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CLEJAN, LIVIU ANTON

STUDIES ON THE BIOSYNTHESIS OF CYTOCHROME B IN SACCHAROMYCES CEREVISIAE

City University of New York PH.D. 1980

STUDIES ON THE BIOSYNTHESIS OF CYTOCHROME b IN SACCHAROMYCES CEREVISIAE

by

Liviu A. Clejan

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

-1980-

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

4/29/80
Date

Diana J. Beattie
Chairman of Examining Committee

5/6/80
Date

Jason Gilbert
Executive Officer

Arthur Ceberbaum

Edith D. Goldberg

Jan F. Hogg

Supervisory Committee

ABSTRACT

STUDIES ON THE BIOSYNTHESIS OF CYTOCHROME *b* IN *SACCHAROMYCES CEREVISIAE*

by

Liviu A. Clejan

Adviser: Professor Diana S. Beattie

Cytochrome *b* was isolated by immunoprecipitation from extracts of mitochondria isolated from yeast cells labeled *in vivo*. Analysis of the immunoprecipitates of sodium dodecylsulfate (SDS) gel electrophoresis revealed a major labeled band with an apparent molecular weight of 31,000, corresponding to cytochrome *b*. No cytochrome *b* apoprotein was present in a cytoplasmic petite mutant which lacks mitochondrial protein synthesis. Two additional, high molecular weight labeled polypeptides were observed in immunoprecipitates obtained from cells labeled with [^{3}H] leucine, either in growing conditions or *in vivo*, under non-growing conditions in the absence or presence of cycloheximide. These results suggest mitochondrial origin for these polypeptides. The time course of labeling of the mitochondrial translation products suggests that these high molecular weight polypeptides are not precursors of cytochrome *b*.

Mitochondrial translation products obtained from yeast cells labeled *in vivo* in the presence of cycloheximide were separated by SDS polyacrylamide gel electrophoresis. The labeled band, with a molecular weight of 30,000 corresponding to cytochrome *b*, was excised and subsequently transferred to a second gel. After electrophoretic separation, two labeled polypeptides with apparent molecular weights of 67,000 and 27,000 became visible in addition to the cytochrome *b* band of 30,000 molecular weight. Heating of the dissociated mitochondria prior to transfer resulted in an increase in the amount of the labeled polypeptides migrating with a molecular weight of 67,000. Longer exposure during autoradiography of the gels of mitochondrial translation products resulted in the appearance of a double band with an apparent molecular weight of 67,000. Limited proteolysis of this 67,000 dalton protein with *Staphylococcus aureus* V8 protease revealed a peptide map similar to that obtained after proteolysis of cytochrome *b*. These results suggest that the polypeptide with an apparent molecular weight of 67,000 represents an aggregate of cytochrome *b* that is either present as such in the membrane or is formed *in vitro* during the experimental manipulations to prepare mitochondria for gel electrophoresis.

Mitochondrial translation products were examined in yeast cells labeled *in vivo* for short times at 10^{o}-15^{o} C in the presence of cycloheximide. Polyacrylamide gel electrophoresis revealed a labeled protein with an apparent molecular weight of 58,000 to 60,000. A labeled protein of this mobility

was not apparent in cells labeled at 30° C. A 30 min chase with unlabeled methionine at either 10° C or 30° C did not result in any decrease in labeling of this protein indicating that it is a stable product. The 58,000 to 60,000 dalton protein has an identical mobility as a high molecular weight protein present in the immunoprecipitates formed with labeled mitochondria and the specific antiserum against cytochrome b. In addition, the 58,000 to 60,000 dalton protein displays an anomalous migration behavior in gels of different acrylamide concentrations as does cytochrome b. These data suggest that the 58,000 to 60,000 mitochondrial translation product may be a stable dimer of cytochrome b synthesized at low temperatures in intact cells.

The presence of the apoprotein of cytochrome b has been demonstrated in a mutant of Saccharomyces cerevisiae lacking δ-aminolevulinic acid synthase, and hence devoid of heme. The apoprotein of cytochrome b present in the mutant was identical with cytochrome b of control cells (mutant cells grown in the presence of δ-aminolevulinic acid) by the following criteria: similar apparent molecular weights in SDS polyacrylamide gel electrophoresis; anomalous migration behavior during electrophoresis in polyacrylamide gels of different porosities; identical gel pattern obtained after immunoprecipitation with specific antiserum against cytochrome b, and identical fingerprints obtained after limited proteolysis with Staphylococcus aureus V8 protease. The kinetics The kinetics of incorporation in vivo of [^{35}S] methionine into

apoprotein of cytochrome b in the mutant suggested that heme deficiency may affect assembly into the membrane of subunits of the cytochrome b-c_1 complex rather than synthesis of cytochrome b.

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Chapter I

INTRODUCTION, BACKGROUND and OBJECTIVES

Interest in the mechanism of mitochondrial formation dates probably from the discovery of mitochondria by cytologists 90 years ago (1). Considered for many decades mainly as a morphological entity, it was not until the fifth decade of this century that interest in mitochondria shifted from the limited sphere of the microscope's focus to the broader attention of geneticists and biochemists.

In 1950, Ephrussi showed that mitochondrial formation was in part governed by extrachromosomal genetic determinants (2) and in 1951-52, Potter et al.(3) and independently, Hogeboom and Schneider (4) noted the presence of DNA in mitochondrial preparations. Since then, the study of mitochondrial biogenesis evolved as a unique area for joint research of biochemists and geneticists. The pioneer genetic studies from Ephrussi's laboratory preceeded in time the biochemical work on the isolation and characterization of the synthetic machinery, electron transfer and energy coupling complexes of the organelle, but it is now clear that the two approaches are "tightly coupled". Progress in one domain converge with the achievements in the other towards an overall understanding of mitochondrial formation.

1. The origin of mitochondria

One of the earliest questions in the study of mitochondrial biogenesis was the origin of the organelle.

In the early fifties and sixties it was suggested that mitochondria originated from other intracellular

structures and almost each structure such as the nucleus, the nucleolus, the Golgi apparatus,the microsomal or the cell membrane, respectively,have been proposed as a possible candidate (reviewed in 5, 6). These suggestions were based mainly on morphological observations of positively stained membranes and are difficult to accept today, after the introduction of negative stain techniques which revealed proformed structural differences between the various intracellular membrane systems.

The hypothesis of "de novo" formation of mitochondria must be considered only as having a historical interest since none of the experimental observations claimed to support it were capable of proving the exclusive formation of the organelle from individual, non-particulate precursors.

In contrast with the above hypothesis, there is convincing experimental evidence supporting the concept, generally accepted today, that mitochondria are formed by a process of growth and division.

In one of the first biochemical experiment which indicated this,Luck (7) used a choline-deficient, exponentially grown mutant of Neurospora crassa.The externally added radioactive choline was incorporated into the phospholipids and served as a marker for formation of mitochondria. After growing in medium containing the labeled choline, the cells were transferred to a nonlabeled growth medium and the distribution of the label in the isolated mitochondria was followed by quantitative autoradiography. The random

distribution of the label in the mitochondria together with the diminishing average grain count as a function of growth in the nonlabeled medium were considered as proof of mitochondrial formation by division.

2. The multisubunit complexes of the respiratory chain.

As a result of a continuous effort to characterize biochemically the unique device by which mitochondria accomplish their respiratory function, Hatefi et al. (8) succeeded in isolating five protein-lipid complexes from the inner membrane of beef heart mitochondria. Four of these complexes are involved in electron transfer and the fifth in the respiration coupled ATP synthesis. These complexes are rather tightly imbedded in the mitochondrial membrane and the fractionation procedures involve in general the use of deoxycholate and cholate in conjunction with KCI for membrane solubilization followed by ammonium acetate and ammonium sulfate for the precipitation of the desired complex. Each multisubunit complex of the oxidation chain is catalytically active within a particular segment of the overall oxidation reaction and the sequence in which electrons are carried from one complex to the other is determined by the oxidation-reduction midpoint potentials of the electron carriers (9).

Complex I (NADH: Coenzyme Q reductase) catalyses the reduction of coenzyme Q by NADH. This sequence constitutes site I of energy-coupling. It contains covalently bound FMN and in most organisms at least four non-heme iron

centers (10). Phospholipids are essential for activity. The bovine complex may contain as many as fifteen polypeptide subunits and has a molecular weight as high as 850,000 daltons (10). It is rotenone and piericidin A sensitive in contrast to the yeast complex (11).

Complex II (succinate: Coenzyme Q reductase) contains covalently bound FAD, at least two non-heme iron centers (12) and possibly a b type cytochrome with an λ max of 565 nm (13). Succinate dehydrogenase is a component of the complex. It has a molecular weight of 97,000 daltons (13) and contains FAD and two ferredoxin like non-heme iron centers.

Complex III (Coenzyme QH_2: cytochrome c reductase) catalyzes the reduction of cytochrome c by $CoQH_2$ and forms the site II energy coupling segment. Currently, the subunit composition of the b-c_1 complex in yeast has been rather well-characterized. Less known are the topographical localization of the subunits in the membrane and the functional role of each component.

An important requirement for the characterization of a multisubunit complex is its isolation in an active form. Recently, in contrast to several previous studies performed on enzymatically inactive complexes, a functionally active b-c_1 complex has been isolated from yeast by Siedow et al (14). In this laboratory (15) the complex was shown to consist of 10 polypeptides, 9 cytoplasmically made polypeptides and one polypeptide, cytochrome b, which is translated on mitochondrial

ribosomes. The following apparent molecular weights of the components were determined by SDS gel electrophoresis: 2 core proteins (50,000 and 40,000); one cytochrome $\underline{c}_1$ (31,000); one cytochrome $\underline{b}$ (30,000), and 6 polypeptides with 24,000 (iron-sulfur protein); 17,000, 16,000, 12,000, 8,400 and 5,800. The complex isolated in this laboratory was able to catalyze the reduction of cytochrome $\underline{c}$ by a reduced synthetic analogue of CoQ and was inhibited by antimycin A or Diuron. Previously, a functionally inactive $\underline{b}$-$\underline{c}_1$ complex was isolated from yeast by Katan *et al* (16). It appears that at least some of the cytoplasmically translated products are synthesized as larger precursors. Thus, Côté *et al* (17) showed that the 24,000 dalton polypeptide is translated as a larger precursor form and results from this laboratory suggest that this might be the case for the two core proteins (unpublished results).

One of the most interesting components of the $\underline{b}$-$\underline{c}_1$ complex is cytochrome $\underline{b}$. In this respect, the existence of more than one type of cytochrome $\underline{b}$ per complex remains a prominent subject of debate in the literature, more than 20 years after Chance reported in 1958 the presence of three spectrally distinct cytochrome $\underline{b}$ bands in submitochondrial particles of beef heart mitochondria (18). The classical cytochrome $\underline{b}$ absorbs at 562 nm upon reduction with succinate, has a half reduction potential of $E_{m,7} = + 50$ mV and is

designated as cytochrome $\underline{b}_K$. Two other peaks, at 558 and 566 nm were obtained by reduction with succinate in the presence of ATP or antimycin and were assigned to a different, high energy form of cytochrome $\underline{b}$, designated as $\underline{b}_T$ (from "energy transducing"). The cytochrome $\underline{b}$ which absorbs at 566 nm has an $E_{m,7}$ of - 50 mV. The presence of the cytochrome $\underline{b}$ peaks was confirmed subsequently but the sharp functional differentiation has been challenged by the reports that the reducibility of all species of cytochrome $\underline{b}$ is increased by ATP (19). Spectral and potentiometric evidence was presented in support of the existence of two $\underline{b}$ cytochromes, in beef heart mitochondria (20) and of three $\underline{b}$ cytochromes in rat liver (21), in pigeon heart (22, 23) and in plant mitochondria (24).

The chemical evidence for the existence of more than one cytochrome $\underline{b}$ species is supported by the findings that isolated $\underline{b}$-$\underline{c}_1$ complexes from beef heart (25), N.crassa (26) and yeast (16) contained 2 moles of $\underline{b}$ heme per mole of $\underline{c}_1$ heme.

However, when cytochrome $\underline{b}$ is purified from the $\underline{b}$-$\underline{c}_1$ complex, only one spectral component is detectable (27, 28). Furthermore, genetic evidence does not suggest the existence of more than one cytochrome $\underline{b}$ species. Thus, most mutants in the genome segment that codes for apocytochrome $\underline{b}$ (cob mutants) have lost apocytochrome $\underline{b}$ as a result of a single reversible mutation and it was emphasized that there was never observed the concomitant presence of apocytochrome $\underline{b}$ and a fragment of

the apocytochrome in a cob mutant (29).

Still another possibility to consider would be Wikstrom's suggestion that different spectral properties of the same chemical cytochrome b molecule may arise from differences in the membrane environment due to the different membrane locations of the cytochrome b (30).

Complex IV (cytochrome c oxidase) catalyzes the oxidation of reduced cytochrome c by molecular oxygen and constitutes the site III of energy coupling. It is inhibited by carbon monoxide, cyanide and azide. It contains two moles of heme a, one of which can react with O_2 or CO (cytochrome a_3) and two copper atoms. It is not known which subunit(s) (31) of the yeast complex is linked to the heme. It was claimed that in beef heart mitochondria the heme might be associated with subunits I, II, and IV (32), or with subunit V (33). The amino acid sequence of subunits II, IV, VII and VIII from beef heart mitochondria was obtained (34). Based on sequence homologies with the azurins, plastocyanins and stellacyanins, known copper-carrying proteins, Steffens and Buse (35) suggested that subunit II might be the copper-carrying polypeptide in cytochrome oxidase.

The purified complex from yeast contains seven polypeptide subunits; the three larger are synthesized in the mitochondria, the four smallest in the cytoplasm (36, 37). The apparent molecular weights of the subunits are: Subunit I: 40,000 ; subunit II: 33,000 ; subunit III: 22,000 ;

subunit IV: 14,500; subunit V: 12,700; subunit VI: 12,700; subunit VII: 4,600 .

Poyton and McKemmie (38) reported that the four cytoplasmically translated subunits are imported into the mitochondria as a polyprotein precursor to the subunits IV - VII and is processed only after its association with the inner mitocondrial membrane. In addition, Sevarino and Poyton (39) showed recently that subunit II is synthesized in an in vitro system as a precursor with a transient "leader sequence" which is processed posttranslationally.

The Oligomycin-Sensitive ATPase (Complex V) catalyzes in vitro the oligomycin and rutamycin sensitive hydrolysis of ATP. It is believed to catalyze the terminal step in oxidative phosphorylation during which ATP is synthesized. The yeast enzyme has been purified by Tzagoloff et al (40). It is composed of 10 subunits, of which six are of cytoplasmic origin. Five of these assemble in the F_1 catalytic oligomer subunit facing the matrix side of the inner membrane and the sixth is necessary for F_1 to bind to the membrane (the oligomycin-sensitivity-conferring protein). Four hydrophobic subunits, membrane imbedded, are of mitochondrial origin and assemble in the F_o oligomer which acts apparently as a proton conducting channel. The smallest hydrophobic subunit with a molecular weight of 8,000 has been sequenced (41) and shown to bind the ATPase inhibitor DCCD at the glutamic acid residue in position 59. Oligomycin, another inhibitor of this enzyme, binds apparently

at different but interacting site on the same subunit (42). Maccechini et al (43) reported that three of the F_1-subunits are translated as larger precursors in a cell-free system and processed post-translationally in the mitochondria to the mature form.

Coenzyme Q (ubiquinone) was postulated by Green (44) to act as a lipid soluble, mobile electron carrier between complexes I and II and Complex III. It is present in yeast as CoQ6 (45).

Cytochrome c transfers electrons from complex III to complex IV. It is very water soluble, and loosely bound, hence easy to remove from the membrane. S. cerevisiae contains two isozymes of cytochrome c. Isozyme I comprises 95% of the total cytochrome c (46).

3. The Mitochondrial Genetic System.

Mitochondria have an autonomous genome, distinct from nuclear DNA, but the formation of the organelle is under the precisely coordinated control of the two genetic systems. It is now demonstrated that mt DNA is able to replicate and that it codes for the three classes of RNA (rRNA, mRNA and tRNA), which are distinct from their nuclear counterparts (47, 48). Eight-ten mitochondrial polypeptides are translated on mitochondrial ribosomes (48). Some of the characteristics of mt DNA and the map of yeast mt DNA will be described below.

3.1. mt DNA

Almost all mt DNA's appear as closed circular duplexes of about 10^7 daltons (47). Exceptions are the ciliates in which mt DNA was found to be linear with unique ends. Yeast mt DNA seems to have a contour of 25 μm with a molecular weight of 5×10^7 daltons and contains about 75,000 base pairs. Bernardi *et al.* (49, 50) showed that yeast mt DNA has an unusually high (A-T) content (82%) and suggested that these regions, amounting to half of the genome serve as spacers. These regions are interspersed with GC rich sequences, clustered in 70 sites along the genome.

It has been suggested that these clustered sequences might have a possible promoter role. Other possible involvements of some of these rich GC sequences in the process of DNA replication, or termination of transcription, or mRNA processing, respectively, have been suggested recently by Cosson and Tzagoloff (51).

3.2. mt DNA Replication.

The rather detailed knowledge of the sequences which occur during mt DNA replication became possible by the isolation of mt DNA molecules from mammalian cells and their analysis by electron microscopy. The initiation of replication is characterized by the synthesis of a 7S single strand DNA fragment which displaces the opposite strand with formation of a "D-loop" (52). The process continues with the extension of the 7S DNA and expansion of the D-loop. The heavy strand

is replicated first and a large segment of this strand has to be replicated before the replication of the light strand starts.

3.3. Transcripts of mt DNA

Mitochondrial DNA is transcribed *in vivo* and in yeast it seems that the transcription is partly symmetrical (47). A most important finding in genetics in the last three years was that there are relatively long stretches of base sequences in the DNA which do not appear in the processed mRNA. The gene is split. In yeast, the primary transcripts of the genes coding for 21SrRNA, cytochrome *b* and subunit I of cytochrome oxidase, respectively, are processed by splicing. Segments transcribed from intervening sequences are removed during this process and are not expressed in the final products, while the remaining transcripts termed exons are joined together and are expressed in the final protein products (47, 53). Whereas the exons specify the primary structure of the gene product, the role of the introns, the DNA segments that are interspersed between the exons is not yet understood. Slonimski (54) has proposed that the introns might be involved in the correct splicing of the primary transcripts or in pre mRNA processing.

3.4. Mitochondrial ribosomes

The large and small subunits of the mitochondrial ribosomes sediment in sucrose gradients with sedimentation coefficients of 54S and 38S (56), in contrast to their cytoplasmic counterparts which have sedimentation coefficients of 60S and 40S respectively. All proteins of the large mitochondrial ribosomal subunit are of cytoplasmic origin. Of about 33 proteins

of the small ribosomal subunit only one Var-1, is a product of mitochondrial protein synthesis (57). To date, the following polypeptides have been identified as products of synthesis on mitochondrial ribosomes: Var-1, the three large subunits of cytochrome oxidase, cytochrome b, and the four subunits (F_o) of the intrinsic membrane component of ATPase (53).

3.5. The mt DNA map. Mutants.

Mapping and sequencing of mt DNA has presented major challenges to geneticists and molecular biologists. It is clear that the considerable progress achieved in this respect in yeast reflects the unusual features of this organism which have been ingeniously exploited by investigators. The physical and genetic mapping of mt DNA in yeast was mainly possible due to the isolation and detailed characterization of a wide spectrum of mitochondrial mutants. Three major types of mutants served as useful tools in the analysis of yeast mt DNA: the ρ^- mutants, the antibiotic resistant mutants and the mit^- mutants (53).

3.5.1. The ρ^- mutants

These were the first described cytoplasmic mutants in yeast (58) and called petites due to the formation of small colonies. Petites are unable to synthesize mitochondrial proteins as a result of large deletions (50-99.9%) of their mt DNA. They can however retain the segments which in the wild type are translated to the corresponding products. The petite mutation is inherited in a non-Mendelian manner and petites cannot revert. Probably the most unique property of these mutants

is that the retained segment is replicated by the cell in a repetitive, tandem manner, without major errors, to the original size of the genome, providing thus pure and enriched mt DNA segments. Furthermore, the genetic characterization of the segment retained is made possible by mating the mutant with wild type cells and transfer of the markers to the cells. In contrast with ρ^- mutants, ρ^o cells have a total deletion of mt DNA. Furthermore, ρ^o mutants have to be distinguished from PET mutants which have nuclear mutations that block respiration but retain mitochondrial protein synthesis.

Petite mutants retaining known segments of structural genes coding for ATPase subunit 9, apocytochrome b and cytochrome oxidase subunit II respectively, were used by Macino et al (59) for sequence determination with restriction endonucleases. These studies lead to the discovery that in a eukaryote the concept of the universality of the genetic code is not applicable: Yeast mitochondria possess a suppressor $tRNA^{TRP}$ and hence the UGA termination codon is used for tryptophan.

