NaCl, 0.07% $MgSO_4.7H_2O$, 0.1% KH_2PO_4, 0.12% $(NH_4)_2SO_4$ and 0.5 mg % $FeCl_3$.

For the isolation of mitochondria by breakage of yeast cells with glass beads, cells were grown to early stationary phase, harvested and washed once with distilled water and suspended in chilled buffer containing 0.65 M sorbitol, 10 mM potassium phosphate, pH 6.8, 2 mM EDTA, 0.1mM $MgCl_2$, 20 mM KCl, 0.1% bovine serum albumin and 1 mM PMSF. The cells were broken by shaking with glass beads 2 times for 45 sec in a Bronwell shaker (152). Small aliquots of cells were broken by placing them in Eppendorf tubes containing glass beads and using an

Eppendorf adaptor in the shaker. Unbroken cells and cellular debris were removed by centrifugation at 5000 g for 5min and mitochondria were pelleted from the supernatant by centrifugation at 18,000 g for 20 mins. This mitochondria was used for spectral analysis and western blotting.

For isolation of mitochondria from spheroplasts, cells were grown to early log phase (110-130 klett units), harvested, washed once with distilled water and preincubated for 10-15 min at 30^{0}C in a medium containing 100mM Tris-HCl, pH 9.4, containing 2-mercaptoethanol (35µl/ 10 ml buffer). The cells were reisolated and suspended in a medium containing 1.2 M sorbitol / 20 mM KPi, pH 7.4, and 2 mercaptoethanol (35µl/10 ml). Zymolyase 5000, suspended in the same buffer was added to the cell suspension at a concentration of 3-4 mg zymolyase / g cells (wet weight). The mixture was incubated with shaking at 30^{0}C for 40-60 min until the optical absorbance at 800nm (of a 1:100 water diluted cell suspension) was decreased to 10 % of the initial 0 min observation. The spheroplasts thus obtained were isolated by centrifugation at 1500 g, washed twice in the same buffer and resuspended in chilled buffer containing 0.6 M mannitol/20 mM Hepes KOH, pH 7.4, 1mM PMSF, 1mg/ml BSA. The suspension was homogenised on ice by 10-15 strokes in a tight fitting dounce homogenizer. The cellular debris was removed by centrifugation at 1500 g and the mitochondria pelleted from the supernatant by centrifugation at 9000 g. The mitochondria thus obtained was washed twice with the same buffer but without containing BSA and PMSF. This mitochondria was used for import studies (153).

Submitochondrial particles were prepared by suspending the mitochondrial pellet in 0.1mM sodium phosphate buffer, pH7.5, 1mM EDTA and 1mM PMSF and sonicating in three 20 sec bursts using a Branson

sonifier at a power setting of 4.5 (154). The suspension was centrifuged at 8000 g for 10 min and submitochondrial particles were pelleted from the supernatant by centrifugation at 100,000 g for 30 min.

C. SPECTRAL ANALYSIS

Washed mitochondrial pellets were suspended in 0.65 M sorbitol, buffer, pH 6.8 at a concentration of 4 mg mitochondrial proteins /ml . Difference spectra were obtained between 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 was used (129). The samples were scanned between 530 nm and 650 nm.

D. RADIOACTIVE LABELING OF CELLS

Labeling of cells with (^{3}H) leucine

Yeast cells were grown to mid log phase, harvested washed once with water and resuspended at a concentration of 200 mg/ ml in labeling medium . This medium consisted of the salts normally present in the semisynthetic growth medium but the concentration of the galactose and yeast extract were reduced to 0.3% and 0.05% respectively. The cells were incubated at 30^{0} C for 5 min when labeling was initiated by addition of (^{3}H) leucine (400 µci/ml). After 1h the label was chased for 5 min with 2 mM unlabeled leucine, the reaction stopped by chilling at 0^{0} C, the cells were broken with glass beads and mitochondria were prepared (142).

Labeling of cells with (^{35}S) methionine

Yeast cells wre suspended in a low sulfate medium which contained salts, vitamins, trace elements, 0.5 µg/ml sulfate and 0.3% galactose at a

concentration of 200 mg/ml. The cells were then pulsed with 200 uci./mlof (^{35}S) methionine and 5 or15 min later the label was chased with 10 mM unlabeled methionine. At various time intervals after the addition of (^{35}S) methionine an aliquot of cells was removed and the cells broken immediately with glass beads . The broken cells were chilled to 4^{0}C and mitochondria prepared (142).

Cells were also pulse labeled in the presence of CCCP (carbonyl cyanide chlorophenyl methyl hydrazone) to accumulate the precursors to core protein I and the iron sulfur protein (ISP) (89). Yeast cells suspended in low sulfate medium (100mg/ml) were preincubated in 20uM CCCP (ethanolic solution) for 5 min prior to a 5 min pulse with (^{35}S) methionine. The membrane potential was then restored by the addition of 0.05% 2-mercaptoethanol (v/v) and the label chased from 10 min to 60 min with 10 mM unlabeled methionine. Reactions were stopped by the addition of 20% TCA (w/v).

Labeling of cells with (^{35}S) methionine in presence of cycloheximide

For the electrophoretic analysis of mitochondrial translation products, cells were incubated for 5 min at 30^{0}C in low sulfate medium . Cycloheximide (200µg/ ml) was added and the incubation continued for another 5 min with shaking . (^{35}S) methionine (200µci/ml) was added and the reaction stopped after 30 min by addition of 10 mM methionine and chilling to 4 ^{0}C . Mitochondria were prepared as described above by breakage of cells with glass beads.

Double labeling of Cells

Yeast cells were also double labeled. Cells were first uniformly labeled with (^3H) leucine by including 4 mCi of label per litre of growth medium. Cells were harvested in the mid log phase, resuspended in low

sulfate medium (200mg/ml), and then pulsed with (^{35}S) methionine (200 uci/ml) for 30 min. The label was chased with10 mM unlabeled methionine and at various time points after the chase an aliquot of cells was removed, cooled on ice, cychloheximide (100µg/ml) and PMSF (1mM) added and the cells broken immediately with glass beads for mitochondrial preparation.

E. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND AUTORADIOGRAPHY

SDS- PAGE was performed on analytical slab gels (12-15cm. long, 1.2mm thick) using the discontinuous SDS-Tris buffer system of Lammli (155). The running gels were either 10, 12.5 or 15%, while the stacking gel was 5% arcylamide. The molecular weight standards used were obtained from Pharmacia and contained phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soyabean trypsin inhibitor (20,100) and α-lactalbumin (14,400). Gels were stained for 1 hr. 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 dissociation buffer for solubilizing proteins for electrophoresis contained 50 mM Tris- Hcl, pH 6.8, 10 % glycerol, 2 mM EDTA, 5% SDS, 5% 2 mercaptoethanol and 1 mM PMSF (131).

For fluorography of gels containing radioactive samples the method of Chamberlain was used (156). The gels were stained with Coomassie blue, destained and soaked in water for 30 mins to remove the acetic acid, thus preventing possible precipitation of sodium salicylate. The gels were then soaked for 30 min in 1M sodium salicylate, dried and exposed to Kodak NS-5T X-ray film at -70^{0}C.

For a quantitative determination of radioactivity in a band the

location of the band in the gel was determined from the autoradiogram. The corresponding position was excised from the gel and digested in 0.5ml of 30 % H_2O_2 at 65^0 C for 36 hrs (142). Liquiscint was then added and the vials counted in a Beckman scintillaiton counter.

F. WESTERN BLOTTING (IMMUNOBLOTTING)

Protein determination of mitochondrial or submiochondrial particle samples was done by the method of Lowry et al. (157), using bovine serum albumin as standard. Approximately 200 µg of mitochondrial proteins from wild type and mutant cells were loaded into each lane of a 10 or 12.5% slab gel and electrophoresis performed. The proteins were then transferred electrophoretically onto nitrocellulose paper by the method of Towbin et al (158). The transfer buffer contained 120 mM glycine, 24.8 mM Tris and 20% methanol (pH 8.4). The total transfer time was 2 h and the voltage used was 50 mV for the first and 100mV for the second hour.

The subunits of complex III were visualized on nitrocellulose paper by double antigen -antibody labeling, using horse radish peroxidase coupled to goat anti-rabbit (GAR-HRP) as the second antibody. The blotted nitrocellulse paper was first incubated for 30 min, with gentle shaking, in blocking solution containing 3% gelatin in TBS (20mM Tris, 500mM NaCl, pH 7.5). It was then immuno-incubated with monospecific antibody (at a dilution of 1:50) in 1% gelatin TBS . After incubation for 16 h, the paper was rinsed with distilled water, washed twice, 10 min each time, in TBS and Tween TBS (TBS containing 0.05% Tween 20) and then incubated in the second antibody solution which contained 20 µl of GAR-HRP conjugate in 60 ml of 1% gelatin TBS (1:3000 dilution). After incubation for 1h, the

membrane was washed twice, as before, in TBS and Tween TBS and immersed in freshly made HRP color development solution. Purple bands became visible within 5-15 min. The color development solution was prepared by adding 60 mg of HRP color developer (4 chloro-1-naphthol) in 20 ml ice cold methanol to 60 µl of ice cold 30 % H_2O_2 in 100 ml TBS, all the mixing being done at room temperature. For a quantitative determination of the amount of complex III suibunits present in each mutant, the nitrocellulose paper was dipped in paraffin oil and scanned in an E-C apparatus densitometer (EC910).

G. ISOLATION OF TOTAL RNA

Total yeast RNA was isolated by the method of McAda and Douglas from wild type yeast strain 777-3A (159). The growth medium was a semisynthetic medium containing 2% lactate,1% glycerol and adenine 80mg /l. Total yeast RNA was also prepared from the mutant strain Box 6-2 and the parent 777-3A when the growth medium contained 3% galatose instead of lactate and glycerol. Cells were grown to early log phase (110-160 Klett units). The culture flasks were transferred to an ice-bath and rapidly cooled for 15 min prior to harvesting at 4^0C. The pellet was washed once with ice cold water and resuspended to 10ml/gm wet weight at room temperature in 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5% SDS (NETS) and rapidly frozen by pouring liquid nitrogen into the suspension. The frozen pellet was crushed to a fine powder in a cooled sterile blender, the powder thawed at 30^0 C in the presence of phenol-chloroform-isoamyl alcohol (50:50:1) with rapid shaking for 20 min. After centrifugation to resolve the two phases the upper aqueous phase was saved and the lower phenol layer was washed twice more with

NETS buffer. The aqueous layers from each washing were pooled and nucleic acids precipitated by the addition of 0.11 volume of 2 M acetate, pH 5.2 and 3 volumes of ice cold 100% ethanol at -20^0 C for 16 hrs. The precipitated nucleic acids were pelleted, dried *in vacuo,* dissolved in 5-10 ml water and RNA precipitated by the addition of an equal volume of 6M LiCl at -20^0 C for 3-4 hr. The LiCl precipitated RNA was pelleted by centrifugation, resuspended in 0.2M sodium acetate, pH 7.5 and after two additional precipitation with 75 % ethanol, stored at -80^0C in sodium acetate and ethanol mixture.

H. PROTEIN SYNTHESIS *IN VITRO*

The RNA was translated in a cell free protein synthesizing system of rabbit reticulocyte lysate obtained from New England Nuclear (160). The amount of RNA required to give maximum protein synthesis was determined for each preparation (161). Starting from 0 time to the end of translation 3µl samples were withdrawn from the tube containing translation mixture with added mRNA at different concentrations or control tube containing no mRNA, and added to 0.5 ml of 1M NaOH / H_2O_2 mixture. The mixtures were incubated at 37^0C for 10 min and then 2 ml of ice cold 25 % trichloroacetic acid was added and the samples left on ice for 1h. The precipitated proteins were filtered through glass fibre discs (Whatman GF/A), washed with 8% TCA, dried in an oven and counted using a toluene scintillant. Thus each 125 µl of reaction mixture contained 50 µl lysate, 1.25 -2 A_{260} O.D units of total yeast RNA, 225 µci of (^{35}S) methionine, 100mm KCl, 1 mM $MgCl_2$, 7.5mg creatine Kinase, 10mM creatine phosphate to give optimal protein synthesis. Protein synthesis was

allowed to proceed for 60 min, at 37^{0}C at the end of which the reaction was rapidly cooled to 0^{0}C. For immunoprecipitation of the precursors the mixture was dissociated with 3% SDS at 95^0 C.

