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FORMATION AND COMPOSITION OF THE YEAST MITOCHONDRIAL RESPIRATORY CHAIN

by

Gregory G. Brown

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

- 1977 -

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

5/17/77
Date

Diana S. Beattie
Chairman of Examining Committee

5/17/77
Date

Terry Ann Krulwich
Executive Officer

Arthur Cederbaum

Richard Needleman

Terry Ann Krulwich

Tsung-Chiu Li

The City University of New York

ABSTRACT

The induction of enzymes of the mitochondrial respiratory chain and cytochrome formation were examined during aerobic adaptation of yeast cultures grown anaerobically. Coordinate increases in the activities of succinate dehydrogenase, cytochrome oxidase and succinate-coenzyme QH_2- and antimycin-sensitive NADH-cytochrome c reductases were observed immediately upon aeration. The formation of cytochromes b, c_1 and a - a_3, however, preceeded the development of coenzyme QH_2-cytochrome c reductase or cytochrome oxidase activities. Addition of cycloheximide to the culture prior to aeration prevented the induction of all enzymes, while addition of chloramphenicol blocked the formation of cytochrome oxidase but inhibited to different extents the induction of the three cytochrome c reductases. Induction of cytochrome b was more sensitive to inhibition by chloramphenicol than was cytochrome c_1.

The relationships between coenzyme QH_2: cytochrome c reductase, cytochromes b and c_1, and NADH: and succinate: cytochrome c reductases were further investigated with a coenzyme Q analog and a series of coenzyme Q deficient yeast strains. The reduction of cytochrome c by the reduced form of the 6-decyl analog of coenzyme Q follows first order kinetics with respect to cytochrome c and increases in a linear manner with added mitochondrial protein. The activity is completely sensitive to antimycin A in whole cell extracts of yeast as well as in isolated mitochondria and fraction-

ates with markers for the mitochondrial electron transport chain. The presence of both cytochromes *b* and c_1 in an approximately 2:1 ratio appears essential for enzymatic activity. Reduced coenzyme QH_2: cytochrome *c* reductase obeys Michaelis-Menten kinetics when assayed in mitochondria obtained from a yeast strain lacking coenzyme Q. Both NADH and succinate: cytochrome *c* reductase activities were not detectable in six coenzyme Q-deficient strains tested, but were restored after addition of the oxidized form of the coenzyme Q analog. No marked difference in the concentration of the analog required to restore the two activities was observed.

The possible accumulation of proteins synthesized by either cytoplasmic or mitochondrial ribosomes was investigated by determining the antibiotic-resistant enzyme induction when cycloheximide or chloramphenicol were added at various times during aeration. The results obtained suggested that a considerable accumulation of proteins synthesized in the mitochondria occurs for both cytochrome oxidase and the cytochrome *b*-c_1 complex. By contrast, almost no accumulation of proteins synthesized in the cytoplasm for either enzyme complex occurred during the first thirty minutes of aeration. After this time, the pattern of accumulation of cytoplasmically made proteins differed for the two enzyme complexes. The accumulation of precursor proteins was also investigated by the sequential addition of cycloheximide and chloramphenicol (or the reverse order) to cultures during respiratory adaptation.

Increases in both cytochrome oxidase and coenzyme QH_2-cytochrome *c* reductase were observed when yeast were aerated in cycloheximide for seventy minutes and then in chloramphenicol suggesting that a pool of proteins synthesized in the mitochondria had accumulated for both enzyme complexes. When yeast were aerated in chloramphenicol for seventy minutes followed by cycloheximide, an increase in cytochrome oxidase activity was observed but not in coenzyme QH_2-cytochrome *c* reductase. These results suggest that a pool of cytoplasmically made proteins had accumulated in a usable form for cytochrome oxidase but not for the cytochrome b-c_1 complex when mitochondrial protein synthesis was blocked by chloramphenicol. The significance of these differences on the mechanism of formation of these two different complexes of the respiratory chain is discussed.

To my Father and my Mother

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CHAPTER I

INTRODUCTION

GENERAL INTRODUCTION

Inquiries into the mechanism of mitochondrial biogenesis probably date from the discovery of the organelle itself. Although as late as 1964, it was hypothesized that mitochondria arose from the extension of other cellular membranes (1) or were synthesized _de novo_ from submicroscopic components (2), it is now generally recognized that mitochondria undergo a growth and division process analagous to that of the cell itself. Perhaps the most convincing evidence in this line came from the experiments of Luck (3). _Neurospora crassa_ were labeled with radioactive choline, and the fate of the label was traced by electron microscope radioautography. Upon removal of the radioactivity, the label remaining distributed itself evenly over the entire population of mitochondria during the subsequent growth of the cells. This result was reconcilable only with a growth and division process. The observations of dumbbell shaped (4) and partitioned mitochondria (5) provided further evidence for this hypthesis.

The discoveries of mitochondrial DNA (6) and mitochondrial protein synthesis (7) suggested that the mitochondrion possessed a certain genetic autonomy in its growth and division process. It is now generally recognized that the formation of mitochondria requires the coordinate operation of two distinct genetic systems. The degree to which mitochondria

are autonomous and the means of coordination between the two genetic systems remain major challenges to workers in this field.

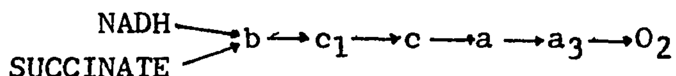
The yeast Saccharomyces Cerevisiae is an organism ideally suited to the study of mitochondrial biogenesis. It is unicellular and eukaryotic and possesses mitochondria which are similar in ultrastructural appearance to those seen in animal tissues (8). It is classified as a facultative anaerobe, being capable of growth on fermentable carbon sources (such as glucose) under anaerobic conditions and on fermentable or non-fermentable carbon sources under aerobic conditions. When yeast are grown on a non-fermentable carbon source the respiratory activity of the cells is necessarily high, and electron micrographs of such cells show a relative abundance of mitochondrial profiles with clearly discernible cristae (9). Certain growth conditions such as high glucose concentrations (10) or anaerobiosis (11) lower the respiratory capacity of the cells and decrease the numbers and types of mitochondrial profiles (12, 13). The ability of the cells to grow in the absence of an aerobic metabolism has facilitated the isolation of many respiratory deficient mutants (14). The respiratory activity of the yeast cell, therefore, can be manipulated by many physiological and genetic techniques.

The major topic of this thesis will be the use of one such mode of experimental manipulation, the aeration of anaerobically grown yeast, to study the formation of several enzymes of the respiratory chain. Since these enzymes are

located within the inner mitochondrial membrane, it is hoped that this study sheds some light on the mechanism of formation of the membrane itself.

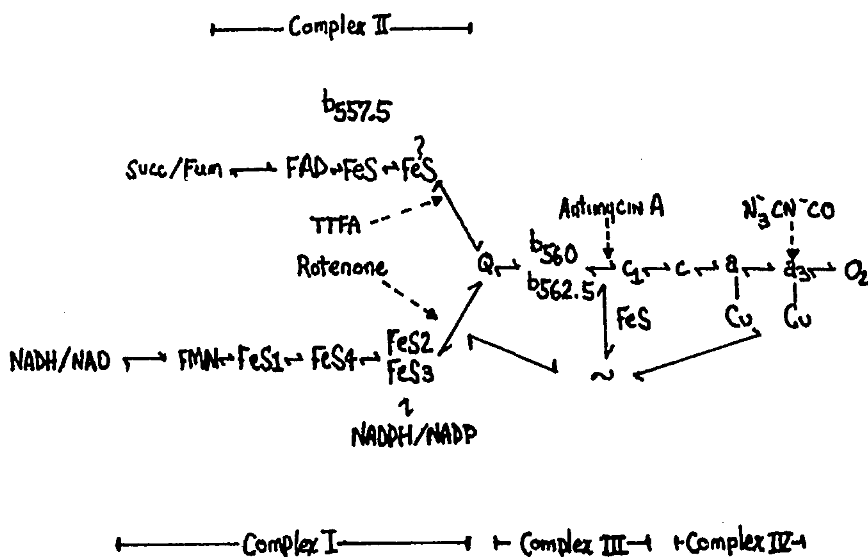
THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

The observation that yeast contain spectropically detectable components capable of undergoing reversible oxidation and reduction, was reported by Keilin in 1925 (15). He termed the absorption bands occurring at 603, 564, and 549nm, cytochromes a, b, and c, respectively. Subsequent spectroscopic studies led to the identification of a new cytochrome component, cytochrome c_1 (16) and to the separation of cytochrome a into two functionally distinguishable components, cytochromes a and a_3 (17). Measurement of their oxidation reduction midpoint potentials and rates of oxidation during respiration by Chance (18) and others, allowed for the ordering of the cytochrome in terms of the sequence of electron transfer as follows:



In mamalian mitochondria, coupling of the oxidation reactions to phosphorylation of ADP was proposed by Chance to take place at three sites: site I, between NADH and cytochrome b; site II, between cytochrome b and cytochrome c and site III, between cytochrome c and O_2. In Saccharomyces mitochondria, coupling differs in that site I phosphorylation does not occur. (19)

In addition to the cytochromes, the electron transfer chain is now recognized to consist of many other components. The current concept of the chain, as it was originally postulated by Green (20) and subsequently by Hatefi et al. (21) is that it is composed of four distinct lipid protein complexes. The protein composition of each complex is distinct and each complex is capable of carrying out a particular segment of the overall oxidation reaction. According to Hatefi et al., the manner in which the complexes participate in electron transfer may be visualized as follows:



Complex I (NADH: Coenzyme Q reductase) catalyzes the reduction of coenzyme Q by NADH. It contains covalently bound FMN, and in most organisms at least four non-heme iron centers (22). Phospholipids are essential for activity and the bovine complex may contain as many as fifteen polypeptide subunits and have a molecular weight as high as 850,000

daltons (22). It is inhibited by piericidin A and rotenone and the loss of piericidin sensitivity is accompanied by loss of non-heme iron centers 1 and 2 (23). The Saccharomyces complex is not sensitive to rotenone and piericidin A (24), and does not contain certain non-heme iron centers present in the bovine complex (25). These observations imply the involvement of non-heme iron centers 1 and 2 in coupling at site I (26).

Complex II (succinate: coenzyme Q reductase) contains covalently bound FAD, at least two, and possibly three, non-heme iron centers (27), and possibly a b type cytochrome λ_{max} 565 nm (21). Succinate dehydrogenase is a component of the complex which is sensitive to mercurial compounds, has a molecular weight of 97,000 (21) and contains FAD and the two ferrodoxin like non-heme iron centers.

Complex III (coenzyme QH_2: cytochrome c reductase) catalyzes the reduction of cytochrome c by reduced coenzyme Q. It has been studied extensively by Rieske (28). The complex contains 2 moles of cytochrome b heme and one mole of non-heme iron per mole of cytochrome c_1 heme, and is strongly inhibited by the compounds antimycin A and hydroxyquinoline oxide, which prevent electron transfer from cytochrome b to cytochrome c_1. It is composed of seven subunits which are numbered according to increasing mobility in SDS polyacrylamide gels. The dissociability of the complex by bile salts and other methods and the distinct spectroscopic properties of individual components has allowed the assignment of the

following functional roles to the subunits: I and II core proteins; subunit III, cytochrome $\underline{c}_1$; subunit IV, iron sulfur protein; subunits V and VI, cytochromes $\underline{b}$ and $\underline{c}_1$; subunits VII, antimycin binding protein. There is, however, general disagreement as to the assigned functional roles.

Cytochrome $\underline{b}$ is present in two spectroscopically distinct forms: $\underline{b}_{562}$ and $\underline{b}_{565}$. The two forms appear to have different redox potentials (29). Cytochrome $\underline{c}_1$ has been obtained in highly purified form from beef heart (30) and from yeast (31). Yeast cytochrome $\underline{c}_1$ has a molecular weight of 31,000 daltons. Cytochrome $\underline{b}$ has been purified from Neurospora crassa (32) and S. cerevisiae (33) and has a molecular weight of 28,000 daltons. Although a complex retaining enzymatic activity has not yet been purified from yeast, Katan et al., have reported the purification of a seven protein subunit complex from S. cerevesiae containing cytochromes $\underline{b}$, $\underline{c}_1$, and non-heme iron in a stoichiometic ratios of 2:1:1 (34).

Complex IV (cytochrome oxidase) catalyzes the oxidation by molecular oxygen of reduced cytochrome $\underline{c}$. It is strongly inhibited by carbon monoxide, cyanide and azide. The enzyme contains two moles of heme $\underline{a}$, one of which is capable of reacting with molecular oxygen or carbon monoxide (cytochrome $\underline{a}_3$) and two copper atoms (35). The enzyme has been purified from yeast by Mason and Schatz (36) and Rubin and Tzagoloff (37). It contains seven polypeptide subunits. The subunits have been assigned molecular weights by Poyton and Schatz (38) as follows: subunit I 37.5 - 41.6 k; subunit II

28.3 - 33.6 k; subunit III 21.7 - 24.2 k; subunit IV 14.1 - 14.7 k; subunit V 12.4 - 13.8 k; subunit VI 12.4 - 13.1 k; subunit VII 4.5 - 5.2 k.

Rutamycin Sensitive ATPase catalyzes the rutamycin and oligomycin sensitive hydrolysis of ATP to ADP and inorganic phosphate. The enzyme is believed to be responsible for the terminal step in oxidative phosphorylation. The yeast enzyme has been purified and extensively studied by Tzagoloff (39) and co-workers. It is believed to contain nine protein subunits with molecular weights of 58.5, 54.0, 38.5, 31.0, 29.0, 22.0, 18.5, 12.5 and 7.5 x 10^3 daltons. The four largest subunits and subunit 8 (12.0 k daltons) comprise the catalytic portion of the molecule (F_1). The remaining subunits are involved with the conferral of rutamycin sensitivity (F_0) and the binding of F_1 to the membrane. The smallest subunit is a proteolipid.

Coenzyme Q (ubiquinone) was postulated by Green (20) to act as a lipid soluble, mobile, electron carrier between complexes I and II and complex III. Co Q is present in yeast as Co Q_6 (40) (has a chain of 6 isoprene units at position 5). In assaying complexes I, II and III, it is convenient to use lower molecular weight homologues on analogues of Co Q. Coenzyme Q has been shown to be required for the oxidation of NADH (41). Its role in succinate oxidation (42) and its mobility in the membrane (43, 44) have been the subject of some disagreement.

Cytochrome c transfers electrons from complex III to

complex IV. In contrast to the other cytochromes, it is highly water soluble and is only loosely bound to the mitochondrial membrane. Cytochrome c of S. cerevisiae exists as two isozymes, I and II. (45)

THE TWO SYSTEMS FOR PROTEIN SYNTHESIS

Mitochondrial DNA

In 1963 Nass and Nass showed that mitochondria contained DNAse susceptible filaments (6). Subsequently, Reich and Luck (46) showed the presence of a DNA species distinct from nuclear DNA in Neurospora mitochondria and Schatz et al. (47) showed highly purified yeast mitochondria contained a considerable amount of DNA. Since these original demonstrations of its existence, numerous studies on mtDNA have been carried out. Mitochondrial DNA appears to exist universally as a closed circular molecule (48) and occassionally dimers and trimers of interlocked circles (catenanes) are observed (49). The contour lengths of mtDNA from animal species varies from 4 to 6μ (50) and is considerably longer in plants and protists. The contour length of S. cerevisiae mtDNA is 25μ. Contour lengths and base compositions appear to be the same for all cells of a given species, but the base compositions of mtDNA for very similar organisms may vary widely, although such variation is confined to certain regions of the genome (51). Studies with a wide variety of organisms indicate that the base composition of mtDNA is independent of that of nuclear DNA (50). A substantial amount of mtDNA may exist as the

"D loop" replicative intermediate (52).