3.5.2. Antibiotic Resistant Mutants.

These mutants arise spontaneously or can be induced and the resistance is due to point mutations or small deletions in mt DNA. The easy isolation of these mutants and their use in combination with petite deletions permitted the assignment of five drug resistance loci on yeast mt DNA in the following order: Cap 1-ery 1-oli 1- ana 1- par 1- cap 1 (60), in which

cap= chloramphenicol, ery= erythromycin, oli= oligomycin, ana= antimycin,par= paromomycin. Recombination studies of these markers indicate that they are far apart in the genome and can be considered unlinked. A second locus for oli was described by Avner et al (61), not linked with oli-1. The cap and ery resistance in these cells is apparently caused by lesion in the large mitochondrial ribosomal subunit (62).

3.5.3. Mit$^-$ and Syn$^-$ Mutants.

Mit$^-$ mutants have a functional mitochondrial protein synthesizing machinery but lack specifically one or more components of the mitochondrial respiratory chain complexes, Hence, they are not able to grow on non-fermentable carbon sources.

Syn$^-$ mutants lack one or more components of the mitocondrial protein synthetic machinery; the lesion(s) affect(s) one or more of the RNA's (t-RNA, r-RNA, m-RNA).

Mit$^-$ and Syn$^-$ mutants are due to point lesions or deletions ranging from a few to several thousand base pairs (63).

3.5.4. The COB-BOX region on the genome.

Results obtained from both genetic and biochemical studies allowed the construction of a DNA map (Fig.1). The so-called COB-BOX region situated between the oli-1 and oli-2 loci codes for apocytochrome b. The structural gene for apocytochrome b is found in five non-adjacent segments (exons). Mutations in one of these loci result in the lack of apocytochrome b with the concomitant appearance of new products with shorter polypeptide chains, denoting probably a premature termination.

LEGEND TO FIG. 1.

A. The inner ring shows the map location of genetic markers.

ery, cap = loci of mutations conferring erythromycin, respective chloramphenicol resistance.

oxi-1, oxi-2, oxi-3 = loci in segments coding for subunit II, III and I of cytochrome c oxidase.

cob-1, cob-2 = loci associated with cytochrome b coding segment (COB-BOX).

oli-1, oli-2 = oligomycin resistant mutations associated with ATPase subunit 9 coding segment.

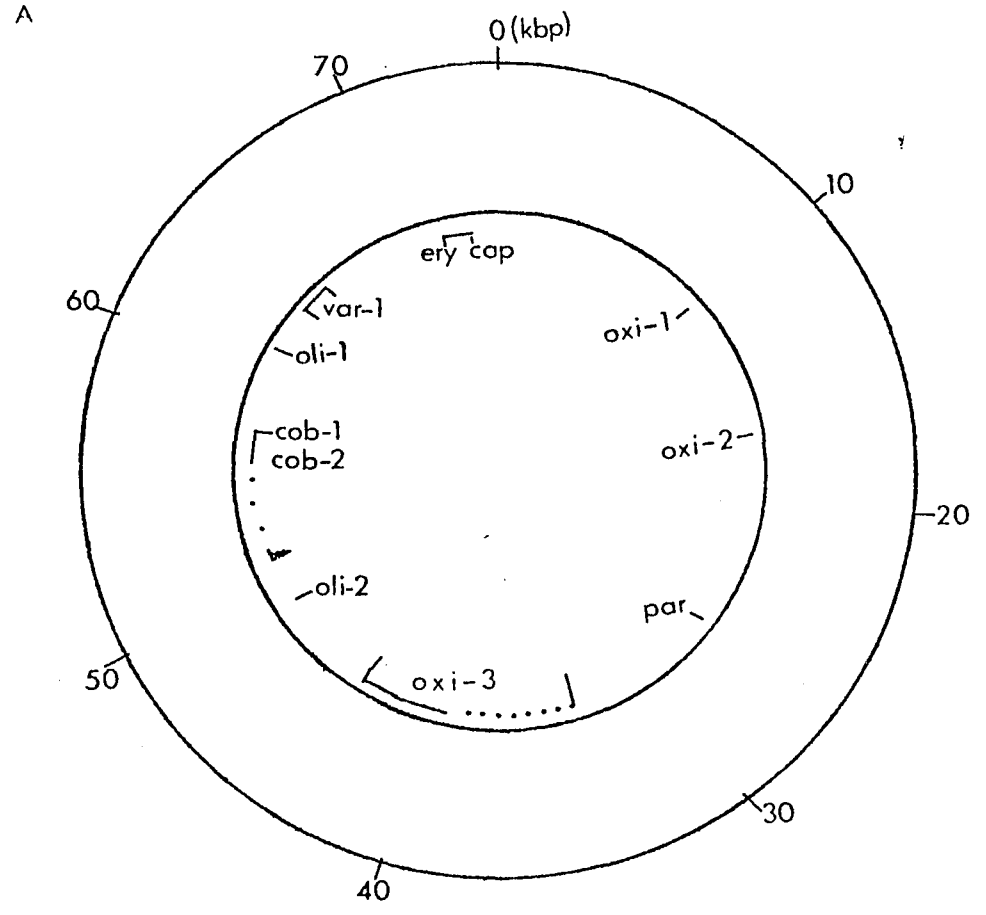
par = loci of mutation conferring paromomycin resistance.

var-1 = segment coding for the ribosome associated protein Var-1.

The length of the genome is expressed on the outer ring in Kilobase pairs.

B. The COB-BOX segment which codes for cytochrome b:

The five hatched clusters (exons) are interspersed with 4 introns (white). Mutants can occur in both. Numbering is historical and not logical. Arrow indicates direction of transcription (refs. 53, 55).


A
0 (kbp)
10
20
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40
50
60
70
ery
cap
var-1
oli-1
cob-1
cob-2
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oxi-2
oxi-3
par

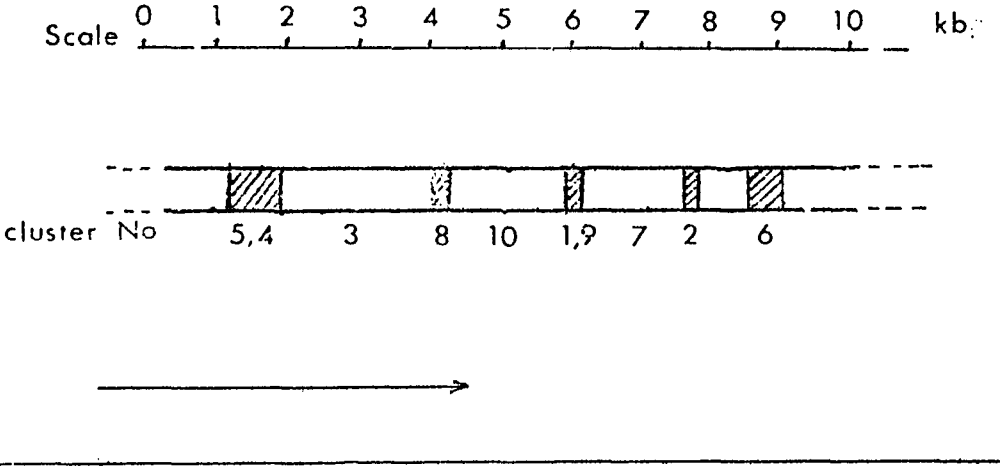
B
Scale
0 1 2 3 4 5 6 7 8 9 10
kb
cluster No
5,4 3 8 10 1,9 7 2 6

Fig. 1

The five non-adjacent clusters of the structural gene are separated by four segments (introns) which might have a role in the regulation of the processing of pre-mRNA for cytochrome b and for cytochrome oxidase subunit I (53). Mutations in these segments sometimes result in new products with molecular weights of 15,000 to 60,000, immunological related to cytochrome b and sometimes lack cytochrome b as well as subunit I of cytochrome oxidase (55). The presence of polypeptides with chains longer than cytochrome b suggest that in these cases translation occurs through sequences not normally translated.

4. Objectives for studies of mitochondrial biogenesis.

The major unsolved problems in the field of mitochondrial biogenesis include: (1) identification of the mitochondrial and cytoplasmic products required for mitochondrial function; (2) determination of the factors that control synthesis of the individual subunits of mitochondrial complexes; (3) elucidation of the regulatory interaction between the mitochondrial and nuclear genetic systems; (4) determination of the mode in which cytoplasmically made products are imported into the membrane of the organelle; (5) determination of factors that control the proper assembly of subunits into functionally active complexes and (6) determination of the function of each subunit component. Such diverse and complex problems present a major challenge and their study must be approached adequately.

5. The eukaryotic cell Saccharomyces cerevisiae, a model system for the study of mitochondrial biogenesis.

Three main types of systems have been used for the study of mitochondrial biogenesis: organized tissues of higher eukaryotes, mammalian tissue culture lines and lower eukaryote cells (yeast and ascomycetes). It was recognized early that the eukaryotic yeast cell S. cerevisiae is particularly suitable for this purpose, since it is a facultative anaerobe and does not need a mitochondrial respiratory chain for replication and survival. Cells are able to grow under conditions which depress formation of the respiratory chain. By changing these conditions, these cells can regain a functional respiratory chain in their mitochondria. The investigator is thus able to maneuver in vivo the formation of functional mitochondria. There are two ways by which this "modulation" of the respiratory chain can be achieved: glucose (or catabolite) repression and respiratory adaptation (anaerobiosis / aerobiosis). Characteristic metabolic effects accompany each of these processes.

6. Glucose (catabolite) repression.

The phenomenon of glucose repression, defined as the repression of the synthesis of certain intracellular enzymes following the addition of glucose to the growth medium was first reported for bacteria by Epps and Gale (64) and for yeast by Spiegelman and Dunn (65). Subsequently, Magasanik (66) called the effect catabolite repression, when it was

observed that not glucose itself but rather rapidly accumulating catabolites repress the enzyme synthesis.

6.1. Effect of glucose repression on mitochondria.

Yeast cells grown aerobically in the presence of rather high concentration of glucose (5% or greater) contain mitochondria which have lower cytochrome content and lower respiratory activity (67, 68). Other fermentable sugars have similar effects and the level of repression depends on the carbon source, glucose repressing more intensely than galactose. (69, 70). In contrast, glycerol and lactate, non-fermentable substrates, stimulate yeast mitochondrial respiratory activity. In the initial exponential stage of growth, when glucose concentration is high, the cells use preferentially the glycolytic pathway for energy production while the product of the glucose degradation, ethanol, accumulates in the cytoplasm. The cells are repressed. Once the glucose is exhausted, growth almost ceases for some hours. During this time respiratory enzymes increase in activity. The cells start to utilize the ethanol and become derepressed. During this period mitochondria form compact organelles with well developed internal membranes and cristae . Full respiratory activity develops concomitant with an increase in cytochrome content. The inability of cells lacking respiratory complexes to grow on non-fermentable substrates has been used frequently in biochemical genetics to test for the existence of a respiratory chain.

While carbon catabolite repression is an important regulatory phenomenon in yeast, the process is poorly understood at the molecular level.

7. Respiratory adaptation. Promitochondria.

Complete repression of respiratory chain activity can be achieved in yeast cells by anaerobic growth. In this case, supplementation with a fermentable carbon source is necessary. A major consequence of anaerobiosis is to block heme synthesis and as a result the formation of a whole array of holocytochromes. Whereas cells can survive in the absence of cytochromes involved in respiration, other non-mitochondrial cytochromes are necessary to maintain cell viability. Thus the absence of cytochrome P450 leads to a blockage in the demethylation of lanosterol and ergosterol cannot be synthesized (71). The lack of cytochrome b_5, a compound of the fatty acid desaturase system, results in the incapacity of the cells to make unsaturated fatty acids (72). Therefore, during anaerobiosis, the cells must be supplemented with ergosterol and unsaturated fatty acids in order to survive. In contrast with the glucose (catabolite) repressed cells, anaerobic cells are completely devoid of a respiratory chain.

The very existence of mitochondria as an organelle in anaerobic grown cells was a subject of controversy. In such cells, Wallace and Linnane (73) were unable to detect mitochondria in electron micrographs and concluded that anaerobic grown yeast do not contain mitochondria. Subsequently however, the existence of promitochondria (thus called to differentiate it from fully respiratory mitochondria) has been demonstrated (74). Promitochondria have been shown to retain both the

mitochondrial genome and the ability to synthesize proteins (75). Specifically, the presence of oligomycin-sensitive ATPase was demonstrated (74). Anaerobic mitochondria also contain NADH, succinate, and malate dehydrogenases, products of cytoplasmic protein synthesis, although at greatly reduced levels (76). It is of interest that apocytochrome $\underline{c}_1$, a cytoplasmically made polypeptide, was not detected immunochemically in anaerobic, promitochondrial membranes (77), suggesting a role of O_2 in the import of the polypeptide into the membrane. When anaerobically grown cells are exposed to O_2, the process of respiratory adaptation induces the formation of cytochromes $\underline{a}$, $\underline{a}_3$, $\underline{b}_1$, $\underline{c}_1$ and $\underline{c}$ and of cytochrome $\underline{c}$ oxidase and the synthesis of Krebs cycle enzymes.

8. Major approaches to the study of mitochondrial biogenesis.

Various approaches which have been used to study mitochondrial biogenesis in yeast are outlined below.

8.1. Biochemical studies. In vivo

Biochemical immunological methods, which have been instrumental mainly in the unequivocal identification of the specific components of mitochondrial enzyme complexes, were first used to elucidate the biogenesis of the ATPase complex (78). Immunotechniques in conjunction with the specific inhibitors of the mitochondrial (chloramphenicol, erythromycin), and cytoplasmic (cycloheximide) translation systems were used in pulse labeling experiments mainly for the identification of

the site of synthesis of the different subunits of mitochondrial enzyme complexes. The result obtained provided for the first time evidence that a mitochondrial complex (ATPase) consisted of subunits synthesized both in the cytoplasm and in the mitochondria (79).

In these experiments a significant stimulation of the ATPase membrane factor was obtained when cells were first incubated with chloramphenicol, permitting an accumulation of cytoplasmic products, the drug removed and labeled leucine added in the presence of cycloheximide the inhibitor of cytoribosomes. A double labeling procedure and an immunoelectrophoretic analysis of the ATPase complex demonstrated that the four subunits of the membrane factor are synthesized by mitoribosomes. (79). Information on the assembly process, on its kinetics and on the regulatory role of the two genetic systems in the formation of the mitochondrial enzyme complexes, respectively, have been mainly obtained by following the development of enzymatic activities and spectral characteristics of these complexes during respiratory adaptation (80, 81) or glucose repression and derepression (70, 82), in the presence or absence of cycloheximide and chloramphenicol, respectively.

8.2. In vitro studies

The capability of isolated mitochondria to incorporate amino acids into proteins was first recognized by McLean et al. (83). The refinement of the method by Ibrahim et al. (84) for yeast made possible the use of in vitro systems to study more

directly the cytoplasmic control of mitochondrial protein synthesis.

8.3. Biochemical genetic studies. Mutants.

Whereas yeast mitochondrial mutants (Mit^-) were instrumental mainly in the construction of mit DNA maps (85), nuclear mutants have been isolated according to the reasoning that the specific product missing as a result of the mutation will provide information on its role in mitochondrial formation. Thus, a nuclear mutant lacking F_1 (a complex of cytoplasmic made subunits involved in the catalytic activity of the ATPase) was shown (86) to have a 10 times lower level of the three mitochondrially synthesized subunits of cytochrome c oxidase and to be unable to grow anaerobically on a fermentable substrate. These results suggested that mitochondrial ATPase might have an essential function in mitochondrial formation even in non-respiring cells. Nuclear mutants with a lesion in the structural gene for δ-aminolevulinic acid synthetase (87) have been useful in studies on the role of heme in mitochondrial biogenesis. Due to the heme deficiency these cells are respiratory deficient, need a fermentable carbon source and, like wild-type anaerobically grown cells, need ergosterol and unsaturated fatty acids for growth (71, 72). Studies in the role of heme in cytochrome c oxidase formation in yeast showed that lack of heme has apparently two effects: it affects the insertion into the membrane of the cytoplasmically made subunits V and VII

which are not detectable and it affects the assembly process of the existing subunits which, apparently remain unassembled.

9. Background studies from this laboratory.

The objectives of this thesis are integrated in an overall project in this laboratory which investigates the biogenesis in yeast of the $CoQH_2$-cytochrome c reductase segment of the respiratory chain (b-c_1 complex) and the biogenesis of cytochrome b.

A major problem in mitochondrial biogenesis is the mechanism by which the two protein synthesizing systems, cytoplasmic and mitochondrial, are controlled such that the mitochondrial respiratory complexes are assembled in a functional manner. In order to gain information on the individual contribution of the two protein synthesizing systems, and on the control mechanism, the formation of the complexes was studied by Kim and Beattie (70) in yeast undergoing glucose derepression. The needs of proteins synthesized at the two sites was estimated by adding at various times during the derepression period,either cycloheximide or chloramphenicol. The results of these experiments suggest that the membrane is assembled in an asynchronous process. Differences were observed in the time-course of formation in the complexes studied. It could be shown that proteins synthesized in the two systems accumulate prior to the formation of the NADH-cytochrome c reductase complex, whereas the assembly of a

functional ATPase complex required concomitant synthesis of proteins in the two systems.

In further experiments by Ibrahim et al. (84) the rate of labeled amino acid incorporation into mitochondrial proteins was followed in partially derepressed cells, three hours after mitochondrial protein synthesis was inhibited by chloramphenicol. The results indicated that after removal of the drug, the rate of labeling of mitochondrial protein was stimulated. These results suggested that during chloramphenicol inhibition, cytoplasmically synthesized proteins might accumulate and upon the removal of the inhibitor stimulate the mitochondrial formation of the complexes.

In a further attempt to learn whether precursors of both synthesizing systems can accumulate in the mitochondria in glucose repressed cells and in order to evaluate their putative role in the formation of the individual enzyme complexes, Lin et al. (88) followed the development of enzymatic activities in glucose derepressed cells, after the sequential addition of inhibitors of the cytoplasmic and mitochondrial protein synthesizing systems. A prerequisite for meaningful results in these experiments was the use of the two drugs for relatively short times of inhibition. Glucose repressed cells were first grown in the presence of cycloheximide for 3 hours to block cytoplasmic protein synthesis. Subsequently, the inhibitor was washed out and chloramphenicol was added to inhibit mitochondrial translation. It was observed that the appearance of enzymatic

activities showed a slight inhibition for ATPase but the rate was equal to the control cells for both cytochrome oxidase and succinic dehydrogenase. These results suggested that glucose repressed cells have accumulated enough proteins made in the cytoplasm to support the formation of the complexes studied despite the immediate inhibitory action of cycloheximide.

In order to define the putative control mechanism of the cytoplasmically made products on mitochondrial formation of complexes Ibrahim and Beattie investigated the effect of chloramphenicol preincubation on the polysome profile of mitochondria (89). In cells preexposed to chloramphenicol, a higher polysome: monosome ratio than in control cells was found, whereas in cycloheximide preincubated cells the ratio was lower. The induction of enzymes of the mitochondrial respiratory chain and cytochrome formation were examined by Brown and Beattie (81) during aerobic adaptation of yeast cultures grown anaerobically. The results obtained showed distinct patterns in the accumulation of cytoplasmically made proteins for cytochrome $\underline{b}$-$\underline{c}_1$ complex and cytochrome $\underline{c}$ oxidase, suggesting differences in the mechanism of formation of these complexes of the respiratory chain.

In order to gain a better understanding on the role of Coenzyme Q in the transfer of electrons from succinate dehydrogenase to cytochrome $\underline{c}$, a subject that previously raised some disagreement (90) and to obtain more information on the controversial existence of a compartmentation of CoQ within the

membrane, Brown and Beattie (91) used nuclear mutants lacking CoQ. The results of reconstitution experiments with a synthetic analogue of CoQ suggested that CoQ is not compartmentalized in the membrane, but that the NADH and succinate dependent reduction of cytochrome c have a different dependence on CoQ.

In in vitro studies, Ibrahim et al. (84) showed that mitochondria isolated from yeast can synthesize, in a protein synthesizing medium, supplemented with an ATP regenerating system, polypeptides equivalent in size to those made in vivo. These results were confirmed subsequently by Poyton (92). Recent studies in this laboratory (93) have provided further evidence that cytoplasmically made products, protein in nature, can exert a stimulatory effect on mitochondrial protein synthesis. These investigations were prompted by an observation reported by Poyton and McKennie (94) that a post-mitochondrial supernatant can restore the protein synthetic activity of isolated mitochondria which have stopped synthesizing proteins. The results obtained in this laboratory showed that a significantly higher stimlation of protein synthesis by mitochondria can be obtained when the post-mitochondrial supernatant was added at the start of the amino acid incorporation reaction. Furthermore, similar stimulatory effects were obtained when post-mitochondrial supernatants from rat liver and E. coli were added to isolated yeast mitochondria suggesting the presence in different species of a common stimulatory factor which may have been conserved during evolution.

