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PURIFICATION AND CHARACTERIZATION OF CYTOCHROME B AND ITS ASSOCIATED PROTEINS FROM YEAST MITOCHONDRIA

by

Thomas J. Domenico

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The Graduate Center of City University, Mt. Sinai Medical Center, N.Y.C.

1983

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

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Chairman of Examining Committee

4-29-83
Date

Executive Officer

Supervisory Committee

ABSTRACT

PURIFICATION AND CHARACTERIZATION OF CYTOCHROME B AND ITS ASSOCIATED PROTEINS FROM YEAST MITOCHONDRIA

by

Thomas J. Domenico

Advisor: Professor Diana S. Beattie

Historically, two forms of cytochrome b with molecular weights of 31,000 and 29,000 have been purified from yeast mitochondria in our laboratory. Both purified proteins cross-reacted with antiserum raised against a major cleavage peptide of cytochrome b purified earlier in our laboratory. The antisera raised against these two forms of cytochrome b were shown to cross-react with each other. Fingerprinting patterns using Staphylococcus aureus V8 protease suggested that these two forms of cytochrome b share a common amino acid sequence with the exception of a 2,000 dalton residue. The intention of this study was to identify the difference between these two forms of cytochrome b and attempt to relate this difference to the two functionally different forms of cytochrome b.

An investigation on the relative concentrations of these two forms in the purification was part of the initial studies. The effect of proteolytic digestion during purification was compared by varying the steps and conditions in the purification and by testing known proteolytic inhibitors (PMSF, TPCK, TLCK, NEM, EDTA). No discernible difference in the relative concentration was recognized, however new modifications were introduced into the purification scheme to optimize the amount of purified products collected while minimizing the conditions where proteolytic digestion can occur. Studies on growth conditions revealed that the specific activity of cytochrome b increased with the age of the growth culture and when mitochondrial utilization was necessary for the growth of the cells (e.g. lactate). In addition, there was only a preferential increase in the 31,000 apoprotein when these cells were grown as obligate aerobes (e.g. lactate). Next, these two purified forms were subjected to characterization studies. They were compared by amino acid analysis, N-terminal analysis, isoelectric focusing and by cyanogen bromide cleavage. The results of these characterizations on the primary structure of these proteins revealed that the 29,000 protein band consisted

of more than one protein and that the majority of this protein isolated does not have sequence homology with the 32,000 protein. This characterization reveals that the 32,000 protein band was a single polypeptide; it has a blocked N-terminal consistent with what has been found for the N. crassa cytochrome b; and it has an isoelectric point consistent with other reported values for yeast cytochrome b.

The 32,000 protein was the only purified form of cytochrome b isolated therefore the immunogenic studies were done on a fresh antibody raised against only the 32,000 polypeptide. Immunotransfer studies and immunoprecipitation studies revealed that this antibody was specific only for the cytochrome b polypeptide and reacts with a variety of mitochondrial preparations and isolations that were previously treated with SDS. Immunoinhibition studies were done on mitoplasts, SMP and cholate solubilized mitochondria. The antibody was found to inhibit only the mitoplast preparations and that only a maximum inhibition of 20% could be obtained. From these immunoinhibition studies, the site of antigenicity of cytochrome b was localized to the outer surface of the inner membrane.

In the literature, much has been written about the

close associations between the membrane proteins and the difficulty of their separations. A closely associated protein of cytochrome b which copurifies with it has a molecular weight of 53,000. Preliminary studies were done on this 53,000 protein to define its function and location in the mitochondrial membrane. This protein requires additional steps in the purification to obtain molecular weight purity due to its tight associations, even in the presence of SDS. An antibody was raised against the 53,000 molecular weight pure protein and immunogenic studies were performed. The immunotransfer studies revealed a specificity for only the 53,000 protein while the immunoprecipitation studies showed that this antibody coprecipitates a 32,000 protein as well. The purified cytochrome apoprotein failed to react with this antiserum indicating that the antigenicity lies only on the 53,000 protein and its close association probably caused the 32,000 protein to precipitate. The immunoinhibition studies revealed that the 53,000 protein is an SDH subunit in yeast and its antigenic site lies on the inner surface of the inner membrane.

DEDICATION

In the memory of Dr. J. Scherer, a man of vision and insight.

To my parents, Sam and Elizabeth, who have consistently helped me to achieve those things which I felt were important in my life.

To my brother, Sam, who has inspired me through my hardest transitions.

To my wife, Lillian, for her patient support and encouragement.

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To all other members of the scientific community that have aided in my development.

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F. Objectives of the Study

A. THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

The years between 1910 and 1950 yielded tremendous advances in the understanding of cellular energy production and its relation to respiration. Wieland(1) investigated the role of dehydrogenase in cellular metabolism. Warburg(2) tackled the process of oxygen "activation" by respiratory enzymes, showing that respiration was inhibited by cyanide and that heme is present in the respiratory enzymes. Engelhardt(3) demonstrated that phosphorylation of ADP and respiration were a coupled process. Warburg(4) examined the role of flavins and pyridine nucleotides in respiration and demonstrated that ATP formation and dehydrogenation are coupled. Krebs et al(5,6) elucidated the urea cycle and the tricarboxylic acid cycle. The use of cellular fractionation led Kennedy and Lehininger(7) to demonstrate that the mitochondrion was the intracellular locus for the Krebs cycle, the β - oxidation of fatty acids and oxidative phosphorylation.

The mitochondrion consists of an outer membrane, an intermembrane space, an inner membrane and a central matrix. Each of these components have distinct structural and metabolic features. Over 100 enzymes have been localized in

mitochondria(8). The mitochondrial matrix is the site of dehydrogenation for pyruvate, fatty acids, ketone bodies, most Kreb cycle intermediates and amino acids, yielding NADH(8). In addition mitochondria participate in metabolic pathways that partially occur in the cytoplasm; these include: fatty acid biosynthesis, ketogenesis, heme biosynthesis and steroidogenesis(9).

The NADH, produced from the dehydrogenation reactions mentioned above, feeds electrons into the respiratory chain which is located in the inner membrane. This process of electron transport leads to the formation of an electrochemical proton gradient across the inner membrane which can be used to drive the phosphorylation of ADP. This electrochemical gradient is also necessary for the transport of: calcium, phosphorous, magnesium, adenine nucleotides, dicarboxylic and tricarboxylic acids and most of the nuclear coded mitochondrial proteins. As early as 1960(10), this inner membrane had been dissected into four distinct lipid protein complexes, each possessing a catalytic role in the oxidation reduction reactions of the electron transfer process. The sequence of electron transfer was arrived at through studies using various substrates, studies using inhibitors to different parts of the electron transfer chain

and studies on midpoint potentials(11). NADH and NADH producing substrates transfer reductants through complex I, whose constituents contain the lowest redox potentials in the respiratory chain. Succinate is oxidized by complex II, transferring electrons to ubiquinone. Complex III is the intermediary complex of the electron transport chain, accepting the reductants from complexes I and II, transferring electrons to complex IV. Complex IV is the terminal complex, transferring its reducing equivalents to molecular oxygen, concomitantly its constituents have the highest redox potentials. Complex V was later isolated(12) from this inner membrane and is responsible for conserving the energy derived from electron transfer in the form of the high energy chemical bond, ATP. Although this complex is not involved in the direct transfer of electrons, its auxiliary function makes it a part of the respiratory chain. A simplified diagram of the respiratory chain is presented in illustration 1.

Complexes I and II are responsible for delivering electrons from a variety of substrates to the ubiquinone of complex III. Complex I, NADH-coenzyme Q reductase, catalyzes the reduction of ubiquinone and ferricyanide by NADH and NAD(P)H producing substrates. Complex I is the first site of oxidative phosphorylation in the respiratory chain.

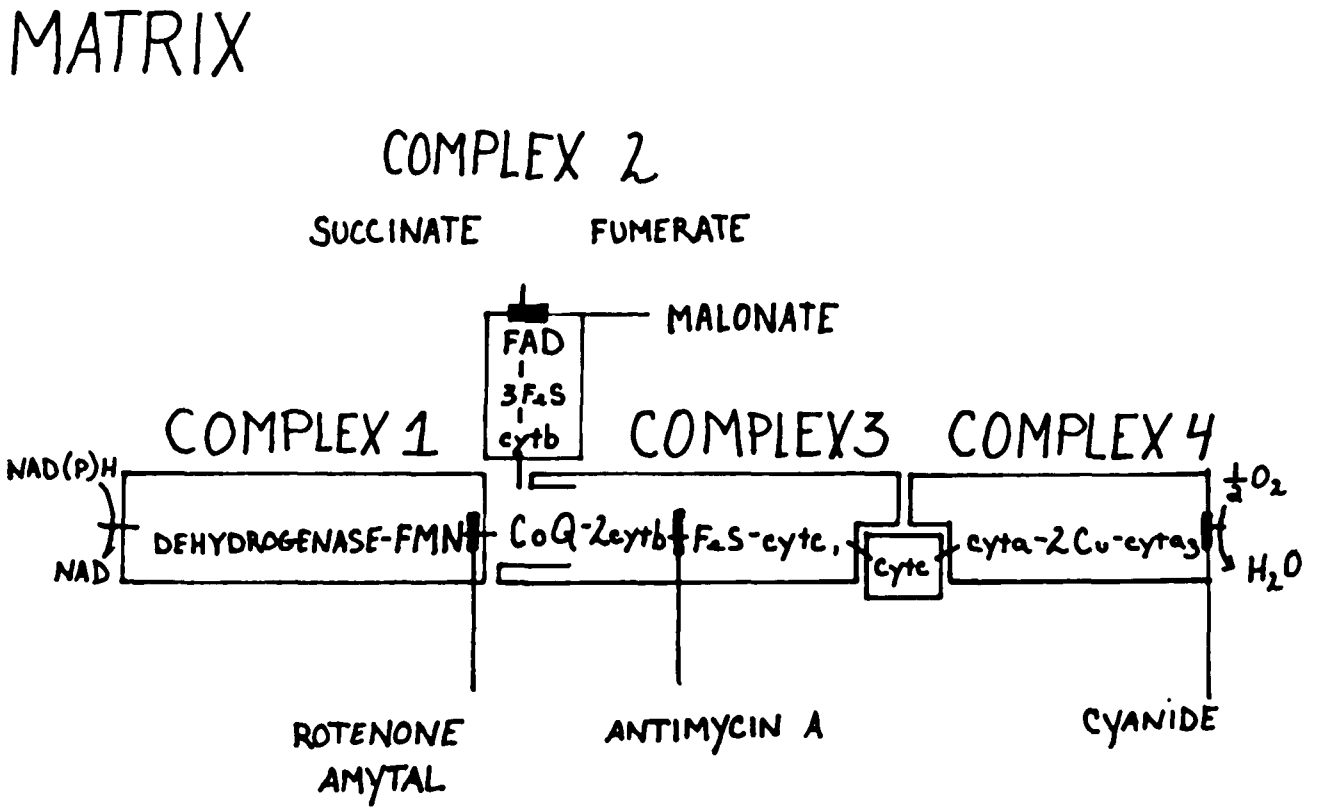

Illustration 1. A representation of the inner membrane respiratory chain. Not included on this diagram is complex 5 which faces the matrix side of the membrane and has sites associated with complex 1, complex 3 and complex 4. Complexes 1, 3 and 4 span the inner membrane whereas complex 2 is projected on the matrix side of the membrane. Included are the salient electron transport features of each of the complexes with specific locations where certain inhibitors have been found to have an effect. This diagram represents the yeast electron transport chain because no FeS clusters are in complex 1, in all other respects it is representative of the mitochondrial inner membrane respiratory chain components found in other organisms.