Mitochondrial DNA in all organism thus far studied appears to code for mitochondrial specific ribosomal RNA and tRNA species. The mitochondrial tRNA species for all amino acids except glutamine and asparagine have been shown to be coded for by mtDNA in S. cerevisiae (53). Similar coding functions have been assigned to mtDNA for all mitochondrial tRNA's in Xenopus (54) and all but glutamine, asparagine, histidine and proline in Hela cells (55). A rigorous demonstration of other coding functions for mtDNA has only recently been accomplished.

Mitochondrial Protein Synthesis

Simpson and his co-workers first demonstrated that mitochondria were capable of protein synthesis (7). Subsequent studies (56-59) confirmed this finding, and eliminated the possibility that the activity observed was due to the presence contaminating bacteria. Kuntzel and Noll (60) and Rifkin et al. (61) demonstrated that Neurospora crassa mitochondria contain ribonucleoprotein particles with a sedimentation coefficient (73^S) different from that of cytoplasmic ribosomes. RNP particles of similar sedimentation coefficient were demonstrated in yeast mitochondria by other groups (62, 63). Grivell et al. (64) were able to demonstrate that the 74^S particles of yeast mitochondria were able to carry out poly u directed synthesis of polyphenylalanine when supplemented with E. coli supernatant factors, indicating that the particles were indeed, ribosomes. Similar results

were obtained by Ibrahim et al. (65) with rat liver mitochondrial 55^S "miniribosomes." Extension of these studies led to the isolation of active yeast mitochondrial polysomes (66).

In addition to their distinct sedimentation, coefficient, mitochondrial ribosomes posses additional properties which distinguish them from their cytoplasmic counterparts. Their action is inhibited by several antibiotics, eg. chloramphenicol and erythromycin, which do not affect protein synthesis on a cytoplasmic ribosomes, and is not affected by cycloheximide, which inhibits cytoplasmic protein synthesis (67). Furthermore, mitochondria possesses a distinct set of tRNA's (68), amino acyl tRNA synthetases (69), and soluble factors (70). A further difference is revealed by the fact that mitochondrial synthesis is initiated with N-formyl methionine, while cytoplasmic protein synthesis is not (71).

Products of Mitochondrial Protein Synthesis

A major objective in the study of mitochondrial biogenesis is the identification and characterization of proteins synthesized by the mitochondrion. Earlier work focused on characterizing the proteins synthesized by isolated mitochondria. These proteins were shown to be associated with the inner mitochondrial membrane (59, 72) - the part of the mitochondria in which the enzymes responsible for oxidative phosphorylation occur. They were demonstrated to be relatively hydrophobic proteins being somewhat insoluble in acqueous systems (73, 74) and containing a higher proportion

of non-polar amino acids (39).

Subsequent work has focused on determining precisely which proteins are synthesized by the mitochondria and what functional role these proteins play in the processes which mitochondria carry out. Research in this vein has been accomplished primarily by two methods:

a) the selective labeling of proteins synthesized by mitochondria coupled with a method of identifying proteins of known functional roles;

b) determining the effect of the absence of mitochondrial protein synthesis on various enzymes and cytochromes suspected to contain products of mitochondrial synthesis.

The identification of mitochondrial products was greatly facilitated by numerous studies of the cytochrome content and enzymatic activities of the cytoplasmic petite cell. This commonly occuring, non-Mendelian inherited mutation in yeast was discovered by Ephrussi's group in 1949 (75). The mutation results from gross alterations (76) and occasional complete absence of mitochondrial DNA, and its presence results in respiratory incompetent cells, devoid of cytochromes $\underline{a}$, $\underline{a}_3$, and $\underline{b}$. Mitochondria from mutant cells are also deficient in the phosphorylation process, although they contain oligomycin insensitive F_1 ATPase (78). These studies were brought into focus with the observation that the pleiotropic effects of the mutation were due to the absence of mitochondrial protein synthesis (79, 80). The cytoplasmic petite or its phenocopy, the chloramphenicol

treated cell (81), therefore provided a means of assessing the composition of mitochondrial membranes formed in the absence of mitochondrial protein synthesis. The studies indicated that oligomycin sensitive ATPase, the cytochrome $\underline{b}$-$\underline{c}_1$ complex and cytochrome oxidase contained products of mitochondrial protein synthesis.

In 1971, Tzagoloff's group demonstrated that subunits 5, 6, 7, and 9 of rutamycin sensitive ATPase were synthesized by the mitochondria by pulse labeling cells in the presence of cycloheximide and rapidly purifying the complex by immunoprecipitation (39). Subsequently Mason and Schatz (82) and Rubin and Tzagoloff (39) were able to show that subunits I, II, and III of cytochrome oxidase were synthesized by the mitochondrion. Recent progress in this area has been made by Weiss and his colleagues and by Lin and Beattie with the demonstration that cytochrome $\underline{b}$ is a product of mitochondrial protein synthesis (32, 33). These proteins constitute the only functionally identified products of mitochondrial protein synthesis to date. Since the number of proteins synthesized by the mitochondrion may be as high as twenty-one in certain strains of yeast (83), the nature of many of the products of mitochondrial protein synthesis remains to be elucidated.

Proteins Synthesized on Cytoplasmic Ribosomes

By far the bulk of mitochondrial proteins appears to be synthesized on cytoplasmic ribosomes. Petite or chloramphenical treated cells have been shown to contain the enzymes of

the Krebs Cycle, NADH dehydrogenase (84, 85) cytochrome $\underline{c}$ (75), cytochrome $\underline{c}_1$ (86) and whatever proteins appear necessary for the maintenance of mitochondrial structure (81). Furthermore, Davey et al. (87) have demonstrated that cells grown for as long as sixteen generations in choloramphenicol retain the capacity for mitochondrial protein synthesis upon removal of the antibiotic. These results have been interpreted to mean that mitochondria do not synthesize proteins required for mitochondrial protein synthesis, such as RNA polymerase, ribosomal proteins and amino acyltRNA synthetases. In a few instances such as with RNA polymerase, this has been shown to be the case.

In terms of specific inner membrane proteins, pulse labeling in the presence of inhibitors of mitochondrial protein synthesis has shown that subunits 1, 2, 3, 4, and 8 of rutamycin sensitive ATPase (29) and subunits IV, V, VI, and VII (39, 82) of cytochrome oxidase and six of the seven subunits of cytochrome $\underline{b}$-$\underline{c}_1$ complex (89) are synthesized on cytoplasmic ribosomes. Similarly, apo-cytochrome $\underline{c}_1$ has been shown to be present in the mitochondria of petites. Thus respiratory complexes III and IV and rutamycin sensitive ATPase may be viewed as being composed of subunits with different origins, which are present in the enzymes stoichiometric ratios.

The mechanism by which proteins translated on cytoplasmic ribosomes enter into or pass through the mitochondrial membrane is at present unknown. Butow (91) has postuated

that the vectorial synthesis hypothesis might apply to the synthesis of mitochondrial proteins on cytoplasmic ribosomes. In support of this contention a class of ribosomes which are loosely bound to mitochondrial membranes have been isolated. The nascent chains on such ribosomes are more precipitable by antibody directed against F_1 ATPase than by antibody to glyceraldehyde 3 phosphate dehydrogenase. More detinitive proof for this hypothesis, however, is lacking.

Do Mitochondrial Genes Code for the Products of Mitochondrial Protein Synthesis?

The facts that mitochondria synthesize proteins and contain DNA do not necessarily imply that the two are related. It is conceivable, for example, that cytoplasmic mRNA's are imported into the mitochondria and therefore that some mitochondrially translated proteins are the product of nuclear genes. The bulk of the evidence, however, supports the thesis that the products of mitochondrial protein synthesis are the products of mitochondrial DNA. Mahler and Perlman (92) concluded that mitochondrial transcription and translation are related since the effects of ethidium bromide a specific inhibitor of mitochondrial transcription are highly similar to the effects of chloramphenicol on a variety of parameters of mitochondrial formation. Further evidence in this vein came from the experiments of Dawdidowicz and Mahler (93), who found that the mitochondrial polysome formation was not affected by conditions which prevented the appearance of cytoplasmic RNA but was affected by

conditions which blocked mitochondrial RNA formation. They concluded that mitochondria were autonomous in their production of messenger RNA. More recent direct evidence has come from Tzagoloff's laboratory (94) where a series of mutants with specific lesions in cytochrome oxidase and cytochrome b were isolated. These mutations were inherited in a non-Mendelian manner, which indicates that they reside in mitochondrial DNA.

An apparent exception appeared to exist in the case of pet 494-1, a mutant isolated by Ebner et al. (95), which was inherited in a Mendelian fashion but lacked a mitochondrial product in cytochrome oxidase (subunit III). This suggested that the mRNA for this subunit was imported from the cytoplasm. Subsequent experiments (96) however, showed that this was not the case since the effects of the mutation could be suppressed by suppressors of nuclear amber mutations. These results were interpreted to indicate that synthesis and/or integration of subunit III require a product of cytoplasmic protein synthesis.

REPRESSION AND INDUCTION OF RESPIRATION IN YEAST

Glucose Repression and Derepression

When *S. cerevisiae* are grown on a fermentable carbon source, the respiratory activity of the cells is low and the cells rely primarily on anaerobic glycolysis for energy. The product of such metabolism, ethanol, can only be metabolized via the respiratory chain and is, therefore, secreted

by the cells. As the cells exhaust the fermentable carbon source, they undergo a transition from a fermentative to a respiratory metabolism which allows for the utilization of the ethanol already present in the growth medium.

The decreased respiration in yeast caused by growth on fermentable carbon sources is termed glucose, or catabolite, repression (10). The degree of repression is largely dependent on the sugar used: glucose represses more severely than other sugars such as galactose (85, 97).

During catabolite derepression, the levels of various enzymes of oxidative metabolism increase considerably (85, 98). Proteins of both mitochondrial and cytoplasmic origin are synthesized during this process, and their synthesis appears to be asynchronous, with respect to both the elaboration of different enzymes and the protein components of individual enzymes (85, 99). In the sequence of synthesis oligomycin sensitive ATPase is first elaborated, followed by the enzymes of the respiratory chain with cytochrome oxidase being the last enzyme to be induced. Derepression appears to take place in two phases: a fermentative phase, in which various respiratory components are elaborated in the absence of respiration, and an oxidative phase which takes place after respiration has begun. The fermentative phase can take place in the presence of respiratory inhibitors such as antimycin A (100).

Anaerobiosis

A second mode of repressing respiratory activity in

yeast is to culture the organism in the absence of oxygen. To achieve this, a fermentable carbon source must be used, and the media must be supplemented with ergosterol and a source of unsaturated fatty acids, since oxygen is required for the synthesis of these compounds (98, 101). Yeast cells grown in this manner are completely devoid of respiratory activity (11).

The anaerobic lipid supplemented yeast cell contains mitochondria-like structures (102), with relatively high levels of oligomycin sensitive ATPase. Necessarily these particles contain mitochondrial DNA and are capable of mitochondrial protein synthesis (103). They also contain NADH, succinate and malate dehydrogenases at reduced levels (103, 104). The particles differ from aerobic mitochondria in that cytochrome oxidase and succinate-cytochrome c reductase activities are completely absent, as is coenzyme Q (105) and cytochromes a, a_3, b, c, and c_1 (11, 103). More recent work has indicated that the particles may contain the five smallest subunits of cytochrome oxidase (82). Apo-cytochrome c_1 is not present in particulate form, by may accumulate in the cytoplasm of the anaerobic yeast. The particles appear capable of performing certain energy linked functions (105).

A major complication in determining the effects of anaerobiosis on the cells is to separate these effects from the specific effects of heme deprivation. Oxygen appears to be required for the enzymatic oxidation of protophorphyrinogen (106) and it would, therefore, seem as if heme synthesis

could not occur under anaerobic conditions. The presence of certain hematin-like compounds in anaerobic yeast (11, 13, 107) seems to complicate this issue. Nevertheless, the apoproteins of iso II cytochrome c and cytochrome c peroxidase appear to accumulate (108, 109) in anaerobic yeast. They are converted into holoenzymes upon areation (110) indicating that their absence as functional or spectrally detectable entitles in anaerobic cells is due to the absence of heme. It is possible that the absorbance peaks detected in anaerobic yeast are due to intermediates in heme biosynthesis which accumulate during anaerobiosis (111). In this regard only one of the absorbance peaks mentioned above,P558 (cytochrome b_1), is associated with the mitochondrial fraction (102) and anaerobic yeast are known to excrete large quantities of coproporphyrin III (112). It is also possible that more than one mechanism for the exidation of protoporphyrinogen exists.

Aside from preventing the apo to holo cytochrome transition, heme deprivation may also prevent the synthesis of the apo-cytochrome. Such a mode of regulation has been proposed in the case of hemoglobin (113). The recent isolation of a series of mutants in heme biosynthesis (114) will undoubtedly provide information on the specific effects of heme deprivation. An additional complication in understanding the effects of anaerobiosis is to distinguish them from the effects of catabolite repression, since the anaerobic growth of yeast requires the use of fermentable carbon sources. In the present studies, this has been accomplished by the use of

galactose, a weakly repressing sugar, and by growing the cells to late stationary phase exhausting the galactose present.

Anaerobic cells cultured in the absence of unsaturated fatty acids also contain mitochondria-like structures (13, 102). Although such structures have been the subject of much investigation (115) the effects of fatty acid deprivation present such extensive complications that they will not be discussed further.

Respiratory Adaption

The process by which anaerobically grown yeast acquire the capacity for respiratory metabolism upon aeration was termed respiratory adaptation by Ephrussi and Slonimiski in 1950. It involves the formation of cytochromes $\underline{a}$, $\underline{a}_3$, $\underline{b}$, $\underline{c}_1$, and $\underline{c}$ (11, 116), the development of various associated enzymatic activities, and the synthesis of Krebs cycle enzymes (104). It is accompanied by changes in certain lipid constitutents of the mitochondria (117), and coenzyme Q appears to be induced by aeration (118). The extent to which the cells are catabolite repressed influences their adaptation, as glucose grown cells adapt more slowly than galactose grown cells (119). The phophorylative capacity of the cells also influences adaptation, as the process is prevented by uncouplers (dinitrophenol), and respiratory inhibitors(azide, cyanide and 2 phenylethanol) (120).

Adaptation is blocked by inhibitors of mitochondrial and cytoplasmic translation and by inhibitors of mitochondrial

transcription (121). It thus appears to require the elaboration of proteins both mitochondrial and cytoplasmic ribosomes. It need not require the synthesis of all the proteins of the respiratory chain, as certain constituents are present during anaerobiosis.

The synthesis of these proteins may take place in an asynchronous manner. In their extensive studies on the development of cytochrome oxidase activity during respiratory adaptation, Chen and Charalampous (122, 123) found that the appearance of cytochromes $\underline{a}$ - $\underline{a}_3$ preceded the development of enzymatic activity and that the appearance of cytochrome $\underline{a}$ preceded that of cytochrome $\underline{a}_3$. Furthermore, the formation of cytoplasmic products seemed to precede the formation of mitochrondrial products.

Schatz and his co-workers have posulated that respiratory adaptation involves the conversion of the mitochondria-like particles in anaerobic yeast into functional mitochondria. In this regard they have termed the anaerobic particles "promitochondria", in analogy to "proplastid" chloroplast precursor of light deprived plants (124). In support of this view, they showed that when promitochondria were specifically labelled with ^{3}H leucine in the presence of cycloheximide and allowed to adapt in unlabelled medium after removal of the antibiotic, the label was associated with cytochrome oxidase stain, as judged by electron microscope autoradiography (125). Respiratory adaptation, according to these authors, does not appear to involve the proliferation of mitochondria, as they found

that mitochondrial ATPase is not induced during adaptation (125).

This last point has been the subject of some disagreement. Somlo (126) has found that the specific activity of oligomycin sensitive ATPase undergoes a three-fold increase during a seventeen-hour adaptation period. Subsequent investigations, however, showed that the newly synthesized ATPase was associated with membranes of different buoyant density than anaerobic ATPase and that newly synthesized succinate cytochrome c reductase and cytochrome oxidase were associated with the ATPase containing membranes of the anaerobic cell (127). These results largely clarified the issue, and strongly supported the notion of promitochondria as precursors of functional mitochondria. Furthermore, they provided an indication that respiratory adaptation may involve a heterogeneity of organelles and organelle precursors. Ainsworth and Tustanoff (128) have made a similar suggestion to explain the shifts in temperature dependence profiles of particulate cytochrome oxidase during respiratory adaptation.