I. IMPORT OF LABELED PROTEINS INTO MITOCHONDRIA

For *in vitro* import of radiolabeled proteins into mitochondria, protein synthesis was stopped by cooling to 4 ^{0}C and centrifugation at 140,000g for 45 min. The supernatant was used for import assays. Mitochondria were isolated from wild type and mutant strains as described previously from spheroplasts. The structural integrity of the mitochondria prepared was examined by measuring respiratory control in an oxygraph (162).

The *in vitro* import assay contained 125-150 ul of translated lysate (approximately 3-4 x 10^6 CPM of TCA insoluble material), which had been centrifuged and adjusted to 0.6 M mannitol-20 mM Hepes /KOH, pH 7.4, 1mM ATP, 1mM $MgCl_2$, 5 mM phosphoenol pyruvate, 4 units of pyruvate kinase, 1mM DTT and 300 ug of mitochondrial protein in a final volume of 0.4 ml. GTP, 5mM, was also added to inhibit the matrix located protease which leaks out from the mitochondria during the incubation (153). The mixture was incubated for 2 min to 60 min at 30 ^{0}C with shaking. The import was terminated by chilling to 4^0 C and adding 1 mM PMSF and 1 mM TLCK. Mitochondria was reisolated by centrifugation at 10,000 g for 10 min, the pellet washed once with 0.6 M mannitol buffer, and then both the pellet and the supernatant were dissociated in 3% SDS for 3 min at 95^{0}C.

J. IMMUNOPRECIPITATION

After dissociation in SDS, supernatants from *in vitro* import assay were mixed with 300 ug of SDS dissociated unlabeled mitochondrial protein. This ensured that all samples subjected to immunoprecipitation contained same amount of mitochondrial antigen (153). Mitochondria obtained from *in vivo* pulse labeling of cells were suspended to 3mg/ml in 0.1M Na-phosphate, pH 8, and dissociated in 3% SDS, 1mM EDTA, 1mM EGTA pH7.4 at 95 ^{0}C for 5min. The protein pellets obtained from centrifugation of CCCP labeled cells (5000 g for 10 min) were dissolved in 3-4 volumes of sample buffer containing 4% SDS, 0.15 M Tris, pH 7.5, 5 mM EDTA, and 0.1% bromophenol blue (89). After neutralization with 1M tris base, the solution was heated for 5min at 95^{0}C. To all the SDS dissociated samples, buffer containing 0.1 M Tris, pH 7.4. 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 1mM PMSF was added such that the final concentration of SDS was reduced to 0.2%. The samples were then centrifuged at 100,000g and incubated with respective antiserum, obtained earlier in this laboratory (130) for 16 h at 4^{0}C. Sepharose- bound protein A (10-15 mg dry weight pre swollen in 150 ul of 50 mM Tris-HCl, pH 7.4,150 mM NaCl) was then added and the mixture incubated with shaking for one more hour at room temperature. The Sepharose beads were collected by centrifugation and washed three times with 50 mM Tris- HCl, pH 7.4, 150 mM Nacl, 2 mM EDTA and 0.2 % Triton X-100 (TNET) and twice with the same buffer without Triton X-100. In the case of *in vitro* experiments the Sepharose beads were washed twice with TNET buffer, once with the same buffer with concentration of NaCl raised to 750 mM and twice more with TNET buffer without Triton X-100 as for *in vivo* experiments.

Washed Sepharose bound immunoprecipitates were suspended in 40 ul of dissociation buffer and boiled for 5 min. The beads were removed by centrifugation at 15,000 g and the supernatants were electrophoresed on analytical slab gels and autoradiography performed as described previously or counted directly in liquiscint.

K. IMMUNOTITRATION

Mitochondria prepared from (^{3}H) leucine labeled cells (from both mutants and the wild type parent) were suspended at 1.5 mg/ml in 0.1M sodium phosphate, pH 8, 1mM PMSF and solubilized by incubation in 1% Triton X-100 at 4^{0} C for 1 h. The unsolubilized membranes were removed by centrifugation at 100,000 g for 45 min. Increasing amounts of complex III antiserum were added to 75 µl aliquots (1.5 mg/ml) of the mitochondrial extracts and immunoprecipitation performed as described above and the washed and dissociated immunoprecipitates were counted directly in liquiscint (142).

L. MATERIALS

L-(^{35}S) methionine (>800 Ci $mmol^{-1}$), and nuclease treated reticulocyte lysate were obtained from New England Nuclear. (^{3}H) leucine (55ci. $mmol^{-}$) was purchased from Amersham. SDS, PMSF, 2-mercaptoethanol, CCCP, pruvate Kinase, coomassie brilliant blue and Triton X-100 were purchased from Sigma. Protein A-Sepharose was from Pharmacia. Zymolyase 5000 was from Miles lab., Inc. Yeast extract and yeast nitrogen base (without amino acids) were from Difco. N, N' methylene -bis -acrylamide, GAR-HRP, HRP colour developer, gelatin and Nitrocellulose filters were purchased from Biorad. Acrylamide was from

serva. Glass beads were from VWR. All other chemicals were of the highest purity commercialy available .

The yeast strains were from the Slonimski collection. The antisera to cytochrome c_1 was a generous gift of Dr. Gottfried Schatz (Biozentrum, Basel, Switzerland).

Chapter III

RESULTS

A. ABSENCE OF CYTOCHROME B IN THE MUTANTS

Prior to an examimation of the various subunits of complex III in the cytochrome b^- mutants, it was necessary to establish that cytochrome b was indeed missing in these mitochondria. Two different approaches, one involving spectral analysis and the other an examination of the products of mitochondrial protein synthesis, were employed.

1. Differance spectra of Mitochondria from the wild type and mutant cells

Each of the strains, the wild type 777-3A, and the mutants, Box6-2, Box 5-1, Box4-1 and Box 1-2 were grown in galactose medium as described under experimental procedures to early stationary phase and were harvested at an identical cell density. The difference spectra, of these mitochondria (dithionite-reduced minus ferricyanide oxidized) revealed the presence of cytochrome b with an absorption maximum at 562 nm in the wild type but indicated the complete lack of any material absorbing at this wave length in the mutants (Fig. 4). Cytochrome a-a_3 and cytochrome c with an absorption maxima at 603 nm and 552 nm respectively, were present in all the mutants.

2. Mitochondrial translation products in cytochrome b^- mutants

Previously it was reported that mutants lacking any spectrally detectable cytochrome b did not synthesize any apoprotein of cytochrome b but instead synthesized novel peptides antigenically related to cytochrome b in some mutants (49, 51, 58-60). The 4 mutants plus the wild type cells were labeled with (^{35}S) methionine in the presence of sufficient

cycloheximide to block cytoplasmic protein synthesis so that the products of mitochondrial protein synthesis could be observed. When mitochondria containing equal amounts of radioactivity were loaded on both 10 and15 % acrylamide gels, the absence of labeled bands corresponding to the apoprotein of cytochrome b in the 4 mutants were clear (Fig. 5). It should be noted that in the 10% acrylamide gel cytochrome b, in the wild type mitochondria, migrates with an apparent molecular weight of 30,000 while in the 15 % acrylamide it migrates with an apparent molecular weight of 34,000, due to the well established anomalous migration behavior of the proteins in SDS gels (152). It is also clear from Figure 5 that mutant Box 6-2 synthesized a novel peptide of 27,000 Da, while Box 1-2 synthesized one of 18,500 Da confirming earlier reports (42). No novel polypeptides were observed in the mitochondria from either Box 4-1 or 5-1, although the latter showed a decreased synthesis of subunit 1 of cytochrome oxidase. The results of all these experiments indicated that none of these mutants synthesize the mature form of cytochrome b.

B. DETECTION OF CYTOCHROME C_1 IN THE CYTOCHROME B LACKING MUTANTS

1. Spectral analysis

Examination of the spectra of Figure 4 indicated that the mitochondria from the mutants contained c type cytochromes with an absorption maximum at 553 nm. Calculation of the amount of these cytochromes per gm of mitochondrial protein using the extinction coefficient for cytochrome c_1 revealed that the mutant had comparable amounts of this hemeprotein with the exception of Box 1-2 in which the cytochrome $c_1$content was decreased 26 % (Table 1).

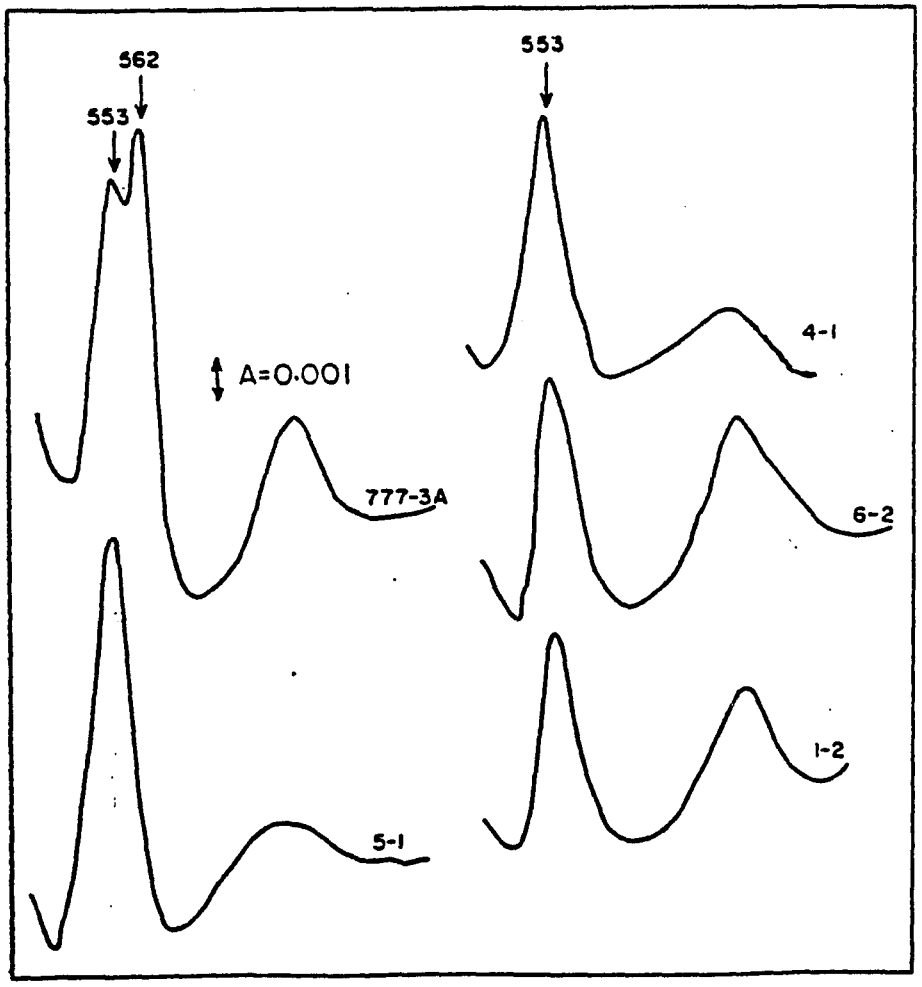


Figure 4. Difference Spectra of Mitochondria from the Wild Type and Mutant Yeast Strains. Difference spectra of the cytochrome b mutants and the wild type parent were obtained using dithionite reduced mitochondrial suspension containing 4 mg protein per ml against ferricyanide oxidized samples.