10. Objectives of the present thesis.

The first objective of this thesis was prompted by the observation, made in this laboratory, that some specific mitochondrial products in the range of 58-60,000 and 67,000 molecular weight, yet not identified or described in the literature, were coimmunoprecipitated with cytochrome b by the specific antiserum to cytochrome b. The elucidation of the nature of these products and their relationship to cytochrome b represented one of the goals of this study. The immunostudies on the biogenesis of cytochrome b and the results which preceeded and determined this investigation will be described in Chapter Two. Chapter Three will present and discuss the data which suggest the formation of an aggregate of cytochrome b_1 observed after gel electrophoresis. Chapter Four will contain data on the detection at low temperature of a possible dimer of cytochrome b.

The second purpose of the present thesis was to define the role that heme, as a cofactor, might have in the synthesis of apocytochrome b and in the assembly of the subunits that form the b-c_1 complex.

Interest in this research was generated by previous reports in the literature suggesting that the absence of heme can affect in various forms the formation of the respective holoprotein, depending on the biological system (95-105).

Specifically, in yeast, it was reported that the lack of heme impairs respectively, the import into the mitochondria of

the cytoplasmically made apocytochrome c_1 and the insertion into the membrane of the cytoplasmically translated subunits V and VII of cytochrome c oxidase. Also, in the absence of heme, the presence of the mitochondrially formed subunit I of cytochrome c oxidase is barely detectable and subunits I - III remain apparently unassembled in the complex (105).

It was therefore of interest to learn if the lack of heme exerts any effect on the biogenesis of the mitohondrially translated apocytochrome b. The results of this research are described in the fifth chapter.

Chapter II

IMMUNOELECTROPHORETIC ISOLATION OF CYTOCHROME b FROM YEAST MITOCHONDRIA

ABSTRACT

Cytochrome b was isolated by immunoprecipitation from extracts of mitochondria isolated from yeast cells labeled in vivo. Analysis of the immunoprecipitates by sodium dodecylsulfate gel electrophoresis revealed a major labeled band with an apparent molecular weight of 31,000, corresponding to cytochrome b. No cytochrome b apoprotein was present in a cytoplasmic petite mutant which lacks mitochondrial protein synthesis. Two additional high molecular weight labeled polypeptides were observed in immunoprecipitates obtained from cells labeled with [^{3}H]leucine, either under growing conditions or in vivo under non-growing conditions in the presence or absence of cycloheximide. These results suggest a mitochondrial site of synthesis for these polypeptides. The time course of labeling of the mitochondrial translation products suggests that these high molecular weight polypeptides are not precursors of cytochrome b.

INTRODUCTION

This study was prompted in order to establish whether minute amounts of cytochrome b could be isolated from the mitochondrial membrane with an immune serum to apocytochrome b and subsequently to investigate its biogenesis. Whereas immunomethods have been employed previously in yeast in studies on the formation of the cytochrome oxidase complex (106), of the ATPase (79) and on an isolated b-c_1 complex (107), no similar attempt was known to have been made in the study of the biogenesis of cytochrome b.

MATERIALS AND METHODS

Strains of yeast

Most experiments were performed with the haploid strain D273-10B. Certain experiments were accomplished with a diploid strain isolated in the laboratory and used for several years.

Growth and Collection of cells

Cells were grown aerobically at 30° C in a liquid culture medium (20% volume of the total flask volume), by shaking at 200 rpm on a New Brunswick shaker. The culture medium consisted of the following ingredients in 950 ml H_2O: 3g yeast extract (2 g was used when cells were labeled in growth conditions), 0.4 g $CaCl_2$, 0.5 g NaCl, 0.7 g $MgSO_4.7H_2O$, 1 g KH_2PO_4, 1.2 g $(NH_4)_2SO_4$, 5 mg $FeCl_3$ (108), and 3% galactose as carbon source. Cells were precultured in a 50-125 ml flasks for 8 to 9 h and following an optical density determination (Klett colorimeter, filter 620) they were transferred to 250-500 ml of growth medium and grown for approximately 10 generations (15 h) to early stationary phase (450-500 Klett units). For each strain of cells a growth curve was determined in advance. Cells were collected by a 5 min centrifugation at 3000 x g (at 6-8° C). Usually, 8 g wet weight cells were obtained from one liter of culture medium.

Cell Breakage

All operations were performed at 4°-6° C. Cells were resuspended in STE buffer (0.25 M Sucrose, 20 mM Tris-HCl, pH 7.5,

1 mM EDTA) containing 1 mM phenylmethylsulfonylfluoride (PMSF), to minimize proteolysis. A fresh stock solution of 100 mM PMSF dissolved in dimethylsulfoxide (DMSO) or 10 mM PMSF in ethanol was used. Suspensions of 1-15 g wet weight cells were transferred to 50 ml Braun flasks and approximately 20 ml glass beads (0.45 - 0.50 mm diameter) were added for breakage.
Cthe cells were disrupted in a Braun mechanical cell homogenizer (Model MSK) for 3 to 20 sec at 4000 rpm. The flasks were kept for one minute intervals in ice in between disruption.

For quantities of cells below 1 g wet weight, 1.5 ml Eppendorf tubes (filled 1/3 with glass beads) were used for breakage. The tubes were placed in a multiholder adaptor (109) which was introduced into the Braun homogenizer and shaken for 2 to 3 min.

In some experiments, small quantities of cells were disrupted with glass beads in 16 x 150 mm glass tubes by shaking for 2 to 5 min on a Vortex at maximum speed (105).

Subcellular fractionation

The suspension of disrupted cells was brought to approximately 30 ml with STE buffer and centrifuged 3 times at 2000 x g for 10 min to obtain the cell homogenate free of debris. Mitochondria were obtained by centrifuging the supernatant at 19,000 x g for 20 min. The pellet was resuspended with a glass-glass homogenizer in STE-PMSF buffer and the centrifugation and resuspension repeated twice. The final pellet was the source of "washed mitochondria". The usual yield in wild-type strains following the cell breakage in Braun

homogenizer was:

total mitochondrial weight: 15-16% of the cell wet weight

total mitochondrial protein: 0.3-0.4% of the cell wet weight

To obtain submitochondrial particles, (SMP) the washed mitochondria was resuspended in 0.1 M sodium phosphate buffer, pH 7.5, 1 mM EDTA, and 1 mM PMSF, to a concentration of 10 mg protein/ml and sonicated at 4-6^{o} C 3 to 4 times for 15 sec with 1 min intervals with a Branson ultrasonicator. The cell homogenate was centrifuged for 5 min at 6000 x g. SMP were obtained by centrifuging the supernatant for 30 min at 100,000 x g. The pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and 1 mM PMSF.

Spectral determination of cytochrome b

For spectral determination, mitochondria or SMP were suspended in 0.1 M sodium phosphate buffer, pH 7.5, containing 2% sodium cholate and adjusted to 5-8 mg protein/ml. Difference absorption spectra were obtained with a Cary model 15 at room temperature as described previously (81).

Labeling procedures

In order to label uniformly all cell proteins, cells were grown for approximately 10 generations in semi-synthetic medium in the presence of 5 $\mu Ci.ml^{-1}$ [4,5-^{3}H] leucine (55 $Ci.mmole^{-1}$). In labeling experiments in which two types of strains (wild-type and mutant, respectively) were labeled under growing conditions, the wild-type cells were grown in the presence of 2 $\mu Ci.ml^{-1}$ [^{14}C] leucine (330 $mCi.mmole^{-1}$). Equal counts of extracted mitochondria from each type of strain were

mixed and the mixture immunoprecipitated and analyzed by electrophoresis.

Labeling of cells in vivo in non-growing conditions was performed in pulse or pulse-chase experiments respectively. The harvested, three times water washed cells were suspended at 250 mg/ml in 0.05 M sodium phosphate buffer, pH 7.4,containing 0.1% glucose and incubated for 20 min at 30° C, in the absence or presence of cycloheximide, 100 µg/ml, respectively. Fifty µCi/ml of [^{3}H] leucine was added and incubation continued for 30 min. Unlabeled leucine 10 mM, was used in chase experiments for an additional 1 h.

Mitochondria were prepared and immunoprecipitates of cytochrome b were analyzed by disc gel electrophoresis.

Labeling of cells under non-growing conditions with ^{35}S methionine (usually 1000 Ci.mmole^{-1}), was used to analyze the mitochondrial translation products by slab gel electrophoresis and autoradiography. The harvested, distilled H_2O washed cells were suspended at 200 mg/ml in incorporation medium. This medium contained: 0.04 g $CaCl_2$, 0.05 g NaCl, 0.06 g $MgCl_2$, 0.1 g KH_2PO_4, 0.048 g NH_4Cl, 0.5 mg $FeCl_3$ and 2 g dextrose/100 ml. Following equilibration at 30° C for 10 min, 1 mg cycloheximide/ml was added and the incubation continued for 5 min with shaking. Labeling was started with ^{35}S methionine (100 µCi/100 mg wet weight cells) and stopped by addition of ice chilled incorporation medium containing 10 mM unlabeled methionine and cycloheximide (0.5 mg/ml).

Counterimmunoelectrophoresis (CIEP) was performed essentially as described by Crowle (110). A 1 mm layer of 1% agarose in 50 mM barbital buffer, pH 8.5, was cast on a microscope slide. Two rows of 2 mm diameter wells were punched in the slide, 6 mm apart in the direction of electrophoresis. The extract containing the antigen was placed in the cathodic well and 10 µl of antiserum or control serum in the anodic well. A long time run (8 to 12 h) at a low current intensity (2 mA/slide) was essential in order to obtain a visible precipitin line. After electrophoresis, the slides were rinsed for 2 to 3 days with shaking at 37° C in repeatedly changed 0.10 M sodium chloride, 0.05 M sodium phosphate buffer, pH 7.5, and subsequently dried and stained with Coomassie blue.

Preparation of Antiserum to Cytochrome b

Purified yeast cytochrome b (28) in 0.02 M sodium phosphate buffer and 0.5% Tween 80 was homogenized with an equal volume of Freund's complete adjuvant. A rabbit was injected with a total of 5.4 mg of protein in three equal doses, subcutaneously into one footpad, in the back and in the neck region. Each injection was one week apart. After 2.5 weeks, the rabbit received a booster injection of 1 mg of protein intravenously. Samples of blood were collected weekly after the booster injection and serum obtained by centrifugation at 1000 x g for 30 min at room temperature. Control serum was collected from the same rabbit prior to immunization.

Solubilization of Mitochondria or SMP for Immunoassays.

Throughout these studies, several solubilization procedures were developed for immunoassays and their usefulness evaluated. according to the magnitude of the extracted cytochrome b, determined spectrally.

The following solubilizing mixtures were tried:

1) KCl, 1M containing sodium cholate, 3 mg/ml protein (final concentrations) (111).
2) NaCl, 150 mM, containing 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS. (107).
3) Lubrol WX 1%.
4) Tris-acetate buffer, 5 mM, pH 7.5, containing 1% Triton X-100, 1M KCl (37).

Aliquots of mitochondrial or SMP suspensions in 0.1 M sodium phosphate, pH 7.5, containing 10 mg protein/ml were extracted for 2 h at 4^{o} C with one of the above mixtures. Also, a 14 h extraction (with extract medium #1) with stirring was performed, according to Ebner et al. (111). The non-solubilized material was removed by centrifugation, either at 10,000 x g for 10 min or at 100,000 x g for 30 min.

Immunotitration and Analysis of the Immunoprecipitates.

Immunotitrations and immunoprecipitation were performed either in siliconized glass tubes or plastic tubes to prevent non-specific adsorptions. For immunotitrations, labeled mitochondrial or SMP suspensions in 0.1 M sodium phosphate buffer, pH 7.5, were adjusted to 10 mg protein/ml and extracted with 1% Lubrol WX. In subsequent experiments, a mixture of 1%

Lubrol- 0.5% deoxycholate was used (112).

Immunotitration curves were performed by adding increasing amounts of antiserum to constant aliquots of extracts containing antigen. After immunoprecipitation was allowed to occur for 1 h at room temperature and overnight at 4° C, the precipitates were centrifuged at 10,000 x g for 5 min, washed three times with 0.5 ml sodium phosphate buffer, pH 7.5, containing 1% Lubrol, solubilized in capped tubes for 60 min at 50° in 0.2 ml NCS tissue solubilizer (Amersham) containing 10% H_2O (v:v) and counted in 10 ml scintillation fluid ACS (Amersham) in a Packard scintillation counter. Results were expressed as percent of counts in immunoprecipitate to initial total counts.

The optimum antibody: antigen ratio determined from the immunotitration curve was used to obtain an immunoprecipitate which was analyzed on 10% disc gel electrophoresis. For this purpose the three times washed immunoprecipitate was solubilized for 20 min at 70° C in a mixture of 0.02 sodium phosphate buffer, pH 7.2, containing 2% SDS, 2% β-mercapto-ethanol and 10% glycerol. After electrophoresis, the gel was sliced with a Gilson automatic slicer and the slices were digested overnight at 60° C with 30% hydrogen peroxide and subsequently counted.

During these studies, the discontinuous SDS-Tris buffer system described by Laemmli (113) was introduced. In this case the immunoprecipitate was solubilized in the medium described by Douglas and Butow (114) modified to contain

0.05 M Tris.Cl, pH 6.8, 1 mM EDTA, 5% SDS, 5% β-mercaptoethanol and 10% glycerol.

Separation and identification of the labeled mitochondrial products by slab gel electrophoresis. Autoradiography, fluorography and scanning of the bands.

Slab gels (10 cm long, 1.2 mm thick) were prepared according to Studier with minor modifications (115) and left overnight for aging. The discontinuous Tris buffer system described by Laemmli (113) was used for electrophoresis. The stacking gels were 5% acrylamide and the running gels were 10% acrylamide. In some cases, 7.5% to 15% acrylamide gels or 10 to 15% gradient gels were used. After electrophoresis, the slab gels were stained for a minimum of 1 h with 1.25% Coomassie blue in 45% methanol: 9.7% acetic acid and either destained by soaking overnight or with a Canalco destainer. The gels were dried for autoradiography on Whatman 3 MM filter paper by either using a drying device similar to that described by Fairbanks et al.(116) or a Biorad gel dryer. The dried gels were exposed at -70° C to Kodak NS-5T X-ray film for different periods of time depending on the number of counts loaded per lane. Usually 40,000 counts required one week of exposure.

Fluorography was performed with 2,5-diphenyloxazol(PPO)-dimethylsulfoxide (DMSO) according to the method of Bonner and Laskey (117).

Autoradiograms were scanned with a Canalco Model G microdensitometer.

The "low molecular weight" Pharmacia Kit, used as a calibration standard, covered the molecular weight range 14,400-94,000.

Determination of hot TCA insoluble protein was performed according to Mans and Novelli (118) using 3 MM Whatman disc filter paper.

Protein concentration was determined according to Lowry et al. (119). When mitochondria or SMP suspension were used, deoxycholate at final concentration of 1% was added to the samples and standards.

RESULTS

In order to study the biosynthesis of cytochrome b by immunotechniques it was first necessary to characterize the antiserum. It was shown previously in this laboratory by double diffusion analysis that purified cytochrome b produced a sharp single precipitin line with the antiserum while the preimmune serum failed to produce any precipitin line (28). The further characterization of the immuneserum and of the immune reaction is described below:

Counterimmunoelectrophoresis (CIEP)

CIEP was used as one approach to characterize the antibody. In CIEP, unlike in immunodiffusion, the immune reaction takes place subsequent to the electrophoretic separation of the nonimmunoglobulins and albumin fractions of the immune serum. As a result only the immunoglobulins come in contact with the antigen in the agarose matrix and sensitivity is increased. Counter immunoelectrophoresis of the purified cytochrome b peptide or of a preparation partially purified to fraction 8(f-8) (28) against the antiserum revealed only one precipitin line (Fig. 2). No precipitin line was observed when the same fractions were electrophoresed against control serum. The observation that the precipitin line is formed closer to the well containing antibody when pure cytochrome b is used rather than fraction 8 results from the considerably greater amount of antigen present in that fraction.

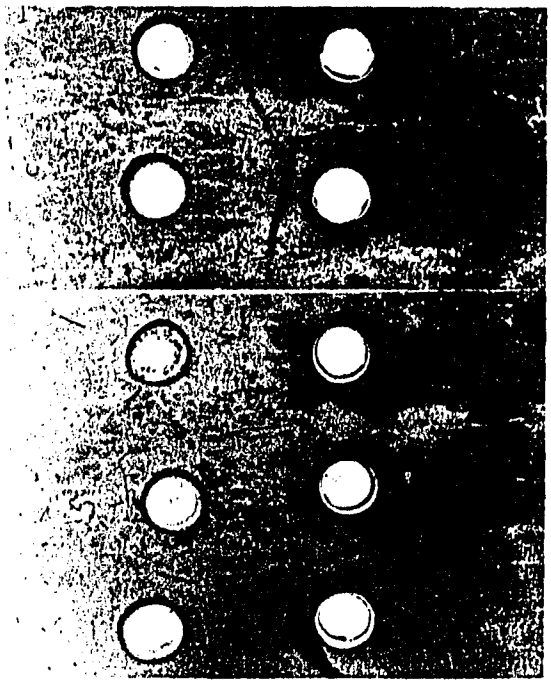

Fig. 2. Counter immunoelectrophoresis of cytochrome b. Solutions (10 µl) containing antigen were placed in the cathodic well (left-hand side of Fig. 2) in the following order from the top: (1,3) partially purified cytochrome b (fraction 8). (2,4) purified cytochrome b (fraction 9) and (5) ovalbumin. Different samples (10 µl) of serum were placed in the anodic wells (right-hand side of Fig. 2) in the following order from the top: (1,2) antiserum against cytochrome b, (3,4) preimmune serum and (5) antiserum to ovalbumin.

Solubilization of the mitochondrial membrane for immunoassays.

A prerequisite step for immunoassay is proper solubilization of the antigen (i.e. cytochrome b). The efficiency of various detergents to extract cytochrome b is shown in Table I. The extracts were separated from the unsolubilized material either by centrifugation at 10,000 x g for 10 min or at 100,000 x g for 20 min (111).

The results in Table I show that less than 15% of the cytochrome b, determined spectrally at 562 nm, was recovered in the 100,000 x g supernatant, regardless of the extract medium used. Hence, only low speed centrifugations were used in subsequent experiments. No reproducible spectra were obtained with extract medium #4 (Tris-acetate buffer, 5 mM, pH 7.5, 1% Triton X-100, 1 M KCl, sodium cholate, 3 mg/mg protein) and medium #2 (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS), medium #3 (1 % Lubrol WX) solubilized and maintained in the extract the highest amount of cytochrome b. Also, the ratios $\frac{\text{cyt b}}{\text{cyt a.a}_3}$ and $\frac{\text{cyt b}}{\text{cyt (a+c}_1\text{)}}$ were higher in the Lubrol 1% extracts. Therefore 1% Lubrol extracts were used for immunoassays. Subsequently 1% Lubrol - 0.5% sodium deoxycholate was introduced, when it was observed that this detergent mixture (112) resulted in a lower non-specific precipitation with control serum in immunotitrations.

TABLE I

PROTEIN AND CYT b RECOVERED IN 105,000g X 30 MIN AND 10,000g x 10 MIN SUPERNATANTS

Solubilization Medium	105,000g x 30 min					10,000 g x 10 min						
	Protein		Peak Height in cm			Protein		Peak Height in cm			cyt b / cyt a	cyt b / cyt a + c
	mg/ml	%	cyt a	cyt b	cyt c	mg/ml	%	cyt a	cyt b	cyt c		
#1 Supernatant	4.5	45.0	-	-	-	5.9	59	10.0	6.5	10.5	0.65	0.32
Pellet		55.0	2.0	10.5	9.0		41	0.0	7.0	0		
#2 Supernatant	3.72	37.2	2.3	3.4	6.5	6.0	60	8.5	13.5	11.	1.59	0.65
Pellet		62.8	14.0	20.5	11.0		40	-	-	-		
#3 Supernatant	4.60	46.0	1.5	1.5	4.5	7.5	75	11.5	19.3	16.5	1.68	0.69
Pellet		54.0	12.0	8.8	7.4		25	1.0	1.5	1.0		
#4 Supernatant	5.60	56.0	-	-	-	-	-	-	-	-	-	-
Pellet		44.0										

[a]SMP were solubilized for 2 hours at 4° with the following extraction media;

#1 - sodium cholate, 3 mg /mg protein and KCl, 1M (final concentration)

#2 - sodium chloride, 150 mM, containing 1% Triton X-100, 0.5% Deoxycholate, 0.1% SDS

#3 - Lubrol WX 1% (final concentration)

#4 - Tris-acetate buffer, 5 mM,pH 7.5, 1% Triton X-100, 1M KCl.

Extracts were obtained either by centrifugation at 105,000 g for 30 min or at 10,000g for 10 min. Cytochromes were determined spectrally in the supernatant and pellets. Protein was assayed in the supernatants.

Immunotitration of cytochrome b

To establish whether the antiserum to cytochrome b could be used to isolate cytochrome b, extracts of SMP obtained from cells uniformly labeled in vivo with [^{3}H] leucine were titrated with antiserum.

Table II lists the specific activities of an SMP preparation which had been extracted with either 1% or 2% Lubrol, prior to immunotitration.