In light of the above mentioned considerations, the formation of mitochondrial electron transfer chain during respiratory adaptation appears to involve four processes:

(1) The synthesis, de novo, of some of the polypeptide subunits of the different enzymes of the chain.

(2) The conversion of apo-hemoproteins into holo hemoproteins by newly synthesized heme.

(3) The synthesis of coenzyme Q and certain other lipid components essential for activity.

(4) The integration of these components into pre-existing promitochondria.

The precise sequence in which events (2), (3), and (4) take place is unclear, and the integration and synthesis of membrane proteins may be coordinated processes.

REGULATION OF MITOCHONDRIAL MEMBRANE FORMATION

Regulation by Heme

The possible regulation of mitochondrial biogenisis by heme in yeast has been suggested by Sugimura (129) and in rat liver by Beattie (130). Beattie showed that the cytochrome content and rate of protein synthesis of rat liver in mitochondria could be increased by the administration of porphyrinogenic drugs. Since this effect could be abolished by inhibitors of heme biosynthesis, it appeared as if the effect was due to the elevated levels of cellular heme, caused by the drugs. Recently, Cabral et al. (131) have shown that the rate of mitochondrial protein synthesis is markedly reduced in a mutant lacking ALA synthetase (132). Furthermore, the mutant mitochondria are incapable of carrying out the ATP-P_i exchange reaction, and lack a functional respiratory chain. Cytochrome oxidase activity is absent from these mitochondria and certain subunits of the enzyme appear to be missing.

A role for heme synthesis in the regulation of cytochrome oxidase formation during respiratory adaptation, was further suggested by Charlampous (112). It was discovered that

cytochrome oxidase induction could take place in the absence of air if the cells were previously aerated in the presence of chloramphenicol, acriflavin, or cycloheximide (132). The procedure allowed for the production of heme but not of certain protein subunits of the enzyme. Subsequent investigation showed that the anaerobic production of cytochrome oxidase could be prevented if coproporphyrin III was added to the incubation medium. It was also shown that coproporphyrin III accumulates during anaerobic growth. Charalampous postulated that coproporphyrin was responsible for preventing the synthesis of the protein subunits of the enzyme during anaerobic growth. Oxygen was shown to abolish the inhibitory effects of coproporphyrin, and it was postulated that oxygen acted to inactivivate a coproporphyrin-repressor complex present in the anaerobic yeast. This mechanism pertained to suppression of both mitochondrial and cytoplasmic products, and was specific for cytochrome oxidase, as coproporphyrin did not prevent the anaerobic induction of cytochrome $\underline{b}$ or $\underline{c}_1$ (133).

The role for coproporphyrin as the repressor of cytochrome oxidase synthesis in the anaerobic cell is not definite. Groot and Poyton (134) have claimed that subunits I and II of cytochrome oxidase are not synthesized by isolated mitochondria in the absence of air, but subunit III is. They explained their results by postulating that O_2 exerts a positive control at the translational level.

Heme deficiency may also have effects at the integration level. Ross and Schatz (91) have shown that the apo-protein

of cytochrome $\underline{c}_1$ may accumulate in the cytoplasm of the heme deficient mutant mentioned previously, as it does in the anaerobic cell. This result provides further evidence that the effects of anaerobiosis on the mitochondria may be due to its effects on heme biosynthesis.

Cooperation of Two Genetic Systems as a Mode of Regulation

The presence of subunits of cytoplasmic and mitochondrial origin in number of mitochondrial enzymes suggests that some mechanism (s) exists to insure their proportionate production. A first indication of the nature of this type of regulation came from the experiments of Tzagoloff (136), who found that the rate of mitochondrial protein synthesis could be greatly increased if cells were first preincubated in chloramphenicol, which allowed for the accumulation of proteins synthesized in the cytoplasm. It was postulated that this accumulation of cytoplasmic products stimulated the production of their mitochondria complements. Furthermore, since F_1 accumulated in a non-particulate form it was suggested that the mitochondrially made subunits were required for the proper integration of the cytoplasmic counterparts. Subsequently, Kim and Beattie, in yeast undergoing glucose derepression showed that the cytoplasmic products of NADH cytochrome $\underline{c}$ reductase and cytochrome oxidase accumulated prior to the synthesis of mitochondrial products of the enzyme (85). It was shown that a preliminary exposure of cells to chloramphenicol increased the rate of induction of coenzyme QH_2: cytochrome $\underline{c}$ reductase (137). If cycloheximide was substituted for chloramphenicol,

the rate of induction was below that of the control. These experiments were consistent with the concept that subunits of the complex made on cytoplasmic ribosomes stimulate the production of complementary subunits on mitochondrial ribosomes. Furthermore, Ibrahim et al. (66) showed that mitochondrial protein synthesis in vitro could be stimulated by the accumulation of proteins synthesized in the cytoplasm. This stimulation appeared to apply only to certain mitochondrial products, an observation consistent with the observation that the rate of cytochrome oxidase induction was not increased by preliminary exposure to chloranphemicol. In an attempt to define the nature of the control phenomena, Ibrahim and Beattie recently investigated the effect of chloramphemicol preincubation on the polysome profile of mitochondria. It was found that cells previously exposed to chloramphemicol have a higher polysome: monosome ratio than control cells, whereas in cells exposed to cycloheximide, the ratio is lower (66). These results have been interpreted as indicating that the proposed regulation resides at the level of translation initiation or transcription.

By contrast, Rouslin and Schatz (139) have shown that during respiratory adaptation cytoplasmic products do not accumulate in a usable form when mitochondrial protein synthesis is blocked - an observation inconsistent with the above proposed mode of regulation. Furthermore, other modes of regulation of this type such as the mitochondrial production of a repressor (140) have been proposed. The present study was undertaken in an effort to clarify these issues.

CHAPTER II

MATERIALS AND METHODS

Strains of Yeast - Saccharomyces cerevisiae

D273-10B, prototrophic (ρ^+) and coenzyme Q deficient mutants derived from it (141) were obtained from Dr. A Tzagoloff; 585-11C (ρ^o, a, lys) was obtained from Dr. R. Needleman, and was used as a petite tester. For purification studies commercial (Budweiser) yeast was used. Experiments on respiratory adaptation were done with a diploid strain of S. cerevisiae used in this laboratory for several years.

Growth and Collection of Cells

Cells were grown routinely at 30° in semisynthetic medium with galactose as carbon source. Cells were aerated by vigorous shaking (200 rpm/min.) on a New Brunswick shaker. Cells were grown anaerobically at 30° in the medium described by Chen and Charalampous (122) (with the omission of Tween 20) in a rubber-stoppered carboy, equipped with gas inlet and outlet lines, an inoculation port and sampling valve. Prior to inoculation the culture medium was saturated with highly purified nitrogen. The initial inoculation consisted of 50 mg dry weight of cells, from a stationary phase aerobic culture into a liter of anaerobic culture medium. Highly purified nitrogen was passed through the culture for six to eight hours after inoculation. At this time the stream of nitrogen was shut off and anaerobiosis was maintained by submerging the outlet line in water. Cells were maintained in suspension by gentle shaking. Stationary phase (56 to 60

grams dry weight per liter) was reached after 20 hours. The anaerobic doubling time was approximately 100 minutes. To eliminate any possible effect of catabolite repression on respiratory adaptation, 24 hour cultures, at late stationary phase, were used for respiratory adaptation. Cultures were chilled on ice and cells were harvested

Estimation of Reversion and Petite Production

The percentage of revertants in cultures of respiratory deficient strains was determined by plating approximately 200 cells on solid YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar), incubating the plates at 30° for two days and then repicating them onto plates containing solid YPGE medium (1% yeast extract, 2% peptone, 3% ethanol, 3% glycerol, 2% agar). Colonies capable of growth on YPGE were scored as respiratory competant revertants. The percentage of cells in the population was estimated from the number of small colonies after growth for two days on YPD (141). The technique was checked by crossing cells from both large and small colonies with a petite tester (ρ^{o}) strain of opposite mating type. In general, only cells from the larger colonies give rise to diploids capable of growth on YPGE.

Protocol for Experiments Involving Sequential Exposure of Cells to Antibiotics

Cells were harvested, incubated under nitrogen and aerated as described above. After seventy minutes aeration in the presence of cycloheximide (10 μg/ml) or chloramphenicol

(2 mg/ml), an aliquot of the medium was removed from the aeration vessel, centrifuged at 2500 x g for two minutes and washed four times by repeated resuspension and centrifugation in ice cold adaptation buffer minus galactose, containing the antibiotic used in the second incubation. Cells were resuspended in the same medium pre-warmed to 30° and aeration was continued. Controls were washed in adaptation medium minus galactose and resuspended in the same medium.

Cell Breakage and Subcellular Fractionation

Cells were resuspended in MTE buffer (.25M mannitol, 50mM Tris-Cl, pH 7.6, 1mM EDTA). Cell breakage was accomplished by shaking the suspension with glass beads (0.45-0.50 mM) in a Bronwill mechanical cell homogenizer (Braun model MSK) for twenty seconds at 4,000 revolutions per minute. Cell extracts were obtained by centrifuging the homogenate at 1,000 x g for twenty minutes and saving the supernatant fluid. Mitochondria were prepared from the cell extract by centrifuging the 1000 x g supernatant at 19,000 x g for twenty to thirty minutes depending on the volume of the sample (380,000 x g-min average). The pellet was resuspended with a teflon-glass homogenizer in MTE and centrifuged at 19,000 x g for twenty min. This final pellet was resuspended in 2 mls of MTE and used as a source of washed mitochondria.

When samples were to be used for spectroscopic determination of cytochrome content, this procedure was modified slightly. The aliquots withdrawn were larger and cell breakage was achieved by breaking the cell suspension with glass beads

two times for thirty seconds each time. In purification studies, eight pounds of compressed commercial yeast were frozen in liquid nitrogen, broken with a Waring blender, and purification fractions prepared as described by Lin and Beattie (33) by centrifugation at 2500 x g for two minutes. Anaerobic cultures were removed from the flask by forcing them through the sampling valve under nitrogen pressure. Cells were washed once with ice cold distilled water (100 mls/1 liter of culture, nitrogen saturated for anaerobic cells) and resuspended in the appropriate medium.

Respiratory Adaptation

Washed cells were resuspended in adaptation buffer (67 mM KH_2PO_4, 20mM sodium potassium phthalate buffer, pH 4.2, 2% glycerol, 2% ethanol, 6 mM galactose) at a density equal to that of the stationary phase culture. Cells were preincubated in adaptation medium under nitrogen for 2½ to 3 hours before adaptation was initiated by spargeing the culture with air at a flow rate of 4 cubic feet per hour. Where studied, chloramphenicol (2 mg/ml) and cycloheximide (25 μg/ml) were routinely added one half hour before aeration.

In experiments involving the addition of chloramphenicol or cycloheximide at various times during the induction, an aliquot of the culture being aerated was withdrawn, antibiotic was added, and aeration was continued in a new flask.

In order to determine net antibiotic resistant enzyme induction, aliquots of culture being aerated were withdrawn at various times after aeration, centrifuged, and the resulting

cell pellets resuspended in adaptation buffer containing the appropriate antibiotic. Aeration of the resuspended cells was continued for four hours in a manifold apparatus designed to give approximately equal aeration to all flasks. At various times after aeration, aliquots of the adapting culture were withdrawn and cycloheximide was added to give a final concentration of 25 ug/ml. Cells were pelleted by centrifugation at 3000 x g for one minute, and resuspended in ice cold MTE-cyclo buffer (0.25 mannitol, 50 mM Tris-Cl, pH 7.6, 1 mM EDTA, 25 ug/ml cycloheximide).

<u>Preparation of Cytochrome c Depleted Submitochondrial Particles and Spectroscopic Determination of Cytochrome Content</u>

Cytochromes <u>c</u> and $\underline{b}_2$ depleted particles were prepared essentially by the method of Flury et al. (142), Washed mitochondria, prepared as described aboved, were centrifuged at 19,000 x g for twenty minutes, suspended in sonication buffer 0.5 M sucrose, 50mM Tris-Cl, 1mM EDTA pH 8.0) at a concentration of 4 to 6 mg protein/ml and sonicated in a Heat-Systems-Ultrasonics sonifier at 90-100 watts for 10 seconds. The sonicate was centrifuged at 105,000 xg for one hour and the pellet was resuspended in 5 ml of sonication buffer and centrifuged once at 12,000 x g for twenty minutes. The supernatant fluid was centrifuged again at 105,000 x g for one hour. The resulting pellet was washed four times by repeated resuspension in 50 ml Tris-Cl, pH 8.0, 1M KCl and centrifugation at 105,000 x g for one hour. After the final centrifugation the pellet was resuspended in 0.5M sucrose,

50mM Tris-Cl, pH 8.0, 1.0% deoxycholate, 0.1% sodium dodecyl sulfate at a concentration of from 2 to 4.2 mg protein/ml for spectral analysis. This procedure allowed for the quantitative recovery of cytochromes a + a_3 with complete removal of spectrophotometrically detectable cytochrome c. Samples prepared during the purification of cytochrome b were appropriately diluted for spectral analysis.

Difference spectra were taken at room temperature with a Cary model 15 recording spectrophotometer, by scanning dithionite reduced minus ferricyanide oxidized samples from 500 to 630 nm. Cytochromes a + a_3 were determined by the method of Claisse et al. (143) from the absorbance at 603 nm relative to the baseline from 580 to 630 nm using an extinction coefficient of $14mM^{-1}cm^{-1}$. Cytochromes b and c_1 were determined by modifying the method of Claisse and Pajot (86) so as to apply it to spectra of cytochrome c depleted particles taken at room temperature.

The following equations were used: $x_2 + i_{23}\, x_3 = A_2$

$$i_{32}\, x_2 + x_3 = A_3$$

Where A_2 and A_3 are the actual absorbances at 553 and 562 nm, respectively, X_2 and X_3 are the absorbances due to cytochrome c_1 at 553 nm and cytochrome b at 562 nm, respectively and i_{23} and i_{32} are the interference coefficents of cytochrome b on cytochrome c_1 and cytochrome c_1 on cytochrome b respectively. i_{23} was calculated from the spectrum of purified cytochrome b (33) as 0.27, and i_{23} from the published spectrum of purified cytochrome c_1 (31) as 0.10.

Thus $X_2 = \frac{A_2 - .27A_3}{.97}$ was used to determine the absorbance due to cytochrome $\underline{c}_1$ at 553 nm and $X_3 = \frac{A_3 - .1A_2}{.97}$ was used to determine the absorbance due to cytochrome b at 562nm. The concentrations of the cytochromes were calculated from these absorbances using extinction coefficents of 19.8 $mM^{-1}cm^{-1}$ for cytochrome $\underline{c}_1$ and 22.8 mM^{-1} cm^{-1} for cytochrome b. Cytochrome concentrations are expressed in terms of nmoles cytochrome/mg protein of KCl extracted submitochondrial particles.

Difference spectra in the ubiquinone region (240-320 nm) were obtained by the method of Kröger and Klingenberg (144).

Enzymatic assays. All assays were performed in a Gilford model 1240 recording spectrophotometer at 30^0.

Succinate dehydrogenase was determined by the method of King (145) as modified by Kim and Beattie (85), except that 5 mM sodium azide was substituted for potasium cyanide.