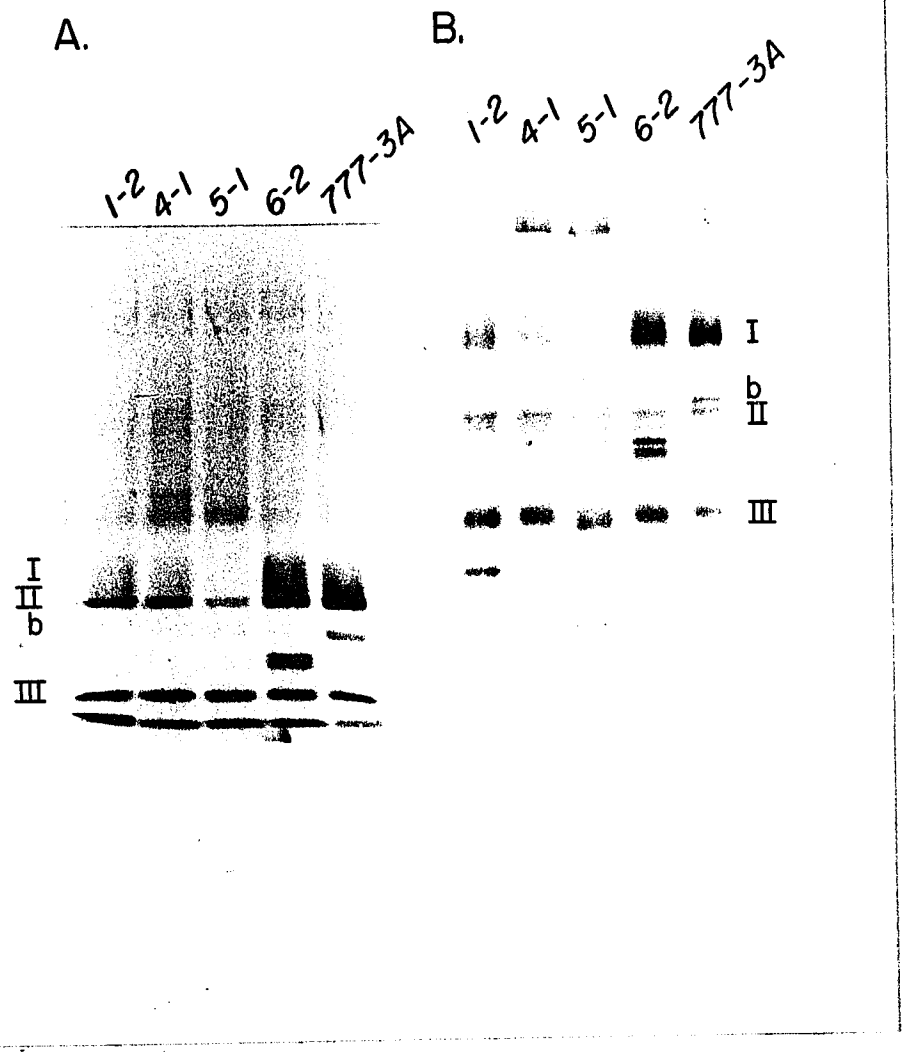


Figure 5. Mitochondrial Translation Products in Cytochrome b⁻ Mutants
Cells were labeled with (^{35}S) methionine in the presence of cycloheximide, as described under experimental procedures and the mitochondria analyzed on either 10% (A) or 15 % (B) polyacrylamide gels. Each lane contained 50,000 CPM of dissociated mitochondria.

TABLE I

CYTOCHROME CONTENT OF YEAST STRAINS

Strains	Cytochrome b n moles/mg	Cytochrome a - a_3 n moles/mg	Cytochrome c + c_1 n moles/mg
777-3A	0.1269	0.0293	0.0957
Box 6-2 (W-267)	0	0.0293	0.0837
Box 5-1 (G-392)	0	0.0097	0.1196
Box 4-1 (G-1334)	0	0.0195	0.0837
Box 1-2 (G-2286)	0	0.039	0.071

Note: The cytochrome content of the mutants 6-2, 5-1, 4-1 and their wild type parent 777-3A was compared in mitochondrial samples from these strains, as described in the legend to figure 4. To quantitate the content of cytochrome $c+c_1$, cytochrome b and cytochrome oxidase the molar extinction coefficients, 20 $mM^{-1}Cm^{-1}$, 25.6 $mM^{-1}Cm^{-1}$ and 12 $mM\,Cm^{-1}$ were used respectively.

2. Immunoblotting and Immunoprecipitation analysis

In a second approach, the antibody against cytochrome c_1 was used in both the immunoprecipitation and immunoblotting experiments to confirm the presence of this protein in the cytochrome b lacking mutants. The gels of the immunoprecipitate indicate that the antibody used was specific for cytochrome c_1, as only one band was present in the gel (Fig. 6). To quantitate the amount of the immunoprecipitate the gels were scanned in a densitometer; however, to avoid possible inaccuracies in scanning which might result because the radioactive band in the immunoprecipitate spread on the gel, the radioactive band was also excised from the gel and counted. Such an analysis indicated that more radioactivity due to cytochrome c_1 was immunoprecipitated from mutants Box 6-2 and 5-1 than from the wild type, while 18 and 49% decreases were observed in mutants Box 4-1 and 1-2 respectively (Table II).

Immmunblotting experiments were also performed using an antibody raised against the intact complex III and again indicated that the apoprotein of cytochrome c_1 was present in the mutants (Fig. 7). In addition, it should be noted in the immunoblot using the antibody against complex III that cytochrome b (subunit III) was present in the wild type (777-3A) but absent in all of the mutants. The immunoblots were quantitated by densitometer scans of the nitrocellulose paper made translucent by dipping in paraffin oil and integrating the area under the curve (Table III). The amounts of cytocrome c_1 in the 4 mutants appeared to be slightly decreased relative to the wild type but interestingly the

decreases correlated well with the results obtained by spectral analysis (Table 1). The results of these three experiments indicate that cytochrome c_1 is present in the same or only slightly decreased amounts in the mutants lacking cytochrome b as compared to the wild type cells.

C. DETECTION OF CORE PROTEIN I, CORE PROTEIN II AND THE IRON SULFUR PROTEIN IN MUTANTS LACKING CYTOCHROME B

Subunit specific antisera against subunts I, II, and V were also used in both immunoprecipitation and immunoblotting experiments to establish the presence of these subunits in mitochondria lacking cytochrome b. Again, it should be stressed that all of the subunit specific antisera used in these experiments immunoprecipitated a single polypeptide with molecular weight corresponding to the protein used as the antigen (130,142). Quantitation of the immunoprecipitates of core protein I, by excising and counting the labeled band as described above for cytochrome c_1, indicated that the amount of this protein in the mutants was decreased 50 % compared to the wild type (Figure 6, Table II). This same antiserum in the immunoblotting experiments reacted nonspecifically with some other proteins on the gel (data not shown); however, densitometer scanning of the nitrocellulose paper confirmed the lowered amounts in the mutants of a protein with a molecular weight of 49,000 corresponding to core protein I (Table III).

Analysis of the mitochondria from the mutants lacking cytochrome b with the antiserum against core protein II using both the immunoprecipitation and immunoblotting techniques revealed that the amounts of this protein were decreased even more than 50% in the mutants

relative to the wild type cell (Figure 6, Table II, Figure 7, Table III). Mutant Box 6-2 appeared to be the most severely affected of the mutants as only 8% of core protein II was present in this strain compared to the wild type. It should be noted that the higher molecular weight band observed in Fig. 6 in the immunoprecipitates of the wild type 777-3A was a contaminant which was eliminated in subsequent experiments.

Similarly decreased amounts of the iron-sulfur protein (subunit V) were observed in the 4 mutants relative to the wild type using both the immunoprecipitation and immunoblotting approaches. While complete agreement in the calculated decreases was not achieved in the two methods, it is clear that significantly decreased (30-64%) amounts of this protein are present in the mutant mitochondria lacking cytochrome b (Figure 6, Table II; Figure 7, Table III).

D. DETECTION OF COMPLEX III BY IMMUNOTITRATION OF MITOCHONDRIAL EXTRACTS FROM CYTOCHROME B^- MUTANTS

Lowered amounts of complex III in the mutants lacking cytochrome b were also observed in immunotitration curves obtained with detergent solubilized mitochondria. Yeast cells were grown in (^{3}H) leucine and their mitochondria solubilized with 1% Triton X-100 prior to treatment with increasing amounts of antiserum against complex III. The immunoprecipitation curve indicated the presence of less than 25 % of complex III protein in the mutants compared to the wild type cells (Fig. 8).

E. DETECTION OF SUBUNITS OF COMPLEX III IN SUBMITOCHONDRIAL PARTICLES FROM WILD TYPE AND CYTOCHROME B^- MUTANTS

In order to determine whether the subunits of complex III are firmly

bound to the mitochondrial membrane in the mutants lacking cytochrome b, the mitochondria were suspended in phosphate buffer and sonicated to obtain submitochondrial particles (SMPs). The presence of different subunits of complex III was tested by immunoblotting experiments using antisera against the individual subunits and against complex III. It should be noted that a comparison of mitochondria and SMPs from the wild type indicated that sonication did not cause any decrease of the subunit proteins of complex III from the mitochondrial membrane. By contrast the gels of Fig. 7B and the quantitated scans (Fig. 9, Table III) indicated that sonication resulted in even greater decreases in the amounts of core protein I, core protein II and the iron sulfur protein present in the membrane of the SMPs as compared to the starting mitochondria from the mutants. The immunoblots with the antiserum against complex III, however revealed that there was absolutely no loss in cytochrome c_1 in the mutants after sonication (Fig. 7, Table III) . These results suggest that in the absence of cytochrome b in the mitochondrial membrane, core proteins I and II as well as the iron sulfur protein can be released by sonicaton, while cytochrome c_1 is more tightly bound to the membrane and hence not released.