As shown in the immunotitration curve in Fig.3, at the optimum antibody: antigen ratio, no more significant radioactivity could be isolated, either by addition of more antiserum or by increasing the time of incubation. Addition of control serum resulted in a rather considerable precipitation of radioactive material. This radioactivity represents mainly non-specific precipitation as indicated by the observation that addition of increasing amounts of control serum did not cause any further precipitation of radioactive material.

Furthermore, the results obtained by counter immuno-electrophoresis (Fig.2) have revealed that no precipitin lines are formed between control serum and extracts of mitochondria as are observed with the specific antiserum. Indeed, the non-specific precipitation of proteins from mitochondrial extracts probably reflects the extremely hydrophobic nature of mitochondrial proteins as addition of saline alone results in some precipitation of radioactive material.

Following the characterization of the extraction conditions of cytochrome b from the membrane and of the immunoreaction,

TABLE II

PROTEIN CONTENT AND SPECIFIC ACTIVITY OF LUBROL MITOCHONDRIAL EXTRACTS

	PROTEIN mg/ml		COUNTS/min/mg PROTEIN	
	1% LUBROL	2% LUBROL	1% LUBROL	2% LUBROL
Prior to extraction	10	10	3.08×10^7	2.58×10^7
After extraction (1 hr) (centrifugation 10 min at 10,000 g)	7.9	8.7	3.16×10^6	2.98×10^6

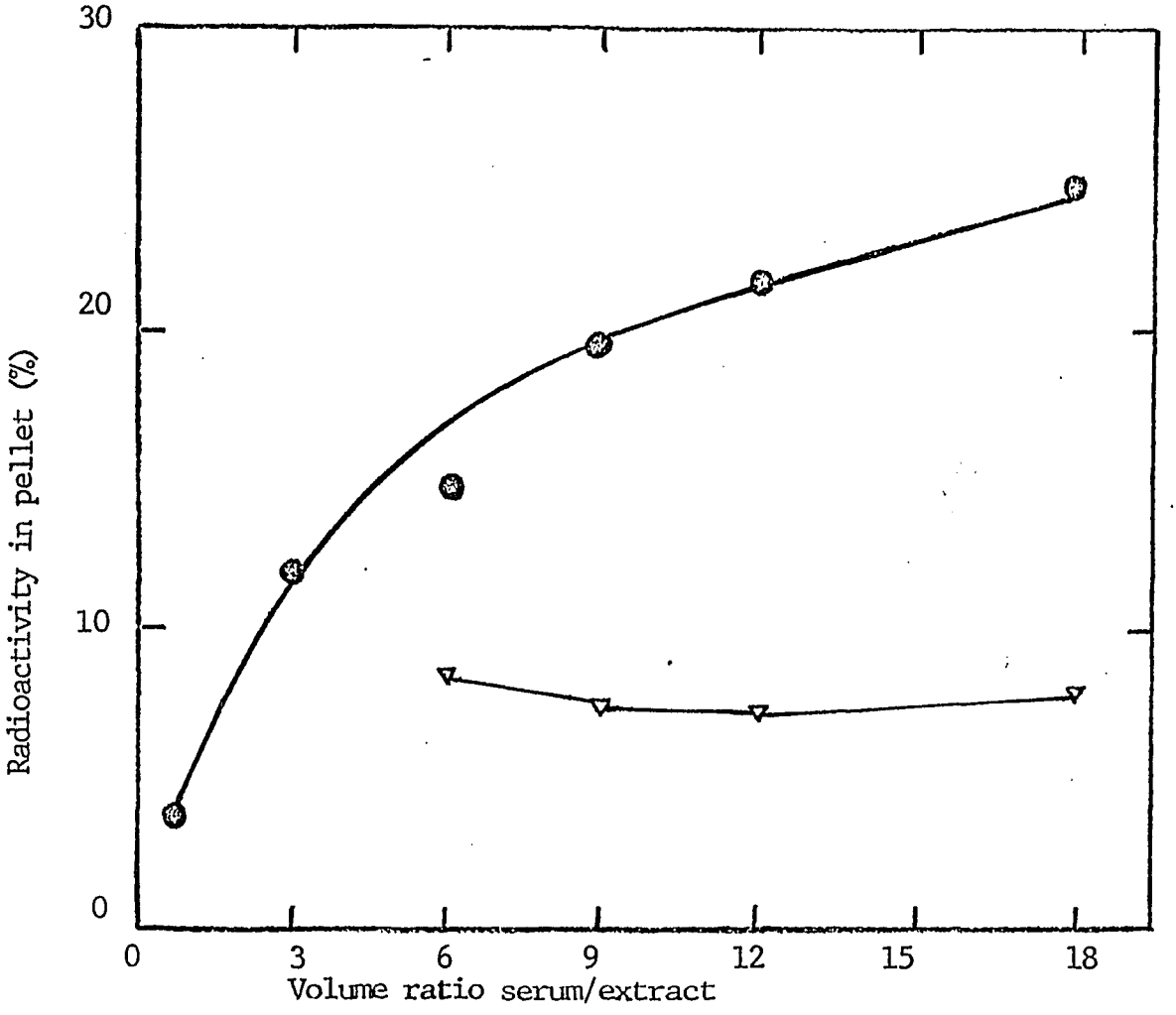

Fig. 3. Titration of extracts of submitochondrial particles with antiserum against cytochrome b. Mitochondria were prepared from wild-type cells of strain D-273-10B grown in semi-synthetic medium containing 0.2% yeast extract and 5.0 mCi/l of [^{3}H]-leucine and extracted with 2% Lubrol as described under Materials and Methods. Increasing amounts of antiserum to cytochrome b were added to 10-µl aliquots of mitochondrial extracts containing 2800000 - 3000000 counts x min^{-1} x mg^{-1}. (● ---- ●) 2% Lubrol extract. Increasing amounts of preimmune serum (Δ—Δ) were also added to the aliquots of mitochondrial extracts.

analysis of the immunoprecipitates by electrophoresis was performed.

Electrophoretic analysis of cytochrome b immunoprecipitated from labeled yeast submitochondrial particles (SMP).

The electrophoretic profile of an immunoprecipitate of cytochrome b, obtained from yeast cells grown for 10 generations in the presence of [^{3}H] leucine is shown in Fig. 4. Most of the radioactive label is present in a band with an apparent molecular weight of 31,000 d. Two labeled bands of molecular weight higher than cytochrome b are coprecipitated in the strain D273-10B with the anticytochrome b serum. These bands were also present in the immunoprecipitates from SMP which were obtained either in the presence of absence of PMSF, the protease inhibitor in the preparation medium. Some of this labeled material may represent non-specific precipitation, as a smaller labeled band corresponding to the band with an apparent molecular weight of 67,000 is also precipitated by the control serum.

In order to learn whether these higher molecular weight bands are translated on mitochondrial ribosomes and to gain information as to whether they are precursors with cytochrome b, a pulse-chase experiment was performed in non-growing conditions in the presence of cycloheximide, the inhibitor of cytoribosomes and of chloramphenicol, the inhibitor of mitoribosomes, respectively. As seen in Fig. 5 the labeling of these high molecular weight polypeptides is insensitive to cycloheximide and sensitive to chloramphenicol.

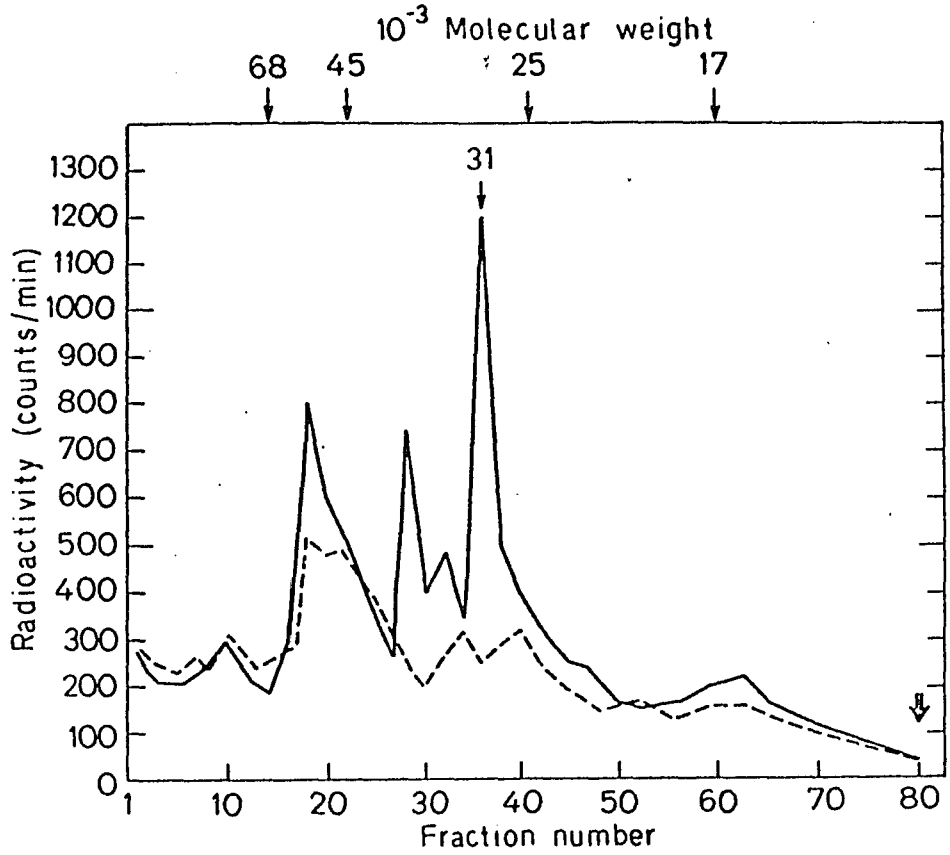

Fig. 4. Gel electrophoresis of immunoprecipitated cytochrome b. Cytochrome b was immunoprecipitated from the Lubrol extract of ^{3}H-labeled mitochondria obtained from wild-type cells of strain D-273-10B grown in semi-synthetic medium containing 0.2% yeast extract and 5.0 mCi/l of [^{3}H]leucine. Immunoprecipitation and gel electrophoresis were performed as described under Materials and Methods. (———) Antiserum to cytochrome b; (— — —) control serum. Arrow indicates the position of the tracker dye.

Some non-specific radioactivity is present in the immunoprecipitate formed by using mitochondria obtained from yeast labeled in the presence of chloramphenicol; however, no sharp peaks are observed corresponding in molecular weight to the labeled bands present in immunoprecipitates obtained from control cells or those labeled in the presence of cycloheximide. Furthermore, these higher molecular labeled bands were not chased by incubation with 10 mM non-labeled leucine for 1 hour.

Absence of cytochrome b in a cytoplasmic petite mutant.

It was reported by Lin, et al. (120) that cytochrome b is synthesized on mitochondrial ribosomes. This was shown when cells were labeled in vivo for 20 min with [^{3}H] leucine, in a non growing medium in the absence or in the presence of cycloheximide, the specific inhibitor of cytoplasmic protein synthesis. Cytochrome b was subsequently isolated by combining immunoprecipitation and gel electrophoresis as described above. After gel electrophoresis, a labeled band corresponding to cytochrome b was present either in the absence or in the presence of cycloheximide. By contrast, labeling of cytochrome b was decreased to 18-26% in the presence of acriflavin, erythromycin or chloramphenicol, (120) confirming that cytochrome b is a product of mitochondrial ribosomes. If cytochrome b is a product of mitochondrial protein synthesis, the apoprotein should not be present in cytoplasmic petite mutants which have lost the ability for mitochondrial protein synthesis. The cytoplasmic petite mutant was grown in the presence of [^{3}H] leucine and its mitochondria

FIGURE 5: Immunoprecipitates formed with labeled mitochondria and cytochrome b antiserum. Washed, early stationary phase yeast cells were suspended at 250 mg/mL in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1% glucose and 2.3% ethanol (A) plus either (B) 100 μg of cycloheximide/mL or (C) 4 mg of chloramphenicol/mL. After 10 min incubation at 30°C, 50 μCi of [^{3}H] leucine/mL was added and the incubation continued for 30 min. Unlabeled leucine (10 mM) was then added for another hour. Cells were harvested and mitochondrial extracts prepared, as described under Materials and Methods. Mitochondrial suspensions at 10 mg of protein/mL were treated according to Lin et al. (120), with either preimmune serum (O—O) or with specific anticytochrome b serum (●—●), and the immunoprecipitate was allowed to form at 4°C overnight. The mixture was centrifuged at 10000g for 10 min and then washed and depolymerized. The immunoprecipitates were analyzed by disk electrophoresis in 10% acrylamide. The arrows indicate calculated molecular weights of 50,000 and 31,000.

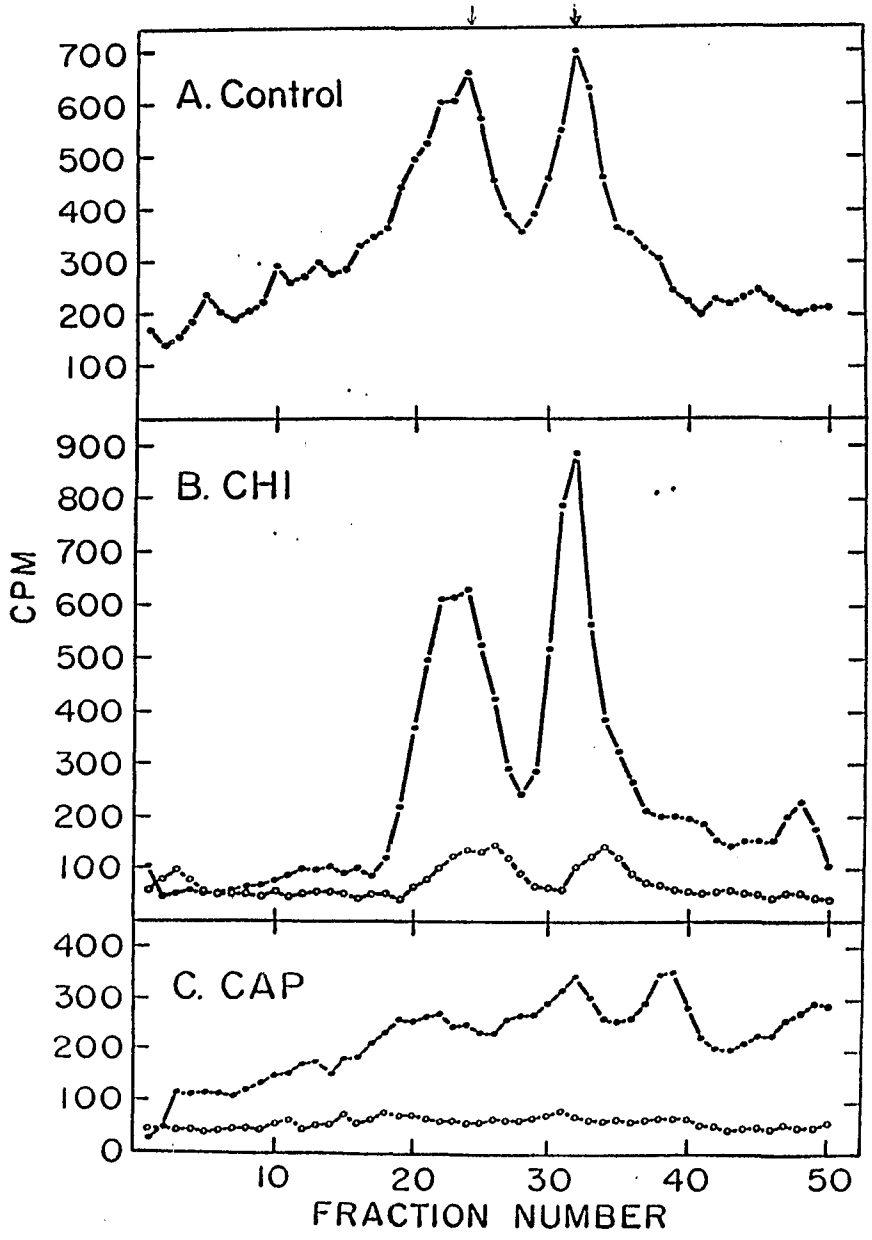
A. Control
B. CHI
C. CAP
CPM
FRACTION NUMBER
700
600
500
400
300
200
100
900
800
700
600
500
400
300
200
100
400
300
200
100
0
10
20
30
40
50

FIG. 5

mixed with mitochondria from wild-type cells which had been grown in [^{14}C] leucine. Extracts of the mixed mitochondria were then immunoprecipitated with antiserum to cytochrome b and subjected to sodium dodecyl sulfate electrophoresis. The results of Fig.6 clearly indicate that no apoprotein of cytochrome b is present in the cytoplasmic petites as would be expected for a product of mitochondrial protein synthesis. It also should be noted that the molecular weight of the major labeled band in the wild-type is 31,000. The counts observed throughout the gel in the petite are identical to that observed when control serum is used and hence represents non-specific precipitation.

Time course of labeling of mitochondrial translation products with [^{35}S] methionine.

To learn whether the high molecular weight proteins were possible precursors of cytochrome b, the time course of labeling of mitochondrial products was examined by varying the time of the pulse with radioactive methionine from 3 to 30 min. Cytochrome b as well as the major subunits of cytochrome oxidase was labeled very rapidly, reaching a maximum after 3 min (Fig.7A). The amount of label present in subunits I (band 5) and II (band 6) of cytochrome oxidase as well as in Var-1 (band 4) remained constant when the time of labeling was increased from 3 to 30 min. By contrast, the amount of label in the bands corresponding to both cytochrome b (band 7) and subunit III (band 8) of cytochrome oxidase decreased 50% or more with increasing time of incubation. The decrease in labeling in these two polypeptides was also observed during a 20 min chase with unlabeled methionine after an initial 10 min pulse labeled with ^{35}S methionine.

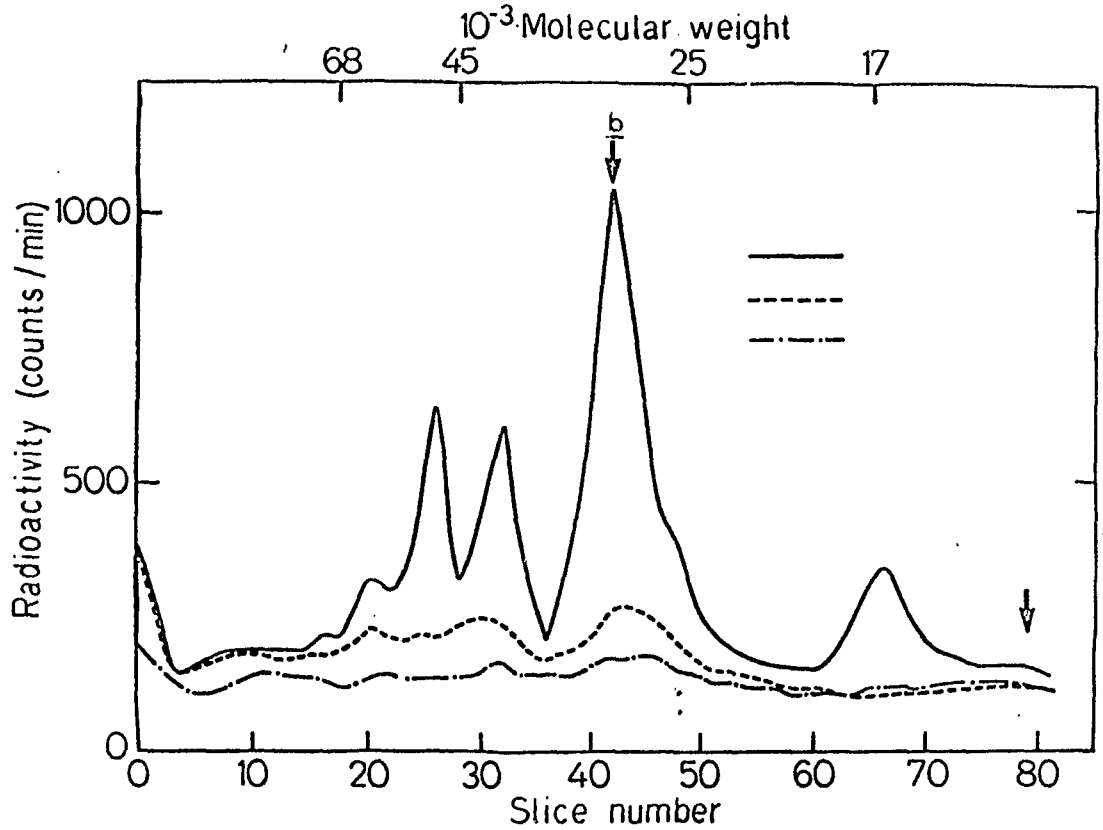

Fig. 6. Absence of cytochrome b in a cytoplasmic petite mutant. The wild-type strain D-273-10B and a cytoplasmic petite derived from it were grown in semi-synthetic medium containing 0.2% yeast extract and 0.5 mCi/l of uniformly labeled [^{14}C] leucine or 5 mCi/l [^{3}H] leucine respectively. Mitochondria were prepared from each type of cells, mixed and extracted with 2% Lubrol. Cytochrome b was immunoprecipitated and the precipitates analyzed by gel electrophoresis as described in Materials and Methods. (———) Wild type cells labeled with [^{14}C] leucine; (----) cytoplasmic petite mutant cells labeled with [^{3}H] leucine. (•—•) Control serum. Arrow indicates position of the tracker dye.