Cytochrome c reductase assays were performed in a 1 ml curvette of 1cm path length containing 600 ug of cytochrome c, 2 mM EDTA, 5 mM sodium azide, 1 mg bovine serum albumin and 25 mM sodium phosphate buffer, pH 7.6, Sodium succinate and NADH were added to give final concentrations of 10 mM or 0.5 mM respectively. Antimycin A was added to give a final concentration of 5 μg/ml except where indicated. DBH_2 was prepared by reduction of DB by the method of Rieske (146). It was added as a 5 mg/ml solution to give a final concentration of 25 μg/ml. Reduced DB and antimycin

A were added as solutions in ethanol. In studies of the reconstitution of NADH and succinate cytochrome c reductase in coenzyme Q deficiene mutants DB in the oxidized form was added as a 1 mg/ml solution in ethanol.

The rate of cytochrome c reduction was monitored by following the increase in absorption at 550 nm. An extinction coefficent of 18.5 $mM^{-1} \times cm^{-1}$ was used to calculate the rate in terms of nmoles cytochrome c reduced per minute.

Cytochrome oxidase was assayed by the method of Wharton and Tzagoloff (24). Reduced cytochrome c was prepared by reducing 50 mg of cytochrome c with ascorbic acid followed by passage through a sephadex G-25 column previously equilibrated with 20 mM potassium phosphate buffer, pH 7.0. Specific activities are expressed in terms of the initial pseudo first order rate constant (25).

All enzyme activities were linear with protein within the ranges used. Although the absolute specific activities varied from experiment to experiment, the trends observed were consistant.

Protein determination. Protein was estimated by the method of Lowry et al. (148) using bovine serum albumin as the standard.

Chemicals

Yeast extract and "bacto" peptone were obtained from Difco. Mannitol, glycerol, dextrose, $FeCl_3$, $CaCl_2$ and sodium ozide were purchased from Fisher. Galactose (purified),

bovine serum albumin NADH, sodium deoxcholate, sodium cholate cycloheximide, chloramphenicol, ergosterol, dichlorophendindophenol, magnesium sulfate, cytochrome _c_ (horse heart type VI), sodium succinate, succinic acid, and "Tris" were from Sigma. Enzyme grade Ammonium Sulfate and Sucrose were obtained from Schwarz Mann. Antimycin A was obtained from Ayrest Laboratories. Highly purified nitrogen was obtained by passing prepurified nitrogen (Matheson) through a "Deoxo" gas purifier (Engelhard Industries) to remove residual traces of oxygen. Antimycin A was obtained from Ayrest Laboratories. 6 decyl, 2, 3, dimethoxy, 5 methyl, 1, 4 benzoquinone was the generous gift of Dr. Karl Folkers.

CHAPTER III

INDUCTION OF RESPIRATORY CHAIN COMPONENTS DURING RESPIRATORY ADAPTATION IN YEAST

RESULTS AND DISCUSSION

Induction of Respiratory Chain Complexes

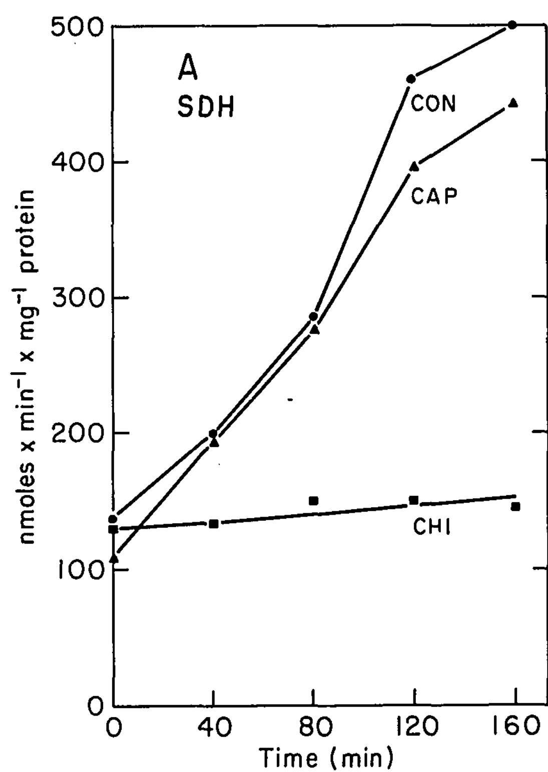
Prior to investigating the regulation of respiratory chain formation during aerobic adaptation, it was necessary to establish the kinetics of induction of the different enzyme complexes as well as the effects of the specific inhibitors of mitochondrial and cytoplasmic protein synthesis. As seen in Figure 1, the activity of succinic dehydrogenase and cytochrome oxidase began to increase immediately upon aeration. Succinic dehydrogenase was present in the anaerobic cell in significant amounts, and its maximum inducible level was reached somewhat earlier than that of cytochrome oxidase. Induction of both enzymes was prevented by the addition of cycloheximide thirty minutes prior to aeration, but only cytochrome oxidase induction was blocked by chloramphenicol addition at this time. These results indicate that subunits of cytochrome oxidase are synthesized on mitochondrial as well as cytoplasmic ribosomes, while subunits of succinic dehydrogenase are synthesized entirely on cytoplasmic ribosomes in agreement with previous studies (103, 21).

The formation of the cytochrome $\underline{b}$ - $\underline{c}_1$ complex was monitored by using three different substrates as electron donors with cytochrome $\underline{c}$ as the electron acceptor. An analogue of coenzyme Q_2, 6 decyl, 2, 3 dimethoxy, 5 methyl, 1, 4 benzoquinone (DB), was used as an immediate electron donor

to the complex. This analogue, recently synthesized by Folker's group (149), reduces cytochrome c in the presence of beef heart complex III at rates similar to those obtained with coenzyme Q_2, with nearly complete antimycin A sensitivity. When mitochondria obtained from either anaerobic or aerated cells are used as a source of enzyme, the reduction of cytochrome c by the analogue is completely sensitive to antimycin A. The enzymatic rates obtained with this assay, therefore, are presumed to accurately reflect the formation of active cytochrome b-c_1 complex. This assumption is further explored in the subsequent chapter. In addition, succinate and NADH, substrates which act via the two primary flavoprotein dehydrogenases, were used as electron donors. In this manner, a profile of induction can be obtained which provides information on the presence of an active cytochrome b-c_1 complex and its ability to receive electrons from the two dehydrogenase complexes.

Induction of succinate, NADH, and coenzyme QH_2-cytochrome c reductases also began immediately upon aeration (Figure 2). Addition of cycloheximide prior to aeration prevented the increase of all three enzymatic activities; however, addition of chloramphenicol resulted in different effects on the induction of the three activities. This antibiotic completely prevented the formation of NADH-cytochrome c reductase but only slightly inhibited the induction of succinate-cytochrome c reductase. By contrast the activity of coenzyme QH_2-cytochrome c reductase increased at a slow rate for 120 minutes

Fig. 1. Kinetics of Induction of (A) succinate dehyrogenase and (B) cytochrome oxidase. Cells were grown, preincubated, and aerated as described in materials and methods. At various times after aeration, samples were withdrawn and mitochondria were prepared. Specific activities as determined in isolated mitochondria are indicated. (●——●) no inhibitor, (▲——▲) chloramphenicol (2 mg/ml) added 30 minutes prior to aeration, (■——■) cycloheximide (25 μg/ml) added 30 minutes prior to aeration.



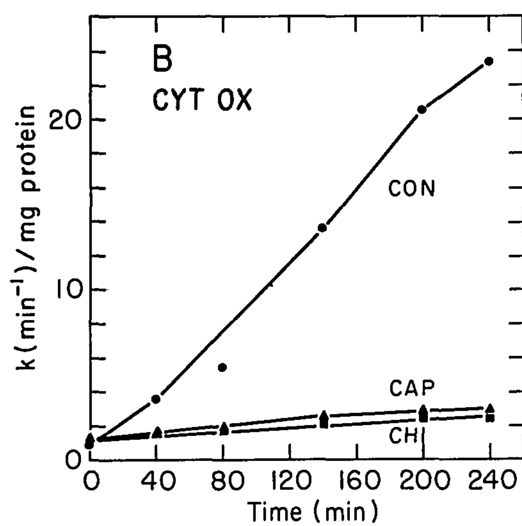


Figure 1

before leveling off when chloramphenicol was added prior to aeration. This small but significant chloramphenicol - resistant increase during aeration probably results from some prior synthesis of proteins for this complex within the mitochondria during anaerobic growth. The effectiveness of chloramphenicol as an inhibitor of mitochondrial protein synthesis is indicated by its complete inhibition of cytochrome oxidase induction (Figure 1a).

The data presented here also confirms previous studies (5,11) performed on yeast undergoing glucose derepression indicating the different response to chloramphenicol of succinate and NADH - cytochrome c reductase. One explanation for these results is to postulate the presence of a "split chain", in which electrons pass from succinate to cytochrome c via a chloramphenicol-insensitive site, and from NADH and coenzyme QH_2 to cytochrome c by a chloramphenicol-sensitive site. Similar "split chains" have been postulated to exist in yeast (150), and animal mitochondria (151). An alternative explanation is based on the observation that coenzyme Q is absent in promitochondria (104), and is produced during adaptation (117). It is possible that during induction this compound is rate-limiting for one or both of the enzymes. In this case, a differential affinity of the primary dehydrogenases for coenzyme Q would give rise to what is, in effect, a "split chain" in the chloramphenicol-treated cell, in which the rate-limiting component for succinate: cytochrome c reductase is the b-c_1 complex and the rate-limiting component for NADH:

Fig. 2. Kinetics of induction of (A) succinate-cytochrome c reductase, (B) NADH-cytochrome c reductase and (C) coenzyme QH_2- cytochrome c reductase. All operations were performed as described in the legend to Figure 1. (●——●) no inhibitor, (▲——▲) chloramphenicol (2mg/ml) added 30 minutes prior to aeration, (■——■) cycloheximide (25 μg/ml) added 30 minutes prior to aeration.

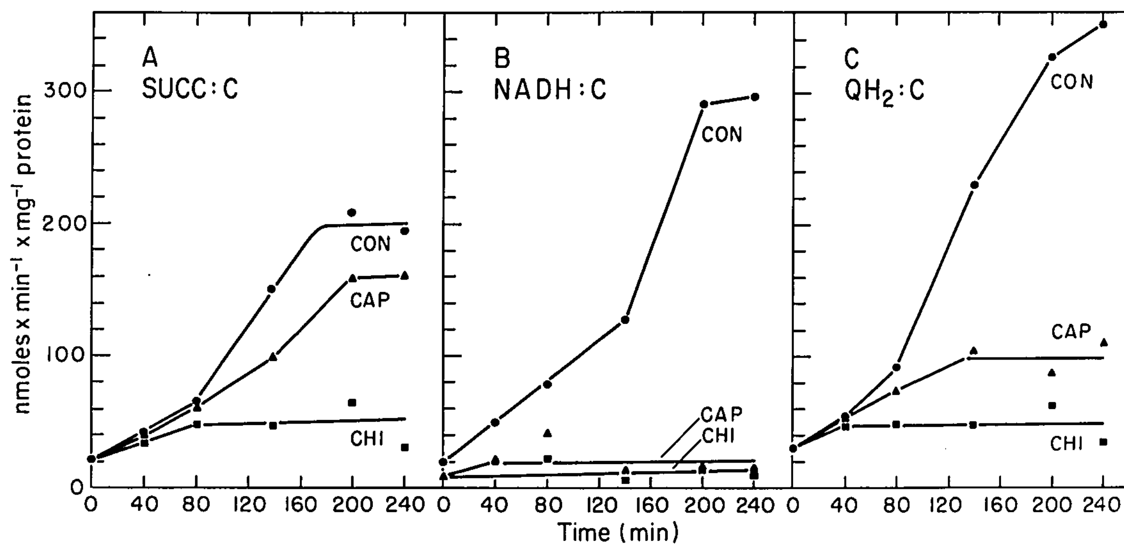


Figure 2

cytochrome c reductase is coenzyme Q.

The induction profiles obtained for either succinate-cytochrome c reductase or cytochrome oxidase were virtually identical when either washed mitochondria or the 1000 xg supernatant fluid of whole cell extracts was used as a source of enzyme. This result indicates that the enzymatic activities determined in mitochondria prepared as described in this thesis, accurately reflect the enzymatic activities in whole cell extracts.

The time course of induction of cytochromes a, b, and c_1 was also determined using KCl-extracted submitochondrial particles for spectral analysis. Since cytochromes c and b_2 are completely removed by this treatment (142), their spectral interference is minimized and hence the content of cytochromes b and c_1 can be precisely determined. Figure 3A indicates that cytochromes a and a_3 appear in the mitochondrial membrane prior to cytochrome oxidase activity as previously reported by Chen and Charalampous (122). As seen in Figure 3b, cytochromes b and c_1 appear in the membrane simultaneously, prior to the development of coenzyme QH_2-cytochrome c reductase activity. The lag period between their appearance and the appearance of enzymatic activity is somewhat longer than that seen with cytochromes $a+a_3$ and cytochrome oxidase activity.

The effects of chloramphenicol and cycloheximide on the development of cytochromes aa_3, b, and c_1 are presented in figure 4. Spectral analysis of cytochrome c and b_2 depleted submitochondrial particles obtained from anaerobic cells and cells aerated for three hours (fig. 4, A and B) show the virtual

absence of cytochromes $\underline{aa}_3$, $\underline{b}$, and $\underline{c}_1$ in the anaerobic cell and the induction of the same upon aeration. Chloramphenicol addition (fig. 4C) allowed for the induction of all three cytochromes, but resulted in a marked reduction of cytochromes $\underline{aa}_3$ and $\underline{b}$ relative to cytochrome $\underline{c}_1$. Cycloheximide addition also allowed for the induction of these cytochromes, but the induction of cytochrome $\underline{c}_1$ was markedly reduced relative to the induction observed in chloramphenicol. These results suggest that the apo-proteins for all these cytochromes in small amounts exist in the anaerobic cell. The greater sensitivity to chloramphenicol of cytochrome $\underline{b}$ induction relative to cytochrome $\underline{c}_1$ induction is consistant with the proposed sites of synthesis of these proteins. (32, 33, 91). The possible presence of the apo-protein of cytochrome $\underline{b}$ in the anaerobic cell may account for the slight resistance of the induction of coenzyme QH_2: cytochrome $\underline{c}$ reductase activity to chloramphenicol.

Fig. 3. Inductions of (A) cytochromes $\underline{a}+\underline{a}_3$ and cytochrome oxidase activity and (B) cytochromes $\underline{b}$, $\underline{c}_1$ and coenzyme QH_2-cytochrome $\underline{c}$ reductase activity. All operations up to the preparation of mitochondria were performed as described in the legend to Figure 1. Aliquots of washed mitochondria were removed for enzymatic activity determinations, and KCl washed submitochondrial particles were prepared from the remaining mitochondrial fractions. The cytochrome content of these particles were determined as described in materials and methods. (A) ●——● cytochrome oxidase activity, ○——○ cytochromes $\underline{a}$-$\underline{a}_3$ content, (B) ●——● coenzyme QH_2: cytochrome $\underline{c}$ reductase activity, ○——○ cytochrome $\underline{b}$ content, ▲——▲ cytochrome $\underline{c}_1$ content.