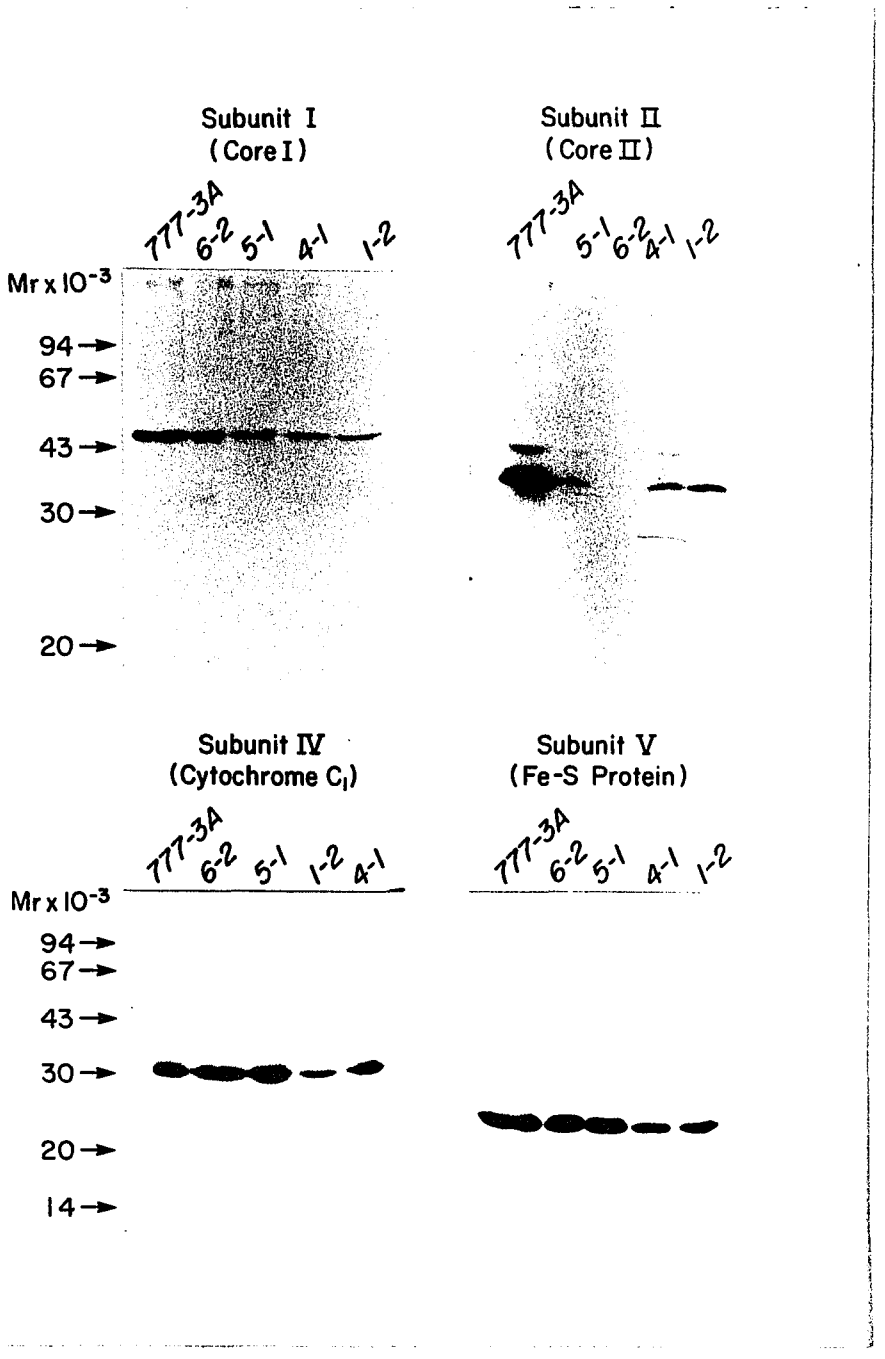


Figure 6. Immunoprecipitation of Subunits I, II, IV, and V of Complex III. Mitochondria from equal aliquots of (^{35}S) methionine labeled cells (for subunits I, IV and V) or (^{3}H) leucine labeled cells (for subunit II) were immunoprecipitated with their respective antiserum prior to electrophoresis on 10 % gels (subunits I and II) or 12 % gels (subunits IV and V) and slab gel autoradigraphy.

TABLE II

SUBUNIT COMPOSITION OF COMPLEX III IN MITOCHONDRIA FROM WILD TYPE AND CYTOCHROME B^- YEAST STRAINS DETERMINED BY IMMUNOPRECIPITAION

Yeast strains	Core protein I		Core protein II		Cytochrome c_1		Iron-Sulfur protein	
	CPM	%	CPM	%	CPM	%	CPM	%
777-3A	1376	100	3174	100	2855	100	4465	100
Box 6-2	698	50	252	8	3641	127	2857	64
Box 5-1	669	48	603	19	3742	131	2907	65
Box 4-1	690	50	952	30	2327	81.5	2036	45
Box 1-2	685	49	1174	37	1456	51	1730	39

Note: The location of subunits I, II, IV and V on the gel was determined from the autoradiogram on figure 6. The corresponding areas were excised from the gel, digested in 30% H_2O_2 and counted (142). The counts in 777-3A were taken as the 100 % value.

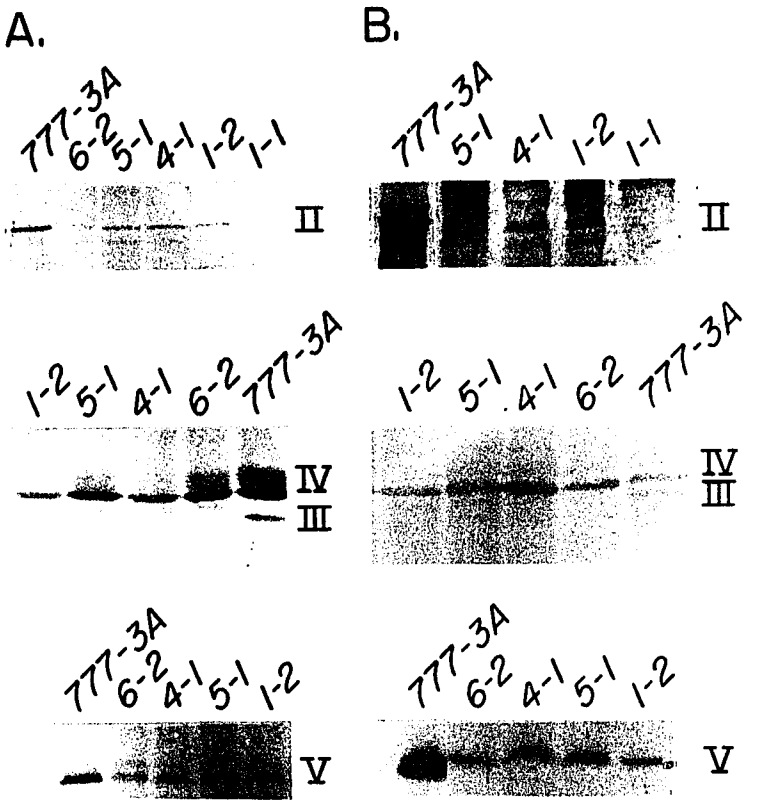


Figure 7. Immunoblotting Analysis of the Subunits of Complex III in Mitochondria and Submitochondrial Particles by Double antibody Labeling. Samples of mitochondria or SMPS prepared from the cytochrome b^- mutants Box 6-2, Box 5-1, Box 4-1, Box1-1, and Box 1-2 and their wild type parent 777-3A were dissociated overnight in buffer containing 0.05M Tris-HCl, pH 6.8, 10% glycerol, 2mM EDTA, 5% SDS and 2-mercaptoethanol. The separated proteins were transferred to nitrocellulose paper by the method of Towbin et al (156). By double antibody labeling using antiserum to either subunit II or V or to the entire complex III the antigens were stained in the blots as described under experimental procedures. (A) Mitochondria (B) Submitochondrial particles. Mitochondrial or SMPs (200 µg) were loaded in the respective lanes.

TABLE III

SUBUNIT COMPOSITON OF COMPLEX III IN MITOCHONDRIAL AND SUB-MITOCHONDRIAL PARTICLES FROM WILD TYPE AND CYTOCHROME B^- YEAST STRAINS BY IMMUNOBLOT ANALYSIS.

Yeast	Core protein I		Core protein II		Cytochrome c_1		Iron-Sulfur	
strains	Mito.	SMP	Mito.	SMP	Mito.	SMP	Mito.	SMP
777-3A	100	100	100	100	100	100	100	100
Box 6-2	N.D.	N.D.	8	a	86	95	41	20
Box 5-1	50	10	38	10	83	100	63	24
Box 4-1	44	10	35	11	76	97	52	23
Box 1-2	21	5	35	2	74	90	46	17

Note: Densitometer scans of the immunoblots shown in Fig.7 (A, B) were prepared after the papers were made translucent by dipping in paraffin oil. The area under the peak corresponding to the respective subunits of 777-3A was taken as the 100 % value. The relative percentage of the subunits present in each mutant was calculated by comparing them to the wild type. N.D. = not detected; a = not determined.

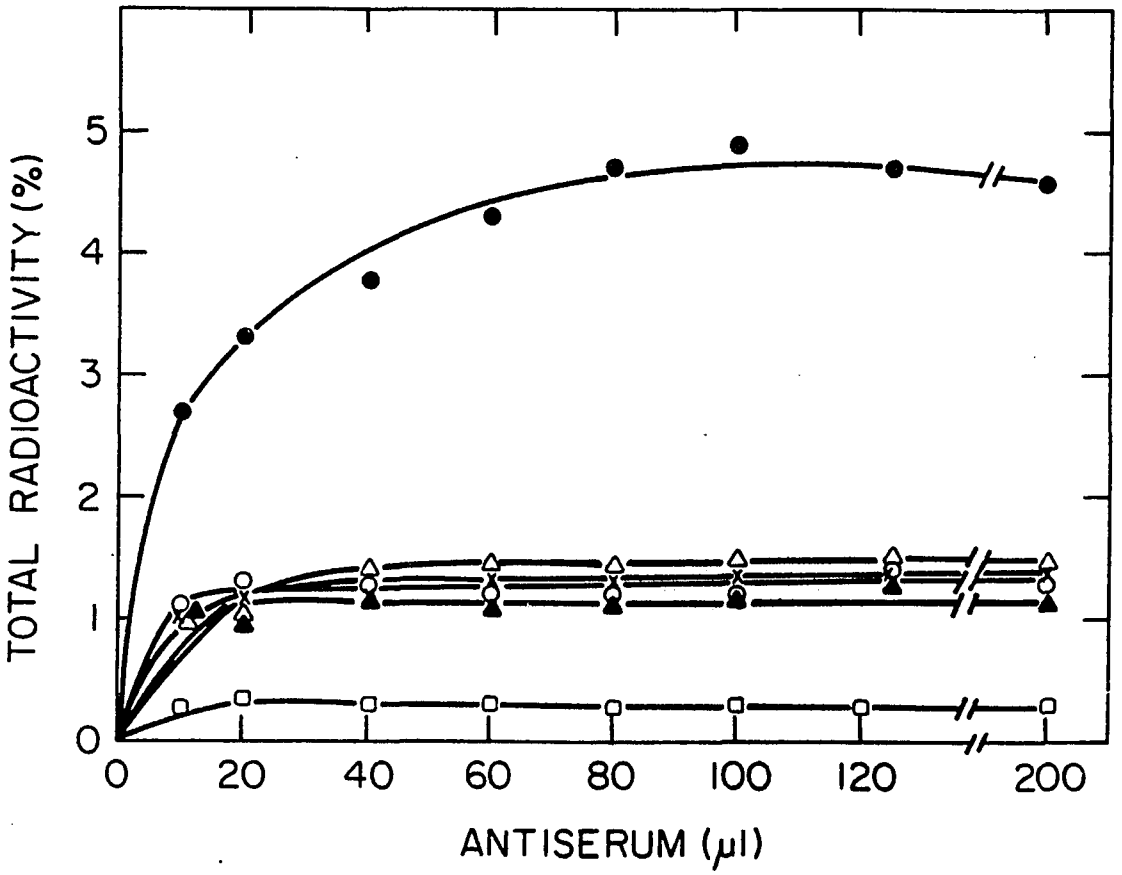


Figure 8. Titration of Mitochondrial Extracts with Antiserum against Complex III. Mitochondria were prepared from cytochrome b^- mutants and their parent 777-3 A cells which were labeled with (^3H) leucine and extracted with 1% Triton x-100 and centifuged for 100,000 x g for 45 min. Increasing amounts of antiserum were added to 75 µl aliquots of the mitochondrial extracts and the precipitates counted. ●——● 777-3A; Δ——Δ 1-1; o——o 4-1; ■——■ 6-2; ✕——✕ 3-2; □——□ control.