Figure 7. Time-course of labeling of mitochondrial translation products with [^{35}S] methionine. Cells were labeled in the presence of cycloheximide, as described under Materials and Methods for the periods of time indicated, with or without a 20-min chase. The labeled mitochondrial translation products were separated on 10% polyacrylamide slab gels and analyzed by scanning the autoradiograms with a Canalco microdensitometer. The numbers over the arrows identify the following: (1) an unknown polypeptide of 67 000 daltons; (2) an unknown polypeptide of 50 000 daltons; (3) VAR 3; (4) VAR 1; (5) cytochrome oxidase, subunit I; (6) cytochrome oxidase, subunit II; (7) cytochrome b; (8) cytochrome oxidase, subunit III. (A) The autoradiogram of mitochondrial labeled polypeptides was scanned at an arbitrary sensitivity scale. (B) The higher molecular weight mitochondrial polypeptides were analyzed at an expanded sensitivity scale.

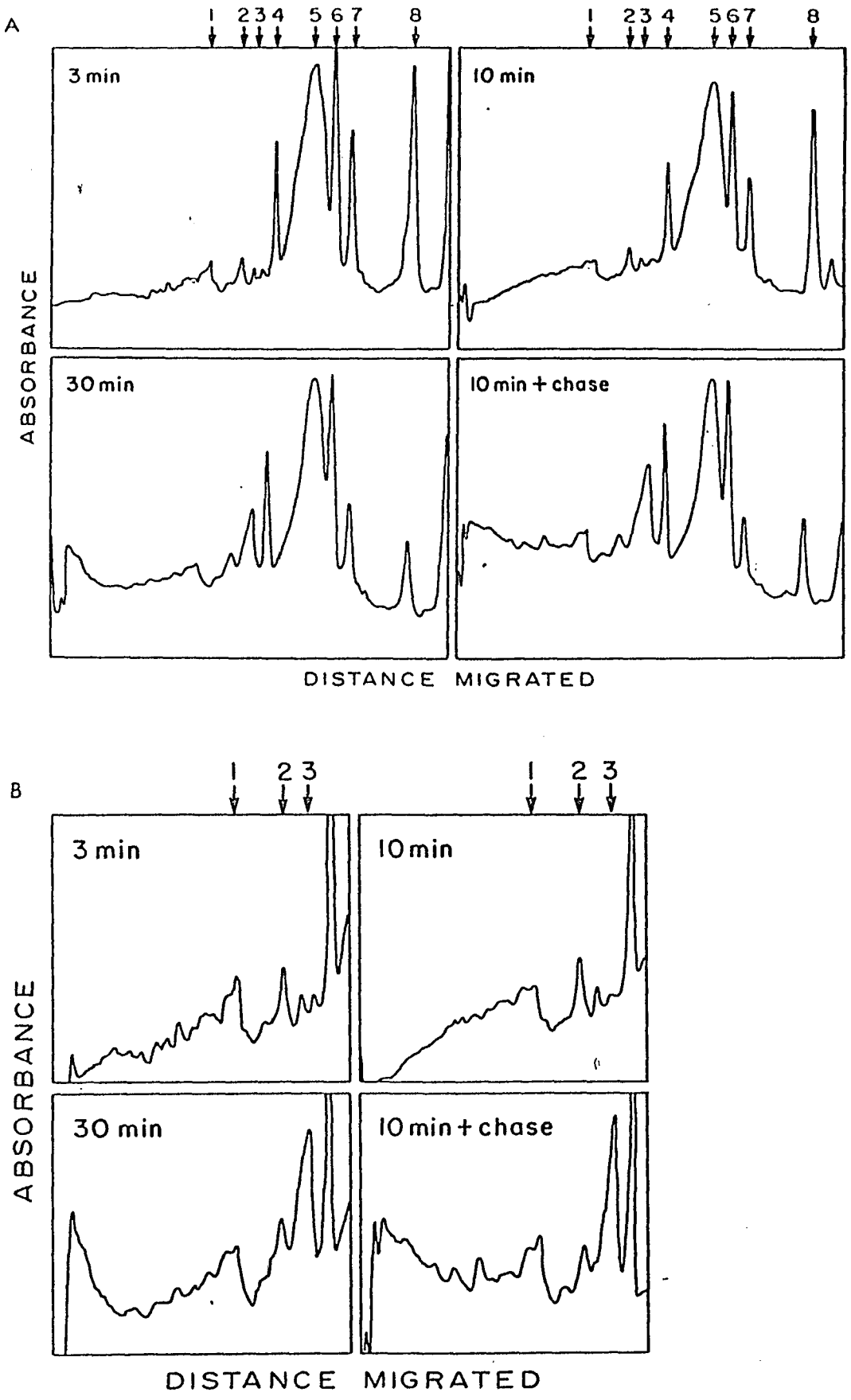
A
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
3 min
10 min
30 min
10 min + chase
ABSORBANCE
DISTANCE MIGRATED
B
1 2 3
1 2 3
3 min
10 min
30 min
10 min + chase
ABSORBANCE
DISTANCE MIGRATED

FIG. 7

The time course of labeling of the subunit of the oligomycin-sensitive ATPase, Var-3 (band 3), differs completely from that of the other mitochondrial translation products. After a pulse of 3 or 10 min, two very small bands are visible in the region of the gel corresponding to Var-3; however, when the time of incubation was increased to 30 min, one large highly labeled band appeared in the gel. A similar increase in the labeling of Var-3 was observed during the 20 min chase after the initial 10 min pulse. Changes in the labeling of subunit I of cytochrome oxidase were also apparent during increasing time of incubation. The labeled band present in the autoradiogram becomes more homogeneous and less diffuse over the gel despite the maintenance of the same peak absorbance.

The time course of labeling of the polypeptides of molecular weight greater than that of Var-1 was more closely examined by using an expanded scale on the gel scanner (Fig.7B). Two polypeptides (band 1) which migrate very close together with a molecular weight of approximately 67,000 were labeled rapidly with a 3 min pulse and maintained a constant amount of label throughout the incubation. By contrast, a less highly labeled polypeptide (band 2) with a molecular weight of approximately 50,000 was labeled to the same extent after a 3- or 10 min pulse, but continuing the incubation time to 30 min or addition of a 20 min chase with unlabeled methionine resulted in a significant decrease in labeling of this polypeptide.

DISCUSSION

Several groups have shown that formation of the reduced ubiquinone; cytochrome c reductase portion of the respiratory chain in yeast required the coordinated synthesis of proteins both in the mitochondria and in the cytoplasm (27, 88, 145). Furthermore, the mitochondrial synthesis of proteins for the cytochrome b-c_1 complex appears to be controlled by proteins synthesized in the cytoplasm (82, 107). As an initial step in the attempts to understand the basis of this regulation, this study has concentrated on one component of this complex, cytochrome b. In a recent paper (28) a method for purification of cytochrome b from yeast mitochondria and some properties of the purified protein were reported. In the present study cytochrome b was purified from small quantities of labeled extracts of yeast mitochondria by specific immunoprecipitation techniques. The data indicate that the antibodies prepared against purified cytochrome b of yeast mitochondria are specific. Single precipitin lines were observed in double immunodiffusion (120) and counter immunoelectrophoresis experiments when antiserum cytochrome b was tested against the pure antigen providing further evidence that the purified preparation of cytochrome b (28) is indeed homogeneous.

Analysis of radioactively labeled immunoprecipitates by sodium dodecyl gel electrophoresis has provided direct evidence that cytochrome b is translated on mitochondrial ribosomes. Incorporation of [^{3}H]leucine into cytochrome b in yeast cells is resistant to cycloheximide, an inhibitor of cytoplasmic protein synthesis, and blocked by inhibitors of mitochondrial

protein synthesis such as acriflavin, erythromycin and chloramphenicol. It should be noted that the specific antiserum against cytochrome b precipitates over 90% of the cytochrome b so that the inhibition noted in the immunoprecipitate is quantitative. Furthermore, no cytochrome b is present in cytoplasmic petite mutants which lack mitochondrial protein synthesis. These results are in complete agreement with those of Weiss and Ziganke (27) who demonstrated that cytochrome b in N. crassa was synthesized on mitochondrial ribosomes. Recently, Katan et al. (107) have also reported that labeling of cytochrome b present in an isolated cytochrome b-c_1 complex was insensitive to cycloheximide and blocked by chloramphenicol suggesting its synthesis on mitochondrial ribosomes.

The use of immunoprecipitation for the isolation of minute amounts of insoluble membrane proteins raises the possibility that even pure antisera may precipitate additional proteins, if these are tightly bound to the solubilized antigen. This approach has already been successfully used to probe the subunit composition of oligomycin-sensitive ATPase (79) and cytochrome oxidase (121, 37). In the present study two polypeptides of higher molecular weight other than cytochrome b are coprecipitated by the antiserum to cytochrome b when the haploid strain D-273-10B was used in the labeling studies (cf. Fig. 4 and 5). Incorporation of [^{3}H]leucine into these polypeptides is sensitive to chloramphenicol, resistant to

cycloheximide (cf. Fig. 4 and 5) and absent in the cytoplasmic petite suggesting the mitochondrial origin of these polypeptides. The possibility that these other proteins may be subunits of either cytochrome oxidase or oligomycin-sensitive ATPase which are synthesized in the mitochondria, has not been ruled out completely. Indeed, it is possible that these higher molecular weight proteins are very hydrophobic and merely adhere to the immunoprecipitate formed between cytochrome b and its antibody and hence are completely non-specific. On the other hand, it is tempting to speculate that these polypeptides of mitochondrial origin are part of the cytochrome b-c_1 complex; however, Katan et al. (107) have reported that cytochrome b is the only polypeptide of the cytochrome b-c_1 complex to be synthesized on mitochondrial ribosomes. By contrast, it has been reported that the three largest subunits of molecular weights 42,000, 33,000, 27,500 of a cytochrome b complex isolated from yeast mitochondria are translated on mitochondrial ribosomes (122). It is interesting to note that during the purification of cytochrome b two polypeptides in the 40-60,000 molecular weight range were present in fraction 8 obtained after protease digestion and ammonium sulfate precipitation (28). These polypeptides are removed from cytochrome b during the final sucrose gradient fractionation.

The higher molecular weight polypeptides which coprecipitated with cytochrome b also do not appear to be precursors of cytochrome b. Addition of a short or long chase with unlabeled

amino acid did not alter the amount of radioactivity in the higher molecular weight polypeptides of the immunoprecipitate relative to that in cytochrome b. Varying the time of pulse labeling in the presence of cycloheximide from 3 to 30 min also did not indicate any precursor-product relationship between the high and low molecular weight products of mitochondrial protein synthesis.

The present study has indicated, however, that mitochondrial translation products are labeled in vivo with very different kinetics. One explanation for the loss of label in cytochrome b and other proteins is that of Costantino and Attardi (123) who suggested that cytoplasmically synthesized proteins may be necessary to stabilize mitochondrial products in HeLa cells.

The identity of these higher molecular weight polypeptides which coprecipitate with cytochrome b remains unclear, but several possible explanations are suggested by the experimental results. These polypeptides may be migrating in sodium dodecyl sulfate-acrylamide gels at or near the true molecular weight of cytochrome b, as determined from the Ferguson plots. Alternately, they may represent cytochrome b present in the dimeric form. Perhaps, when cytochrome b has been precipitated as an immune complex with its antibody, the further solubilization with detergent is affected. Alternately, these associated polypeptides may be non-specifically trapped in the antigen-antibody complex and, hence, be unrelated to cytochrome b.

CHAPTER III

AGGREGATES OF YEAST MITOCHONDRIAL CYTOCHROME *b* OBSERVED AFTER ELECTROPHORESIS

ABSTRACT

Mitochondrial translation products obtained from yeast cells labeled *in vivo* in the presence of cycloheximide were separated by dodecylsulfate polyacrylamide gel electrophoresis. The labeled band, with a molecular weight of 30,000 corresponding to cytochrome *b*, was excised and subsequently transferred to a second gel. After electrophoretic separation, two labeled polypeptides with apparent molecular weights of 67,000 and 27,000 became visible in addition to the cytochrome *b* band of 30,000 molecular weight. Heating of the cytochrome *b* band prior to transfer resulted in an increase in the amount of the labeled polypeptides migrating with a molecular weight of 67,000.

Longer exposure during autoradiography of the gels of mitochondrial translation products resulted in the appearance of a double band with an apparent molecular weight of 67,000. Limited proteolysis of this 67,000 dalton protein with *Staphylococcus aureus* V8 protease revealed a peptide map similar to that obtained after proteolysis of cytochrome *b*. These results suggest that the polypeptide with an apparent molecular weight of 67,000 represents an aggregate of cytochrome *b* that is either present as such in the membrane or is formed *in vitro* during the experimental manipulations to prepare mitochondria for gel electrophoresis.

INTRODUCTION

Mitochondria have the ability to synthesize 8-10 hydrophobic proteins located in the inner mitochondrial membrane (6). These mitochondrial translation products have been shown in yeast to include the three large subunits of cytochrome oxidase (121,124), 3-4 subunits of the oligomycin-sensitive ATPase complex (40), cytochrome b (27,107,120) and VAR-1, a subunit of the 38S mitochondrial ribosome (125,126). To date, these polypeptides are the only ones which have been positively identified as products of protein synthesis on mitochondrial ribosomes; however, suggestions have been made that other mitochondrially synthesized proteins may exist, especially of higher molecular weight (114). Indeed, examinations of published gels indicates the cycloheximide-resistant synthesis of several proteins which apparently are not labeled as rapidly or as extensively as the majority of mitochondrial products (127,128).

The data reported in the previous section showed the presence of polypeptides of higher molecular weight, other than cytochrome b, which are co-precipitated by the specific antiserum to cytochrome b. These proteins are products of mitochondrial protein synthesis but do not appear to be associated with cytochrome b in a partially purified preparation or to be precursors of cytochrome b (129). In the current study, the presence of aggregates of cytochrome b, with an apparent molecular weight of 67,000 has been demonstrated. These results suggest that some mitochondrially synthesized proteins of higher molecular weight may be aggregates of known mitochondrial products.

MATERIALS AND METHODS

Materials. Acrylamide, bisacrylamide and TEMED (N,N,N',N'-tetramethylene-

diamine) were from Eastman. Cycloheximide, phenylmethanesulfonyl fluoride, 2-mercaptoethanol were purchased from Sigma. Staphylococcus aureus V8 protease was a Miles Laboratories product. [^{35}S] methionine (specific activity 1000 Ci·mmol^{-1}) was obtained from Amersham. The molecular weight standard proteins (range 14,400-94,000 daltons) were from Pharmacia Fine Chemicals. The chemicals were of highest purity available.

Methods. The growth of yeast, the in vivo labeling of cells, the preparation of mitochondria and the dodecylsulfate (SDS)[1] polyacrylamide slab gel electrophoresis of the mitochondrial translation products were performed according to the procedures described in Chapter II, Materials and Methods. Linear concentrations of polyacrylamide were used for electrophoresis. The mitochondria were dissociated overnight at room temperature in the medium described by Douglas and Butow (114) but contained 5% SDS and 5% 2-mercaptoethanol. For the limited proteolytic digestion of the electrophoretically separated polypeptides, the method of Cleveland et al. (130) was followed with the modifications described by Cabral et al. (131). In this method, radiochemically pure protein isolated in minute amounts by electrophoresis in the first gel is proteolytically digested in the matrix of the second gel and the labeled products which result during the limited proteolysis are analyzed by SDS gel electrophoresis. The position of the polypeptides, either to be digested or transferred only to a second gel, was localized precisely in the following way: after electrophoresis, the whole gel was quickly stained with Coomassie Blue 0.025g% in methanol: acetic acid (4.9:1) and destained in a CANALCO slab gel destaining apparatus. One part of the gel which contained the duplicates of the samples that had to undergo digestion (or to be transferred only) was cut

apart, dried and autoradiographed for 14-16 hours. This portion of the gel contained on one of its edges half of the lane on which the marker proteins were separated. The positions of these proteins were marked with [^{35}S] methionine along the dried gel and served to match the autoradiograph with the undried portion of the gel. The undried gel contained the second half ot the lane with the markers and the polypeptides to be digested or transferred only. The excised slice was equilibrated for 30-45 min., with occasional shaking, in 10 ml soaking buffer consisting of Tris HCl, 0.125M, pH 6.8, SDS 0.1%, EDTA 1 mM, dithiothreitol 1 mM and subsequently introduced in the well of the second slab gel with the aid of an 0.25 mm thick, bent 5 µl micropipette. The slice was gently pressed on the surface of the stacking gel to remove small, trapped bubbles of air. The proteolytic enzyme was dissolved at the desired concentration (usually 1 mg/0.1 ml) in the following buffer: Tris. HCl, 0.125 M pH 6.8, SDS 0.5%, glycerol 10%, Bromphenolblue 0.1 mg% and added in the same well on the top of the already introduced gel slice. The wells were subsequently filled with a soaking buffer (above) containing 10% glycerol and electrophoresis was started. When the dye reached half distance between the start and the level of the running gel (1.2 cm) the current was turned off and limited digestion of the polypeptides in the excised slices was achieved at 37°C for 30 min. in the matrix of the stacking gel. After digestion, electrophoresis was started again at 40 mA/gel in the 15% polyacrylamide gel. Alternately, digestion was performed in plastic tubes for 60 min. at 37°C and the whole digestion mixture was transferred to the second slab gel for electrophoretic analysis of the resulted products. S. aureus V8 protease 100 µg or stepwise dilutions of 1-100 µg protease were used for digestion.

RESULTS

Prior to proteolytic digestion by the method of Cleveland *et al.* (60), it is necessary to transfer the radioactive band corresponding to a given protein from the original gel to the stacking gel of a new slab gel as described in Materials and Methods. The band corresponding to cytochrome *b*, indicated by the arrow in Figure 8 is widely separated from other labeled proteins when total mitochondrial translation products are subjected to SDS-electrophoresis in 10% acrylamide gels. This separation permits the excision of cytochrome *b* from the original gel without contamination by other proteins, especially subunit II of cytochrome oxidase. Autoradiography of the gel after the excisions confirmed that no other radioactive bands were removed. After electrophoresis of the excised band in a new gel without proteolytic digestion, several bands other than cytochrome *b* were observed, depending on the concentration of acrylamide in the second gel. In a 12% acrylamide gel, a labeled band with a molecular weight of 67,000 was present in addition to the band of 31,000 daltons corresponding to cytochrome *b* (Figure 9A). Similar results were observed when the original band was transferred to a 10% acrylamide gel. Electrophoresis of the excised cytochrome *b* band on either a 15% (Figure 9B) or 8% acrylamide gel revealed the presence of a third labeled band with a molecular weight approximately 3000 less than that of cytochrome *b*. It should be recalled that cytochrome *b* migrates with an apparent molecular weight greater than 30,000 in a 15% acrylamide gel and less than 30,000 in a 8% gel (129,132). Again, a labeled band with a molecular weight of 67,000 is clearly visible in addition to the band corresponding to cytochrome *b*.

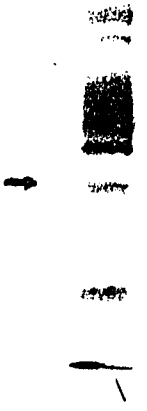

Figure 8. Autoradiogram of yeast mitochondria labeled *in vivo* with [^{35}S] methionine in the presence of cycloheximide and separated on a 10% polyacrylamide slab gel, as described in the procedures in Chapter II. The arrow indicates the position of cytochrome b.

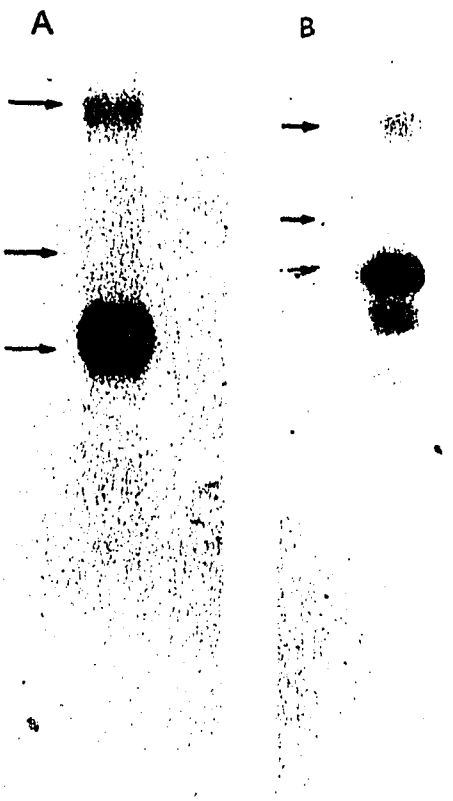

Figure 9. Electrophoresis of cytochrome b excised from a 10% polyacrylamide gel and transferred to a second slab gel for a subsequent electrophoresis: (A) 12% polyacrylamide gel; (B) 15% polyacrylamide gel. Arrows indicate the position of standards with molecular weights of 67,000, 43,000 and 30,000.