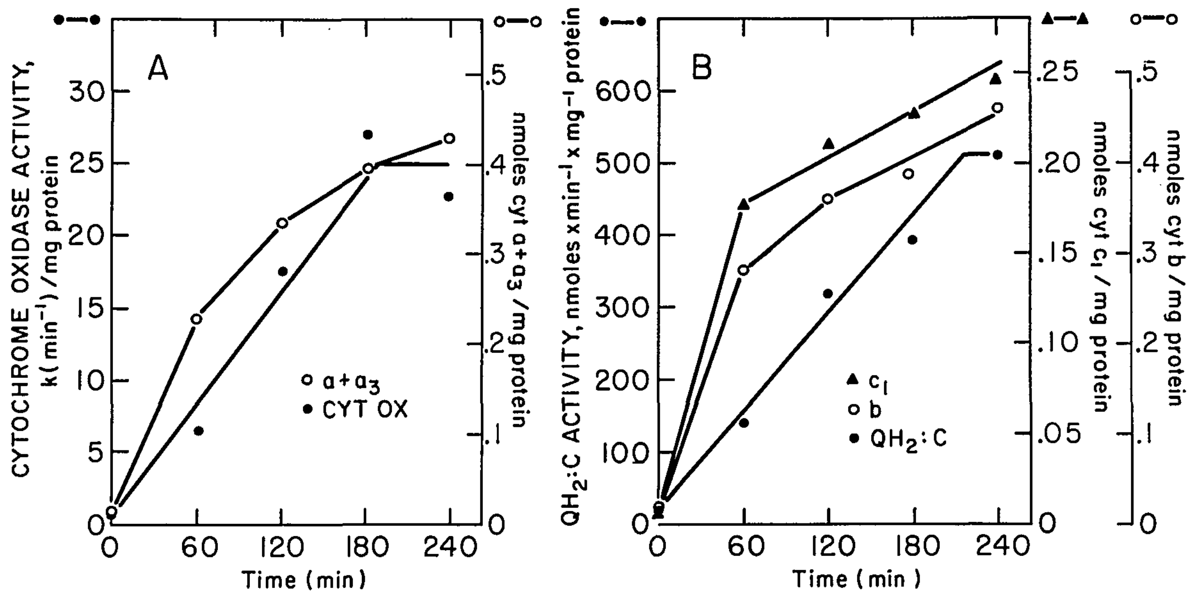
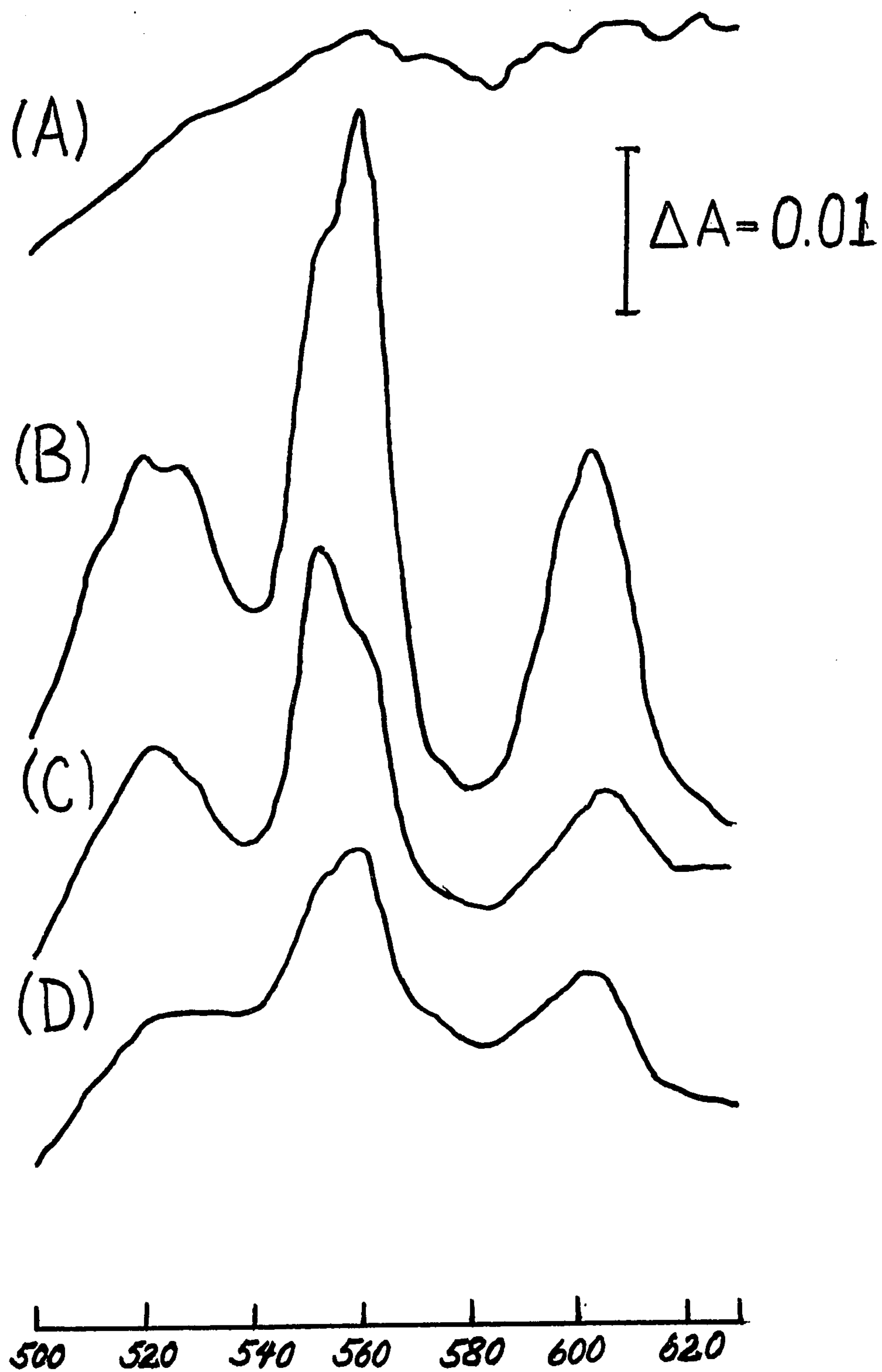


Figure 3

Fig. 4. Reduced vs. oxidized difference spectra of cytochrome *c* and b_2 depleted submitochrondrial particles: (A) particles isolated from anaerobic yeast prior to aeration; (B) particles from the same culture after three hours of aeration; (C) after four hours aeration in chloramphenicol; (D) after four hours aeration in cycloheximide. Protein concentrations: (A) 0.8 mg/ml; (B) 3.9 mg/ml; (C) 4.4 mg/ml; (D) 2.8 mg/ml.

FIGURE 4



CHAPTER IV

THE ROLE OF COENZYME Q IN MITOCHONDRIAL ELECTRON TRANSPORT

INTRODUCTION

The discrepancy between the chloramphenicol sensitivity of induction of NADH: and Succinate cytochrome c reductase activities prompted more extensive investigation of the relationship of these two activities to coenzyme QH_2-cytochrome c reductase activity. In addition, the role of coenzyme Q in the two activities was explored.

While it has long been accepted that coenzyme Q acts as a mediator of electron transfer between the NADH dehydrogenase complex and complex III, the role of coenzyme Q in the transfer of electrons from the succinate dehydrogenase complex has been the subject of some disagreement (42). Furthermore, a functional heterogeneity and compartmentation of coenzyme Q within the mitochondria has been suggested to explain the results obtained in reconstitution experiments with coenzyme Q- depleted mitochondria and submitochondrial particles (44, 152). For example, maximum succinate oxidase activity can be achieved at lower concentrations of coenzyme Q than can NADH oxidase activity. In addition, the two oxidase activities differ with respect to the stimulatory and inhibitory effects of analogs and homologs of coenzyme Q (43, 152, 153, 154). Kröger and Klingenberg (155), however, have maintained that no such compartmentation exists because the coenzyme Q pool appears kinetically homogenous.

The recent development by Folker's group of stable, low molecular weight analogs of coenzyme Q has provided a new tool to probe coenzyme Q function (149). One of these analogs, DB in which the isoprenoid side chain has been replaced with a n-decyl group, was used to assay the formation of the $\underline{bc}_1$ complex. In the present study, a detailed investigation of the catalytic activity of complex III using DB as substrate was undertaken. The analog was also used to reconstitute NADH and succinate cytochrome $\underline{c}$ reductase activities in a series of coenzyme Q defecient mutants of yeast. The results obtained indicate that reduced DB can be used to monitor accurately the catalytic activity of the cytochrome $\underline{b}$-$\underline{c}_1$ complex. The antimycin A sensitive reduction of cytochrome $\underline{c}$ by both NADH and succinate requires coenzyme Q with a similar concentration dependence. No evidence for the "functional heterogenity" of coenzyme Q has been obtained.

RESULTS

Reduction of cytochrome $\underline{c}$ by DBH_2

Before investigating the role of coenzyme Q in the respiratory chain using the analog DB, it was first necessary to show that reduced DB interacts specifically with the cytochrome $\underline{b}$-$\underline{c}_1$ complex. When yeast mitochondria are added to a cuvette containing excess DBH_2, cytochrome $\underline{c}$, azide, and buffer, the rate of cytochrome $\underline{c}$ reduction may be monitored by measuring the increase in absorbance at 550nm. As seen in Fig. 5A, the initial slopes of the absorbance _vs_. time

curves bear a linear relationship to the amount of added mitochondrial protein. Both the extrapolated and experimental rates of cytochrome c reduction in the absence of added mitochondria are comparable.

The analysis of absorbance vs. time curves for various concentrations of mitochondria was further examined by the method of Guggenheim (156). The logarithm of changes in absorbance, ΔA_t, occurring in a relatively short period of time (in this case t=38 sec) is plotted as a function of t, the time after the addition of mitochondria during which ΔA_t occurs (Fig. 5B). The straight lines obtained indicate that the reduction of cytochrome c follows first order kinetics during the initial part of the reaction over a four-fold range of protein concentrations. The pseudo first order rate constants obtained from the slopes of these lines are directly proportional to added mitochondrial protein (Fig. 5C).

The initial rates of cytochrome c reduction, calculated by multiplying the experimentally measured first order rate constants by the concentration of cytochrome c, are in good agreement with the observed rates. These results indicate that the catalytic activity of the cytochrome b-c_1 complex may be estimated conveniently by subtracting the non-enzymatic rate of cytochrome c reduction from the initial reduction rate obtained in the presence of mitochondria. Alternately, a first order rate constant for cytochrome c reduction can be determined. For most purposes, the measurement of initial velocities is preferable because of its simplicity; however,

Fig. 5. Kinetics of the reduction of cytochrome c by DBH_2 (A) Initial rates of cytochrome c reduction obtained with increasing amounts of added mitochondrial protein. (B) Analysis of the absorbance vs. time curves used to obtain the data in (A) by the Guggenheim method. Amounts of added mitochondrial protein in micrograms indicated by parenthesis. $\Delta A_{t=38}$ seconds. (C) First order rate constants, determined from slopes of the lines in (B) vs. added mitochondrial protein. Mitochondria from strain D273-10B were used.

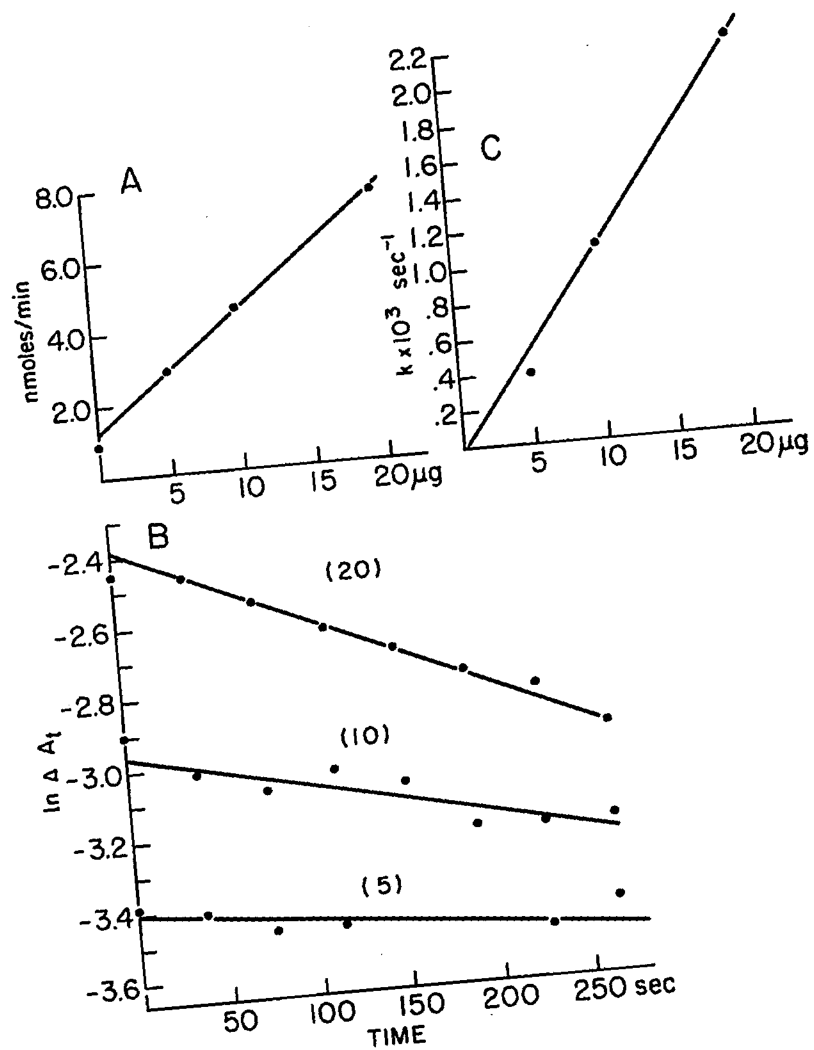


Figure 5

its accuracy is limited at higher protein concentrations, where measurement of initial velocities may not be feasible.

Substrate Specificity of DBH_2

A major problem encountered with many substances used as substrates to assay the $\underline{b}$-$\underline{c}_1$ complex is their lack of specificity. Much of the apparent activity measured in whole cell extracts results from the reduction of cytochromes $\underline{c}$ by extra-mitochondrial electron transport chains. Although corrections for non-mitochondrial activity can be made, these large differences can introduce substantial errors into the estimation of mitochondrial activity. Using the analog DBH_2 as a substrate circumvents most of these difficulties. The enzymatic activity of the cytochrome $\underline{b}$-$\underline{c}_1$ complex measured in whole cell extracts or in isolated mitochondria using DBH_2 as substrate was completely inhibited by antimycin A (Table I). Furthermore, upon fractionation, this activity sediments with cytochrome oxidase and succinate-cytochrome $\underline{c}$ reductase, markers for the mitochondrial respiratory chain. These results suggest that DBH_2 selectively reduces the mitochondrial cytochrome $\underline{b}$-$\underline{c}_1$ complex and that reduction of extra-mitochondrial cytochromes (e.g., cytochrome $\underline{b}_5$) is negligible.

The relationship between DBH_2: cytochrome $\underline{c}$ reductase activity and mitochondrial cytochrome content was further examined in order to determine to what extent DBH_2: cytochrome $\underline{c}$ reductase reflects the activity of the cytochrome $\underline{b}$-$\underline{c}_1$ complex. Various fractions obtained during the purification of cytochrome $\underline{b}$ were examined for DBH_2: cytochrome $\underline{c}$ reductase

TABLE I

Specific Activity and Antimycin A Sensitivity of Succinate and DBH_2: Cytochrome c reductase in Cell Free Extracts and Mitochondria

	Cell Free Extract		Mitochondria		Purification
	nmoles/min or K(min^{-1}) mg protein	%Inhibition by Antimycin A	nmoles/min or K(min^{-1}) mg protein	%Inhibition by Antimycin A	(fold)
DBH_2: Cyt c reductase	67.4	100%	386	100%	5.7
Succinate: Cyt c reductase	26.4	93%	180	100%	6.8
Cytochrome oxidase	6.5	-	29.6	-	4.5

Cell free extracts and mitochondria were prepared from a culture of strain D273-10B. Succinate and DBH_2 cytochrome c reductase activities were determined in both fractions in the presence or absence of antimycin A (5ug/ml). Values for DBH_2 cytochrome c reductase are corrected for non-enzymatic reduction.

activity. As seen in Table II, the specific activity of DBH_2: cytochrome c reductase more than doubled when submitochondrial particles were prepared from mitochondria and further increased with specific cytochrome content during extraction of submitochondrial particles with bile salts and KCl. When this detergent-solubilized preparation was treated with ammonium sulfate, an insoluble fraction was obtained in which the specific content of both cytochromes b and c_1 was increased, but DBH_2: cytochrome c reductase activity was completely absent. The ratio of cytochrome b to c_1 is considerably greater in this fraction than that observed in the cholate-solubilized fraction or in intact mitochondria (as seen in figure 4B), suggesting that the b-c_1 complex is no longer intact. The presence of cytochrome c_1 in the fraction may have resulted from the coprecipitation of this protein with cytochrome b, an interpretation consistent with the observation that ammonium sulfate in the presence of bile salts cleaves the isolated complex (28). In the final step of the purification yielding spectrally pure cytochrome c, cytochrome c_1 is completely absent. These results provide further evidence that DBH_2: cytochrome c reductase activity is a reflection of the activity of cytochrome b-c_1 complex, and suggests that this complex in yeast is similar to that of beef heart, at least in terms of its dissociability by bile salts and ammonium sulfate.