The first enzyme carrier of the complex is an NADH dehydrogenase, which contains noncovalently bound FMN and catalyzes the transfer of electrons from NADH to the next member of the electron transport chain. This flavoprotein with NAD(P)H dehydrogenase activity also possesses a NAD(P)H-NAD transhydrogenase activity. Another electron carrier of this complex is an iron sulfur protein with EPR signals characteristic of the iron sulfur center 2 of complex II(13).

This EPR signal has been observed in all of the organisms studied with the exception of yeast(14,15). The complex I isolated from beef heart mitochondria was found to contain fourteen subunits(16).

Complex II, succinate coenzyme Q reductase, catalyzes the substrate reduction of ubiquinone by succinate. Localized in complex II is succinate dehydrogenase, which generally has a far greater activity than the other enzymes of the tricarboxylic acid cycle. In beef heart, succinate dehydrogenase consists of two subunits with molecular weights of 70,000 and 27,000. The larger subunit is a flavoprotein with a covalently bound FAD. The nonheme iron and labile sulfide are also contained in the succinate dehydrogenase on the smaller subunit. From nonheme iron-labile sulfide contents and EPR studies, three iron sulfur centers were identified, two being Fe_2S_2 clusters and one as a Fe_4S_4 cluster(17). A low potential cytochrome $\underline{b}$ (b_{557}) contains two polypeptides of molecular weights 15,500 and 13,500 in equimolar amounts(19). This preparation of cytochrome $\underline{b}$ (b_{557}) can be reconstituted with succinate dehydrogenase to reform the active complex.

Complex III, coenzyme QH_2 cytochrome $\underline{c}$ reductase, catalyzes the reduction of cytochrome $\underline{c}$ by reduced ubiquinone. This complex contains quinones with an isoprenoid side chain,

that in nature has anywhere from 5-10 repeating units depending on the organism studied(20). To reconstitute the specific activities of complex III, investigators will often use as substrate, the more water soluble analogs which have a n-pentyl or n-decyl side chain in place of the repeating isoprenoid chain. Cytochromes b and c_1 are electron transferring proteins of this complex with the prosthetic group, protoporphyrin IX, bound non-covalently and covalently respectively, These cytochromes are distinguished by their respective redox potentials and differential absorption spectra. Another electron transferring polypeptide in complex III is the iron sulfur protein, which contains nonheme iron and labile sulfide in a single Fe_2S_2 cluster(21). The core proteins are the two largest subunits in complex III and are believed to have a structural role, rather than being enzymatically functional(22). This complex has at least seven polypeptides with molecular weights ranging from 15,000 to 50,000. Active complex III has been isolated from beef heart (23) and yeast(24) mitochondria by extraction with cholate and/or deoxycholate. These procedures require ammonium sulfate fractionation to remove the contaminating succinate dehydrogenase from complex III. Alternate methods using a milder detergent, TritonX-100 and column chromatography

were also utilized and will be discussed in greater detail in the next section. Active complex III has been isolated as dimers with molecular weights ranging from 440,000 to 480,000. Cytochrome b is the only subunit in complex III which is coded on the mitochondrial genome. This complex contains the second site of oxidative phosphorylation in the respiratory chain.

Complex IV, cytochrome c oxidase, is the terminal complex in the electron transfer chain and it catalyzes the oxidation of reduced cytochrome c by molecular oxygen. This complex contains the third site of oxidative phosphorylation. Complex IV contains two copper atoms and a mole each of heme a and heme a_3. Heme a_3 is the terminal cytochrome and reacts with molecular oxygen or carbon monoxide. A remarkable consistency of subunit structure has been maintained for this complex in beef heart(26), locust(27), N. crassa(28) and S. cerevisiae(29). The cytochrome oxidase complex isolated from each of the above sources, was found to contain seven subunits and these subunits have nearly the same molecular weight. The three largest subunits (40,000, 33,000, 22,000) are mitochondrial translation products while the remaining four smaller subunits (14,500, 13,700, 12,500, 4,600) are coded on nuclear genes. Three of the four smaller

subunits are synthesized as precursors(30). The pattern of processing nuclear coded membrane proteins upon insertion into the membrane, has been described for a number of inner membrane proteins and will be detailed in section E. The mitochondrially made subunit II is also synthesized as a precursor form and it is processed post-translationally(31).

Complex V catalyzes the synthesis of ATP in a process which is inhibited by oligomycin. The hydrolysis of ATP also occurs on this complex. The purified complex from yeast contains ten polypeptides(32). Five of the six cytoplasmically made polypeptides are assembled in F_1 which faces into the matrix and catalyzes ATP hydrolysis and synthesis. The sixth subunit forms the stalk that is responsible for the binding of F_1 to the membrane. The membrane factor (F_0) is composed of at least three hydrophobic subunits and functions as a proton conduction channel, containing the site for oligomycin sensitivity. Three of the five cytoplasmically made subunits of F_1, are imported into the mitochondria as precursors(33,34).

B. THE PROPERTIES AND FUNCTION OF COMPLEX III

Complex III is the only complex of the respiratory chain that responds to the redox states of the other complexes rather than to the availability of substrate. Whether it regulates the electron flow from the other complexes or is modulated by them has yet to be determined. Complex III was first isolated from beef heart mitochondria by Hatefi et al(35) in 1962, using combinations of cholate/deoxycholate and ammonium sulfate for its solubilization and fractionation. Other purifications reported for beef heart(36,37,38) follow this basic design, with modifications,have helped in elucidating the properties of the complex. A deviation from the above methods was reported(39), where antimycin had been added to stabilize the complex. Antimycin is known to prevent the dissociation of heme from cytochrome b apoprotein during chromatography in high ionic strength buffers. A good heme content value was obtained for cytochrome b but the complex was recognized to be inactive and devoid of the nonheme iron protein. Affinity chromatography was the method of choice for the isolation of complex III from N. crassa(40). The binding and then release of complex III from the column is based on the conversion of ferricytochrome c to ferrocytochrome c.

The conditions used to isolate complex III from beef heart and N. crassa were unsuccessful when applied to yeast mitochondria. It was difficult to obtain a complete separation of complex III from complex II. In the first successful separation of complex III in yeast(41) antimycin was used, rendering this complex inactive. In addition, the iron sulfur protein was found to be absent from this complex. An active complex III in yeast has since been isolated and its minimal subunit structure appears to be seven polypeptides. These subunits consist of two core proteins at 49,000 and 40,000; cytochrome b at 31,000; cytochrome c_1 at 29,000; an iron sulfur protein at 24,500 and two lower molecular weight polypeptides of unknown function(42,43). Complex III has a stoichometry of : two of the smaller core protein and one of the larger; two forms of cytochrome b; a cytochrome c_1; an iron sulfur protein and one of each of the two lower molecular weight subunits. The stoichometry of the core proteins were quantitated in beef heart complex III and in mitochondria using radioinimunoassay(22). Antibodies to these core proteins do not inhibit electron transport and they have no oligomycin or uncoupler effects on oxidizing NADH. Crosslinking and surface labelling studies in beef heart gave additional information on the orientation of

the core proteins(44). The smaller core protein spans the membrane while the larger core protein is only exposed to the outer surface of this inner membrane. DABS labelling of the two core proteins in yeast(45) suggest that both proteins span the bilayer of the inner mitochondrial membrane. The functional role of the two lower molecular weight proteins, 17,000 and 15,000 in complex III is unknown. Either or both of these subunits have been implicated in functioning as an antimycin binding protein(46) or as a ubiquinone binding protein(47) or as being associated with cytochrome $\underline{c}_1$ and cytochrome $\underline{b}$(48).

Cytochrome $\underline{b}$, cytochrome $\underline{c}_1$ and iron sulfur protein are the components of complex III, that are directly involved in the transfer of electrons through the complex. Cytochrome $\underline{c}_1$ is the terminal electron transferring protein in this complex and functions to transfer electrons to cytochrome $\underline{c}$. It contains a covalently bound protoporphyrin IX as its prosthetic group and is located on the outer surface of the inner membrane. Cytochrome $\underline{c}_1$ has a characteristic reduced absorbtion spectra with an α-peak at 553nm and a β-peak at 523nm. Cytochrome $\underline{c}_1$ displays a distinct EPR signal in its oxidized state at g=3.49. The iron sulfur cluster of complex III has a redox potential nearing that of cytochrome $\underline{c}_1$, 30mV more positive and both proteins are reduced

by ascorbate. Rieske (49) first identified this iron sulfur center in beef heart complex III. In yeast, the EPR spectra exhibits three resonances with nominal g values of 2.036, 1.89 and 1.79, similar to what had been observed by Rieske (43). This Rieske iron sulfur protein is of the Fe_2S_2 type. EPR studies have been done on mutants of cytochrome b, mutants of subunit III in cytochrome oxidase and mitochondrial DNA depleted petite mutants (rho°). In the absence of a functional respiratory chain the iron sulfur centers of complex II were depressed an average of 75% in all the mutants tested. In the case of the Rieske cluster of complex III, no signal was observed in the mutants of cytochrome b and rho° while they were present in the mutants of cytochrome oxidase subunit III. Therefore, the proper assembly of this iron sulfur center appears closely linked to the structural integrity of cytochrome b in the complex(15). A concomitant observation is that cytochrome c_1 is unaffected under these same conditions.

C. THE PROPERTIES OF PURIFIED CYTOCHROME b

Cytochrome b is an electron transferring dimeric protein, containing non-covalently bound protoporphyrin IX as its prosthetic group. It has a monomeric molecular weight between 30,000 and 35,000 under SDS solubilization conditions. Heme content studies reveal a stoichiometry of the cytochrome b to cytochrome c_1 in complex III of 2:1. In mitochondria and in submitochondrial particles (SMP), cytochrome b has been identified spectrally and potentiometrically to exist in two forms. In preparations of complex III, these two forms are retained and have been distinguished by their different contributions to the absorption spectra, by their different midpoint potentials and by their characteristic EPR signals. EPR studies on complex III from yeast have revealed the presence of two low spin hemoproteins which are distinct from cytochrome c_1 (43). The g values observed for these two contributions from cytochrome b are g=3.60 and g=3.78 which are similar to what has been found in beef heart mitochondria(50). An associated signal at g=2.95 is sometimes observed and is related to non-physiological modifications of cytochrome b.