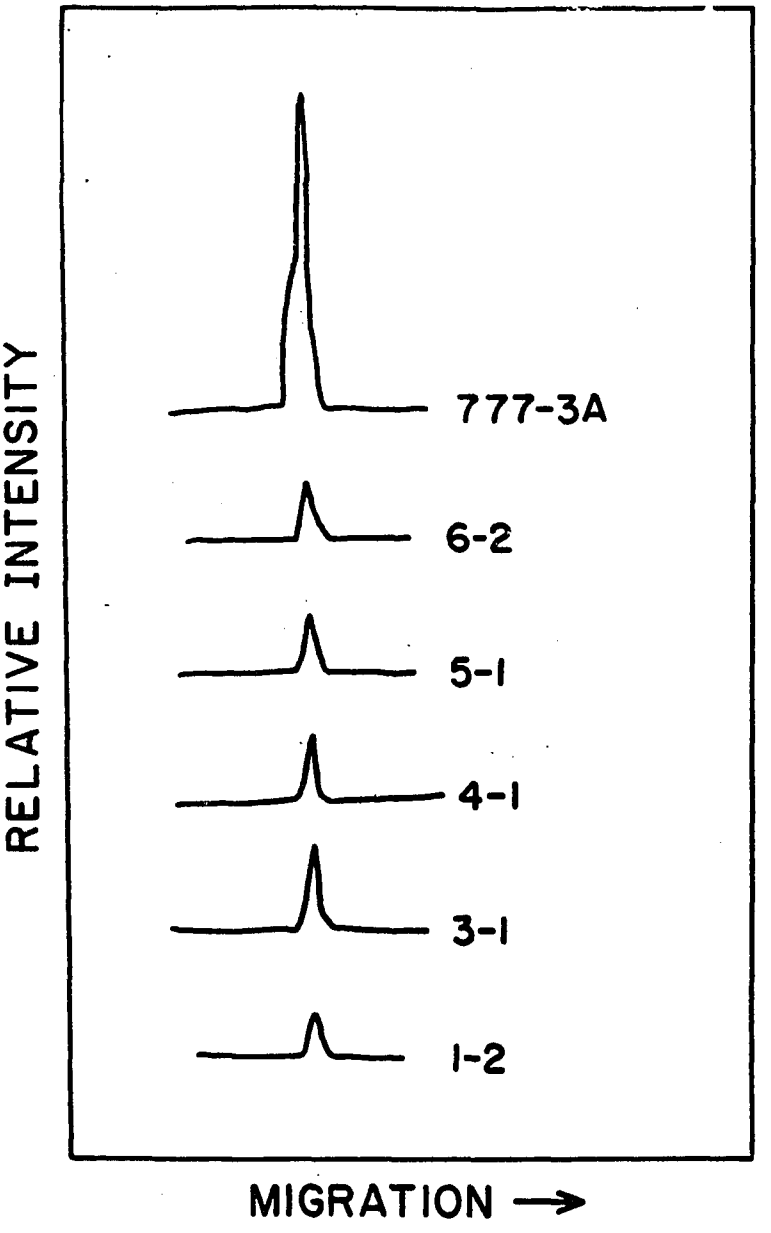


Figure 9. Immunoblotting Analysis of Subunit V of Complex III in Submitochondrial Particles. SMPs (200 µg protein) were analyzed on 12.5 % gels and transferred to nitrocellulose paper; subunit V specific antiserum was used in the double antibody labeling method as described under legend to figure 7. The immunoblot was scanned in a densitometer after dipping in parraffin oil to make it translucent.

F. ANALYSIS OF THE PRECURSORS OF CORE PROTEIN I AND THE IRON SULFUR PROTEIN

The cytochrome b deficient mutant Box 6-2 was chosen for the next series of studies as this mutant contained significantly lowered amounts of core proteins I and II as well as the iron sulfur protein. Initially it was necessary to establish that the synthesis of the precursors was not hindered in the mutant leading to the lowered amounts of these proteins in the membrane. It has been shown in both yeast (89) and *Neurospora crassa* (144) that CCCP, an uncoupler of oxidative phosphorylation, blocks the import of several cytoplasmically synthesized subunits of complex III, resulting in an accumulation of the precursor form of these proteins. In the present study both mutant Box 6-2 and the wild type parent 777-3A were pulse labeled for 10min with (^{35}S) methionine in the presence of CCCP. The immunoprecipitates, obtained using subunit specific antiserum against core protein I, when analyzed on 10 % gels revealed that the precursor to core protein I, which is about 500 Da larger than the mature protein (143), accumulated in similar amounts in both strains (Fig 10, lane 1 & 4). When the membrane potential was restored upon the addition of 2-mercaptoethanol, and the (^{35} S) label chased for 60 min radioactivity detected in the mature form was much diminished in the mutant relative to the wild type (lane 2 & 5). If the cells were pulse labeled in the absence of CCCP for 10 min and the label chased for 60 min once again the mature 47 KDa form of core protein I appeared less in the mutant relative to the wild type (lane 3 vs lane 6). These results suggest that the precursor to core protein I may not be processed correctly or alternatively that the mature form once produced is rapidly degraded.

Using similar techniques to block the import of the iron sulfur

protein, it was earlier reported from this laboratory that the precursor form of this protein has a molecular weight of 28,000, 4000 Da. larger than that of the mature form (142) and Table 1V. The precursor is first processed in an energy requiring step into an intermediate form which is 1500 Da larger than the mature form. Subsequently the intermediate form is processed in the mitocondrial membrane to the mature form. In the presence of CCCP the precursor form of the iron sulfur protein accumulated in similar amounts in mutant Box 6-2 and the wild type (Fig.11A, lane 3 vs. 6) suggesting that synthesis of this protein is not impaired in the mutant. However, as observed for core protein I, the mature form appeared much diminished in the mutant after a 10 mins chase (lane 1, 2 vs. 4, 5). In a different experiment cells were pulse labeled for 10 mins in the absence of CCCP at 18^0C, in order to slow down the synthetic process and hence accumulate the precursor (Fig.11B). The precursor form of the iron sulfur protein could be only seen on very long exposure to X-Ray film in the wild type. Again the intermediate and the mature form appeared much diminished in the mutant compared to he wild type (lane 9, 10 vs.7, 8).

G. AMOUNT OF MATURE FORM IN THE MITOCHONDRIA OF THE MUTANT AND THE WILD TYPE STRAINS

In order to compare the kinetics of the assembly of the mature form of the iron sulfur protein present in the wild type and mutant strains, mitochondria were prepared from cells labeled with (^{35}S) methionine for 5 min and chased for various time points with cold methionine (Fig.12A). At all time points during the chase, the mutant mitochondria contained much lower amounts of the iron sulfur protein than

the wild type. By 40 min much more protein had accumulated in the mitochondria of both strains, however, the mutants showed lesser amounts (1&4, 2&5, 3&6).

In another experiment cells were pulse labeled with methionine for 15 min and chased for 25 min (Fig 12B, 7-10). Again the results indicated that significantly lowered amounts of the mature form of the iron sulfur protein were present in the mutant.

Similarly, it was earlier reported in section C of this chapter that the mature form of the core protein I was present in lower amounts in the mutant when mitochondria were isolated from (^{35}S) labeled cells of Box 6-2 and 777-3A and analyzed by immunoprecipitation.

By contrast the amount of cytochrome c_1, present in mutant Box 6-2 was identical to that of the wild type. When cells were labeled with methionine for 10 min and subsequently chased for 25 min the mature form of cytochrome c_1 actually appeared in greater amount in the mitocondria isolated from Box 6-2 (Fig 13). These results suggest that the mutant mitochondria lacking cytochrome b can establish the membrane potential necessary to process imported proteins. Hence the lack of processing of core protein I and the iron sulfur protein in the mutant is not a consequence of a lowered membrane potential.

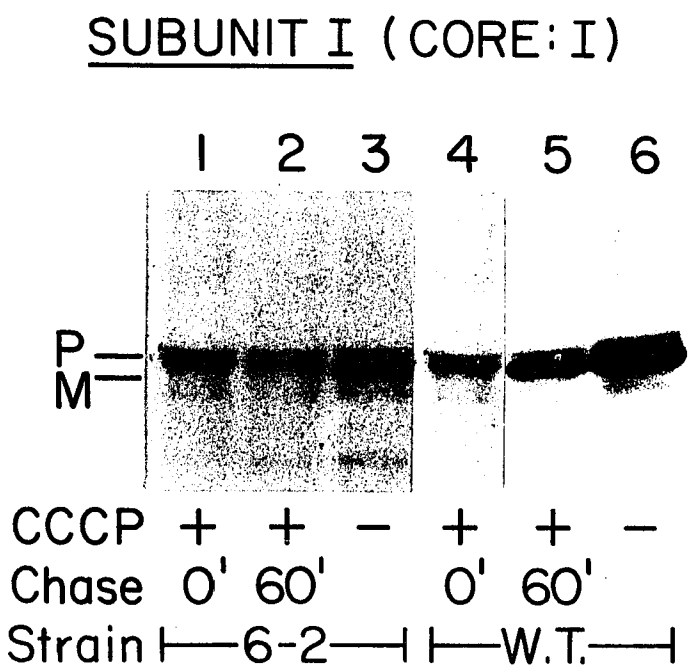


Figure 10. Comparison of Two Forms of Core Protein I in Mutant Box 6-2 and Wild Type Cells. Yeast cells from mutant Box 6-2 and the wild type parent 777-3A were pulsed with (^{35}S) methionine for 10 min in the absence of CCCP (lanes 3 and 6) or presence of 20μM CCCP (lanes 1, 2 and 4, 5). To the CCCP treated cells 0.05% 2-mercaptoethanol(v/v) was added prior to a chase of 60 min. The label was chased with 10 mM unlabeled methionine and at 0 min and 60 min equal aliquots were removed, mixed with 20% trichloroacetic acid and solubilized with 4% SDS as described under Experimental Procedures.

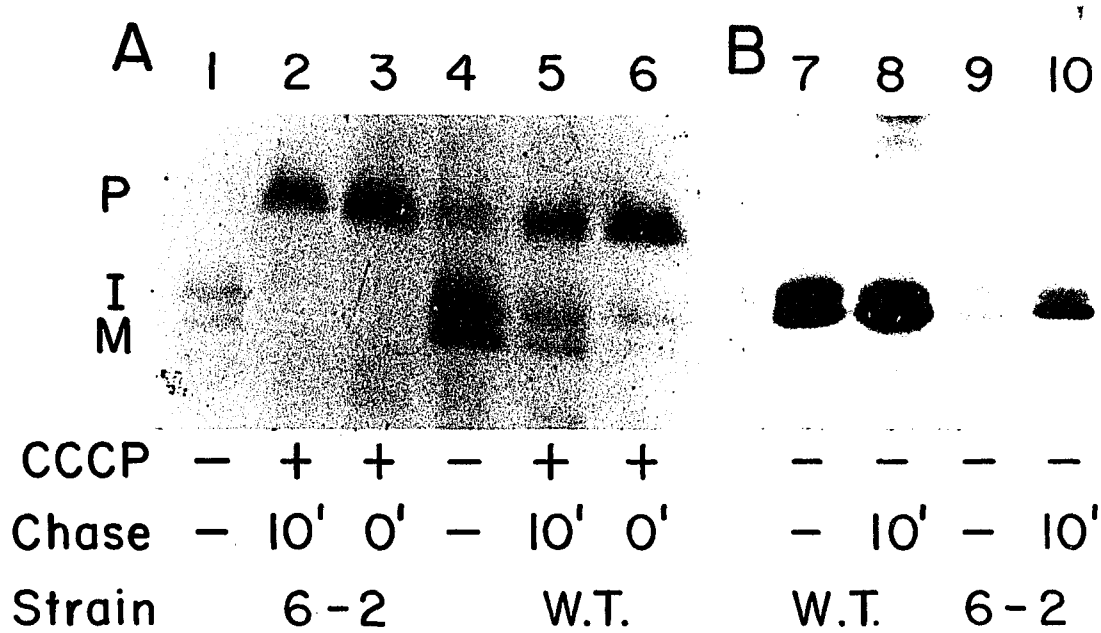


Figure 11. Comparison of Precursor and Mature forms of the Iron-sulfur Protein in Mutant Box 6-2 and Wild Type Cells. Mutant Box 6-2 and wild type parent cells were pulsed with (^{35}S) methionine for 10 min in the absence of 20μM CCCP (A) lanes 1 and 4 or presence of CCCP (lanes 2, 3 and 5, 6) as described under legend to Figure 10. The membrane potential was reestablished by the addition 0.05% 2-mercaptoethanol in the CCCP treated cells and the pulse was chased with for 10 min. The two strains were also pulse labeled at 18^{0} C in the absence of CCCP and chased for 10 min (B). Equal aliquots were removed at 0 min (lanes 7 and 9) and 10 min (lanes 8 and 10), mixed with 20 % trichloroacetic acid and solubilized as described earlier.