Properties of coenzyme Q deficient mutants

The recent isolation of a number of mutants presumed to

TABLE II

DBH$_2$: Cytochrome c reductase activity and spectral properties of various fractions prepared during purification of cytochrome b

	$\underline{a}+\underline{a}_3$	$\underline{b}$	$\underline{c}_1$	$\underline{b}$-$\underline{c}_1$	DBH$_2$: $\underline{c}$ reductase
Mitochondria	-	-	-	-	270
Submitochondrial particles	.289	.224	-*	-	636
F_4 (deoxycholate KCl extraction	.902	.872	.500	1.74	3316
F_5 (cholate $AmSO_4$ Fraction)	1.100	4.08	.990	4.12	0
F_6 ($\underline{b}$-$\underline{c}_1$ separation)	.529	5.01	0	-	0

Cytochrome $\underline{b}$ was purified from commercial yeast according to the procedure of Lin and Beattie (33). At various stages during the purification aliquots were removed for the determination of cytochrome content and DBH$_2$: cytochrome $\underline{c}$ reductase activity. Cytochrome content is expressed as nmoles/mg protein; DBH$_2$: cytochrome $\underline{c}$ reductase as nmoles cytochrome $\underline{c}$ reduced min/mg protein.

* The cytochrome $\underline{c}_1$ content of sub mitochondrial particles could not be accurately estimated because of the presence of cytochrome $\underline{c}$.

be deficient in coenzyme Q biosynthesis (141) permitted studies of the effectiveness of DB as a replacement for coenzyme Q in promoting electron transfer from the primary dehydrogenases to the cytochrome $\underline{b}$-$\underline{c}_1$ complex. After a preliminary screening of thirteen strains, six were selected for further studies on the basis of their relative long-term stability. These six mutants, which fall into five different complementation groups, have a complete, or near complete, absence of both NADH and succinate: cytochrome $\underline{c}$ reductase activity in the absence of DB, with the exception of strain E2-247. This strain has an appreciable succinate: cytochrome $\underline{c}$ reductase activity in the absence of added quinone; however, a high percentage of revertant cells were present in the culture used. The activities of both NADH and succinate: cytochrome $\underline{c}$ reductases were markedly stimulated in all strains upon addition of 10 ug of DB to the assay medium. It should be noted that the DB stimulated succinate: cytochrome $\underline{c}$ reductase activities of the mutants were not as high as those observed in the wild type. Similarly, the activities of DBH_2: cytochrome $\underline{c}$ reductase and cytochrome oxidase were also lower in the mutants, probably a result of the inability of respiratory deficient cells to undergo complete catabolite derepression (98).

The ability of DB to promote electron transfer from NADH or succinate to cytochrome $\underline{c}$ in these mutants is further confirmation that the defect in these mutants is the absence of coenzyme Q and not another component of the respiratory chain.

Further direct evidence for this conclusion has been obtained by spectral analysis of petroleum ether methanol extracts of mitochondria from both wild type and mutant cells. As seen in Fig. 6, the wild type cells contain spectrally detectable coenzyme Q, while extracts of three mutant mitochondria do not.

Reconstitution of NADH and succinate - cytochrome c reductases by DB

An investigation of the reconstitution of NADH and succinate-cytochrome c reductases in the coenzyme Q deficient mutants, using DB, was undertaken in an attempt to clarify the considerable discrepancies in the literature with regard to the role of coenzyme Q in NADH and succinate oxidation (42—44, 150-153). The restoration of NADH and succinate: cytochrome c reductase activity in the mutant E3-24 with increasing concentrations of DB (Fig. 7) shows a sigmoidal dependence on added quinone. The NADH dependent activity reaches a three-fold higher maximum velocity than the succinate dependent, and requires a higher concentration of DB to reach half maximal velocity. It should be mentioned that succinate: cytochrome c reductase activity showed a marked lag upon addition of coenzyme Q before attaining a constant velocity. This lag may result from the well documented activation of succinic dehydrogenase by reduced quinone (157) as the lag was eliminated by preincubation of mitochondria in the presence of succinate and DB. The rates presented in Fig. 3A were obtained after the lag period.

By contrast, the substrate dependence of the DBH_2: cyto-

TABLE III

Properties of presumptive mutants in coenzyme Q biosynthesis

Strain	Complementation group*	% revertant	% petite	NADH: C		Succ: C		DBH_2 C	Cyt. Ox.
				-DB	+DB	-DB	+DB		
N9-57	II	0	44	5.8	56.0	1.0	21.8	78.5	2.20
E3-24	V	0	7.5	5.4	62.3	0	24.0	66.3	3.00
E3-71	I	0	10	6.5	51.0	0	11.8	71.7	2.98
E2-247	III	29	12.9	7.6	73.0	5.8	30.1	75.0	3.10
E1-237	IV	0	3.1	5.8	56.0	0	11.4	35.0	4.72
E2-249	V	0	5.1	13.1	64.7	0	13.0	98.7	2.60
D273-10B	wt	-	1	-	-	180	180	386	29.6

* From Tzagoloff et al. (141)

Cells of mutant and wild type strain were grown to stationary phase on galactose media and revision and petite frequency were determined. Mitochondria were prepared and assayed for enzymatic activity.

DB stimulated rates were > 90% inhibited by antimycin A (5 ug/ml) and DBH_2: cytochrome c reductase activity was completely inhibited by antimycin A. To reconstitute NADH:c and Succ:c, 10ug of DB were added as an ethanolic solution.

Fig. 6. Spectra of petroleum ether-methanol extracts of wild type (D273-10B) and coenzyme Q deficient mitochondria. Mitochondria were extracted by the method of Kröger and Klingenberg (1966). Dried extracts were dissolved in 3 mls of ethanol: cyclohexane (1:4υ/υ), and 1 ml aliquots were reduced with 10 μg of $NaBH_4$ or oxidized with 10 μg of $FeCl_3$. Solid lines (——) indicate reduced vs oxidized samples. Dotted lines (---) indicate oxidized vs oxidized samples. Amounts of mitochondrial protein extracted D273-10B, 1.0 mg; E1-237, E3-24, E2-249, 2 mg.

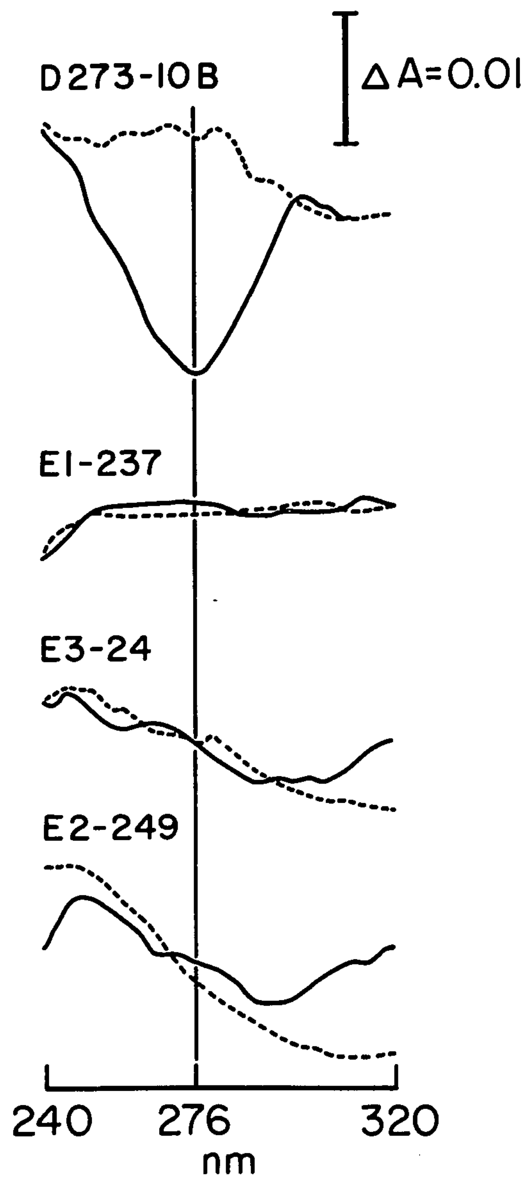


Figure 6

chrome *c* reductase activity is markedly different. At substrate concentrations varying from 0 to 50 μg per ml., a hyperbolic relationship between substrate concentration and specific activity is obtained. Typical Michaelis-Menten kinetics are observed when the data are analyzed by a double reciprocal plot (Fig. 7B). A comparison of Figs. 7A and 7B indicates that the maximum specific activities of NADH or succinate: cytochrome *c* reductases are considerably lower than the activities of DBH_2: cytochrome *c* reductase obtained with DBH_2 at less than saturating concentrations. The V_{max} for the latter enzymatic activity, as estimated from the double reciprocal plot, is 555 nmoles min/mg protein, a value four times and twelve times higher than the maximal velocities attainable with NADH and succinate, respectively. Furthermore, DBH_2: cytochrome *c* reductase activity continues to increase at concentrations of the analog that are considerably higher than those necessary to reconstitute NADH and succinate: cytochrome *c* reductase.

These results may reflect the lower affinity of complex III for the reduced form of the analog than that of complexes I and II for the oxidized form. This interpretation is complicated by the presence of some oxidized analog in the preparation of DBH_2 used in this experiment; hence, the actual substrate concentration would be somewhat lower than that indicated in Fig. 3B where substrate concentration is expressed in terms of total DB content. Since the addition of the oxidized form of the analog does not influence DBH_2: cytochrome *c* reductase activity, we assume that its presence does not influence the nature of the substrate dependence.

Fig. 7. Concentration dependence of cytochrome c reductase activities on added DB or DBH_2. (A) Specific activity of NADH (●——●) and succinate (O——O) cytochrome c reductase in mitochondria of strain E3-24 vs concentration of DB (ug/ml). 42.5 ug of mitochondrial protein were added. (B) Specific activity of DBH_2 cytochrome c reductase in mitochondria of strain E3-24, vs increasing concentrations of reduced DB (ug/ml) Insert: Double reciprocal plot of same. 17 μg of mitochondrial protein were added.

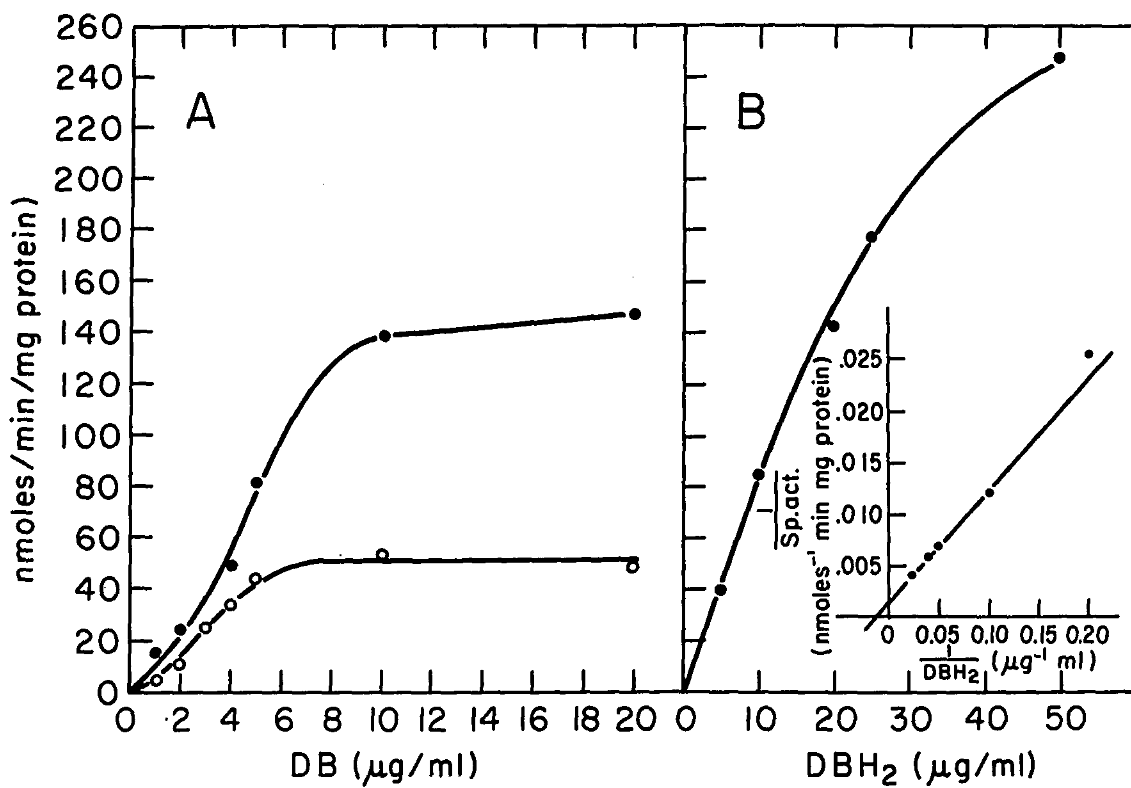


Figure 7

DISCUSSION

The activity of Complex III of the respiratory chain has proven difficult to assay properly because readily available substrates lack specificity. Most substrates reduce extra-mitochondrial cytochromes or reduce cytochrome c non-enzymatically. Use of DBH_2, the synthetic analog of coenzyme Q, as a substrate in the assay described in this paper circumvents both of these difficulties. The non-enzymatic rates of cytochrome c reduction are low and can be simply corrected. Furthermore, the reaction is highly specific for the mitochondrial b-c_1 complex as it is completely sensitive to antimycin A in unfractionated cell extracts and co-purifies with markers for the electron transport chain upon fractionation. The activity appears to require cytochrome b and c_1 in an approximately 2:1 ratio, as purification procedures which alter this ratio result in complete loss of activity.

One possible explanation for the different sensitivity of succinate and NADH cytochrome c reductases induction to chloramphenicol is that the two enzymes have different dependences on coenzyme Q which also increases during respiratory adaptation. Our experimental approach to this problem was to use the coenzyme Q analog, DB, in its oxidized form to reconstitute these activities in mitochondria obtained from a coenzyme Q deficient strain of yeast. Hence, the dependence of these two enzymes on DB could be measured directly. Furthermore, the specific activities obtained could be compared to

that of DBH_2: cytochrome c reductase. The results obtained in the reconstitution experiments have indicated that both succinate and NADH oxidation are completely dependent on added quinone and that Complex III is normally not rate limiting for either NADH or succinate: cytochrome c reductase activity. At low concentrations of quinone it is not possible to discern from these experiments whether the reduction or oxidation of DB is rate limiting. A convenient explanation for the sigmoid curves obtained in Fig. 3A, however, is to postulate that at low concentrations the oxidation of the reduced form is rate limiting by virtue of the lower affinity of the oxidizing enzyme for substrate. At higher concentrations, the primary dehydrogenases with their lower V_{max} would become rate limiting.

The results obtained in the reconstitution experiments using coenzyme Q-deficient mutants of yeast has allowed us to make several interesting observations pertaining to the role of coenzyme Q in mitochondrial electron transport. Slater's group (42) have postulated the existence of a compound other than coenzyme Q which is capable of restoring respiration to CoQ depleted particles with succinate but not with NADH as substrate. This result was interpreted to mean that coenzyme Q was not essential for succinate oxidase activity. By contrast, the present study indicates that coenzyme Q is absolutely essential for the oxidation of succinate as succinate: cytochrome c reductase activity is absent in all strains free of revertants. Furthermore, since the coenzyme

Q deficient strains fell into five of the six known different genetic complementation groups, it is unlikely that an intermediate in coenzyme Q biosynthesis or a compound synthesized from an intermediate, can replace the quinone in this function. These experiments do not, however, rule out the possibility that the compound described by Albracht et al. (42) has coenzyme Q as a biosynthetic precursor.