These observations suggested that protein(s) which had migrated with an apparent molecular weight of 30,000 in the presence of SDS contained a polypeptide with an apparent molecular weight of 67,000 after a second electrophoresis. A possible explanation for this observation is that cytochrome b aggregates during the experimental manipulations, especially during fixation of the gel with methanol and acetic acid and migrates as if it were a larger protein. To test this possibility, mitochondria were heated for 20 min at 60° in dissociation buffer prior to the initial electrophoresis. Our usual procedure is to leave the mitochondria in dissociating buffer overnight at room temperature. As seen in Figure 10, the band migrating at 67,000 was more pronounced after this treatment when compared to a similar band containing cytochrome b from mitochondria which had not been heated prior to the initial electrophoresis.

These results suggested that the proteins of higher molecular weight which were observed previously in the gels of mitochondrial translation products and in immunoprecipitates formed with the specific antiserum against cytochrome b, might indeed be aggregates of cytochrome b (Chapter II). The autoradiogram of Figure 11 clearly indicates a labeled doublet with a molecular weight similar to that of bovine serum albumin. The gel of Figure 11 was exposed to the X-ray film for a longer time than usual in order to visualize these bands. Hence, the major labeled proteins are overexposed. The area of the gel with 67,000 molecular weight was also excised and transferred to another gel for electrophoresis. Only one band with a molecular weight of 67,000 was observed (Figure 10). No material of lower molecular weight was observed, suggesting that if these proteins do consist of aggregates of cytochrome b, they do not dissociate in SDS

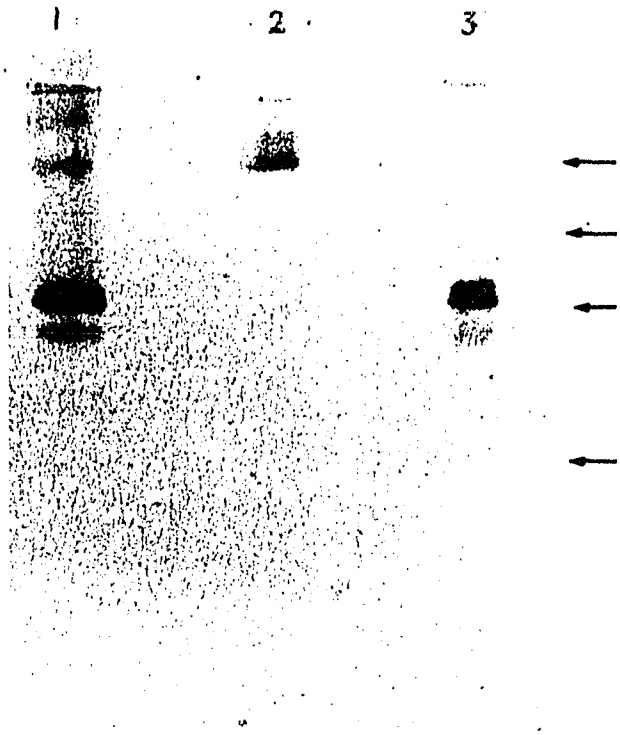

Figure 10. Electrophoresis of cytochrome b and the polypeptide of 67,000 molecular weight excised from a 10% polyacrylamide gel and transferred to a 15% polyacrylamide gel for electrophoresis. Lane 1: mitochondria were dissociated for 20 min at 60° C prior to electrophoresis in 10% polyacrylamide. The band corresponding to cytochrome b was subsequently transferred to a 15% polyacrylamide gel for electrophoresis. Lane 2: polypeptide of 67,000 molecular weight transferred to a 15% polyacrylamide gel. Lane 3: mitochondria were dissociated at room temperature overnight prior to electrophoresis in a 10% polyacrylamide gel. Cytochrome b was transferred to a 15% gel. Arrows indicate molecular weight standards of 67,000, 43,000, 30,000 and 20,100, respectively.

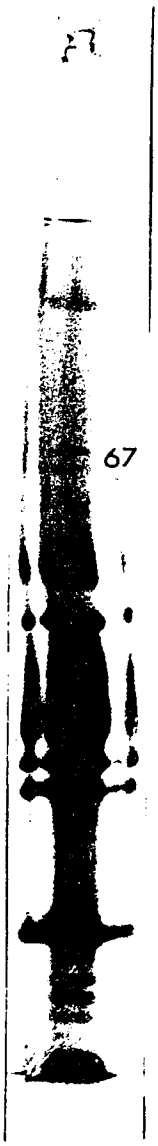

Figure 11. Autoradiogram of mitochondrial translation products after longer exposure to reveal the double polypeptide in the 67,000 dalton region.

and glycerol under the usual conditions.

A direct comparison of the mitochondrial translation products and cytochrome b was next attempted after proteolytic digestion with S. aureus V-8 protease. The high molecular weight proteins were not readily digested as is clear in Figures 12 and 13. In some cases, larger amounts of protease were added and the time of digestion increased. A comparison of the peptide pattern after digestion of cytochrome b and the 67,000 molecular weight doublet proteins indicated several similar peptides (Figure 12). One prominent band appearing in the digest of the 67,000 dalton protein(s) has an identical migration as undigested cytochrome b. At least 5-6 other bands with similar molecular weights were present. In a subsequent experiment, the two bands with a molecular weight close to 67,000 (Figure 13 lanes 3 and 4) were separated prior to digestion in comparison with cytochrome b (Figure 13, lanes 1 and 2). While considerable material of 67,000 molecular weight was not digested at least 4 bands in the digest were of similar mobility.

DISCUSSION

The present study was prompted in order to define the identity of the polypeptides of higher molecular weight which are present in the immunoprecipitates formed with the specific antiserum against cytochrome b (Chapter II, 120, 129). The results obtained suggest that certain of these polypeptides which migrate with an apparent molecular weight of 67,000 represent aggregates of cytochrome b which do not dissociate completely in SDS. This conclusion is based on the following observations: when the labeled band corresponding to cytochrome b is excised from a 10% polyacrylamide slab gel and transferred to a new gel for electrophoresis,

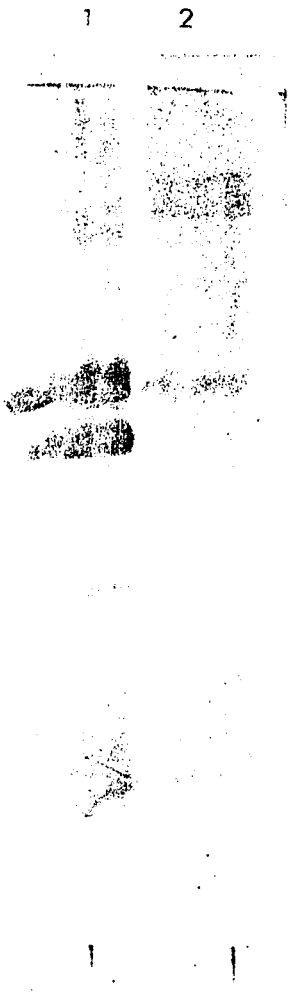

Figure 12 Peptide maps of digests of cytochrome b and the 67,000 molecular weight polypeptide. Limited proteolytic digestion was achieved with 100 μg of S. aureus V-8 protease, as described in Materials and Methods. Lane 1: cytochrome b. Lane 2: 67,000 molecular weight polypeptide.

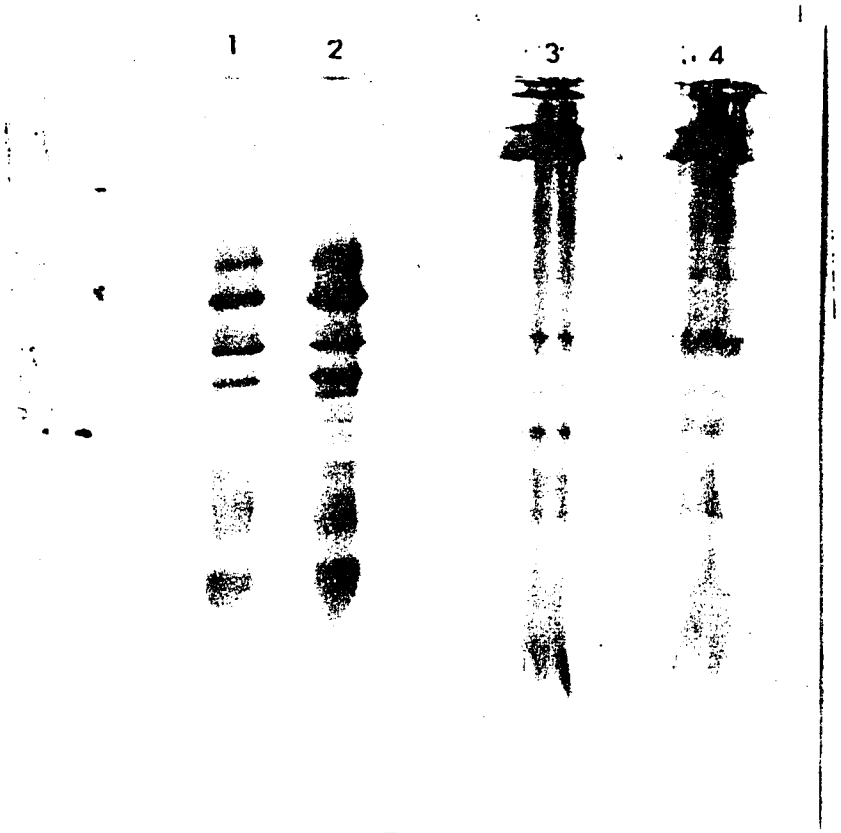

Figure 13 Peptide maps of digests of different concentrations of cytochrome b and the doublet band with an apparent molecular weight of 67,000. Limited digestion was achieved with 50 µg of S aureus V-8 protease, as described in Materials and Methods. Lane 1: cytochrome b, 48,000 counts per lane. Lane 2: cytochrome b, 60,000 counts per lane. Lanes 3 and 4: double band of 67,000 molecular weight, 48,000 and 60,000 counts per lane, respectively.

a band migrating with an apparent molecular weight of 67,000 is clearly visible. Similar results were observed in different strains of yeast including mutants which lack the 30,000 dalton cytochrome b peptide but have new translation products of lower molecular weight (133). Secondly, gels of total mitochondrial translation products contain a double band of approximately 67,000 molecular weight clearly visible with longer exposure during autoradiography. Limited proteolysis of this double band with S. aureus protease revealed similar peptides to those obtained after proteolysis of cytochrome b suggesting that the protein in the double band of 67,000 molecular weight is indeed identical to cytochrome b and hence represents an aggregate of cytochrome b.

The greater prominence of these higher molecular weight proteins in the immunoprecipitates, as compared to total mitochondrial translation products, may have resulted because of the procedures involved in immunoprecipitation. Previous studies (132,134) had indicated that boiling in dissociation medium caused the formation of aggregates of cytochrome b which would not enter the gel. We have reported similar observations (129) but did not observe an increase in material migrating at a molecular weight of 67,000 after boiling. In this context, it should also be noted that when cytochrome b is separated from complex III, labeled polypeptides with molecular weights of 31,000 and 67,000 were observed (129). The higher molecular weight labeled proteins may be aggregates of cytochrome b formed during the preparation which involves cholate extraction of mitochondria followed by ammonium sulfate precipitation.

It should be stressed that these aggregates do not appear nonspecific as only one labeled protein of 67,000 molecular weight is observed after the transfer to the second gel and not many labeled high

molecular weight proteins. In addition, a protein migrating as a doublet at this molecular weight is observed in gels of total mitochondrial translation products *in vivo*.

The results of the present study do not preclude the possibility that cytochrome *b* may exist as a polymer in the mitochondrial membrane. The existence of a dimer of cytochrome *b* has been proposed in *Neurospora* mitochondria (135). Indeed, in an isolated complex III there is a 2:1 molar ratio of cytochrome *b* to cytochrome c_1 (135,14,15). Although 67,000 is more than double the 30,000 molecular weight of cytochrome *b*, the anomalous migration behavior of this protein in polyacrylamide prevents a precise calculation of these molecular weights. An additional explanation for these high molecular weight proteins is that they represent complexes of cytochrome *b* and other proteins of complex III. One possible candidate is the protein associated with cytochrome *b* during purification (136). Incomplete dissociation of either this protein or some other protein from cytochrome *b* in the presence of SDS might result in material migrating with a higher apparent molecular weight. In any event, caution is necessary in examining labeling patterns after SDS gel electrophoresis. These data indicate clearly that treating samples with SDS does not always cause them to dissociate to monomers.

CHAPTER IV

THE SYNTHESIS OF POSSIBLE DIMERS OF CYTOCHROME *b* IN YEAST CELLS AT LOW TEMPERATURES

ABSTRACT

Mitochondrial translation products were examined in yeast cells labeled *in vivo* for short times at 10°C -15°C in the presence of cycloheximide. Polyacrylamide gel electrophoresis revealed a labeled protein with an apparent molecular weight of 58,000 to 60,000. A labeled protein of this mobility was not apparent in cells labeled at 30°C. A 30-minute chase with unlabeled methionine at either 10°C or 30°C did not result in any decrease in labeling of this protein indicating that it is a stable product. The 58,000 to 60,000 dalton protein has an identical mobility as a high molecular weight protein present in the immunoprecipitates formed with labeled mitochondria and the specific antiserum against cytochrome *b*. In addition, the 58,000 to 60,000 dalton protein displays an anomalous migration behavior in gels of different acrylamide concentrations as does cytochrome *b*. These data suggest that the 58,000 to 60,000 mitochondrial translation product may be a stable dimer of cytochrome *b* synthesized at low temperatures in intact cells.

INTRODUCTION

Previous studies on the biogenesis of cytochrome b in yeast mitochondria raised important questions as to the identity of the one to two polypeptides of higher molecular weight other than cytochrome b which are co-precipitated by the specific antiserum raised against cytochrome b (Chapter II and III). The proteins, with apparent molecular weights of 55-60,000 determined by sodium dodecylsulfate (SDS)[1] polyacrylamide gel electrophoresis, are products of mitochondrial protein synthesis; however, they do not appear to be associated with cytochrome b in a partially purified preparation (129) nor are they an integral part of an enzymatically-active cytochrome b-c_1 complex isolated from yeast (15). In addition, these high molecular weight mitochondrially-translated proteins do not appear to be precursors of cytochrome b, as the addition of a short or long chase of unlabelled amino acid did not alter the extent of labeling of these proteins relative to cytochrome b in the immunoprecipitate (129). In the previous chapter the labeled protein with a 67,000 molecular weight was shown to consist of aggregates of cytochrome b which might be formed *in vitro* during the experimental manipulations to prepare mitochondria for gel electrophoresis.

In the present study, the biogenesis of the proteins with a molecular weight in the 55-60,000 range was further investigated. A mitochondrial translation product of this molecular weight is present in the mitochondrial membrane of yeast cells pulse-labeled at temperatures of 8-15°C but is either not labeled or is faintly labeled at 30°C. It is suggested that the polypeptide with an apparent molecular weight of 58,000 to 60,000 may be a dimer of cytochrome b which is formed during biosynthesis.

MATERIALS AND METHODS

Growth of Yeast

Diploid wild-type strain of Saccharomyces cerevisiae was grown aerobically on liquid medium containing 3% galactose (w/v), harvested at a density of approximately 1 x 10^7 cells/ml and washed three times with distilled water.

Preparation of Mitochondria

The in vivo labeling of cells with [^{35}S]methionine in non-growing conditions was performed at 10°, 15° and 30°, respectively. The subsequent preparation of mitochondria by mechanical agitation was performed as described in Chapter II, Materials and Methods. After the pulse label, the cells were added to 10 volumes incorporation medium containing 10 mM methionine and 0.5 mg cycloheximide per ml at 0°. All subsequent operations were performed as rapidly as possible at 4°.

Electrophoresis and Autoradiography

Slab gels (10 cm long, 1.0 mm thick) were prepared according to the methods described in Chapter III. Gels were stained, dried and auto radiographed as described in Chapter II.

Ferguson Plots

The relative mobilities of cytochrome b, of the 60K molecular weight polypeptide and of the standard proteins were plotted against the acrylamide concentration, according to Ferguson (137). The "free mobilities" of the proteins were obtained by extrapolating the respective lines to "zero" concentration of acrylamide.

Presence of a Novel High Molecular Weight Mitochondrial Translation Product

Previous attempts to demonstrate a precursor-product relationship between the proteins of higher-molecular weight present in the immunoprecipitates formed with antiserum raised against cytochrome b and the antigen were unsuccessful. Neither a 20 min or one hour chase with unlabeled leucine after the original 30 min pulse resulted in any decrease in the radioactivity in the polypeptides of a higher molecular weight as compared to the labeling of the 31,000 dalton polypeptide corresponding to cytochrome b (Chapter II). Furthermore, varying the time course of labeling of the total mitochondrial translation products from 3 to 30 minutes revealed that both cytochrome b and a protein with a molecular weight greater than 55,000 were labeled very rapidly after a 3 or 10 min pulse, but continuing the incubation for 30 min did not result in any significant decrease in the labeling of this band.

These results suggested that further decreasing the rate of mitochondrial protein synthesis *in vivo* by lowering the temperature at which the yeast cells were labeled might reveal a putative precursor. As indicated in Figure 14 a new labeled protein with a molecular weight of 60,000 was prominently present in autoradiographs of mitochondria isolated from yeast cells pulse-labeled with [^{35}S]methionine for 4 min at 15°C. Additional labeling studies were performed at temperatures below that of the transition temperature of the membrane. The transition temperature for mitochondrial protein synthesis *in vivo* occurs at 12°C for our strain of yeast. A protein of molecular weight 60,000 was also observed when yeast cells were labeled at 8° or 10°C for 4 min; however, this labeled protein was either not observed or was present as a very faint band in autoradio-

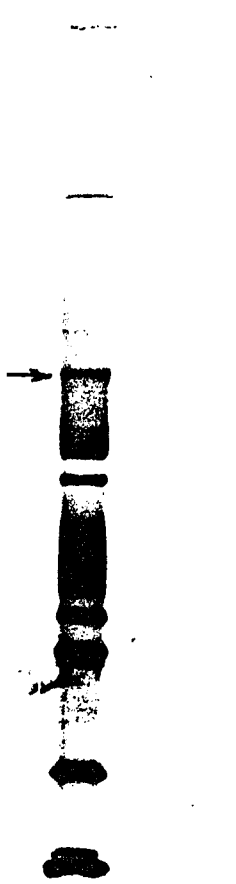

Figure 14 Autoradiogram of total mitochondrial translation products from yeast cells labeled for 4 minutes at 15° as described under Methods. Arrow indicates the presence of new polypeptide migrating with an apparent molecular weight of 60,000.

grams of mitochondria obtained from cells labeled at 30° (Figure 15).

One possible explanation for this observation is that the 58,000 to 60,000 dalton protein is a large precursor,perhaps a dimer, which is not processed in yeast cells maintained at these low temperatures. To test this possibility, yeast cells were pulse-labeled with [^{35}S] methionine in the presence of cycloheximide for 4 min at 10°C and then chased with unlabeled methionine for 30 min at either 10° or 30°. As a control experiment, cells were labeled under identical conditions at 30° and then chased at 30°. Figure 16 indicates that the 58,000 to 60,000 dalton mitochondrial translation product is present in the mitochondrial membranes pulse-labeled at 10° whether the chase was performed at 10° or 30°. In addition to the novel high molecular weight bands, the autoradiograms of Figures 15 and 16 indicate that changes in the labeling pattern of cytochrome b also occur at these low temperatures. The 30,000 dalton band is considerably broader in mitochondria obtained from cells labeled at 10° (Figure 15). In some experiments two distinct labeled bands are apparent in the cells labeled at low temperatures (Figure 16). Similar double bands are also observed when the cells are labeled at 30°, but in that case the lower band is invariably less prominent in the cells labeled at 30° when a direct comparison is made to cells labeled at 10°.

To compare in more detail the kinetics of labeling of the high molecular weight protein and cytochrome b, the time period of labeling at 10° was shortened to 1.5 min. In addition, the effect of addition of the protease inhibitor, PMSF, during the incubation medium was studied. As seen in Figure 17 addition of PMSF to the incubation medium had no effect on the labeling pattern of any of the mitochondrially synthesized proteins. Figure 17 also indicated that both cytochrome b and the 58,000 to 60,000 dalton

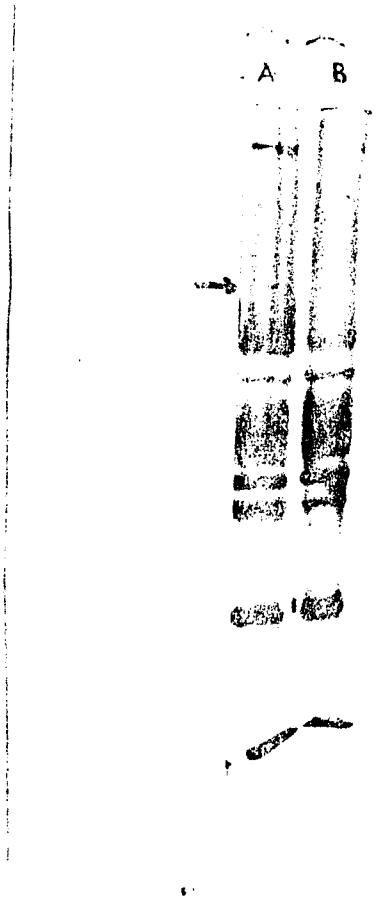

Figure 15, Comparison of mitochondrial translation products from yeast cells labeled at 10° and 30° as described under Methods. Arrow indicates the presence of the labeled polypeptide migrating with an apparent molecular weight of 58,000. Lane A, cells labeled at 10°; Lane B, cell labeled at 30°.