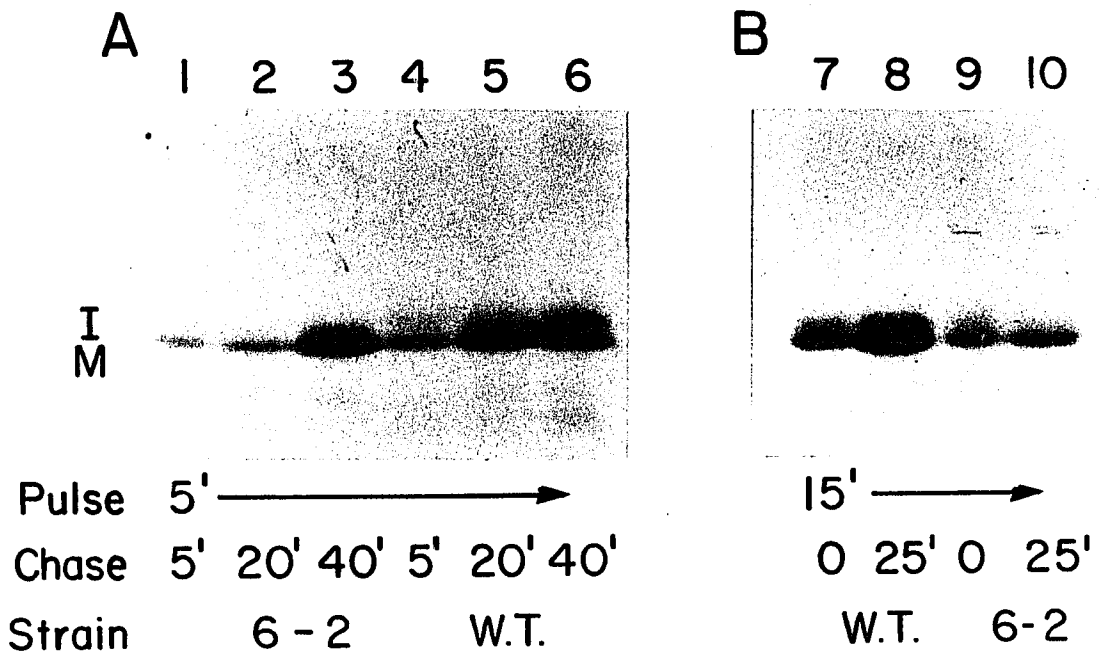


Figure 12. Analysis of Mitochondria Isolated from 777-3A and Box 6-2 for Mature form of Iron-Sulfur Protein. Yeast cells were pulse labeled with (^{35}S) methionine for 5 min (A) or 15 min (B). The pulse was chased with 10 mM unlabeled methionine for 40 min (A) or 25 min (B). At different time points equal aliquots of cells were removed chilled to 4^{0} C and mitochondria prepared immediately as described under Experimental Procedures. The mitochondria were dissociated in 3 % SDS at 95^{0} C and immunoprecipitated with iron-sulfur antiserum.

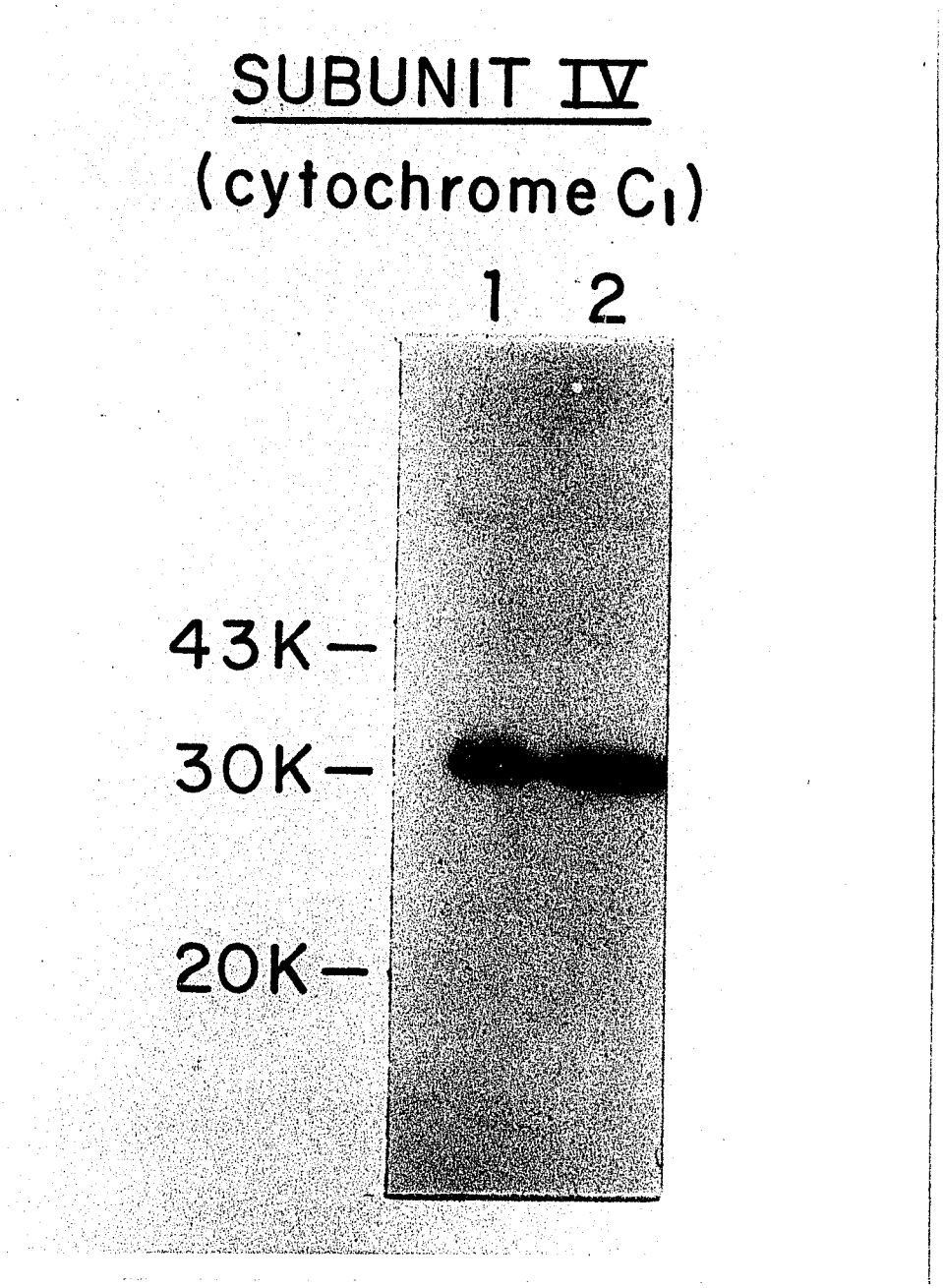


Figure 13. Analysis of Mitochondria Isolated from 777-3A and 6-2 for Mature Form of Cytochrome C_1. Yeast cells of the two strains were pulsed with (^{35}S) methionine. At the end of 25 min the mixtures were cooled to 4^0 C, mitochondria prepared and immunoprecipitated with cytochrome c_1 antiserum as described under legend to Figure 12.

H. ANALYSIS OF mRNA FROM PARENTAL AND CYTOCHROME B^- STRAINS

Although the results described above indicated that the synthesis of the iron sulfur protein was not impaired in the cytochrome b deficient mutant, it was necessary to demonstrate that the mRNA for this protein was not affected in the mutant prior to attempting import experiments. In order to examine this possibility, total yeast RNA was isolated from Box 6-2 and 777-3A strains. Equal amounts of RNA (1.25 O.D units at A_{260nm}) were translated in a cell free system of rabbit reticulocyte lysate at 30 ^{0} C for 60 min. Since apo-cytochrome c_1 and the iron sulfur protein have 4000 and 6000 Da larger precursors respectively (Table IV) and therefore can be clearly distinguished on gel electrophoresis, these subunits were immunoprecipitated using specific antisera. As shown in Fig. 14 similar amounts of precursors were immunoprecipitated in both cases indicating that the reduced level of the iron sulfur protein in the mutant mitochondria did not result from a decreased amount of the mRNA for this protein.

I. *IN VIVO* STABILITY OF CORE PROTEIN I AND THE IRON SULFUR PROTEIN IN THE PARENTAL AND CYTOCHROME B^- STRAINS

The *in vivo* stability of newly synthesized proteins of complex III was compared in order to examine whether increased degradation was the cause of reduced levels of the subunits in the mitochondria. Mutant and wild type yeast strains were grown in the presence of (^{3}H) leucine to mid log phase and the cells were exposed to (^{35}S) methionine for 30 min, followed by the addition cold methionine to stop incorporation of labeled

methionine. At various time points equal aliquots of cells were removed, PMSF and cycloheximide added, cells cooled and broken immediately and mitochondria prepared for immunoprecipitation with antiserum against the iron sulfur protein or core protein I. As shown in Fig.15 A &B the initial ratio of $^{35}S/^{3}H$ was the same for both strains and remained the same until the end of the chase indicating that once processed into the membrane the newly synthesized core portein I and the iron sulfur protein are as stable as that synthesized by the parental strain. This experiment only measures the amount of the mature form and does not indicate any thing about the kinetics of synthesis or processing.

J. *IN VITRO* IMPORT OF RADIOLABELED PRECURSOR OF THE IRON SULFUR PROTEIN AND CYTOCHROME C_1 INTO MITOCHONDRIA ISOLATED FROM WILD TYPE AND MUTANT STRAINS

The lack of degradation of the subunits in the mutant mitochondria suggested that inefficient processing probably was the cause of the appearance of lowered amounts of complex III subunits in the mutant mitochondria. Processing of the subunits into the mitochondria was studied by synthesizing the precursor proteins in rabbit reticulocyte lysate system in the presence of (^{35}S) methionine.

1. Import of the iron-sulfur protein

The import of these labeled proteins into mitochondria was performed in the presence of ATP and an ATP regenerating system. Mitochondria prepared from zymolyase digested cells with a respiratory control ratio of 3 was used. The incubation for import was allowed to proceed for different times at the end of which the mitochondria were reisolated. The mitochondria and the resulting supernatants were

dissociated in 3% SDS and radiolabeled iron sulfur protein isolated by immunoprecipitation. After a 2 min incubation under import conditions the precursor form of the iron sulfur protein could be seen in both strains with the appearance of some mature form in the wild type mitochondria (Fig 16A, lane 1 vs. 4). A 25 min incubation appeared sufficient for the complete import of the iron sulfur precursor protein into the mitochondria of the wild type cells (lane 2 vs.5) as no precursor form of the protein was visible in the supernatant; however, in the mutant strain, even after 40 min the precursor was present in the supernatant indicating that it had not entered the mutant mitochondria (lane 3vs. 6). In a second experiment the import reaction was performed for 15 min at 28 ^{0}C. Under these conditions the import of the iron sulfur protein into mitochondria was not complete in either strains; however, in the mutant greater amounts of the precursor appeared in the mitochondrial fraction (Fig 16B, lane1 vs 2) suggesting that it was bound to the membrane but not processed. The intermediate form of the iron sulfur protein was observed between 8-10 min of import in both the mutant and the wild type (results not shown).

2. Import of cytochrome c_1

The import of the precursor form of cytochrome c_1 was also investigated using the same experimental system. In this case similar amounts of intermediate form could be clearly seen in the mitochondria from both the wild type and the mutant after a 15 min incubation with the precursor (Fig 17, lane 2 vs 5). At the end of 30 min the mature form could be faintly seen. Earlier reports by Schatz et al. (145) have indicated that cytochrome c_1 can be chased only to the intermediate form under *in vitro* import conditions in the yeast. The intermediate and the mature

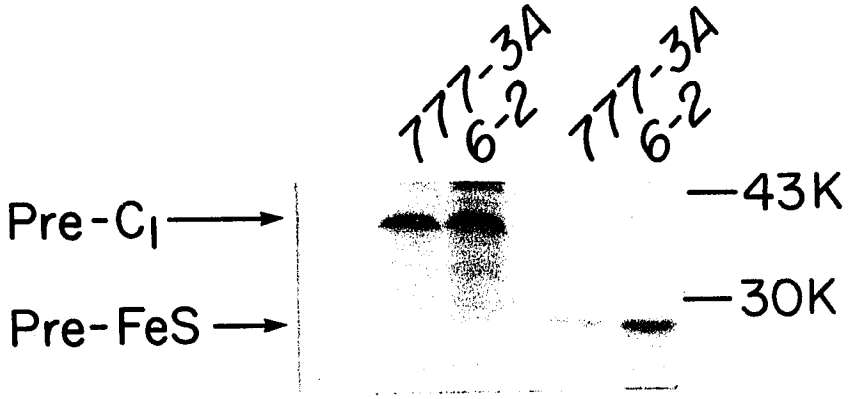


Figure 14. Precursors of Iron-sulfur Protein and Cytochrome C_1 Synthesized *in vitro*. Total yeast RNA was isolated from strains 777-3A and Box 6-2 (157) and translated in a nuclease treated reticulocyte lysate in the presence of (^{35}S) methionine (158). Each 125 µl of reaction mixture contained 50 µl lysate, 1.25 A_{260} units of total yeast RNA and 225 µci of L (^{35}S) methionine. The reacton mixture was incubated at 37^0 C for 60 min at the end of which the reaction was terminated with 3% SDS at 95^0 C. Precursors of cytochrome c_1 and the iron-sulfur protein were immunoprecipitated using the respective antiserum as described under Experimental Procedures.