The existence of two species of cytochrome b is classically supported by observations such as the biphasic response of

the kinetics of cytochrome b oxidation(51) and the red shift with increased reduction of cytochrome b caused by antimycin (52). Cytochrome b shows a pattern of different spectral contributions depending on the state of reduction in the membrane. When succinate is used as substrate or if dithionite is added in limiting amounts, cytochrome b will become partially reduced and the only α-peak observed will be at 562nm When cytochrome b becomes fully reduced, e.g. excess dithionite, an additional α-peak is observed at 565nm. In this fully reduced state the intensity of the α-562 peak has equal contributions from both forms of cytochrome b. There is a midpoint potential difference of nearly 100mV between the two forms. These two types of cytochrome b have also been observed in studies performed at liquid nitrogen temperatures. A higher potential cytochrome b (designated b_k) at 562nm, has been distinguished from the lower potential cytochrome b (designated b_t) at 565nm. The most accepted theories on the functioning of cytochrome b in complex III, utilize these two forms to cycle electrons between the various chemical species of ubiquinone. Mutants were found in S. cerevisiae deficient in low potential, cytochrome b_{565}. From a study of these mutants, it was shown that cytochrome b_{565} is not in the main pathway of the electron transfer chain and it

appears not to be coupled to the oxidative phosphorylation site of complex III(53).

All attempts to isolate these two functional forms of cytochrome b have resulted in the loss of their spectral, potentiometric and EPR differences. Cytochrome b was isolated from beef heart as a dimer with a monomeric molecular weight of 31,000(35). It appears as a single species and unlike cytochrome b in the intact complex, it reacts with carbon monoxide(54). Two forms of cytochrome b have been separated in N. crassa by hydroxyapitite chromatography and again they exhibited sensitivity toward carbon monoxide(55). There was no discernible differences in the spectral characteristics or the molecular weight in these two forms from N. crassa yet their amino acid composition was found to differ to some extent. Cytochrome c_1 migrates in SDS at the same molecular weight as cytochrome b however it was clearly shown that both N. crassa cytochrome b forms are distinct from cytochrome c_1 (56).

In yeast it has been far more difficult to purify the cytochrome b to homogeneity. The methods used on the mitochondria from other organisms failed when applied to yeast. The first reported isolation of cytochrome b from yeast(41) used antimycin to stabilize the heme moiety.

SDS gel electrophoresis showed a major 32,000 molecular weight band and two minor bands at 14,000 and 11,000. A highly purified form of cytochrome b with the prosthetic heme attached, was obtained by solubilization with 3,4,5,6 tetrahydrophthalic anhydride a lysine modifying reagent(57). The EPR signal g=3.70 of this isolated cytochrome b indicates that the strained heme structure of the native cytochrome b is maintained. Yet a single α-peak at 562nm and a single low midpoint potential are observed for this isolated form. From this data, it appears that there is an apparent environmental difference in the two forms of cytochrome b which is removed upon treatment with 3,4,5,6 tetraphthalic anhydride. Under SDS conditions this isolated cytochrome b migrates at a molecular weight of 26,000 with minor amounts of a 53,000 band.

In S. cerevisiae, Chen and Beattie(58) separated two forms of cytochrome b with differing molecular weights, 32,000 and 29,000. Both proteins were found to react with an anticytochrome b antibody, raised against a proteolytic fragment of cytochrome b(59). The antisera raised against each of these isolated proteins were found to be crossreactive. Both antibodies immunoprecipitate the cytochrome b from pulse-labelled, cycloheximide incubated yeast cells, showing

that the antigenic determinants of cytochrome b are present in both purified proteins. Both antibodies were found to partially inhibit the coenzyme QH_2 cytochrome c reductase activity of complex III. One of the intentions of this study is to characterize these two forms of cytochrome b and if possible to relate them back to the functional differences previously described.

D. CYTOCHROME b GENE

The mitochondria has an autonomous genome distinct from the nuclear DNA and is capable of translating its own polypeptides on its unique mitochondrial protein synthesizing system. The genetic code of mitochondrial DNA differs from the universal code in that UGA codes for tryptophan and not termination. In mammalian systems the mitochondrial DNA is a closed circular duplex of about 15,000 base pairs(60). By contrast, mitochondiral DNA of S. cerevisiae is considerably larger, consisting of 75,000 base pairs. The pattern of the membrane protein mitochondrial translation products is similar to both organisms and no additional proteins have been isolated for the S. cerevisiae.

Cytochrome b is the only polypeptide of complex III that is a mitochondrial translation product. The cytochrome b gene has been found in the mitochondria of various organisms in different native forms; it is continuous and has no introns in man(61), it has one intron in Aspergillus(62) and it has two(63) or five(64) introns depending on the yeast strain examined. The yeast mitochondrial gene (box), which specifies the amino acid sequence of cytochrome b, displays a mosaic organization characteristic of many nuclear genes(65).

The location and number of intron/exon regions in the yeast cytochrome b gene "box" are based on genetic and physical mapping of a large number of mutants, characterization of the RNA's and intragenic complementation analysis(66). A comparison of the two gene forms of yeast are seen in illustration 2. The long gene for cytochrome b is three kilobase pairs longer than the short gene yet the translational products are the same. The first four exon regions in the long gene are condensed in the short gene to form one continous exon segment of the gene. The short mosaic "box" gene of the parent strain D273-10B in S. cerevisiae and the continuous cytochrome b genome from human mitochondria have been sequenced. Based upon a homology of sequence nearing 80%, three regions have been defined as the exon regions of the short gene in yeast. In the human mitochondrial DNA, the cytochrome b gene has a reading frame coding for 380 amino acids and is located between two t-RNA's. A comparison of the human and yeast mitochondrial genome is seen in illustration 3.

Over the entire mammalian DNA are a number of open reading frames for proteins that as yet are undefined. The same is true for the yeast mosaic cytochrome b gene. In the short "box" gene strain, D173-10B of S. cerevisiae,

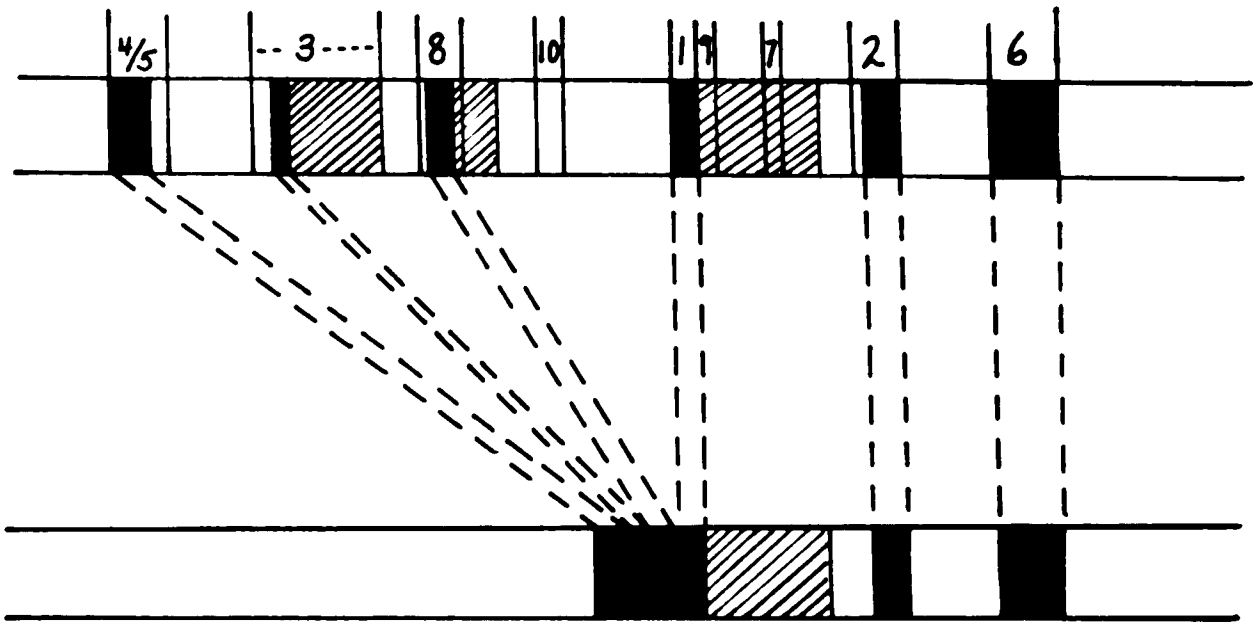

Illustration 2. A comparison of the long gene and the short gene of cytochrome b in S. cerevisiae. The long gene contains six exons which are depicted by darkened regions in the gene. DNA sequencing data reveals three additional regions in the gene with open reading frames as defined by the hatched lines in the diagram. Before the DNA sequence was obtained regions of cytochrome b had been defined by categorizing mutants. These categories are listed above the long gene and are numbered(1-10) in the order they were discovered. The first 4 exon regions of the long gene are condensed into a continuous exon region in the short gene while the remaining portion of the gene is common to both.