Previous reconstitution studies have involved the use of lypholization and pentane extraction to remove coenzyme Q from mitochondria or submitochondrial particles (41, 156). This procedure has the disadvantage that lypholization causes inactivation of NADH oxidase activity (44). Studies with particles obtained after lyophilization and extraction have suggested that restoration of succinate oxidation required lower concentrations of coenzyme Q than does NADH oxidation (44) and that these differences are exaggerated when low molecular weight homologs of coenzyme Q are used (43). These results were interpreted as evidence for the "functional heterogenity" of the coenzyme Q pool (43, 44). By contrast we were unable to find a marked difference in the concentration of DB required to reconstitute NADH and succinate: cytochrome c reductase activities suggesting a common pool of coenzyme Q.

NADH dehydrogenase, or Complex I, of S. cerevisiae differs from that of most other organisms as it lacks the non-heme iron center believed to be involved in site I phosphorylation and is insensitive to rotenone and piericidin A (26). Hence, the site of interaction with coenzyme Q may differ in Complex I of these organisms as compared to S.

cerevisiae resulting in an increase in their relative affinity for quinone. An alternate explanation may be that the lack of complete reactivation of NADH oxidase observed after lyophilization results in an increased dependence on coenzyme Q. For example, it is possible that inactivated particles compete with active particles for the added quinone, thereby giving rise to spuriously high estimates for the amount of CoQ necessary to reactivate NADH oxidase. Thus, the suggestion of a functional heterogenity or compartmentation of the coenzyme Q pool depends on the experimental system employed. It is impossible to decide whether the disparity of these results with those of others results from the use of yeast mitochondria or the use of the lypholization procedure. However, on the basis of kinetic experiments performed with intact particles, Kröger and Klingenberg (155) suggested that the active coenzyme Q fraction was not comparmentalized. These reconstitution experiments with intact mitochondria lead to the same conclusion.

CHAPTER V

ACCUMULATION AND UTILIZATION OF PRECURSORS OF RESPIRATORY CHAIN COMPLEXES DURING AEROBIC ADAPTATION

RESULTS

As was previously mentioned, a discrepancy appears to exist between yeast undergoing glucose derepression and yeast undergoing respiratory adaptation, with regard to the prevailing mode of genetic interregulation. The present experiments were designed to investigate the cooperation between the mitochondrial and cytoplasmic systems for protein synthesis in yeast undergoing respiratory adaptation and compare it to that observed in yeast undergoing glucose derepression. The possible accumulation of proteins synthesized by either system for protein synthesis and the effects of such an accumulation on membrane formation were tested by using the selective inhibitors of protein synthesis, chloramphenicol for the mitochondrial and cycloheximide for the cytoplasmic system. Furthermore, the inductions of several enzymes of the electron transport chain, succinate dehydrogenase, cytochrome oxidase, and coenzyme QH_2-cytochrome c reductase, were compared.

Kinetics of chloramphenicol and cycloheximide-resistant induction of respiratory enzymes.

Previous studies of yeast undergoing glucose derepression have suggested that proteins synthesized on both mitochondrial and cytoplasmic ribosomes may accumulate prior to the formation of active enzyme complexes (5). To investigate this phenomenon during respiratory adaptation, cycloheximide and chloramphenicol were added at various times after aeration had begun and the kinetics of enzyme induction studied. When chloramphenicol was

added after thirty minutes, the activities of both coenzyme QH_2-cytochrome *c* reductase and cytochrome oxidase increased slightly (Figure 8). When chloramphenicol was added after eighty minutes, a considerable and abrupt increase in both activities was observed. By contrast, addition of cycloheximide after thirty minutes of aeration caused an immediate and nearly complete inhibition of induction of both enzymes. When cycloheximide was added after eighty minutes, a gradual increase in activity of both cytochrome oxidase and coenzyme QH_2-cytochrome *c* reductase was observed (Figure 9A and B); however, no increase in succinic dehydrogenase activity occurred after addition of cycloheximide at this time (Fig. 9C), suggesting that cycloheximide must immediately block any further protein synthesis after its addition to the culture. Hence, the continued increases in activity of the other two enzyme complexes in the presence of cycloheximide is apparently related to the integration of proteins synthesized in both compartments into an active enzyme complex. Also, addition of either antibiotic immediately prior to aeration blocked enzyme induction to the same extent as when added thirty minutes prior to aeration. This result provides further evidence that the time lag between the addition of antibiotic and inhibition of protein synthesis is negligible.

The suggestion that protein components of the cytochrome *b*-c_1 complex and cytochrome oxidase may build up prior to the formation of enzymatic activity prompted us to investigate precisely when, during aeration, accumulation of proteins occurred. The experimental approach employed was that used

Fig. 8. Effect of chloramphenicol on (A) cytochrome oxidase, (B) coenzyme QH_2-cytochrome <u>c</u> reductase induction when chloramphenicol was added during the induction process. Conditions were as described in Figure 1. At thirty and eighty minutes after aeration began part of the culture was removed, antibiotic added, and aeration continued. (●——●) no additions, (▲----▲) chloramphenicol added (2 mg/ml). Arrows (↓) indicate the time of chloramphenicol addition.

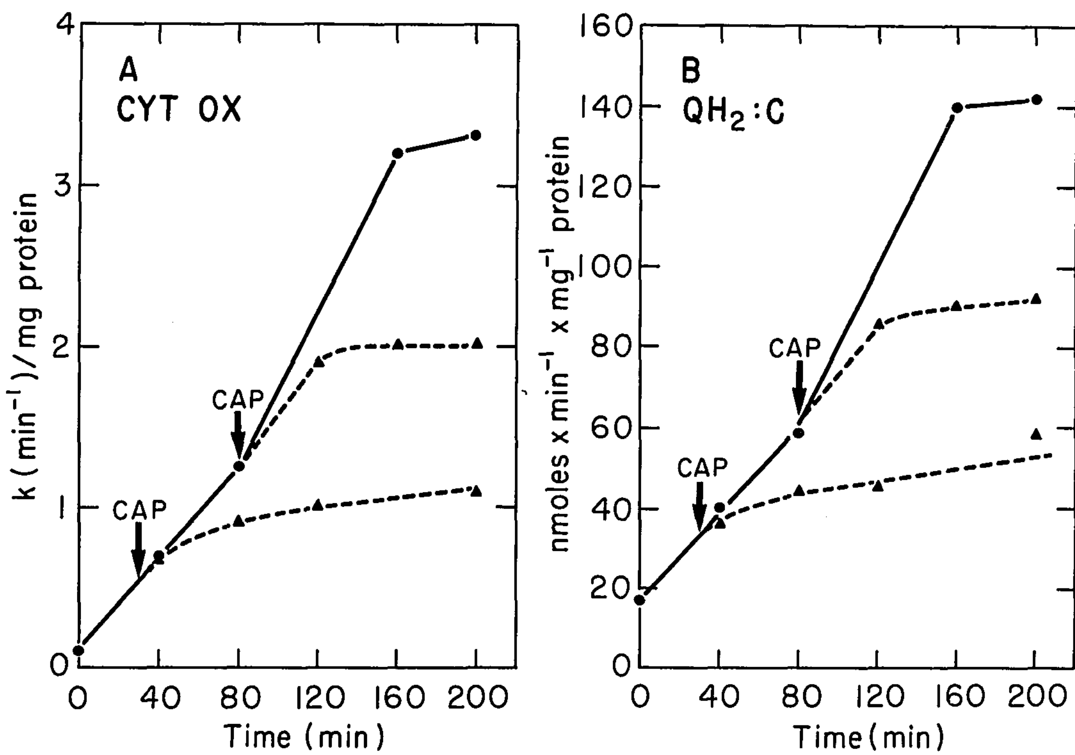


Figure 8

Fig. 9. Effect of cycloheximide on (A) cytochrome oxidase (B) coenzyme QH_2-cytochrome c reductase, (C) succinic dehyrogenase induction when added during the induction process. Conditions were as described in Figure 4, except that cycloheximide was added. (●——●) no additions, (■----■) cycloheximide (25 μg/ml) added. Arrows (↓) indicate the time of cycloheximide addition.

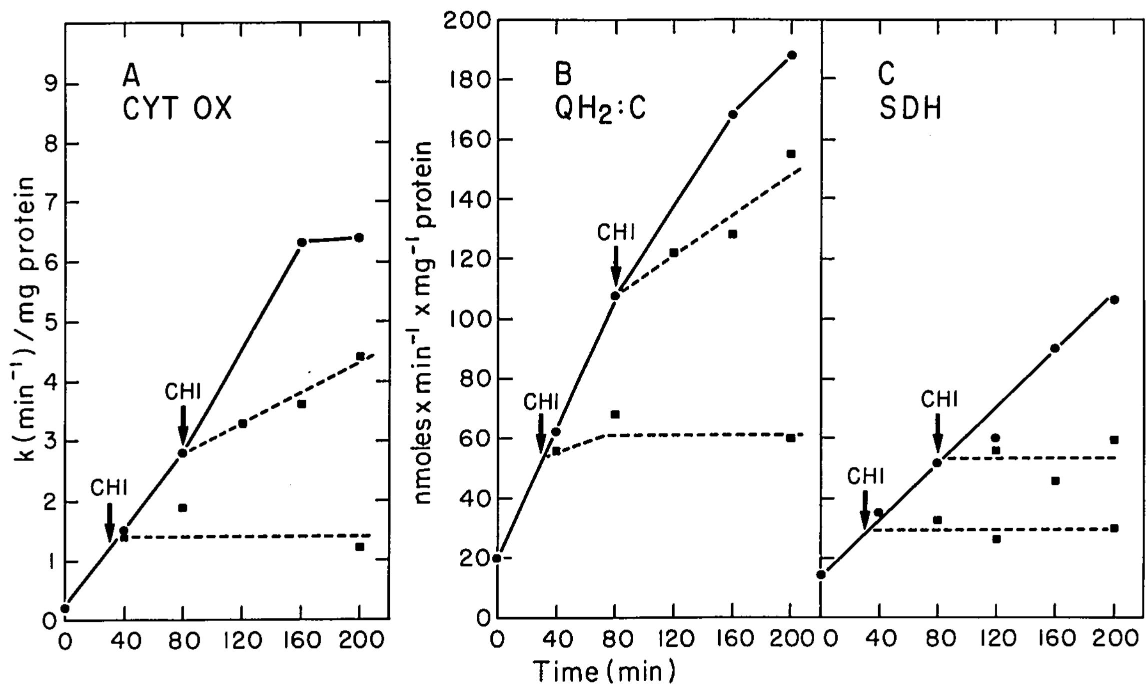


Figure 9

by Chen and Charalampous (122) in their studies of cytochrome oxidase induction. This approach is based on the assumption that the amount of precursor present at any time during the course of aerobic adaptation may be estimated by the total increase in specific activity eventually obtained after addition of antibiotic at that time. Specifically, samples are removed at various times during aeration of the culture and incubated for an additional four hours in air in the presence of either chloramphenicol or cycloheximide, after which enzymatic activities are determined. The amount of precursor proteins which have been previously synthesized on either mitochondrial or cytoplasmic ribosomes at any time during aeration may be estimated by the observed increases in specific activity in the presence of chloramphenicol and cycloheximide respectively, relative to the enzymatic activity present at the time of antibiotic addition. As seen in Figure 10, the accumulation of mitochondrially synthesized proteins for both complexes is observed immediately after aeration has begun. The increase in coenzyme QH_2-cytochrome c reductase activity observed when chloramphenicol was added immediately upon aeration may result from the presence of proteins synthesized in the mitochondria during anaerobic growth. The accumulated pool for coenzyme QH_2-cytochrome c reductase reaches a plateau after ninety minutes of aeration, whereas this pool for cytochrome oxidase is present throughout adaptation. The accumulation of proteins synthesized in the cytoplasm for both enzymes is not observed until the culture has been aerated for thirty minutes. After this period, the pool of

Fig. 10. Pattern of net cycloheximide and chloramphenicol resistant induction of (A) cytochrome oxidase and (B) coenzyme QH_2-cytochrome c reductase during aeration. Anaerobic cells were harvested and preincubated as described in Figure 1. At various times after beginning aeration (abscissa), cells were harvested and processed immediately (●——●) or aerated for an additional four hours in the presence of 2 mg/ml chloramphenicol (▲——▲) or 25 ug/ml cycloheximide (■——■) before processing.

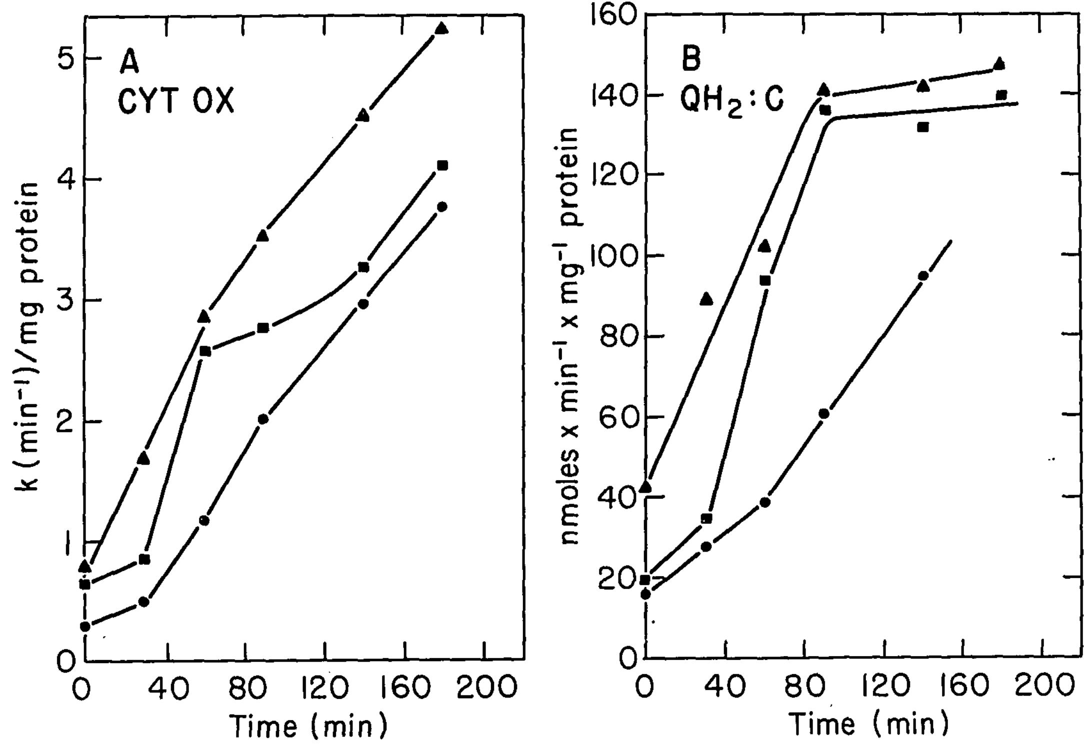


Figure 10

accumulated protein for coenzyme QH_2-cytochrome c reductase becomes progressively larger until ninety minutes of aeration, when it plateaus at the fully induced level. In addition, cycloheximide-resistant induction of cytochrome oxidase is observed only after thirty minutes of aeration; however, after sixty minutes the induction becomes progressively more cycloheximide-sensitive. This result suggests that proteins synthesized on cytoplasmic ribosomes destined to become part of cytochrome oxidase are produced in excess during the early stages of aeration, but at later times are produced at a constant rate, parallel to the rate of overall enzyme induction. Chen and Charalampous observed a similar pattern of cycloheximide-resistant cytochrome oxidase induction in *S. carlsbergensis* (122).

Induction of enzymes upon sequential exposure of cells to antibiotics.