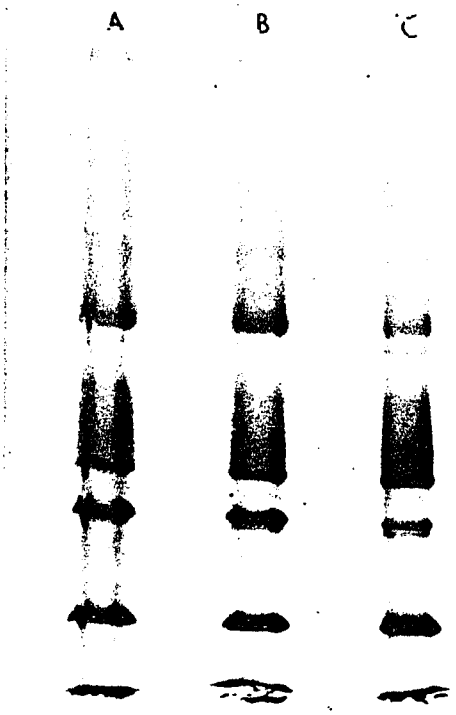

Figure 16 Effect of temperature and chase on the labeling of mitochondrial translation products. Lane A: cells were labeled for 4 minutes at 10° with [^{35}S] methionine followed by a 30 minute chase 10° with unlabeled methionine. Lane B: cells were labeled for 4 minutes at 10° followed by a 30 minute chase at 30°. Lane C: Cells were labeled for 4 minutes at 30° followed by a 30 minute chase at 30°.

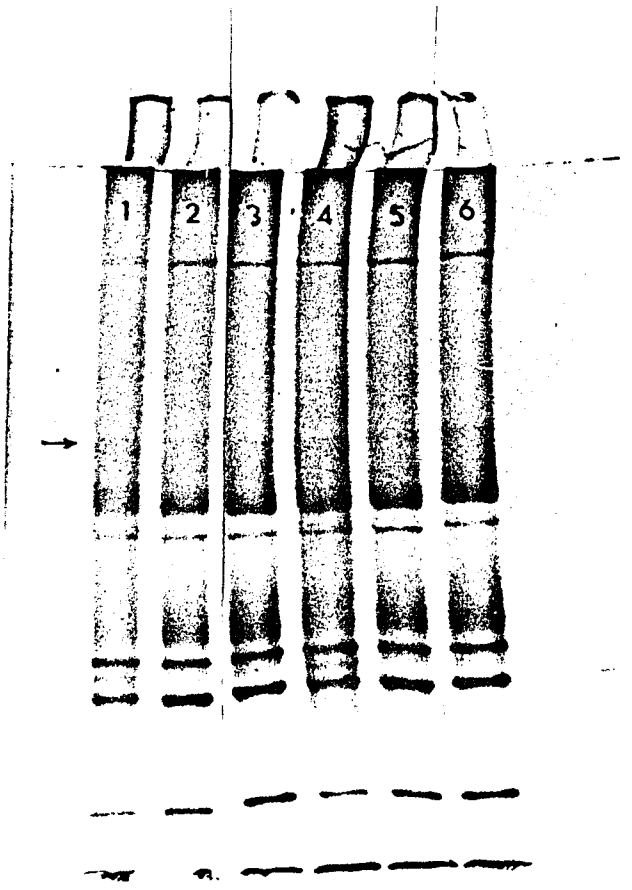

Figure 17 Time course of labeling of mitochondrial translation products in the presence or absence of PMSF. Cells were labeled at 10° for 1.5 minutes [lanes 1 and 4], 3 minutes [lanes 2 and 5] and 5 minutes [lanes 3 and 6] in the presence of [lanes 1-3] or absence [lanes4-6] of PMSF. Arrow indicates the migration of the new polypeptide of higher molecular weight.

protein are synthesized during the first 1.5 min of incubation at 10°C; however, it is apparent that continued synthesis of cytochrome b occurs after an additional 3-5 minutes.

The result obtained in these experiments plus the previous work had revealed that this new mitochondrial translation product has a molecular weight double that of cytochrome b. This observation coupled with the previous studies in which a protein with this apparent molecular weight co-precipitated with cytochrome b using the antiserum raised against cytochrome b (Chapter II, 120) suggested that this new mitochondrial translation product might be a stable dimer of cytochrome b formed during synthesis. As a further test for this possibility, the migration behavior of the 58,000 - 60,000 dalton protein in gels of different acrylamide concentrations was examined. It has been established that cytochrome b exhibits anomalous migration behavior in SDS-polyacrylamide gel electrophoresis as compared to standard proteins or to the majority of mitochondrial translation products (129,132). The high molecular weight protein labeled at 10°C also displays anomalous migration in gels of different acrylamide concentration (Figure 18). The slope for the migration extrapolates to the same free mobility as does that for the cytochrome b analyzed in the same gels.

DISCUSSION

The concept that cytochrome b might exist in the respiratory chain as a dimer has been suggested by a number of experimental approaches (138). Initially, the existence of two, or possibly three b-type cytochromes was proposed on the basis of spectral analysis of mammalian mitochondria (139); however, only two functionally different cytochrome b's can be distinguished in the b-c_1 region of the respiratory chain using several experimental ap-

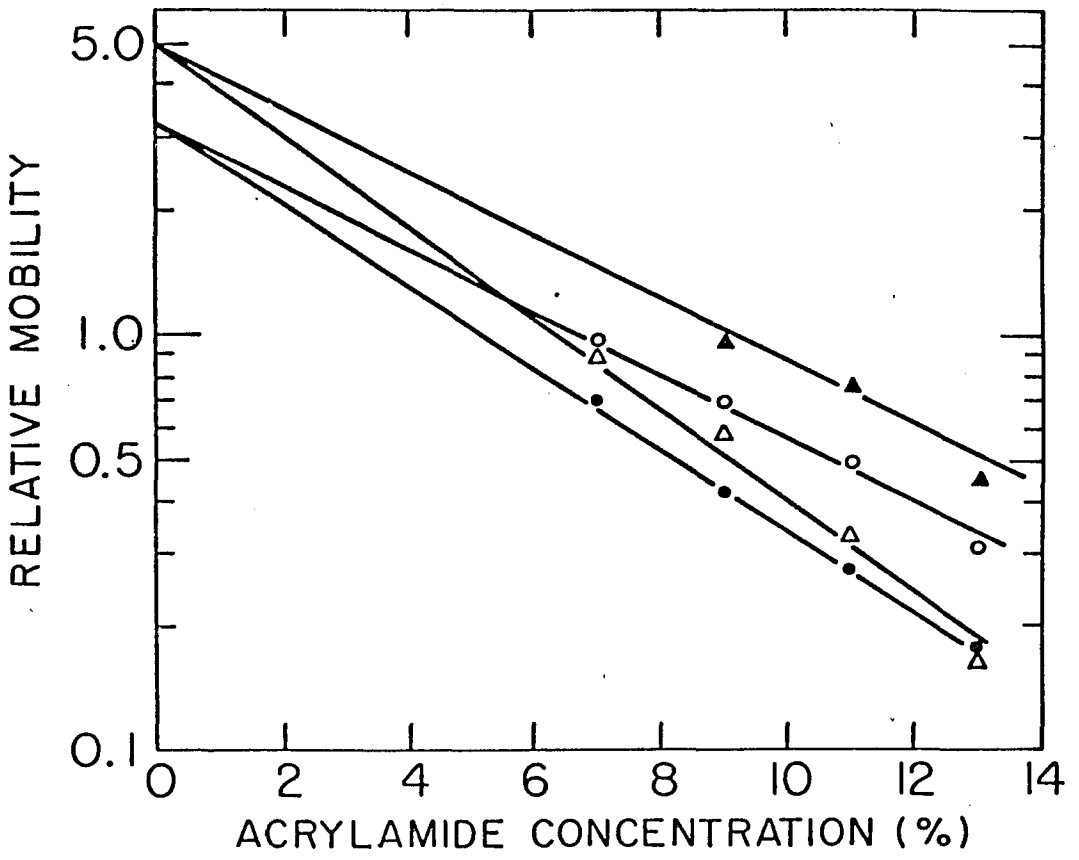

Figure 18 Ferguson plot of cytochrome b and the new high molecular weight mitochondrial translation product. [▲-▲] cytochrome b; [△-△] new polypeptide labeled at 10°; [○-○] ovalbumin, 43,000-dalton standard; [●—●] bovine serum albumin, 67,000-dalton standard.

proaches (140). Despite the evidence for more than one type of cytochrome b in the mitochondrial membrane only one cytochrome b containing polypeptide has been purified and characterized from both yeast (28) and Neurospora crassa (135). An earlier report that two different cytochrome b's with different molecular weights and properties are present in heart mitochondria has not been further investigated (141).

The presence of two cytochrome b's in the mitochondrial membrane with functional differences but with identical molecular weights and properties suggested that the b cytochromes might either exist in close contact with each other or actually occur as a dimer in the membrane. This proposal has been strengthened by the many reports that there are two molecules of cytochrome b per molecule of cytochrome c_1 on a molar basis in isolated Complex III preparations containing both cytochromes b and c_1 (14,15,26). The necessity for this stoichiometry of cytochromes b and c_1 was shown when coenzyme QH_2-cytochrome c reductase activity was investigated (90,142). A change in the 2:1 ratio of these two cytochromes resulted in loss of enzymatic activity. Physical evidence for the existence of a dimer of cytochrome b in a purified preparation was reported by Weiss and Ziganke (27). Cytochrome b isolated from N. crassa had a molecular weight of 30,000 based on heme content or SDS gel electrophoresis, while gel filtration on Sephadex G-75 in cholate suggested a molecular weight of 58,000. A recent study examining the oxidoreduction properties of the two cytochromes b supported the concept that a dimer of cytochrome b may be the functional unit for electron transport in this region of the chain (140). One suggestion has been that each of the two cytochrome b's in Complex III is associated with a different protein of the complex such that its properties are affected differently (135). Indeed, labeling studies

with diazo benzene sulfonate have indicated that cytochrome b may be localized on both sides of the mitochondrial membrane (143) further supporting the notion that cytochrome b dimer may span the membrane.

In the current study, newly-synthesized apparent dimers of cytochrome b were observed in the mitochondrial membrane when protein synthesis had been slowed by lowering the temperature at which the yeast cells were labeled to 15° or below. The evidence supporting the suggestion that the polypeptide with a molecular weight of 58,000 to 60,000 labeled in mitochondria at 10° is indeed a dimer of cytochrome b is suggestive. First, a protein with an identical mobility is observed in immunoprecipitates formed with the specific antiserum raised against monomeric cytochrome b (120, Y.S. Chen and D.S. Beattie, manuscript in preparation). Secondly, this putative dimer displays anomalous migration behavior in acrylamide gels of different porosities as does cytochrome b (129,132). Thirdly, during the purification of cytochrome b by Ultragel chromatography dimers as well as monomers of pure cytochrome b have been observed (Y.S. Chen and D.S. Beattie, manuscript in preparation).

The putative dimer of cytochrome b synthesized at low temperatures appears to be a stable product. A 30 min chase at 30° with unlabeled methionine did not result in any decrease in the labeling of the 58,000 - 60,000 protein; however, this labeled protein was not generally observed in mitochondria obtained from cells labeled at 30°. The possibility that stable dimers are formed during synthesis was also indicated by the observation that two labeled bands were present in the indirect immunoprecipitates formed with the antiserum against cytochrome b and the cell-free translation products obtained with mitochondrial RNA (T. Domenico and D.S. Beattie, manuscript in preparation).

CHAPTER V

SYNTHESIS OF THE APOPROTEIN OF CYTOCHROME b IN HEME DEFICIENT YEAST CELLS

ABSTRACT

The presence of the apoprotein of cytochrome b has been demonstrated in a mutant of Saccharomyces cerevisiae lacking δ-aminolevulinic acid synthase, and hence devoid of heme. The apoprotein of cytochrome b present in the mutant was identical with cytochrome b of control cells (mutant cells grown in the presence of δ-aminolevulinic acid) by the following criteria: similar apparent molecular weights in dodecyl sulfate polyacrylamide gel electrophoresis; anomalous migration behavior during electrophoresis in polyacrylamide gels of different porosities; identical gel pattern obtained after immunoprecipitation with specific antiserum against cytochrome b, and identical fingerprints obtained after limited proteolysis with Staphylococcus aureus V-8 protease. The kinetics of incorporation in vivo of [^{35}S]methionine into apoprotein of cytochrome b in the mutant suggested that heme deficiency may affect assembly into the membrane of subunits of the cytochrome b-c_1 complex rather than synthesis of cytochrome b.

INTRODUCTION

The recent isolation of heme-deficient mutants of S. cerevisiae (87) has permitted investigation of the possible role that heme, as cofactor, might exert on biosynthesis of the apoproteins of heme-containing enzymes. The mutant GL1 (hem1), used in this study, lack δ-aminolevulinic acid synthase (144); when grown in the absence of δ-aminolevulinic acid it has no respiratory activity and lacks all mitochondrial cytochromes as well as other heme compounds. When this mutant is grown in the presence of δ-aminolevulinate the cells are phenotypically identical to wild-type yeast cells, except that their doubling time is longer (144).

Previous studies with this mutant have indicated that heme deficiency results in diverse effects on various enzymes and enzyme complexes. Ross and Schatz (77) reported that accumulation of cytochrome c_1 in the inner mitochondrial membrane is controlled by heme. In heme-deficient cells, however, the apoprotein of cytochrome c_1 could be detected in the cytosol. Likewise, the assembly of cytochrome oxidase is also regulated by heme or one of its precursors (105). In heme-deficient cells, control levels of two of the three mitochondrially translated subunits of cytochrome oxidase (II and III) and one cytoplasmically translated subunit (VI) are present in the mitochondrial membrane. Two subunits, one a mitochondrial product (I) and one a cytoplasmic product (IV), are present in low amounts in mitochondria from the heme-deficient cells, while two subunits are undetectable (V, VII).

In the present study the presence of the apoprotein of cytochrome b has been demonstrated in yeast mutant GL1 grown in the absence of

δ-aminolevulinate. The results obtained in kinetic experiments suggest that assembly of the cytochrome b-c_1 complex is affected by lack of heme but not synthesis of the apoprotein of cytochrome b.

EXPERIMENTAL PROCEDURE

Uniformly labeled L-[^{14}C] leucine (330 mCi,mmole^{-1}), L-[4,5-^{3}H]-leucine (55Ci.mmole^{-1}) and L-[^{35}S] methionine (1000 Ci.mmole^{-1}) were obtained from Amersham. Phenylmethansulfonylfluoride, cycloheximide, sodium dodecyl sulfate and dithiothreitol were from Sigma Chemical Co.; acrylamide and N.N'-methylenbisacrylamide were purchased from Bio-Rad; yeast extract and Yeast Nitrogen Base were Difco Products; Staphylococcus aureus V8 protease was obtained from Miles Laboratories.

Cell Growth - Strain GL1 was grown at 30°C in a semi-synthetic medium containing 0.67% Yeast Nitrogen Base (without amino acids), 0.2% yeast extract, and 0.3% glucose, supplemented with 12mg/l of ergosterol and 0.5% Tween 80, or with 30mg/l of δ-aminolevulinate (77). Parent strain X2180 was grown on the same medium without supplements. Cytochrome b-mutant Strain W-267 (box 6-2) and its parent 777-3A were grown in a medium containing 2% yeast extract, 0.35g/l ammonium sulfate, 80 mg/l adenine, 3% galactose.

Labeling of Cells and Preparation of Mitochondria and Submitochondrial Particles - For the electrophoretic analysis of the mitochondrially translated polypeptides, cells were harvested in late log phase, washed three times with water and resuspended in incubation medium at a concentration of 200 mg wet weight cells per ml. The incubation medium was

the same as the growth medium except that it lacked yeast extract and Ergosterol-Tween 80, and contained 2% glucose. The cells were incubated at 30°C for 5 min with vigorous shaking, cycloheximide (1 mg/ml) was added, and the incubation continued for another 5 min. Labeling was initiated by the addition of [^{35}S] methionine (200 μCi/ml) and stopped after 30 min by addition of 10 mM unlabeled methionine. After an additional 5 min incubation, cells were harvested and washed with 0.5 mg/ml of cycloheximide. In the time course experiments the period of labeling was varied as indicated. For the immunoprecipitation experiments δ-aminolevulinate synthase deficient cells were uniformly labeled by growth in medium containing [^{3}H] leucine (5 mCi l^{-1}) for 10 generations, while the control cells were grown in medium containing [^{14}C] leucine (500 μCi l^{-1}). Mitochondria and submitochondrial particles were prepared as described in Chapter II, Materials and Methods.

Electrophoretic and Fingerprint Analysis - Mitochondria were dissociated overnight at room temperature in a solution of 5% dodecyl sulfate, 2 mM EDTA, 5% 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, and 10% glycerol. Disc and slab gel electrophoresis was performed, and Ferguson plots were drawn as described previously (129). Limited proteolysis of excised slices containing the [^{35}S] methionine-labeled cytochrome b was performed by the method of Cleveland et al. (130). Identification of the polypeptides for digestion was performed by exposing a dried part of the gel for 16-18 h on X-ray film. The non-dried part of the gel containing the polypeptide for digestion was maintained at -20°C. The soaking buffers were prepared according to Cleveland et al. (130) but also contained 1 mM dithiothreitol. Digestion was performed in the stack-

ing gel (2 mm thick) for 30 min at 37°C with S. aureus V8 protease (100 µg per sample), and the digested peptides were subjected to electrophoresis in a gel containing 15% acrylamide. Autoradiography was performed by exposing the dried slab gels on X-ray film Kodak NS 5T at -70°C. The autoradiograms were scanned with a Canalco Model J microdensitometer.

Immunoprecipitation Procedures - Aliquots of submitochondrial particles, obtained from control and mutant cells or containing equal amounts of radioactivity, were mixed, centrifuged for 30 min at 105,000 x g and the resuspended pellet was extracted with 2% Lubrol in a 0.1 M sodium phosphate buffer, pH 7.5. The Lubrol extracts were subjected to immunoprecipitation with an optimum volume of antibody (Chapter II).

RESULTS

The total mitochondrial translation products of yeast can be readily identified after separation by electrophoresis on 10% polyacrylamide slab gels in the presence of dodecyl sulfate. Autoradiography of the dried slab gels (Figure 19) showed the three large subunits of cytochrome c oxidase, cytochrome b, VAR1, a mitochondrial product described by Douglas and Butow (114) and VAR3, a subunit of oligomycin sensitive ATPase (125). Heme-deficient cells of strain GL1 were labeled with [^{35}S]methionine in the presence of cycloheximide, and the mitochondrial translation products were compared to those obtained from cells grown with δ-aminolevulinate (control cells). A polypeptide migrating in front of subunit II of cytochrome c oxidase with the same mobility as a cytochrome b (molecular weight 30,000) was present in cells grown with and without δ-aminolevulinate (Figure 19). In the above experiment the mitochondria

from heme-deficient and heme-sufficient cells prior to electrophoresis had an equal number of counts. However, a lower radioactivity was observed in the band corresponding to cytochrome b in the heme-deficient cells. With the same amount of mitochondrial protein from the two types of cells, the lower counts in the band corresponding to cytochrome b of heme-deficient cells was even more apparent.

The degree of labeling of other mitochondrial translation products in the heme-deficient cells also differed when compared to the control cells (Figure 19). Subunit I of cytochrome oxidase as well as VAR3 in the mitochondria from heme-deficient cells contained much less label than the mitochondria from control cells. However, several mitochondrial translation products, especially VAR1 and a polypeptide with a molecular weight of approximately 67,000 were considerably more highly labeled in the heme-deficient cells as compared to the control.