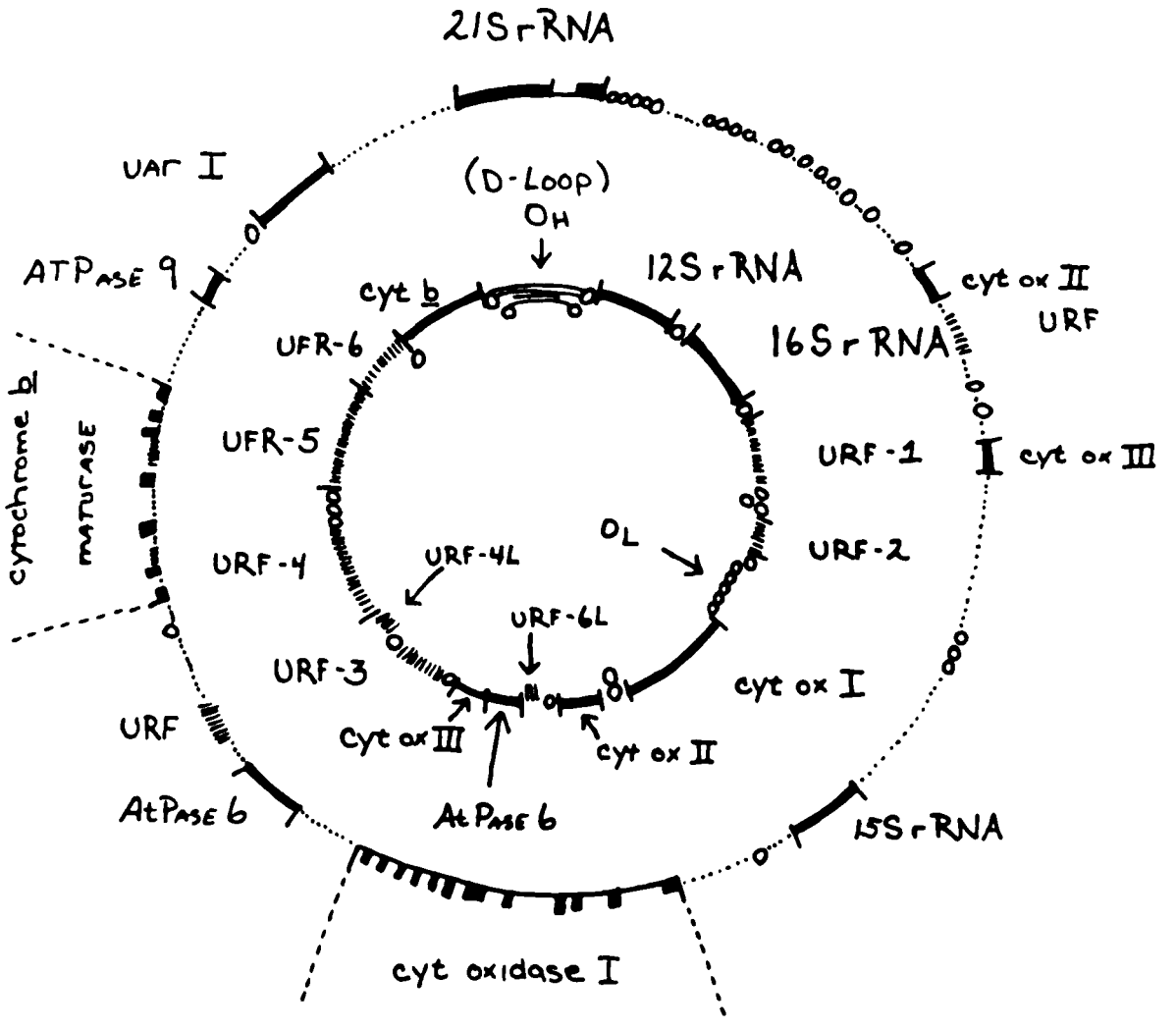

Illustration 3. The organization of yeast and human mitochondrial genomes. The outer circle represents the mitochondrial genome of S. cerevisiae strain KL-14-4A; the inner circle represents the genome of human placental cell mitochondria; drawings are not to scale. The human genes named outside their respective circles are transcribed from the H-strand, while those inside are transcribed from the L-strand. Transfer RNA genes are indicated by open circles. Adapted from Borst and Grivell(77) and Anderson et al(61). Note the split genes for the 21S RNA, cytochrome b, and cyt ox I in the yeast genome and the tRNA "punctuation" in the mammalian genome.

the open reading frames of the cytochrome b gene codes for nearly 700 amino acids. No function has been assigned to the polypeptide of the additional 310 amino acid coding sequence. It has been suggested that the product of this coding region has a maturase function for the cytochrome b mRNA. The mature mRNA, containing only the exon markers of the cytochrome b gene, is 2.1 kilobases(67). A similar transcript has been observed to be the mature apocytochrome b mRNA in yeast strains with the long gene(68). In the long strains 777-3A, KL14-4A and ID41-6, there are inserts totaling three kilobases of DNA into the "box" genome, see illustration 2. Pleotropic mutations that affect the cytochrome b and cytochrome oxidase subunit I genes from being expressed, are localized in the intron 3 region of the insert. The intron 3 region contains an open reading frame for 318 amino acids and this polypeptide is believed to function as a common maturase for both cytochrome b and cytochrome oxidase subunit I mRNA(64).

The organization of the mosaic cytochrome b gene in yeast is under intense investigation and the exact boundaries of the exon regions are not known. The exon boundaries have been defined, based on a best fit with the human gene. Any deletions from or additions to the DNA exon sequence

have been ignored in the yeast cytochrome b gene studies. The coding sequence of unknown function, which is continuous with the exon b_1 region, makes the proper assignment of the exon b_1 boundary a guess based on probability rather than a certainty. The mutants used to verify the intron and exon regions are of the deletion type rather than being point mutations. These mutants define local areas of the "box" genome but they cannot be used to identify the exact exon/intron boundaries. The best estimate of size comes from the human cytochrome b gene which has a continous coding region for a protein of 42,000 molecular weight.

E. BIOGENESIS OF MITOCHONDRIAL IMPORT PROTEINS

In the previous section cytochrome b has been discussed at length and its assembly was shown to affect the proper insertion of iron sulfur protein into the membrane. Greater than 90% of the proteins isolated in the mitochondria are nuclear coded. This section deals with the assembly of nuclear coded proteins into their respective compartments and/or membranes. Blobel and Dobberstein(69) proposed a mechanism for compartmentalization and vectorial transfer of cytoplasmically translated proteins. The original, signal hypothesis states that vectorially transferred proteins are made as larger precursors with leader sequences. This leader sequence enters the membrane and upon cleavage the rest of the polypeptide is either anchored into or traversed through the membrane. This segregation as well as proteolytic processing was found in native and stripped microsomes to occur only during ongoing translation but not after completion of translation. Topics such as recognition sites on the membrane, energy coupled to polypeptide transfer and specificity of the leader sequence, all require further investigation. The mitochondria is an excellent organelle for studying these processes and their variations

because it is known to import proteins, it contains two membranes with two discernible soluble compartments.

Yeast makes an ideal organism for studying the biogenesis of mitochondria because it is a eukaryotic faculative anaerobe. The first important contribution to the study of mitochondrial biogenesis began with the discovery of precursor forms for the three largest subunits of the F_1ATPase(70). These studies were carried out in reticulocyte lysates programmed with yeast RNA (i.e. in vitro) and in pulse-labeled yeast spheroplasts (i.e. in vivo). Each of the three largest F_1 subunits (α,β,γ) were found to be made as larger precursors both in vitro as well as in vivo. These larger precursors made in vivo disappeared with time, if the pulse-labeled spheroplasts were subsequently chased. Extramitochondrial pools of precursor to F_1ATPase can be increased in vivo by adding an uncoupler of oxidative phosphorylation (CCCP) or during growth of the cytoplasmically petite rho mutants(71) or by depleting the ATP pool inside the mitochondria(72). The pools can be decreased by lowering the rate of cytoplasmic protein synthesis (cycloheximide). The F_1-subunit precursors are processed and imported into the mitochondria in the absence of protein synthesis therefore making these mature subunits insensitive to trypsin digestion.

The protease responsible for processing the β-subunit of F_1 ATPase is a mitochondrial matrix protease that is chelator-sensitive and can be reactivated by magnesium addition. A matrix extract can be used to convert the precursor pool of β- subunit F_1ATPase to the mature form but this mature form is sensitive to trypsin digestion and is not capable of being transported into the mitochondria(73).

The F_1ATPase subunits have made a good model to study the import processing events in mitochondria but many variations of this processing have been defined. Cytochrome c peroxidase, an intermembrane space protein also follows a precursor-product relationship however in this case the depletion of ATP from the mitochondria does not stop the processing of this polypeptide(74). The conversion of cytochrome b_2, another intermembrane space protein, is a two step process. The initial conversion of the precursor to the intermediate form is energy dependent and is catalyzed by an o-phenanthroline sensitive protease located in the soluble matrix. The second processing step is o-phenanthroline insensitive and requires neither an energized inner membrane nor a soluble component of the intermembrane space(75). In studies on the bc_1 complex proteins, cytochrome c_1 and the non-heme iron protein are made as larger precursors(74).

Cytochrome c_1 also undergoes a two-step processing event but the binding of heme appears obligatory for converting the intermediate form to the mature polypeptide. The majority of import proteins do not appear to have precursor pools wherein the mature polypeptide is imported. However, the lack of precursor pools can also come from a rapid degradation of precursors in the cytoplasm and/or a rapid incorporation of precursors into the mitochondria.

The in vitro import of precursor polypeptides requires energy in respiring mitochondria, import is blocked either by protonophores or by valinomycin plus potassium, but not by oligomycin. In cyanide inhibited mitochondria, supplemented with external ATP, import is blocked by carboxyatractyloside or by oligomycin. This shows that an electrochemical gradient across the inner mitochondrial membrane, and not ATP itself is required for protein import. A brief trypsin treatment of mitochondria prior to incubation with labeled $F_1\beta$-subunit precursor abolished its uptake into the mitochondria(73). It appears that a protein component, exposed on the cytoplasmic face of the outer membrane is necessary for the uptake of precursors into mitochondria. These surface receptor proteins appear to be heterologous in specificity, since the overloading of one precursor for import does not appear to affect the incorporation rate of another.

F. OBJECTIVES OF THE STUDY

Polypeptides I and III of mammalian cytochrome oxidase exhibit Mr values on SDS gels of 36,000 and 21,000 respectively (76), while their respective genes specify polypeptides of size 57,000 and 30,000(61). As previously discussed a number of cytoplasmically made polypeptides of the inner membrane have been translated as precursors and cleaved upon insertion into the membrane. A precursor product relationship has also been found to exist for the mitochondrial translation product subunit 2 of cytochrome oxidase. Clearly, size estimates obtained by SDS gel electrophoresis must be viewed with considerable caution. A similar ambiguity of molecular weight exists for cytochrome b and it will not be completely resolved until a complete amino acid sequence on the purified protein is obtained. It is evident that a single gene codes for cytochrome b. Therefore, the observed differences described above in the two forms of cytochrome b are either caused by differing environmental constraints or by some post-translational modification. In either case, both forms would contain a homologous amino acid sequence with possible modifications or deletions. The two cytochrome b forms isolated in our laboratory at their different molecular weights, provides a good starting point for doing the amino acid

sequence of yeast cytochrome b. All previously reported isolations of yeast cytochrome b (41,57) have recognized the existence of contaminating protein(s) of differing molecular weights. An aim of this study is to define the differences in these two forms and to learn more about the properties of the cytochrome b polypeptide. The knowledge gained from this study will lead to a better approach for the eventual sequencing of the cytochrome b polypeptide.

Mitochondrial translation products, especially cytochrome b, have been under investigation for a number of years in our laboratory. Yeast cytochrome b was first isolated in this lab using a limited proteolytic digestion to solubilize it from the inner mitochondrial membrane. This polypeptide was nearly 4,000 daltons smaller than what had routinely been observed to be the mature cytochrome b. The mature cytochrome b has a characteristic migration in SDS gel electrophoresis nearing 31,000, depending on gel conditions. The larger of the two antigenically similar forms of cytochrome b is recognized to migrate as the mature cytochrome b species while the 3,000 dalton smaller form is believed to be a post-translational modified form. The smaller cytochrome b polypeptide does not appear in comparable amounts to the mature cytochrome b when mitochondrial translation products

are examined. However, in the purified state both proteins are found in equimolar amounts therefore making a check for absolute purity another necessary aim of this study.

Flavoproteins, the iron sulfur EPR signals, residual SDH activity and minor amounts of subunits from complex II have been described in the various purifications of complex III. The difficulty of removing these contaminants is strong evidence for a tight association between complex II and complex III. In our laboratory, the antibodies derived against the two forms of cytochrome b were both found to immunoprecipitate a 53,000 dalton polypeptide(80). In the purification of the two forms of cytochrome b, this protein copurifies in large amounts but is removed in the preparative gel electrophoresis stage. A recent report on the isolation of a highly purfied form of cytochrome b from yeast, recognizes this protein as a contaminant in the purification(57). As a final part of this study, information will be presented that identifies the function of this tightly associated protein which has not appeared as an integral protein of the bc_1 complex.