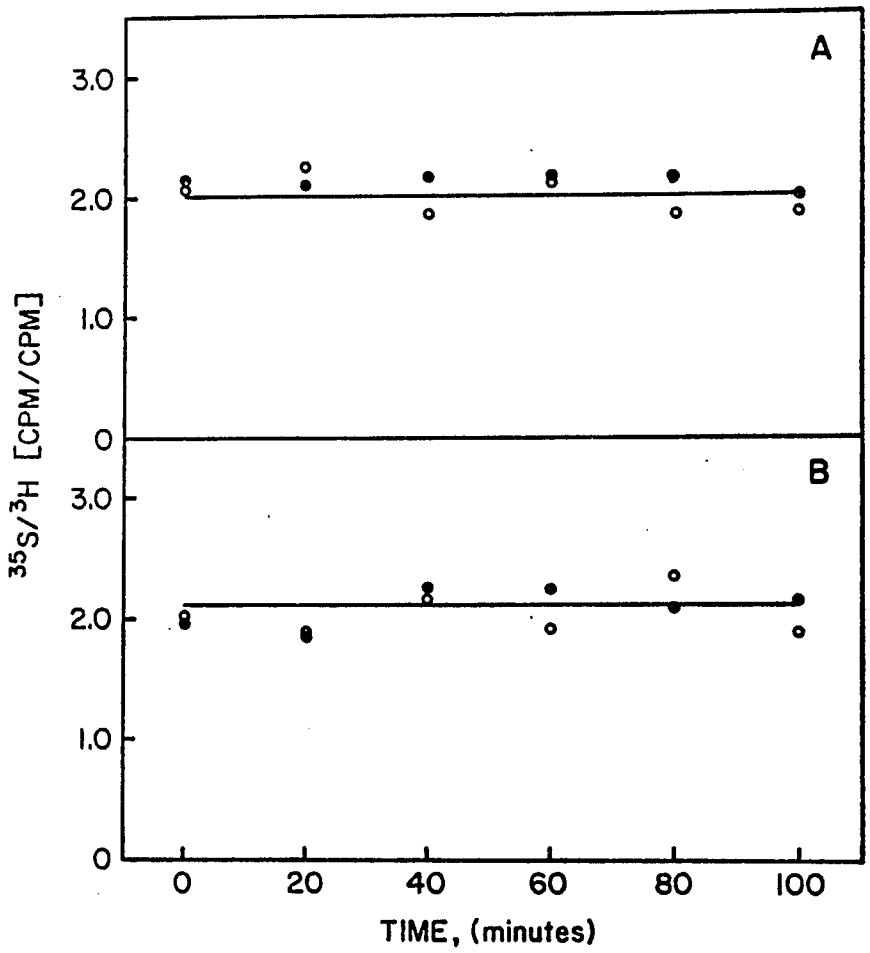


Figure 15. Double Label Analysis of Stability of Core protein I and the Iron-sulfur Protein in Mutant Box 6-2 and Wild Type Cells. The parental and the mutant strains were grown in the presence of (^{3}H) leucine to mid log phase and then labeled with (^{35}S) methionine as described under Experimental Procedures. After 30 min the (^{35}S) label was chased with 10 mM unabeled methionine. Aliquots of cells were taken at the times indicated, chilled and mitochondria prepared. The mitochondria were immunoprecipitated with core protein I (A) or iron-sulfur protein (B) antisera as described under legend to Figure 12 and the precipitate counted. o—o Mutant; •—• Wild Type.

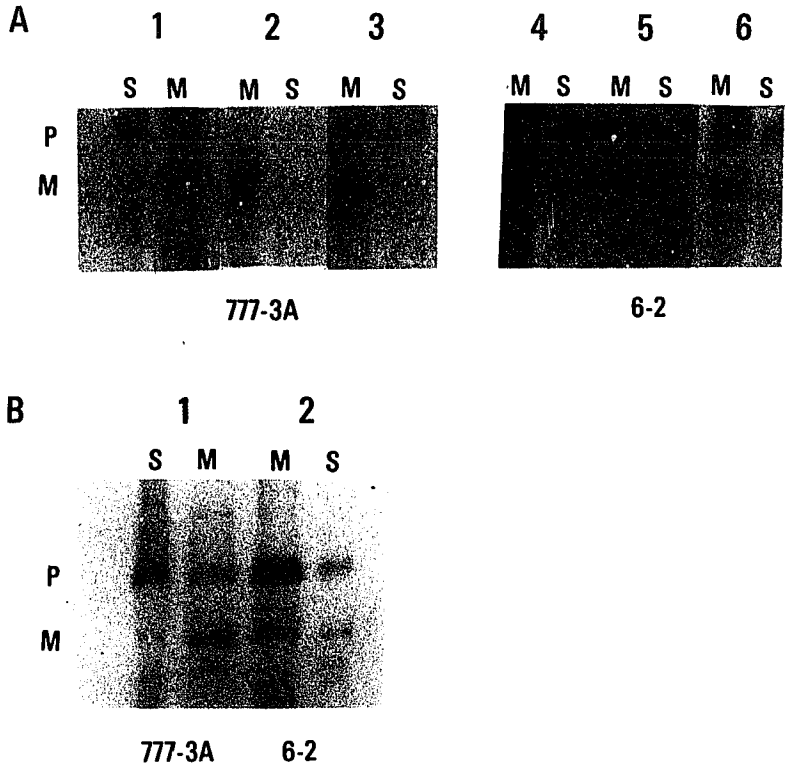


Figure 16. Import of Iron-Sulfur Protein into Mitochondria Isolated from 777-3A and Box 6-2. Total yeast RNA was translated in a rabbit reticulocyte lysate in the presence of (^{35}S) methionine as described under legend to Figure 14 and the mixture was centrifuged for 45 min at 140,000 g to remove the polysomes. Aliquots (125µl) of the centrifuged lysate were incubated with 300 µg of isolated mitochondria from strains 777-3A (A, 1-3) and Box 6-2 (A, 4-6) at 28^0 C as described under Experimental Procedures. The incubations with the mitochondria were for 2 min (1,4), 25 min (2,5) or 40 min (3,6) at the end of which 1mM PMSF and 1mM TLCK were added and the mixture cooled to 4^0 C. The mitochondria were pelleted by centrifugation (10 mins at 10,000g) and pellets (M) and supernatants (S) were dissociated in 3% SDS at 95^0 C and subjected to immunoprecipitation with iron-sulfur antiserum. (B) Aliquots of the centrifuged lysate were also incubated with isolated mitochondria from 777-3A and Box 6-2 for 15 mins, and pellets and supernatants separated and subjected to immunoprecipitation as described above.

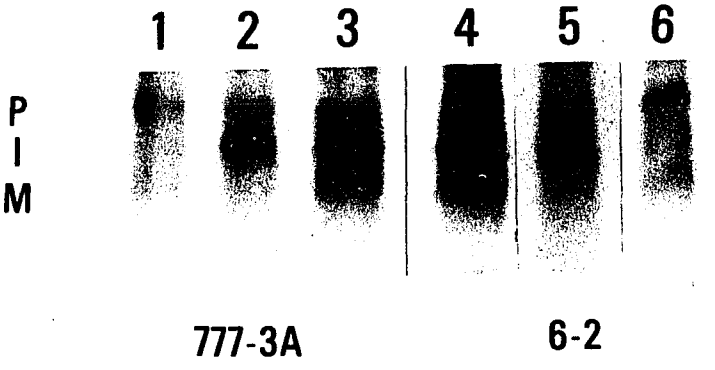


Figure 17. Import of Cytochrome c_1 into Mitochondria Isolated from 777-3A and Box 6-2. Translation *in vitro* and import of labeled precursors into mitochondria was performed as described under legend to Figure 16 for 2 min (lane 1 and 6), 15 min (lane2 and 5) and 30min (lane 3 and 4), at the end of which the import was terminated by addition of 1 mM PMSF and 1mM TLCK and dissociation at 95^0 C with 3.5 % SDS. Samples were subjected to immunoprecipitation with cytochrome c_1 antiserum prior to electrophoresis on a12 % gel and slab-gel autoradiography.

forms appeared imported in similar amounts in the wild type and mutant mitochondria (lane 3 vs 4).

TABLE IV

COMPARISON OF PRECURSOR SIZES FOR SUBUNITS OF COMPLEX III FROM VARIOUS ORGANISMS

	Precursor extension (KDa)		
Subunit	*Neurospora* (a)	Yeast (b,c,d,e,f)	Rat (g, h)
I (core protein I)	1.5	0.5[b, d]	8 - 9
II (core Protein II)	2.5	0.5[b]	–
III (cytochrome b)	–	none[f]	–
IV (cytochrome c_1)			
1st Precursor	7	6[c, d]	2 -3
2nd Precursor	4	4[c, d]	N.D.
V (iron sulfur protein)			
1st Precursor	3	2[b, e]; 4[d, f]	6 - 7
2nd Precursor		1.5[d, f]	N.D.
VI	none	9[b]	–

a. Teintze et al. (1982), (144)
b. Van Loon et al. (1983), (143)
c. Ohashi et al. (1982), (145)
d. Present study
e. Coît'e et al. (1979), (60)
f. Sidhu et al. (1983), (142)
g. Kolrav et al. (1984), (146)
h. Kuzela et al. (1985), (147)
N.D. not detected

Chapter IV

DISCUSSION

The biogenesis and the topography of complex III has been a subject of much research over the last two decades for two main reasons. First, complex III is an energy conserving site and during electron transport causes the electrogenic translocation of protons across the inner mitochondrial membrane. The localization of the various protein components of the complex is therefore of considerable interest to explain the unidirectional proton translocation at this site of the respiratory chain. Second, only one subunit, cytochrome b, is a mitochondrial translation product and hence the complex serves as an ideal model system to study nucleo-cytoplasmic interactions during the assembly of the respiratory chain. Several observations from this laboratory and several other laboratories have indicated that cytochrome b may have a template role in the assembly of the complex III, however, no definitive role of cytochrome b in the assembly of the complex has yet been shown. As a subject for this thesis it was thought that a detailed analysis of the assembly mechanism of the cytoplasmically translated components of complex III in the cytochrome b deficient mutants of yeast would establish what role cytochrome b has in the assembly of the complex and also lend an insight into the mechanism of assembly of complex III in the inner mitochondrial membrane. In the first part of this thesis, the presence of four component subunits of complex III in mutants of the yeast *S.cerevisiae* lacking cytochrome b has been examined. In the second part the kinetics of synthesis of the component subunits and their

subsequent assembly into a complex has been investigated in both the mutant and the wild type strains.