The electrophoretic behavior of cytochrome b and other mitochondrial translation products of heme-deficient cells was examined by dodecyl sulfate gel electrophoresis at concentrations of acrylamide from 7.5% to 15% (Figure 20). The band corresponding to cytochrome b was established by comparison with gel patterns of mutants known to be lacking cytochrome b (133). This step was essential since at certain concentrations of acrylamide cytochrome b migrates more slowly than subunit II of cytochrome oxidase while at others it migrates more rapidly (132,134). Bands identified as cytochrome b, from both heme-deficient and control cells, showed the characteristic anomalous migration behavior of cytochrome b in different concentrations of acrylamide as compared to standard proteins and other mitochondrial translation products (Figure 20). Subunit I of cyto-

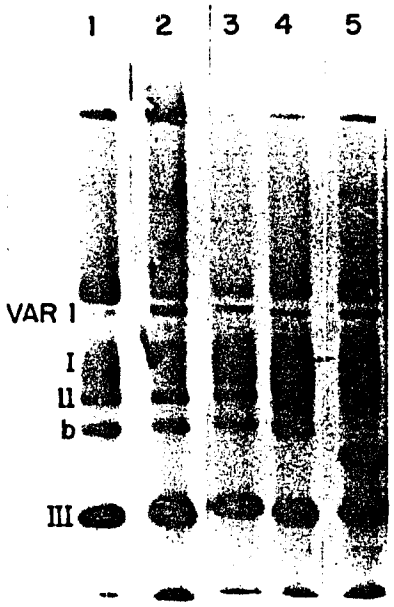

Fig.[19] Autoradiogram of dodecyl sulfate-polyacrylamide gel electrophoresis showing [^{35}S]methionine-labeled mitochondrial translation products. Cells were labeled with [^{35}S]methionine in the presence of cycloheximide, as described in Experimental Procedure, and the mitochondrially synethesized proteins were subjected to dodecyl sulfate -10% polyacrylamide gel electrophoresis. Lanes 1 and 2 are heme-deficient strain GL1 plus or minus δ-aminolevulinate, respectively; 3, wild-type strain X2180; 4, wild-type strain 777-3A; and 5, cytochrome $\underline{b}^-$ mutant W-267 (box 6-2) derived from strain 777-3A. Identification of the bands is discussed under Results. VAR 1-Variant I; I, II and III are mitochondrially translated subunits of cytochrome $\underline{c}$ oxidase; b is position of cytochrome $\underline{b}$.

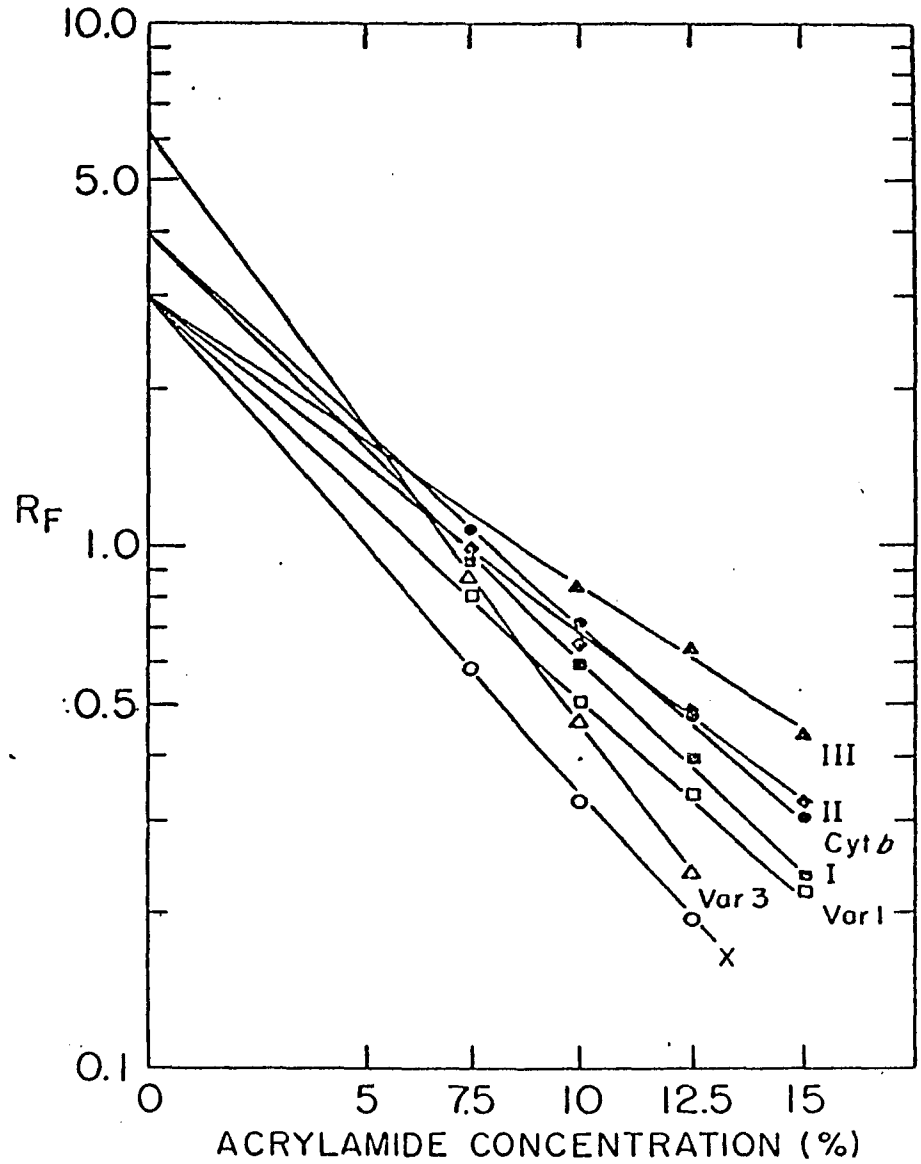

Fig. 20. Anomalous migration of apocytochrome b, of control and mutant cells. The relative mobilities in gels of 7.5%, 10%, 12.5% and 15% polyacrylamide of VAR 3 (△ -- △), VAR 1 (□ -- □), subunits 1 (■ -- ■), II (◆--◆), and III (▲ -- ▲) of cytochrome oxidase, apocytochrome b (● -- ●), and a polypeptide with an apparent molecular weight of 67,000 (○-○) (x), were plotted according to the procedure described previously (6) and extrapolated to "zero" polyacrylamide concentration.

See Fig. 19 for abbreviations.

chrome c oxidase and VAR , the ATPase subunit, also displayed anomalous behavior in the heme-deficient cells as was previously reported for wild-type cells (129,131).

A further indication that the mitochondrial translation product which migrated with an apparent molecular weight of 30,000 in heme-deficient cells was indeed identical to the apoprotein of cytochrome b was obtained by examining the immunoprecipitates formed with specific antibody against cytochrome b (Chapter II). The heme-deficient mutant was grown with ergosterol and Tween 80 in the presence of [^{3}H]leucine, and with δ-aminolevulinate in the presence of [^{14}C]leucine. Submitochondrial particles obtained from both cultures were mixed, extracts of the mixed particles were immunoprecipitated with antiserum to cytochrome b, and the immunoprecipitates were subjected to dodecyl sulfate gel electrophoresis. The results in Figure 21 clearly indicate that the apoprotein of cytochrome b is present in the mitochondria of the heme-deficient cells, and that it migrated with an apparent molecular weight of 30,000. The immunoprecipitates contained two polypeptides of higher molecular weight which have been characterized. However, recent studies have indicated that they are not precursors of cytochrome b, and are not associated with cytochrome b in the cytochrome b-c_1 complex (Chapter II, 129).

As a final and structural characterization of the putative apoprotein of cytochrome b in the heme-deficient cells, a limited proteolysis was performed according to the method of Cleveland et al. (130). The autoradiographic pattern of the digested polypeptides indicated that the polypeptide migrating at 30,000 daltons in the heme-deficient cells

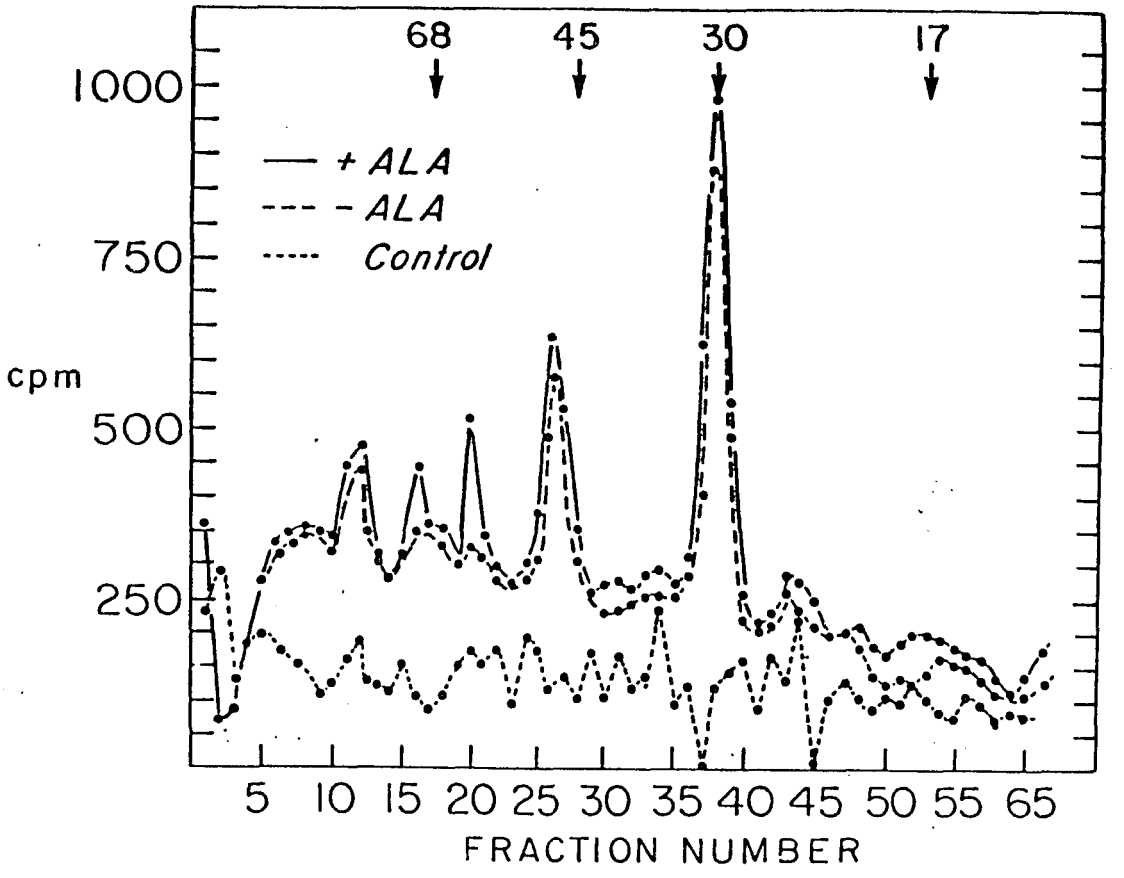

Fig.21 Immunoprecipitates formed with labeled submitochonodrial particles from control and heme-deficient cells. Aliquots of 2% Lubrol extracts containing an equal number of counts were mixed, treated with specific anticytochrome b serum (1:6, v:v), and the immuno-precipitate analyzed by 10% polyacrylamide disc gel electrophoresis. An aliquot of the extract was treated with pre-immune serum (•-•-•) and analyzed under the same conditions; (———) control cells; (- - -) heme-deficient cells.

had the same fingerprint pattern as cytochrome b in the control cells (Figure 22).

In the experiments described above, we consistently observed that specific radioactivities of total mitochondrial protein, and of mitochondrial translation products in heme-deficient cells were always considerably lower than those of control cells (Table III). On the average the decrease in labeling amounted to approximately 33%. The kinetics of labeling of mitochondrial translation products, especially cytochrome b, in heme-deficient cells as compared to controls, was studied by varying pulse-labeling with [^{35}S] methionine from 3 to 30 min. A 10 min pulse was also carried out with labeled methionine followed by a 20 min chase with an excess of unlabeled methionine (Figure 23). After a pulse of 3 or 10 min the peak corresponding to cytochrome b was weakly labeled in the heme-deficient cells. In contrast, cytochrome b was labeled quickly in the control cells, reaching a maximum in 10 min. With longer times of labeling, however, almost as much radioactivity was present in the band corresponding to cytochrome b in heme-deficient cells as in the control cells. Indeed, the band corresponding to cytochrome b was more highly labeled in the heme-deficient cells than in control cells after a 10 min pulse with [^{35}S] methionine and a 20 min chase with unlabeled methionine. Most of the other mitochondrial translation products in the heme-deficient cells were also labeled with similar kinetics as cytochrome b with the exception of subunit III of cytochrome oxidase which was highly labeled after a 3 min pulse. In the control cells, most mitochondrial translation products had lower radioactivity after the chase, whereas in the heme-deficient cells, the opposite was observed i.e., more label was present in the translation products after

Fig. 22 Peptide maps obtained after limited proteolysis of apocytochrome b control and mutant cells. The apocytochrome b was separated by 10% polyacrylamide gel electrophoresis, excised, and transferred to a second gel, where it was subjected to proteolysis with 100 μg of S.aureus V8 protease for 30 min at 37°C. The digested peptides were fractionated on 15% polyacrylamide gel and subjected to autoradiography. Lanes 1 and 2 are from cells grown without and with δ-aminolevulinate.

Table III

Incorporation of [^{35}S]Methionine in vivo into Mitochondria of Heme-deficient cells

Yeast cells from control and heme-deficient cells were incubated with [^{35}S]methionine in the presence of cycloheximide for 30 min as described under Experimental Procedure. Mitochondria were isolated and washed. Aliquots were taken to determine radioactivity and protein.

Mitochondria from	Counts/min/mg protein x 10^{-5}
Control cells	7.9*
Heme-deficient cells	5.3*

* Average of 5 separate experiments.

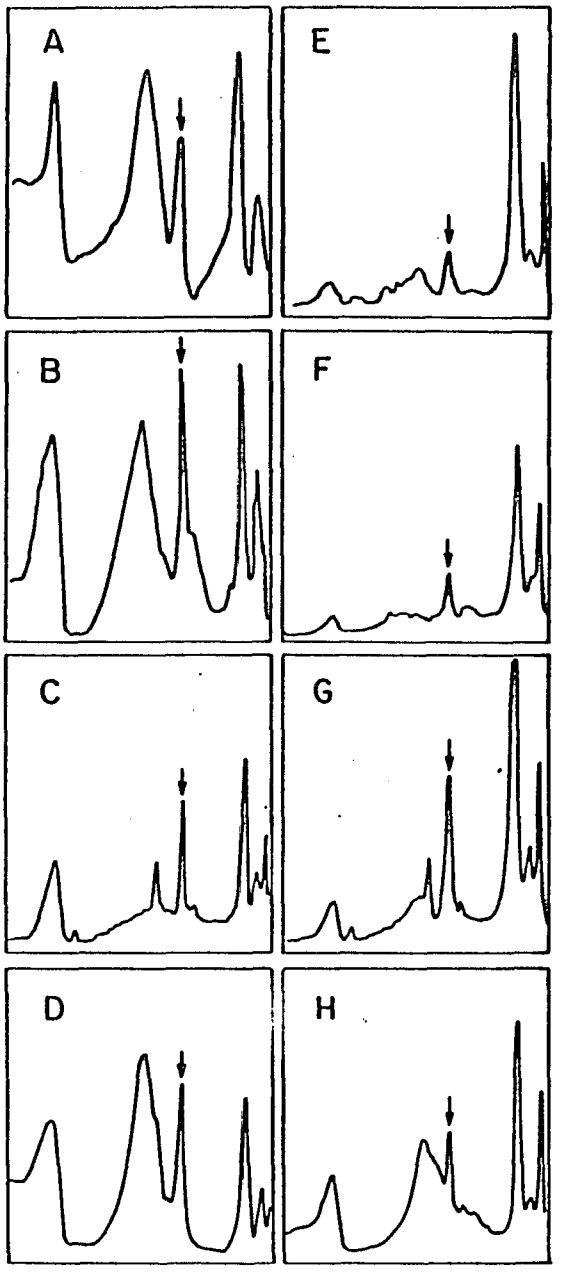

Fig.23 Time course of labeling of mitochondrial translation products. Cells were labeled with [^{35}S] methionine, as described under Experimental Procedure. The labeled translation products were separated on 10% polyacrylamide slab gels, and analyzed by scanning the autoradiograms. A-D, control cells; E-H, mutant cells. A and E, 3 min pulse; B and F, 10 min pulse; C and G, 10 min pulse + 20 min chase; D and H, 30 min pulse. The low molecular weight peptides migrating with the tracker dye were not recorded. Arrow indicates the band corresponding to cytochrome b.

the chase. These results suggest that in heme-deficient yeast assembly of mitochondrial translation products into the membrane is affected more than their synthesis.

DISCUSSION

The results obtained in this study indicate that yeast cells have the ability to synthesize apocytochrome b in the absence of heme. Cytochrome b polypeptide was identical to cytochrome b in control cells by the following criteria: molecular weight after dodecyl sulfate polyacrylamide gel electrophoresis, anomalous migration in acrylamide gels of different porosities, immunoprecipitation of a 30,000 dalton polypeptide with specific cytochrome b antiserum, and fingerprint patterns after limited proteolysis.

The observation that the apoprotein of cytochrome b is present in heme-deficient cells provides another example of the diverse regulatory functions of heme in biosynthetic pathways. Although it is clear that in rabbit reticulocytes the absence of heme results in an inhibition of synthesis at the level of initiation (97-99), the role of heme in other cells is less well understood. For example, the absence of heme in yeast cells appears to have no effect on the synthesis of the apoprotein of cytochrome c peroxidase (100), the level of the apoprotein of cytochrome b in the membrane of a heme-deficient mutant of E. coli (101,102), or the formation of apocytochrome c in Neurospora crassa (103). However, heme apparently regulates the assembly of cytochrome c_1 into the mitochondrial membrane (77) as well as the formation of the holoenzyme of cytochrome c oxidase (105) in yeast. Furthermore, two cytoplasmically made subunits of cytochrome c oxidase (105), catalase A, and catalase T (107) are not detectable in heme-deficient yeast mutants.

In Bacillus subtilis in absence of heme the normal binding of succinic dehydrogenase (SDH) to the bacteria's membrane is impaired. When bacteria cells were grown in the presence of δ-ALA, and the formation of membrane-bound cytochrome c was initiated the SDH attached to the membrane at a normal level, indicating close coupling between holocytochrome formation and SDH membrane binding (95). The absence of heme impairs also the synthesis of nitrate reductase in E. coli and its binding to the membrane (96). As to one of the mechanisms by which heme might control the import of the cytoplasmically made apocytochromes in yeast, it was suggested by Schatz (146) that the heme insertion might result in a covalent irreversible modification in the configuration of the apoenzyme, resulting in an irreversible trapping in the membrane of the imported polypeptide.

The kinetics of incorporation of [^{35}S] methionine into mitochondrial translation products suggests that the rate of mitochondrial protein synthesis may be decreased in the mutant lacking heme. Much longer times were required for maximum labeling to occur in several polypeptides. Furthermore, a long chase after the initial pulse resulted in a decrease in the labeling of cytochrome b in the control cells but an increase in labeling in the mutant. These results may reflect a change in either the processing or assembly of the subunits of the cytochrome b-c_1 complex into the membrane. In the absence of heme, the assembly process may occur more slowly or the different proteins may be integrated incorrectly.

In this context it might be of interest that VAR-1, suggested to be required for the assembly of the 38S mitochondrial ribosomal subunit in yeast (56) was found in this study to be more highly labeled in the mitochondria of the mutant lacking heme as compared to the heme sufficient cells.

CONCLUDING REMARKS

Cytochrome b, an integral hydrophobic protein of the mitochondrial inner membrane, is translated on mitochondrial ribosomes in the yeast Saccharomyces cerevisiae. The first part of this dissertation demonstrates that immunotechniques utilized previously in investigations on the biogenesis of cytochrome c oxidase and ATP-ase complexes are a valuable tool for the isolation of cytochrome b and polypeptides associated or related to it. The data also indicate that cytochrome b has a marked tendency to aggregate even in the presence of sodium dodecyl sulfate and dithiothreitol. In addition, heating samples in sodium dodecyl sulfate prior to electrophoresis enhances the aggregation process. The presence of an aggregate of cytochrome b with an apparent molecular weight of 67,000 has been demonstrated. The data obtained do not exclude the existence of this aggregate in the mitochondrial membrane in vivo.

Kinetic studies of in vivo labeled cells showed a rapid and rather characteristic rate of labeling of each of the mitochondrial translation products. Cytochrome b appears to be synthesized very rapidly in vivo, reaching a maximum labeling after 5 minutes at 30° C. Furthermore, cytochrome b is also extensively labeled relative to other mitochondrial translation products at 10° C, which is below the transition temperature of the membrane. The presence of a 58-60,000 molecular weight polypeptide has also been demonstrated in

mitochondrial translation products of cells labeled below the transition temperature of the mitochondrial membrane. It is suggested that this polypeptide might represent a stable dimer of cytochrome b.

The role of heme in the biosynthesis of cytochrome b was also investigated. The results obtained indicate that heme is not necessary either for the translation of apocytochrome b or for its insertion into the membrane, and suggest that the holoenzyme is formed in a subsequent step. The role of cytochrome b and heme in the assembly of an intact b-c_1 complex is a promising area for further research.

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