Chapter II: Purifications, Methods and Materials

A. Isolation of the Two Forms of Cytochrome b and the 53K Protein.

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Step 2: Submitochondrial Preparation
Step 3: Mitochondrial Membrane Solubilization
Step 4: Spectral Cytochrome b Isolation
Step 5: Preparative Gel Electrophoresis
Step 6: Electroelution and Protein Precipitation
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B. Experimental Procedures

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9. Other Assays Performed

C. Materials

A. THE MODIFIED ISOLATION OF TWO FORMS OF CYTOCHROME b

Step 1: Growth of Yeast and Isolation of Mitochondria

Yeast cells were grown and the mitochondria isolated according to the method of Kim and Beattie(77). The growth media contains 0.3% yeast extract, 0.04% $CaCl_2$, 0.05% NaCl, 0.07% $MgSO_4$ $7H_2O$, 0.12% $(NH_4)_2$ SO_4, 0.5mg.% $FeCl_3$ and was adjusted to PH4.5. Yeast cells were routinely grown on 3% galactose. All buffers and all steps in the purification were maintained at 4°C until the preparative gel electrophoresis step which was performed at room temperature. Centrifuging at 4,000RPM-5 min., cells were harvested at mid-log stage then washed twice with cold distilled water. These washed cells were suspended in STE buffer at a concentration of 3ml /gm wet cells. The STE buffer contains 0.25M sucrose, 20mM tris PH7.5, 1mM EDTA, 1mM PMSF. These cells were broken by Bronwill homogenization using two 25 sec. bursts. After breakage the supernatant was decanted and the glass beads washed with 3ml of STE buffer/gm wet cell, adding these washings to the cell suspension. The suspension was centrifuged at 1000g-7 min., resuspending the pellet in 6 ml of STE/gm wet cells and centrifuging again. The supernatants were combined and centrifuged three times discarding the pellet each time. This cleared supernatant was centrifuged at 18,000g-15 min. to collect the

mitochondrial pellet. This pellet was washed twice with STE buffer and stored frozen.

Step 2: Submitochondrial Particles

To make submitochondrial particles (SMP), the mitochondrial pellet from 30-40 gm. wet cells was resuspended in 25 ml of phosphate buffer and homogenized. The phosphate buffer contains 0.1M sodium phosphate PH7.5, 1mM EDTA, 1mM PMSF. The mitochondria was sonicated for 2 min. in 45 sec., 45 sec., 30 sec. increments, chilling the sample and sonicator tip in between. The Bronson Sonifier with a large tip at a setting of 4.7 was used. The SMP suspension was diluted 1:1 with phosphate buffer and centrifuged three times at 4,300g-10 min. discarding the pellet each time. The clear supernatent was ultracentrifuged at 100,000g-30 min. These pellets were resuspended in 25ml of phosphate buffer, homogenized then ultracentrifuged again.

Step 3: Mitochondrial Membrane Solubilization

The pelleted SMP was resuspended in cold phosphate buffer at a concentration of 10mg /ml and stirred in an ice bath. Solid KCl was added at a concentration of 75mg /ml of solution. Next a 20% solution of sodium cholate PH8.0 was added until a final concentration of 3.5% cholate was reached. This suspension was incubated on ice for 3 hrs. with stirring and finally centrifuged at 17,000g-10 min. This supernatant was incubated overnight on ice then particulates remaining were removed by centrifugation at 35,000g-10 min.

Step 4: Spectral Cytochrome b Isolation

Solid ammonium suflate was slowly added to the rapidly stirring cold supernatant until a final concentration of 10% saturation was reached. The solution was stirred for 15 min. then centrifuged at 35,000g-10 min. and the pellet saved. To the supernatant more ammonium sulfate was added with rapid stirring until a final concentration of 16% saturation was reached. The solution was allowed to stir an additional 15 min. and then was centrifuged at 35,000g-10 min. The pellets were combined homogenized then washed once in 25 ml of phosphate buffer. This final pellet was resuspended in 3-6ml of phosphate buffer, the protein concentration was determined(78) and it was assayed for cytochrome b spectral purity. Spectral purity was assessed on a dual beam spectrophotometer by viewing the differential spectrum. The cytochrome b preparation was assayed at 3mg /ml using K_3FeCN_6 to fully oxidize and dithionite to reduce. The oxidized spectrum was subtracted from the reduced spectrum and the difference spectrum is observed. The sample was considered spectrally pure, if only the α-peak of 562nm and the β-peak of 530nm of cytochrome b were displayed and both peaks were symetrical.

Step 5: Preparative Gel Electrophoresis

Preparative gel electrophoresis is essentially the discontinuous slab gel electrophoresis method described by

Lammeli(79) but scaled up to enable the processing of much larger protein samples. The spectrally pure preparation of cytochrome b was aliquoted into 8mg samples and microfuged at 15,600g-5 min. The pellet was solubilized in working dissociation buffer at a concentration of 10mg /ml and heated at 70°C for 30 min. or at room temperature overnight. Stock dissociation buffer contains 50mM tris PH6.8, 10% glycerol, 2mM EDTA. Working dissociation buffer was prepared fresh and it contained 950ul of stock dissociation buffer, 50ul of 2-mercaptoethanol and 50 mg /ml of SDS. A 1/10 volume of tracker dye [1 mg. bromophenol blue in 10ml of 50% glycerol] was added to the sample prior to loading it in the sample wells.

This electrophoretic apparatus uses a notched glass plate with a height of 24.5cm , a width of 16cm and a spacer thickness of 0.4cm. Blenderm tape seals the sides while the bottom was sealed with a 14% acrylamide 0.5% bisacrylamide gel. 85% of the slab gel bed volume is the lower running gel with a final concentration of 11% acrylamide, 0.39% bisacrylamide, 0.375M tris PH8.8, 0.1% SDS, 0.1% ammonium persulfate and 0.001% TEMED. The upper stacking gel has final concentrations of 5% acrylamide, 0.17% bisacrylamide, 0.125M tris PH6.8, 0.1%SDS, 0.1% ammonium persulfate, and 0.001% TEMED. The reservoir buffer contains 0.385M glycine, 0.05M tris PH8.3 and 0.1% SDS.

The samples can be electrophoresed in 9 hrs. by applying a constant 100mA until 170V is reached and then switching to constant voltage.

Preparative gels were soaked in 300ml of staining solution 0.25% Coomassie blue R-250; 45% methanol; 9% acetic acid for 2 hrs. They were destained rapidly until the banding pattern is visible, by soaking the gel in 25% methanol, 10% acetic acid in a covered tray inside a 50°C shaking water bath. The gels were destained to a clear background in 7.5% methanol, 5% acetic acid at room temperature in the presence of stain absorbent material. Individual protein bands of interest were excised from the gel and cut cross-sectionally into 3-4mm. slices. The slices were rinsed in 2 changes of approximately 75ml each of reservoir buffer, or until the buffer PH remains greater than 8.0. The reservoir buffer was removed and these slices were ready for funnel gel electrophoresis.

Step 6: Electroelution and Protein Precipitation

Funnel gel electrophoresis is a technique developed in our laboratory(58) to elute proteins from the acrylamide slices into solution. The botton of a glass funnel was sealed with parafilm and 12ml of gel solution containing 4.6% acrylamide, 0.16% bisacrylamide, 0.375M tris PH8.8, 0.1% SDS, 0.1% ammonium persulfate and 0.001% TEMED was added. When the gel has hardened, the parafilm was removed

and 12 inches of membrane tubing (M.W. cutoff 6,000) was attached. The tubing was filled with 10ml of reservoir buffer, all the air bubbles were removed and the distal end was closed, making sure there was room for expansion. The cathode wire was placed in the upper portion of the funnel containing the protein slices with approximately 30ml of reservoir buffer. The attached dialysis tubing was submerged in the anode reservoir containing about 500ml of reservoir buffer. Electrophoresis was performed at 4°C and run at a constant 50V for at least 24 hrs.

The eluted protein solution was dialyzed against several 2-liter changes of distilled water over a 48 hr. period at 4°C. The dialysate (approximately 15ml.) was added to 200ml of 100% acetone 0.1NHCl solution and stored at -20°C. The purified protein precipitates between 9 and 48 hrs. from the solution and was collected by centrifugation. This protein precipitate was washed twice with 90% cold acetone, homogenized, aliquoted, and rotary evacuated to dryness then stored at -70°C.

Step 7: Special Adaptations Used to Purify the 53K Protein

The above procedure was followed for the purification of the 29K and 32K proteins. These proteins were determined to be pure when the protein was loaded on a slab gel over a ten fold range of concentration and no other molecular weight proteins were detected. All of the 53K-protein

purified by this method showed minor contaminants of different molecular weights. This crude purification was therefore routinely subjected to the purification steps 5,6 and 7 for a second time. This purified 53K-protein now appeared to have the same molecular weight purity as had been seen for the 32K and 20K proteins.

B. EXPERIMENTAL PROCEDURES

1. Analytical Electrophoretic Procedures

11% acrylamide slab gels were used in purity studies, growth condition studies, molecular weight studies, covalent modifier studies and immunotransfer studies. The same gel preparation and sample dissociation conditions were used here as had been described for preparative gel electrophoresis, see purification step 5. These gels were scaled down to a thickness of 1.2mm with sample lanes, 6mm in width. When different acrylamide concentrations were used e.g. 9% to 15% the bisacylamide adjusts accordingly since both are present in the stock solution at 22.2% and 0.8%, respectively. In the special case where only the bisacylamide was changed, its concentration was cut in half. These gels were electrophoresed at a maximum of 60mA or 175V and were stained for only 1½ hrs.

2. Amino Acid Analysis

All of the amino acids except tryptophan were analyzed by the method of Liu(81). In this procedure two samples were assayed, one being a 6NHCl hydrolysate and the other a 6NHCl hydrolysate would be treated with dithiothreitol and sodium tetrathionate to recover the cysteine in a derivatized form. To two separate 250ug (8.3mmol /ml) samples of purified protein was added 1ml of double distilled 6NHCl. In one of the samples was added 10ul of phenol, 10ul of

ethdithol, it was flushed thoroughly with nitrogen and sealed in a hydrolysate tube. To the other sample, 1mg (2umol /ml) sodium tetrathionite was added then this hydrolysate tube was flushed with nitrogen and sealed. The samples were hydrolyzed for 18-24 hrs. at 110°C then dried under vacuum at 100°C with KOH pellets present in the evacuating chamber. Certain hydrolysates were tried at 12 hrs and 40 hrs also. To the dried tetrathionate treated hydrolysate was added 0.1ml pyridine, 3ml of 4umol /ml dithiothreitol solution. This tube was flushed with nitrogen, sealed and incubated for 1 hr. at 37°C. At the end of this reduction period, about 60mg (200umoles) of sodium tetrathionite was added to the tube and this mixture was allowed to stand for 16 hrs. at room temperature. Lastly, oxygen was bubbled through the solution for 7 min. and after sitting at least 1 hr., this solution was dried by rotary evaporation. Now, both dried samples were diluted with 125ul of 0.02N Na citrate PH2.2 $\pm$0.05, 0.5% thiodiglycerol, 0.1% phenol buffer. The samples were microfuged and 100ul aliquots from each sample were injected into the Beckman 119C amino acid analyzer. The results were compared to a set of amino acid standards and a S-sulfocysteine standard. The amounts of each of the amino acids recovered in both samples were in good agreement with the exception of tyrosine. Therefore the cysteine values were taken from the derivatized sample while tryosine values were only taken from the untreated sample.