The observation that proteins synthesized on both mitochondrial and cytoplasmic ribosomes may accumulate during adaptation prompted us to investigate whether proteins made by either system would accumulate when protein synthesis on the other system was blocked. Initially, the ability of cytoplasmic ribosomes to function in the absence of mitochondrial protein synthesis was investigated by aerating the cells in the presence of chloramphenicol for seventy minutes to permit the accumulation of cytoplasmically synthesized proteins, then washing out the antibiotic and continuing the aeration in the presence of cycloheximide (CAP→CHI). Controls consisted of cells aerated in the absence of antibiotic during

both incubations (CON→CON) or aerated in the presence of antibiotic only during the first incubation (CAP→CON). The accumulation of cytoplasmically-synthesized proteins during the incubation in chloramphenicol would thus appear as cycloheximide-resistant induction of enzyme during the second incubation. As seen in Figures 11A and B, induction of cytochrome oxidase but not coenzyme QH_2-cytochrome c reductase is observed in the presence of cycloheximide after the first incubation in chloramphenicol. It should be noted that the effects of chloramphenicol are completely reversible, as both enzymes are induced after the removal of antibiotic. To ascertain the capacity of mitochondrial protein synthesis to function in the absence of cytoplasmic protein synthesis, the sequence of antibiotic additions was reversed. When cells aerated for seventy minutes in the presence of cycloheximide were washed free of the antibiotic and further aerated in the presence of chloramphenicol (CHI→CAP) both coenzyme QH_2-cytochrome c reductase and cytochrome oxidase were induced (Figure 12) (157).

The results of these double inhibitor experiments suggest that cytoplasmically made proteins for cytochrome oxidase, but not for cytochrome b - c_1 complex, can be accumulated in a usable form in the absence of mitochondrial protein synthesis. By contrast, proteins synthesized in the mitochondria for both enzyme complexes can accumulate in the absence of cytoplasmic protein synthesis and can subsequently be integrated into active enzymes.

Fig. 11. Induction of (A) cytochrome oxidase and (B) coenzyme QH_2-cytochrome c reductase upon sequential exposure of cells to chloramphenicol and cyloheximide. Cells were harvested and preincubated as described in Figure 1. Aeration was begun in the presence (▲——▲) or absence (●——●) of chloramphenicol (2mg/ml). After seventy minutes of aeration cells were washed four times then incubated in the presence or absence of cycloheximide (25 ug/ml). Cells were washed with ice cold media identical to that used in the second aeration period and aliquots were withdrawn and processed during both aeration periods. (●——●) no additions during either period; (△——△) chloramphenicol present during first period, no addition during second period; (▲——▲) chloramphenicol present during first period, cycloheximide present during second period, ¦ ¦ indicates wash period.

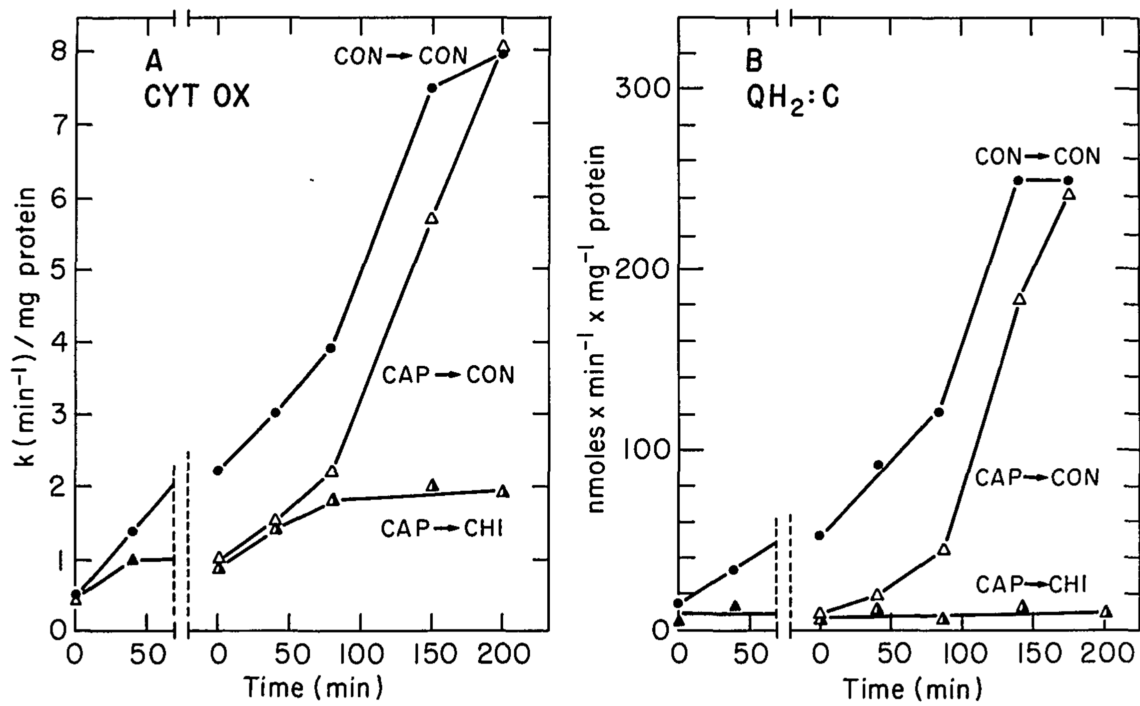


Figure 11

Fig. 12. Induction of (A) cytochrome oxidase and (B) coenzyme QH_2-cytochrome c reductase upon sequential exposure of cells to cycloheximide and chloramphenicol. Cells were harvested and preincubated as described in Figure 1. Aeration was begun in the presence (▲——▲) or absence (●——●) of cycloheximide (10 μg/ml). After seventy minutes, cells were washed four times and then incubated in the presence or absence of chloramphenicol (2 mg/ml). Cells were washed with ice cold media identical to that used in the second aeration period, and aliquots were withdrawn and processed during both periods, (●——●) no additions present during either period; (△——△) cycloheximide present during first period, no addition during second period; (▲——▲) cycloheximide present during first period, chloramphenicol present during second period, ¦ ¦ indicates wash period.

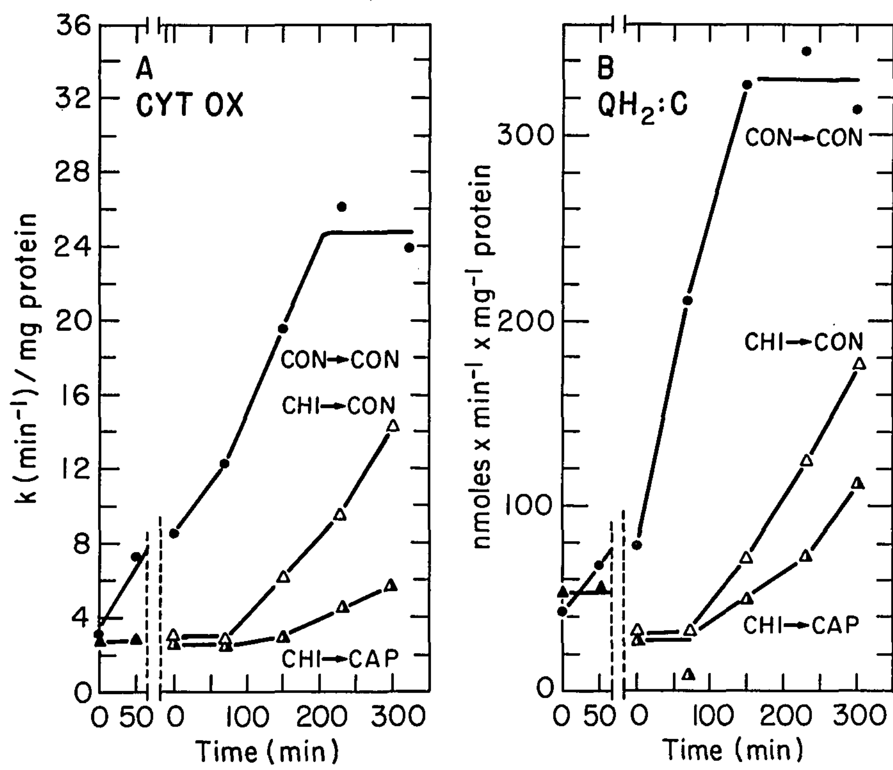


Figure 12

DISCUSSION

The initial approach to understanding the possible interactions between the mitochondrial and cytoplasmic systems for protein synthesis during formation of the yeast mitochondrial membrane involved the analysis of enzyme induction during respiratory adaptation in the presence of inhibitors of protein synthesis. The basic assumption underlying these experiments is that the activity of a mitochondrial enzyme complex containing proteins synthesized both in the mitochondria and the cytoplasm can increase when only one system for protein synthesis is active only by utilization of a pool of proteins previously synthesized by the other inhibited system. The data obtained suggest that a considerable accumulation of proteins synthesized in the mitochondria for both cytochrome oxidase and the $\underline{b}$-$\underline{c}_1$ complex occurs throughout aeration. Greater increases in activity of both enzyme complexes were observed when chloramphenicol was added after eighty rather than thirty minutes of aeration suggesting that the pool of proteins made in the mitochondria increases throughout respiratory adaptation. In addition, protein(s) which are synthesized in the mitochondria and destined to become part of an active cytochrome $\underline{b}$-$\underline{c}_1$ complex appear to accumulate to a slight extent during anaerobic growth as indicated by the chloramphenicol-resistant formation of coenzyme QH_2-cytochrome $\underline{c}$ reductase.

By contrast, almost no accumulation of proteins synthesized in the cytoplasm for either cytochrome oxidase or reduced

coenzyme Q-cytochrome $\underline{c}$ reductase occurs during the first thirty minutes of aeration. Addition of cycloheximide to anaerobic cultures or at any time up to thirty minutes after aeration results in an immediate block in induction of either enzyme complex. After this point during respiratory adaptation, the pattern of cytoplasmic protein synthesis differs for the two enzyme complexes. After ninety minutes of aeration, the pool of accumulated proteins for the $\underline{b}$-$\underline{c}_1$ complex reaches a maximum at the fully induced level suggesting that the observed increases in QH_2-cytochrome $\underline{c}$ reductase activity over the two hundred forty minute aeration period results from the assembly of previously synthesized proteins into an active enzyme complex rather than from the continued synthesis of proteins for the complex in either the cytoplasm or mitochondria. The assembly process for formation of an active cytochrome oxidase complex does not appear to require as long a time since the pool of cycloheximide-resistant proteins for this enzyme complex are apparently produced at the same rate as the increase in enzymatic activity despite a larger accumulation of cytoplasmically synthesized proteins observed after sixty minutes of aeration. Furthermore, a greater time lag is observed between the appearance of cytochrome $\underline{b}$ and $\underline{c}_1$ in the membrane and the development of coenzyme QH_2-cytochrome $\underline{c}$ reductase activity than between the appearance of cytochromes $\underline{a}$ + $\underline{a}_3$ and the development of cytochrome oxidase activity.

The accumulation of precursor proteins synthesized by either cytoplasmic or mitochondrial ribosomes was also

investigated by the sequential addition of cycloheximide and chloramphenicol (or the reverse order) to cultures undergoing respiratory adaption. Increases in both cytochrome oxidase and coenzyme QH_2-cytochrome c reductase activities were observed when yeast cells were aerated in cycloheximide for seventy minutes and then transferred to fresh medium containing chloramphenicol indicating the presence of a pool of mitochondrially made proteins for both enzymes. These results further suggest that the synthesis of proteins in the mitochondria for both enzyme complexes does not require the simultaneous synthesis of proteins in the cytoplasm for these complexes. In addition, after the cycloheximide was removed there was a lag of one hour before enzymatic activity began to increase. This lag may be a reflection of the time necessary for proteins made in the cytoplasm to be transferred into the mitochondria and assembled into an active enzyme complex, as cytoplasmic protein synthesis begins immediately after removal of the antibiotic.

The possible accumulation of proteins in the cytoplasm in the absence of mitochondrial protein synthesis was tested by aerating cells sequentially in chloramphenicol and cycloheximide. The results obtained indicated that a pool of cytoplasmically made proteins for cytochrome oxidase accumulates when mitochondrial protein synthesis is blocked by chloramphenicol. Almost a doubling of enzymatic activity is observed in the CAP→CHI sequence of inhibitor addition. Furthermore, no lag is observed before active cytochrome oxidase begins concomitantly with mitochondrial protein

synthesis. In this context, Poyton and Groot (158) have recently demonstrated that cytoplasmically made subunits of cytochrome oxidase have accumulated in the inner mitochondrial membrane and can be subsequently assembled into the holoenzyme.

The induction of an active $\underline{b}$-$\underline{c}_1$ complex differs significantly from that of cytochrome oxidase, in that no accumulation of proteins made in the cytoplasm is observed when mitochondrial protein synthesis is blocked by chloramphenicol. When yeast cells were incubated in the sequence CAP→CHI, the activity of coenzyme QH_2-cytochrome $\underline{c}$ reductase did not increase above that observed at the end of the first incubation. Furthermore, this result suggests that concomitant mitochondrial protein synthesis is necessary for the cytoplasmic synthesis of proteins destined to become part of this enzyme complex. Perhaps, a protein made in the mitochondria acts as a regulator of cytoplasmic protein synthesis as suggested previously by Küntzel (140). Alternately, this apparent lack of cytoplasmic protein synthesis in the presence of chloramphenicol may simply reflect the assembly process which, as discussed earlier, may be the rate-limiting factor during formation of an active cytochrome $\underline{b}$-$\underline{c}_1$ complex. It may well be that proteins for this complex are indeed synthesized on cytoribosomes in the absence of mitochondrial protein synthesis but are not assembled into the membrane under these conditions and hence are subject to proteolytic digestion. Another possibility is that concomitant mitochondrial protein synthesis is necessary for the proper integration of proteins synthesized in the cytoplasm. In this context, Katan and

Groot (90) have recently reported that two cytoplasmically synthesized polypeptides of a purified cytochrome _b_-$\underline{c}_1$ complex are not tightly integrated into the complex when yeast cells are incubated in the presence of chloramphenicol. Whatever the explanation for the lack of coenzyme Q-cytochrome _c_ reductase induction in the CAP→CHI sequence of aeration, there is no question that biogenesis of this complex differs significantly from that of cytochrome oxidase. A different mechanism must exist for the synthesis and possibly the integration of cytoplasmically-synthesized proteins into an active enzyme complex. The recently reported purifications of both a cytochrome _b_-$\underline{c}_1$ complex (34) and cytochrome _b_ (33) from yeast mitochondria will permit further studies of this phenomenon. It is also of interest that our results on the formation of the cytochrome _b_-$\underline{c}_1$ complex may explain the absence of induction of whole cell oxygen consumption when cells are aerated in the CAP→CHI sequence (139), as cytochrome oxidase is induced under these conditions.

The results of this paper also suggest that the formation of an active cytochrome _b_-$\underline{c}_1$ complex in yeast undergoing glucose depression is apparently subject to a different mode of regulation. When growing cells are allowed to derepress in the CAP→CHI sequence, a large increase in coenzyme QH_2-cytochrome _c_ reductase activity was observed (137). During the first hour in cycloheximide, the activity of this enzyme complex as well as oligomycin-sensitive ATPase nearly doubled when compared to cells grown in chloramphenicol and

then in fresh medium without cycloheximide suggesting that the intramitochondrial synthesis of proteins for these two complexes may be under control of proteins synthesized in the cytoplasm. Subsequent studies had indicated that the rate of mitochondrial protein synthesis both *in vitro* and *in vivo* (138) as well as the labeling of nascent chains on polysomes was stimulated when proteins previously synthesized in the cytoplasm had accumulated (66). The reason for the considerable difference in formation of the cytochrome b-c_1 complex during respiratory adaptation is not immediately apparent. One explanation may be that the oligomycin-sensitive ATPase complex increases in the CAP→CHI sequence during glucose derepression but is not appreciably induced during respiratory adaptation. Formation of the respiratory chain, particularly of the cytochrome b-c_1 segment, may differ when an active ATPase complex is already present.

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