A. EFFECT OF CYTOCHROME B MUTATION ON THE PRESENCE OF CORE PROTEINS I, II, CYTOCHROME C_1 AND THE IRON SULFUR PROTEIN

The cytochrome b mutants chosen for the study map in several exons of the mosaic cytochrome b gene. They lack a spectrally detectable cytochrome b and do not synthesize the corresponding apo-protein. Mutant Box 6-2 maps in exon E (B6 in Fig. 2) and synthesizes a 26.6 kDa fragment of apo-cytochrome b; mutant Box 1-2 maps in exon C (B4) and synthesizes a 18.5 KDa fragment; mutant Box 4-1 maps in exon A (B1) and synthesizes a 13.4 kDa fragment (not detected in our experiments); mutant Box 5-1 maps near exon A (B1) but does not synthesize a novel polypeptide related to cytochrome b (42). A significantly reduced amount of core protein I, core protein II and the iron sulfur protein was observed in the mutants when their respective mitochondria were analyzed by both immunoprecipitation and immunoblotting techniques. Despite the actual locus of the mutation or the size of the novel polypeptide synthesized, these mutants were equally affected in the loss of these subunits. Core protein II seemed to be the most severely affected as less than 40 % of this subunit was present in the mutants relative to the wild type . The loss was 50 % or more for core protein I and the iron sulfur protein I. By contrast cytochrome c_1 was affected to a much lesser extent in these mutants and was present in nearly equivalent and sometimes greater amounts in the mutants compared to the wild type as determined immunologically as well as spectrally. Furthermore, less than 25 % of

complex III proteins compared to the wild type were present in the mutants as determined by immunoprecipitation analysis with complex III antiserum. Recent observations by Ohnishi et al. on similar Box mutants indicated that these mutants fail to give the EPR spectra due to the Reiske iron sulfur cluster of the complex III; clusters associated with complex II were, however, present (163). They concluded that incorporation of the iron sulfur protein was perturbed in the complex as a result of structural alteration in the mitochondrially translated cytochrome b. Ohnishi et al. also reported that cytochrome c_1 was present in all of the mutants they examined. This agrees very well with our results. It must be mentioned that in this study, PMSF was present at all stages of the isolation of mitochondria from the different strains and the samples were maintained at 4^0C to eliminate any possible degradation of the subunits arising during the process of isolation and thus contributing to the diminished presence of the subunits in the membrane. Furthermore, mitochondria were prepared from cells of the different strains which were harvested at the early stationary phase under similar optical density readings at 650 nm. This was done to minimize the effect of glucose/galactose repression leading to decreased amounts of the different subunits. It is well known that the amount of cytochromes present in the cell depends on the stage of the growth of the cell (164,165).

The firmness of binding of core proteins I and II and the iron sulfur protein with the mitochondrial membrane was also examined in the Box mutants by sonication of the mitochondrial samples and preparing submitochondrial particles. Indeed sonication of the mutant mitochondria

resulted in a considerable loss of these subunits, while no decrease in any subunit was observed after sonication of wild type mitochondria. Again, cytochrome c_1 was the exception, as assembly of this protein into the membrane appeared unaffected by the absence of cytochrome b as indicated by the lack of release upon sonication. These results suggested that the apoprotein of cytochrome b may be necessary for the proper assembly of the subunits of complex III into the mitochondrial membrane. Topographically cytochrome b spans the inner membrane (131,132) while cytochrome c_1 is exposed to the cytoplasmic side and interacts directly with cytrochrome c (131, 137, 138). Furthermore, cytochrome b, c_1 and the core protein I are tightly associated in the complex in wild type mitochondria and association of these subunits into the membrane bound complex appears to occur through strong, possibly lipid-protein interaction (132). The association of the iron-sulfur protein and the core protein II to the other subunits of the complex by contrast is through weak interactions and these subunits can be dissociated from the complex by low concentrations of detergent(132). Recent studies in this laboratory on the biogenesis of the complex indicated that cytochrome b and c_1 are the first subunits to assemble into the holoenzyme while core protein I and the ISP are inserted more slowly (142). The assembly of the various subunits of cytochrome oxidase into the holoenzyme also appears to proceed at different rates. Thus subunits II and III are assembled very rapidly while subunit I is the last of the subunits to be integrated into the oxidase. It has been suggested that the rapidly labeled subunits of

cytochrome oxidase may act as a template for the insertion of the remaining subunits of cytochorome oxidase (166). By drawing analogy with cytochrome oxidase it was postulated that the rapidy assembled proteins of complex III, cytochrome b and c_1, especially cytochrome b, may act as a template on which the other polypeptides are assembled (142).

B. KINETICS OF SYNTHESIS AND ASSEMBLY OF CORE PROTEIN I, THE IRON SULFUR PROTEIN AND CYTOCHROME C_1

There may be several alternatives which may be responsible for the decreased presence of the subunits of complex III in the cytochrome b mutants. The possibilities include :

a. a deficiency in the messenger RNA for these proteins

b. a lowered rate of synthesis in the cytoplasam

c. an inefficient import and processing of these precursors into the mitochondria or

d. a more rapid degradation of the mature form of the proteins in absence of a functonal cytochrome b.

A mechanism similar to that observed for the import of the small subunit of ribulose 1,5 -bisphosphate carboxylase into chloroplasts may be operating in the Box mutants. The small subunit of this enzyme is imported normally into the organelle when pools of the chloroplast synthesized large subunit are depleted. However, it cannot reside there for an extended time in the absence of proper assembly with the large subunit, which is synthesized in the chloroplast, and is subsequently rapidly degraded (167).

In order to distinguish between these possibilities mutant Box 6-2

was chosen for these set of studies. The processing of the precursor forms of both core protein I and the iron sulfur protein appears to be defective in the mutant. The time course of processing of precursors which had been accumulated in cells treated with the uncoupler CCCP *in vivo* suggested that the precursors of core protein I and the iron sulfur protein were processed more slowly in the mutants. Furthermore, the amounts of the mature forms of these proteins in mitochondria pulse-labeled under different conditions were also considerably decreased at all times studied confirming the results obtained with an immunological approach (Section A). The import into isolated mitochondria of the precursor forms of the iron sulfur protein synthesized in a rabbit reticulocyte lysate programmed with yeast messenger RNA also suggested a defect in the mutants lacking cytochrome b. While the precursor form of the iron sulfur protein was present in equal amounts in the supernatant fraction after a 2 min incubation in the import medium containing mitochondria from either the wild type or mutant cells, the appearance of the mature form in the mutant mitochondrial fraction was considerably less than that in the wild type mitochondria after either a 25 or 40 minute incubation. It should be noted that 25 minutes appeared to be sufficient for the complete import of the iron sulfur protein into mitochondria from wild type cells.

The synthesis of both the core protein I and the iron sulfur protein appeared to be unaffected in the mutant, as the precursor forms of both proteins accumulated to the same extent when processing *in vivo* was blocked by CCCP. Furthermore, translation of RNA in the reticulocyte lysate indicated that the messenger RNA' s for both cytochrome c_1 and the the iron sulfur protein were present in the mutant and translated

with equal efficiency.

The lack of processing of the core protein I and the iron-sulfur protein in the mutants does not appear to be a consequence of a lowered membrane potential or a defect in the processing enzymes, as the synthesis and the processing of cytochrome c_1 appears to be unaffected in these mutants. In the *in vitro* import system, the intermediate form of cytochrome c_1 was detected in equal intensity in mitochondria from the wild type and the mutant cells. Furthermore, *in vivo* studies indicated that the mature form of this protein appeared in the mitochondrial membrane in equal amounts in both mutant and wild type cells. Schatz et al. have shown that *in vivo* processing of precursors and hence protein import, is not blocked in *rho*$^-$ yeast cells which lack respiration as well as a functional ATPase complex (88). They concluded that these mutants can generate an electrochemical gradient by some process other than respiration or ATP hydrolysis, such as by ATP/ADP exchange via the adenine nucleotide translocator which is coupled to a membrane potential (168). Import of polypeptides into mitochondria, therefore, possibly requires a much smaller membrane potential than that generated by ATP hydrolysis as the potential generated by the translocator is very small.

Recently De Haan et al reported that lowered amounts of the 11 kDa and 14 k Da subunits of complex III were present in the mitochondria from similar cytochrome b lacking mutants (169). They concluded that an increased turnover of these proteins, as a consequence of weakened interaction with the complex, resulting directly or indirectly from the lack of a functional cytochrome b, was responsible for the decreased presence of these proteins in the membrane. In the present study,

however, it was seen using double label analysis to study the stability of core protein I and the iron sulfur protein that there was no degradation of these subunits once the mature form of these proteins was present in the mitochondrial membranes. Once again, care was taken to see that PMSF was present at all stages of isolation of the mitochondria. De Haan et al also reported that the processing of the 14 and 11 kDa proteins was not affected in mutants of yeast which lack subunit II of cytochrome c oxidase, indicating that a mere lack of functional mitochondria does not affect their stability. Ohnishi et al. similarly observed the presence of the Rieske's iron-sulfur cluster in *oxi* mutants carrying deficiencies in the mitochondrially synthesized subunits of cytochrome c oxidase, though these clusters were absent in the Box mutants (163), concluding that a tight correlation exists between elements functionally associated within the same multipotein complex III.

Studies on the assembly of the proton translocating ATPase in bacteria, using several mutations in the ATPase gene (the unc operon) have indicated that the and ß subunit of F_1 must be synthesized prior to the incorporation of the 18 kDa subunit of F_0 into the membrane (170,171). Results from the present study, similarly, suggest that cytochrome b and c_1, the most rapidly synthesized subunits of complex III, must be incorporated properly into the mitochondrial membrane to form a template for the proper processing of the remaining subunits of the complex III.

C. CONCLUDING REMARKS

The mitochondrion in addition to its essential function in the cell of conserving oxidatively derived energy may have a role in other vital

processes in the higher eucaryotes. Patients suffering from the disease ceruloplastic anemia have been shown to have a defect in the mitochondrially contained portion of the heme biosynthetic pathway. In acute conditions of the disease they develop leukemia , a disease thought to involve the nuclear genes. It was recently shown that a mitochondrial gene product is required for the expression of a murine cell surface antigen (172). Wilkie and Evans have also suggested that genetic lesions in mitochondria can promote neoplastic transformations (173). Their suggestion is based on the findings that various petite mutants of yeast which have abnormal mt DNA, show cell surface changes influenced by nuclear DNA, similar to those associated with animal cell cancers. The role of nuclear mitochondrial interactions in the biosynthetic pathways by which the organelle is formed must be resolved. The yeast is highly suitable for such studies and recent advances in yeast biotechnology, especially with the discovery of the 2 µm yeast plasmid, has greatly faciliated research on mitochondrial biogenesis. In addition, the ability of the yeast to sustain fermentable growth has allowed the isolation of several classes of respiratory deficient mutants, e.g., the heme mutants, the coenzyme Q mutants, the box mutants, the cytochrome oxidase mutants, the temperature sensitive mutants having defective import of cytoplasmic synthesized polypeptides and several others. Characterization of these mutants have helped establish the role of a specific gene product, whether of mitochondrial or nuclear origin, in the biogenesis of the mitochondria and also helped in uncovering the details of the assembly process. The box mutants to that extent have made their contribution in establishing the role of cytochrome b in the assembly of complex III.

LIST OF ABBREVIATIONS

ATP:	adenosine triphosphate
BSA:	bovine serum albumin
CCCP:	carbonyl cyanide m-chlorophenyl hydrazone
CHI:	cycloheximide
CPM:	counts per minute
DABS:	diazobenzene sulfonate
DTT:	dithiothreitol
EDTA:	ethylenediamine tetraacetic acid
EGTA:	ethyleneglycol-bis-(amino-ethylether) N,N'-tetraacetic acid
EPR:	electron paramagnetic resonance
GAR-HRP:	goat anti rabbit horse radish peroxidase
GTP:	guanosine -5'- triphosphate
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HRP:	horse radish peroxidase
ISP:	iron sulfur protein
KDa:	kilodaltons
Kbp	kilobase pairs
LSM:	low sulfate medium
mt DNA	mitochondrial DNA
PAGE:	polyacrylamide gel electrophoresis
PMSF:	phenylmethyl sulfonyl fluoride
SDS:	sodium dodecyl sulfate

SMP:	submitiochondrial particles
TBS:	tris buffered saline
TCA:	trichloro acetic acid
TLCK:	N -p-tosyl-L-lysine chloromethyl ketone

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