As a control for the S-sulfocysteine method, insulin was assayed and all six cysteines were recovered.

Tryptophan was determined by an alternate procedure of Liu(82). Tryptophan is destroyed during HCl hydrolysis by oxidation, for this reason methanesulfonic acid, a non-oxidizing strong acid was used instead. To 250ug of the purified protein was added 0.5ml of 4N methanesulfonic acid. The sample was frozen then slowly thawed under vacuum to remove any oxygen present in the sample, then the tube was sealed. The sample was hydrolyzed in the sample then the tube was sealed. The sample was hydrolyzed at 110°C for 24 hrs. then was **neutralized** with 0.1ml of 3.5N NaOH and rotary evacuated to dryness. The residue was dissolved in 150ul of PH2.20 ±0.05 citrate buffer, millipore filtered and 100ul was injected into the Beckman 119C amino acid analyzer. The results of all the amino acids were in good agreement with the HCl hydrolysates and the tryptophan peak was clearly resolved. The only drawback to this procedure is that unknown material from this hydrolysate is absorbed on the resin causing a pressure buildup for later samples. A good practice is to clean the resin immediately after these samples have been analyzed.

3. N-Terminal Dansylation, Deformylation and TLC

Deformylation follows the method of Steffens and Buse(83) while Dansylation was done by the method of Weiner and Weber(84).

To 250ug of purified protein was added 1ml of 0.5N HCl methanol and incubated at room temperature for 1 hr. The sample was rotary evacuated to dryness then it was dissolved in 200ul of coupling buffer (0.5M $NaHCO_3$, PH9.8 NaOH adjusted) with 20ul of 10% SDS., 0.5ml of freshly made dansyl chloride solution (5mg./ml. acetone) was added, the tubes were covered with layers of parafilm and incubated at 37°C for 20 min. The reaction was stopped by adding 300ul of 20% TCA, keeping it on ice for 20 min. then centrifuging. The precipitate was washed in 1ml OF 1N HCl to remove the dansylic acid then 1ml of 6N HCl was added. The hydrolysate was evacuated to remove the oxygen as described in the tryptophan assay and it was hydrolyzed at 110°C for 4-12 hrs. This hydrolysate was dried under vacuum at 100°C.

5ul of pyridine are added to the dried amino acids. This unknown sample was spotted in a corner of the polyamide 5cm x5cm sheet with an extra thin capillary. This spotting is done in several applications with drying in between, making sure the spot is round and no larger than 2mm in diameter. The dansylated standard amino acids were spotted on the reverse side in the same corner. Ascending chromatography was done in four successive, freshly made solvents at room temperature. The spot ascends in 5 min. in solvent I (1.5% formic acid in water) and takes 10 min. to dry. Solvent II (benzene-acetic acid 9:1), solvent III

(ethylacetate-acetic acid-methanol 20:1:1) and solvent IV (o.5M Na_3PO_4 in 25% aqulons ethanol) were all chromatographed in the second dimension. Ascending chromatography in solvent II, solvent III and solvent IV takes 5 min., 5 min., 15 min., respectively with 5 min. of drying in between each solvent. The dansylated amino acids were examined after solvent II, after solvent III and again after solvent IV in order to clearly identify each of the spots. The best results were obtained when the concentration of dansylated amino acid is such that the migrated spots remain circular and that no trailing occurs.

4. Isoelectric Focusing

Isoelectric focusing (IEF) on slab gels is a combined (85,86,87) procedure designed to optimize the separation and staining of these purified proteins. A slab gel casing 140mm x 90mm x 1.2mm was assembled with a sealer gel, as described in the preparative gel electrophoresis section. The IEF gel solution (6M urea, 4.5% acrylamide 0.16% bis, v.7%u/v 3010 ampholines, 0.025% ammonium parsulfate, 0.25% v/v TEMED) is poured into the casing with a water overlay and was allowed to harden for 1 hr. 30ug of purified protein was dissociated by adding 20ul of sample buffer (8M urea, 5% Triton x-100, 1% mercaptoethanol) and incubated at room temperature for 30 min. The gel casing was disassembled with the IEF gel stuck to one of the glass plates then the sealer gel was removed with a razor. The samples were

microfuged and loaded directly on top of the gel near the anode. Acetylated cytochrome c markers were used to visualize the focusing and to verify the migration. The anolyte contains 1.7% phosphoric acid and the catholtye contains 2% ethylene-diamine and 6M urea. The IEF gel was run at a steady 6 watts for 5 hrs on a water cooled jacket. The PH gradient was identified by cutting 1 inch strips from both sides of the gel and slicing each strip into 5mm pieces from the top down. Each piece was put in 1ml of H_2O shaken for 30 min. and the PH determined. The remaining gel containing the proteins was fixed and the ampholines removed by soaking for 2 days in several changes of destaining solution or in 3.5% perchloric acid. The staining and destaining was as described for preparative gel electrophoresis.

5. Cyanogen Bromide Cleavage

Cyanogen bromide cleavage was performed according to the method of Swank and Munkres(88) and was viewed according to the method of Cabral and Schatz(89). The 29K and 32K CNBr cleaved peptides were compared using sperm whale myo-globin(88) as a control for CNBr cleavage. All dried pro-teins were solubilized in 70% formic acid at 1mg/ml. To 10-40ul of each sample was added 1ml of 0.8% CNBr in 70% formic acid was added, incubating as above. All incubated specimens were then dried by rotary evaporation at 45°C.

A slab gel was prepared by making the lower gel solution fresh, to a final concentration of 19% acrylamide, 0.35% bisacrylamide, 0.375M tris PH8.8, 10% glycerol, 0.1% SDS. An upper stacking gel was made by diluting the lower gel solution 1:1 with water. Each solution was solidified by adjusting the final concentration of 0.1% ammonium persulfate and 0.001% TEMED. 40ul of dissociation buffer (previously described)was added to each sample, adjusted to an alkaline PH by addition of tris base crystals, the indicator bromophenol becomes blue, then boiling for 3 min. The samples were loaded on the gel at a constant 10 volts for 20 hrs. then stained and destained as previously described.

6. Immunotransfer "Western Blotting"

All samples tested by immunotransfer (Western Blotting) (90,91,92) were first dissociated and run on slab gels as described under analytical electrophoretic procedures. The gel has a duplicate lane pattern so one side can be transferred while the other side is stained and destained for comparison. A nitrocellulose sheet was presoaked 5 min. with transfer buffer (25mM tris base, 93mM glycine, 10% methanol) and placed over three layers of wetted filter paper. The gel to be transferred was placed directly on top of this nitrocellulose paper and the air spaces between them were removed, then three more layers of wetted filter paper was placed on top. This sandwich in a gel holder was placed in an electric destainer with the nitrocellulose side facing

the anode and the chamber was filled with transfer buffer. The protein transfer was complete within 1 hr. for proteins of this molecular weight. At the end of the transfer, the nitrocellulose sheet was marked for sidedness. Other available sites on the nitrocellulose sheet were filled by incubating at 25ml/lane in 5% newborn calf serum (NBCS) in PBS (150mM NaCL, 10mM NakPi buffer at PH6.8) for 2-3 hrs. with room temperature shaking. Next the transfer sheet was placed in 10% NBCS-PBS at 12.5ml/lane 20ul/lane of anticytochrome b antibody or 75ul/lane of anti 53K antibody was added and then incubated overnight with room temperature shaking. The excess antibody was washed off the transfer with a series of ½ hrs. wash incubations in 0.3% bovine serum albumin (BSA) in PBS. The transfer sheet was again placed in 10% NBCS-PBS at 12.5ml/lane, 20-100ul of [^{125}I] protein a was added depending on its freshness and then incubated 2 hrs. with room temperature shaking. It was washed as before in several changes of 0.3% BSA-PBS until radioactivity in the buffer was barely detected. The dry nitrocellulose paper was protected by plastic wrap and was exposed to XR-5 film for 1-3 days.

7. Immunoprecipitation and [^{3}H] Autoradiography

The yeast cells were grown in 0.67% yeast nitrogen base without amino acids, 0.09% NH_4Cl, 0.2% yeast extract,

of five times the volume of antibody to as low as three times the volume and shaken at room temperature for 1 hr. The protein a cells were collected by centrifugation at 6,000 RPM-10 min. and washed four times with Triton buffer. The cells were suspended in 100-150ul of dissociating buffer and incubated overnight at room temperature. These cells were microfuged and the entire supernatant sample containing 4,000-6,000cpm/mg starting mito protein was loaded onto a lane of the 11% analytical SDS gel. After the gel had been run, it was stained, destained and shaken at room temperature for 20 min. in 200ml of distilled water. The gel was transferred to 150ml of 1M sodium salicylate PH6.0 and shaken at room temperature for 30 min.(93). The excess salicylate solution was rinsed off the gel and the gel was then dried. An autoradiogram was made from the gel by exposing it for 2-4 weeks to XR5 Xray film at -70°C. The molecular weight of each band was determined by comparison against the known molecular weight standards in the gel.

8. IgG Fractionation of the Antisera

The partial purification of an IgG fraction from antisera and control sera follows the method of Chan and Schatz(94). The fraction was prepared by precipitating the sera at room temperature with solid ammonium sulfate to 34% saturation. The precipitate was dissolved in 0.01M sodium phosphate PH7.2 to a total volume of ½ the starting material. This ammonium sulfate fractionation was repeated three times more, remembering to calculate the volume of precipitate as containing 34% ammonium sulfate. The final precipitate was again brought to ½ volume of the starting material and dialyzed (M.W. cut off 6,000) against three times 2-liter changes of phosphate buffer at room temperature over a 15 hr. period. The dialysate was centrifuged at 17,000g.-20 min. at 4°C. The protein concentration of the IgG supernatant fraction was determined and it was stored at -70°C.

8b. Mitoplast, SMP and Cholate solubilized SMP Preparations

Mitoplasts were prepared according to the method of Daum et al(95) and the spheroplasts according to the methods of Reid et al(71) and Glasser et al(73). Yeast cells were grown to early log phase in the media previously described but the galactose concéntration used was only 2%. All buffers and centrifugation steps, in the formation of spheroplasts were kept at room temperature. The cells were harvested at

4,000RPM-5 min., resuspended to 0.25g. cell wet weight/ml in 0.1M tris-SO_4 PH 9.4; 10mM dithiothreitol and incubated at 30°C-5 min. with shaking. The cells were pelleted and resuspended to 0.1g cell wet weight/ml in spheroplast buffer [1.2M sorbitol; 20mM K_2HPO_4 PH7.7; 0.05% yeast extract; 1% galactose]. Zymolyase 5,000 was added to a concentration of 1mg /ml and the cells were incubated at 30°C for 30-60 min. The conversion to spheroplasts was monitored by the autolysis of spheroplasts in water. Spheroplasts were harvested by centrifugation at 3,500RPM-5 min. and washed twice with 1.2M sorbitol.

The isolation of mitochondria and the formation of mitoplasts require 0-4°C so all buffers are chilled, all incubations are done on ice and all centrifugations are at 4°C. The spheroplasts were resuspended in chilled breaking buffer [0.6M mannitol; 20mM Hepes-KOH PH7.4; 0.1% bovine serum albumin; 1mM PMSF] to a concentration of 0.3g cell wet weight/ml The spheroplasts disrupted by 2X10 sec. bursts at low speed in a Waring Blender small cup, diluted with one volume of breaking buffer and centrifuged at 3,500RPM-5 min. This supernatant was centrifuged at 11,000RPM-10 min. to pellet the mitochondria. The mitochondria were homogenized in breaking buffer and centrifuged at 3,500RPM-5 min. The supernatant was centrifuged at 11,000RPM-10 min. to collect

the mitochondria again. The pelleted mitochondria was washed once in mito buffer [0.6M mannitol; 20mM Hepes-KOH PH7.4], centrifuged and the pellet homogenized in 2 ml of mito buffer with a protein determination being done.

The mitochondria were resuspended at a concentration of 6mg protein/ml in mito buffer and this stirring suspension was diluted to a mannitol concentration of 0.1M by adding dropwise 5 volumes of mito hypotonic buffer [10mM tris-HCl PH7.4; 1mM PMSF]. The diluted suspension was stirred gently on a magnetic stirrer for 20 min. then centrifuged at 50,000xg -25 min. The supernatant was checked for the release of cytochrome c and the mitoplast pellet was re-suspended at 10mg protein/ml in 0.4M mannitol buffer (mito buffer: mito hypotonic buffer 2:1).

The SMP used in the inhibition studies were prepared as described in the purification step 2 with a final concentration of 10mg protein/ml of phosphate buffer. The mitoplasts or SMP at 10mg protein/ml can be cholate solubilized by adding 20% v/v sodium cholate PH8.0 until a final concentration of 2% cholate was reached. This cholate solubilization was carried on at 4°C and once added, the suspension was allowed to continue stirring for 30 min.

8c. Enzymatic Assays

In all of the enzymatic assays tested, the Mp, SMP and cholate solubilized samples were mixed with varying amounts of IgG_{53}, IgG_{32} and control IgG. Each mixture was then diluted with its respective buffer until a final concentration of 1mg/ml of mito protein was reached. The samples were incubated at room temperature for 1 hr., put on ice and then assayed for enzymatic activity.

Cytochrome c reductase activity was measured according to the method of Brown and Beattie(96) with modifications. After the IgG fractions were incubated with the appropriate mitochdonrial particulate as described above, 10-30ul aliquots were removed for assay. When succinate was used as the substrate, the sample aliquot was put in 0.8ml of reaction cocktail [0.1mM NaN_3; 0.2mM EDTA; 25mM sodium phosphate PH 7.6]. To this was added 0.1ml of 20mM sodium succinate in cocktail buffers and after a 3 min. incubation, 0.1ml of 50uM cytochrome c was added to start the reaction. In cases where the lag phase was examined there were no preincubations and the reaction was started by adding the sample aliquot to the cuvette containing all of the other constituents. When DBH_2 (see results) was used as the substrate 0.9ml of reagent cocktail, 0.1ml of 50uM cytochrome c

were mixed in a cuvette with the sample aliquot and the addition of 6ul of DBH_2 starts the reaction. Both assays were recorded at 550nm following the change in absorbance with time and the rate being calculated from this linear portion of the graph. The extinction coefficient of $25.6mM^{-1}$ cm^{-1} was used in the calculations(97).

Succinate dehydrogenase activity was assayed according to the method of King(98). The initial reaction rate at 600nm was recorded after addition of mitochondria to the cuvette containing 0.005mM KCN, 50mM phosphate buffer, PH7.6 and 9mM succinate. The extinction coefficient of $21mM^{-1}cm^{-1}$ was used in the calculations(99).

9. Other Assay Conditions

Automated Edman degration was carried out in a Beckman-890B sequener spinning cup with a 0.1M Quadrol program. PITC converts into PTH and is read at 254nm on a Hewlett Pacard HPLC- 1080B. For the proteolytic inhibitor studies; PMSF, NEM, EDTA were used at a concentration of 1mM in all buffers whereas TPCK and TLCK were used at concentrations of 1uM(100).

C. MATERIALS

High purity sodium cholate was purchased from Calbiochem. Double distilled 6N HCl, sodium tetrathionite, S-sulfocysteine standard and 4N methane sulfonic acid were purchased from Pierce Chemical. Acetylated cytochrome c PI markers were purchased from U.S. Biochemical. XR-5-X-omat photographic film was purchased from Kodak. All radioactive isotopes used were supplied by New England Nuclear. DEH_2 was a gift from B. Trumpower. All other chemicals used were of high quality grade from Sigma.

Chapter III: Results

A. Studies on Proteolysis During the Purification

B. Growth Conditions Affecting Cytochrome *b* Production

C. Characterization of the Purified Forms of Cytochrome *b*

D. Studies with the Anti-cytochrome *b* Antibody

E. Defining a Protein closely Associated with Cytochrome *b*

A. STUDIES ON PROTEOLYSIS DURING THE PURIFICATION

In our laboratory cytochrome b was isolated from yeast, in two forms based upon immunological studies (58) and separated according to apparent molecular weight differences. The larger cytochrome b polypeptide was recognized to migrate at the same value as its mitochondrial translation counterpart, Mr=32,000 and will be referred to as the 32K protein. The other cytochrome b polypeptide was approximately 3,000 daltons smaller and will be referred to as the 29K protein. This 29K protein does not appear as a major protein when viewing mitochondrial translation products yet is co-purifies with the 32K protein in equimolar amounts. It became necessary to investigate the possibility that the 29K protein results from proteolytic cleavage during the preparative procedure. My first attempts were to modify the purification procedure in such a way as to minimize conditions where proteolytic digestion can occur. The buffers used in this procecure routinely contain PMSF to inhibit serine proteases but other proteases with o-phenanthroline and TPCK-TLCK sensitivity have also been identified in mitochondrial preparations. As a first attempt, the volume of buffer used

for the isolation was increased during cell breakage and SMP formation, to further dilute any proteolytic enzymes which might be active in the supernatant.

The remaining steps in the purification scheme (see fig. 1) up to the isolation of the spectral cytochrome b stage were as described in the original purification by Chen (58) with minor adjustments affected by dilutional changes. This isolated spectral cytochrome b stage sample still contains several proteins but cytochrome b is the only spectrally detectable hemoprotein. In Chen's purification this sample was then dissolved in SDS and subjected to column chromatography using AcA-44 resin, to identify at what molecular weight the heme moiety resides. Cytochrome b was found to elute as a 30,000 dalton species under these mild SDS conditions. This chromatography step took a few days at room temperature and was unable to separate the proteins in the 30,000 molecular weight range. The column chromatography step was therefore eliminated to avoid sample exposure to warmer temperatures and to minimize the time required to separate the remaining proteins by SDS-preparative gel electrophoresis.

The modified purification as described in figure 1 and detailed in the Methods, section A, contains the conditions

YEAST CELLS

↓ harvesting, Braun homogenization, 3,000 then 17,000g centrifugations

MITOCHONDRIA

↓ sonication, 4,300g then 100,000g centrifugations

SUBMITOCHONDRIAL PARTICLES

↓ 1M KCl, 3.5% Cholate additions, a 4°C incubation with intermittent 17,000g centrifugations

CHOLATE SOLUBILIZED SUPERNATANT I

↓ overnight 4°C incubation, 35,000g centrifugation

CHOLATE SOLUBILIZED SUPERNATANT II

↓ 0-15% ammonium sulfate additions, incubation, 35,000g centrifugation, resuspend pellet

SPECTRAL CYTOCHROME b STAGE

↓ assayed for spectral purity, dissociated and run under SDS preparative gel electrophoresis conditions, band(s) of interest are excised from the destained gel.

ISOLATED MOLECULAR WEIGHT BANDS

↓ proteins are electroeluted into dialysis bags and dialysed.

PURIFIED PROTEIN DIALYSATE

↓ add to 10 volumes of 0.1N HCl acetone and incubate at -20°C overnight, then collect the ppt, wash with 90% acetone and rotary evacuate to dryness.

PURIFIED PROTEINS

Figure 1 - A brief outline for the modified purification of the two forms of cytochrome b. A detailed description is presented in chapter II of the methods section A. The column chromatography and the pooled fraction concentration steps were performed immediately after the spectral cytochrome b stage in the original procedure. These steps were omitted in this modified purification.

conditions found to be optimal in purifying these cytochrome b proteins. It achieves a consistency in heme content and in the relative concentrations of the 29K and 32K proteins from one preparation to the next. Much of the heme content of cytochome b detected spectrally was lost during the cholate extraction step, but this was necessary to insure a complete separation of cytochrome b from cytochrome c_1. Figure 2A is an analytical SDS slab gel showing the purified proteins 29K and 32K and the spectral cytochrome b stage of the purification. The spectral cytochrome b stage sample (Fig. 2A lane 4) contains several proteins but only one spectral cytochrome was detected in a different spectrum. This different spectrum is seen in figure 2B with two symmetrical peaks at the characteristic wavelengths of cytochrome b, namely the α-peak at 562nm and the β-peak at 530 nm.

The possibility exists that other proteins with the same Mr values may co-precipitate with ammonium sulfate. Other procedural variations were therefore explored to find shifts in the relative concentration of the 29K and 32K proteins as viewed on SDS slab gels. In the procedure, the isolated mitochondria was routinely stored at -20° until they were converted into submitochondrial particles. Changes in the method of mitochondrial storage were tried, such as:

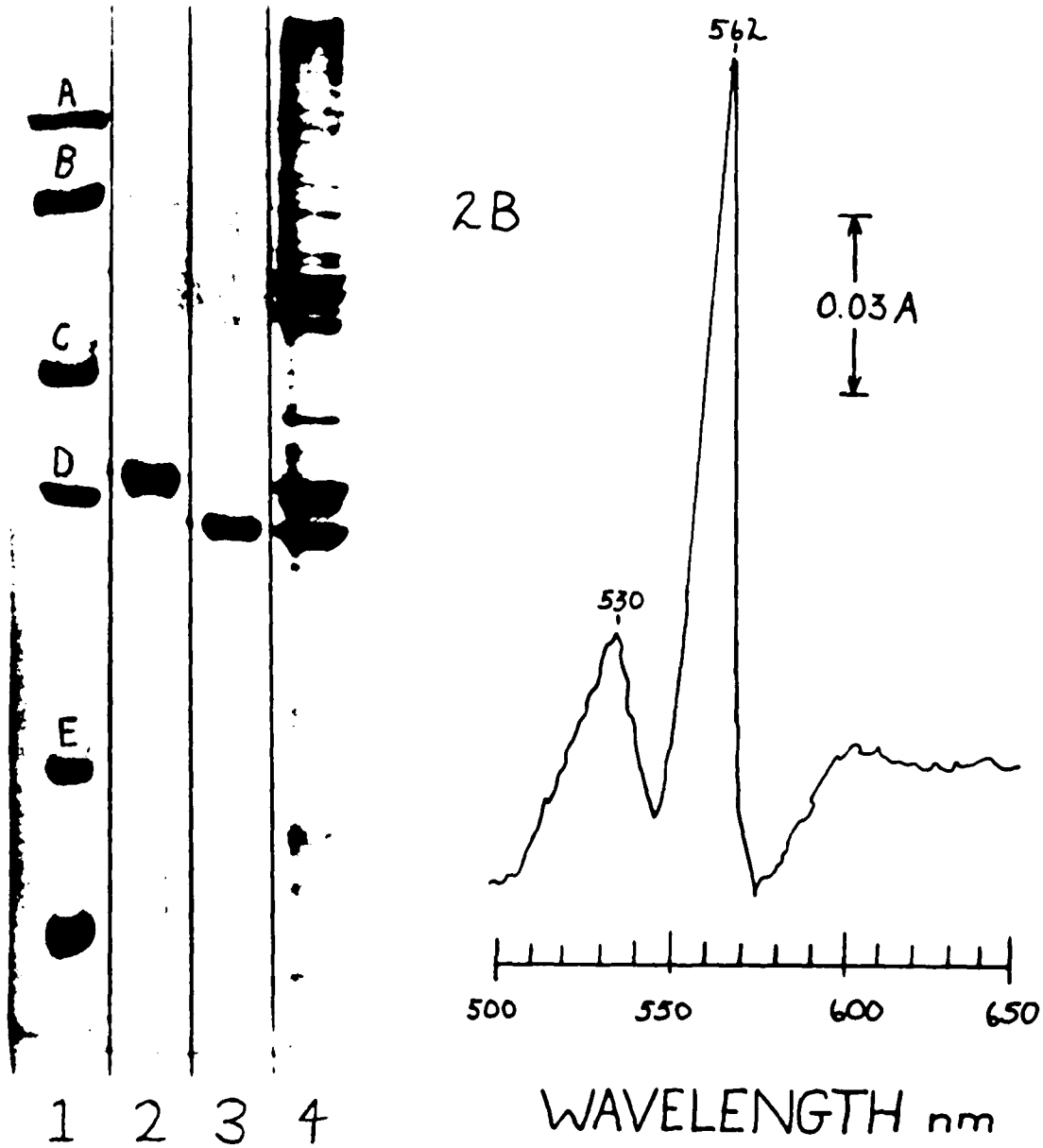


Figure 2A (left) is an 11% SDS analytical slab gel stained with Coomessie blue which depicts the proteins remaining at certain stages in the purification. Lane 1 contains the following molecular weight standards: A) phosphorylase b, 94,000; B) bovine serum albumin, 67,000; C) ovalbumin, 43,000; D) carbonic anhydrase, 30,000; E) soybean trypsin inhibitor, 20,100. Lane 2 contains 10ug of purified 32K, lane 3 has 10ug of purified 29K and lane 4 has 150ug of spectral cytochrome b stage sample

Figure 2B (right) is a difference spectrum of the material used in lane 4, showing cytochrome b (α562, β530) to be the only cytochrome present at this stage of the purification.

4°C incubations for 4 days; 4°C in hypotonic buffer for one day; -70°C for one day or the immediate processing of the mitochondrial pellet. In a similar manner these storage conditions were examined on SMP samples. At other steps in the procedure, additional fractionations with ammonium sulfate were tried, as well as changes in incubation times and temperatures. An additional cholate-KCl solubilization of the spectral cytochrome b stage sample and another ammonium sulfate fractionation were also tried. Under all of the above conditions, the spectral cytochrome b stage appeared unchanged and the relative concentrations of the 29K and 32K proteins, as determined on SDS gels, remained the same.

In the purification PMSF has been routinely used to prevent proteolytic degradation by covalently binding to the active site of serine proteases. The metal chelator EDTA is put in all purification buffers to prevent bacterial contamination and to inactivate metalloproteases. TLCK and TPCK have been found to covalently modify the active histidine group in trypsin and chymotrypsin, respectively causing an inactivation of the proteases. Metal chelators and the above covalent modifiers all have been found to have an inhibitory effect on proteases, endogenous to the mitochondrial preparations. To test the possible effects of proteolysis

during the purification, a number of conditions were compared. This comparison was made using no covalent modifying compounds or only 1-10mM EDTA in all buffers or using 1mM EDTA with PMSF or using 1mM EDTA with PMSF, TPCK, TLCK or using all of the inhibitors with NEM. In all of these cases tried, the relative concentration of the Mr=29K and the Mr=32K protein bands of the spectral cytochrome b stage remained unchanged. From all of the studies described above it appears evident that proteolytic degradation during this modified purification, does not contribute to the intensity of this 29K protein band.

B. GROWTH CONDITIONS AFFECTING CYTOCHROME b PRODUCTION

Yeast cells are faculative anaerobes and growth conditions will affect the production of functional mitochondria. Growth in media containing high concentrations of fermentable substrate (e.g. glucose) leads to a decrease of cellular respiration, this condition is known as glucose repression. Under glucose repressed conditions cells are devoid of respiratory activity and mitochondria-like structures "promitochondria" which contain mtDNA, oligomycin-sensitive AtPase and reduced levels of various NAD linked dehydrogenases are found(101). In these structures there is a complete lack of enzymatic activity found for complexes II, III and IV of the respiratory chain(102). The end products of fermentation are generally pyruvate, lactate and/or ethanol. To reduce these products further to CO_2 and H_2O requires aerobic conditions and the utilization of the mitochondrial electron transport chain. When glucose repressed yeast cells are transferred to aerated media containing non-fermentable substrate, the "promitochondria" undergo a rapid transformation to respiratory competent mitochondria.

Growth on glucose, galactose and lactate have been shown to have different effects on mitochondrial maturation

and on the consumption of oxygen. At the spectral cytochrome b stage, values ranging from 0.39nmol/mg protein to 1.02nmol/mg protein were obtained, depending on the growth conditions of the culture. The heme contents obtained at the spectral cytochrome b stage have shown an increased pattern of heme content as the carbon source used requires the utilization of mitochondrial respiration. Glucose had been shown to depress the mitochondrial respiratory chain and when assayed this cytochrome b preparation had the lowest heme content. Growth on lactate requires mitochondrial respiration and the preparation from these cells was found to have the highest heme content. It was also observed that the closer to stationary phase the cells grew, the higher the heme content values became (see table I). These heme content patterns mimic what was concurrently observed in submitochondrial particles.

The spectral cytochrome b stage samples of the various growth conditions were compared by SDS gel electrophoresis (see figure 3). Samples from the cells for each growth condition were loaded on the gel at the same protein concentration of 150ug/lane. Under all growth conditions, the relative concentration of the 29K and 32K proteins were near unity with the exception of the lactate grown cells (fig. 3,

CARBON SOURCE	GROWTH PHASE	HEME CONTENT	RELATIVE INTENSITY
		nM/mg Protein	%32K:%29K
3% Galactose	Stationary	1.02	90:96
3% Galactose	Late-log	0.78	100:130
3% Galactose	Mid-log	0.62	100:96
10% Glucose	Mid-log	0.39	59:64
5% Lactate	Mid-log	1.02	144:31

Table 1 - A comparison of growth conditions which were found to affect the heme content and the relative concentration between the 29K and the 32K forms of cytochrome b. The samples assayed are all at the spectral cytochrome b stage of purity and all contain 250ug of protein per sample lane as determined by the method of Lowry. The relative concentrations of 29K and 32K are established by densitometric scanning of the Coomassie blue stained SDS-polyacrylamide gel seen in figure 3. An arbitrary value of 100% was assigned to the concentration of the 32K band in mid-log galactose grown cells. The heme content was determined by using the method of Van Gelder(103) by difference spectrum analysis on K_3FeCN_6 oxidized, dithionite reduced samples of each condition.

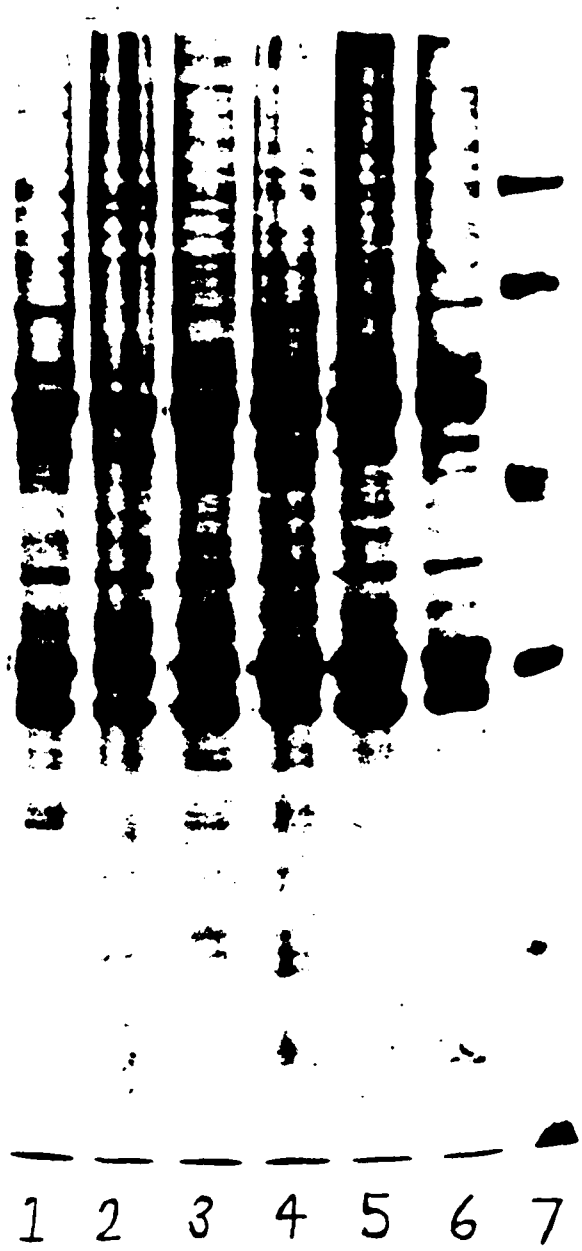


Figure 3 - is an 11% SDS analytical slab gel containing the spectral cytochrome b stage samples with varied growth conditions. Lanes 1, 3, and 6 are samples from stationary, late-log and mid-log galactose grown cells, respectively. Lane 2 is from mid-log glucose grown cells, lane 4 is from mid-log lactate grown cells and lane 5 is from mid-log galactose grown KL-14 cells. Lane 7 contains the set of molecular weight markers of 96,000; 67,000; 43,000; 30,000; 20,000 from the top down.

lane 2). From these studies, it was observed that growth factors do affect the heme b content of the spectrally pure cytochrome b stage but the densitometric scans of the SDS gels reveal that both polypeptides are not necessarily affected equally (table 1). The summary of heme contents and relative intensities of staining for the two bands of cytochrome b are presented in table I. No conclusions could be drawn on the stoichiometry of the two forms of cytochrome b until a check of absolute purity was made for each of these proteins.

C. CHARACTERIZATION OF THE PURIFIED FORMS OF CYTOCHROME b

Cytochrome b was the only hemoprotein detected at the spectral cytochrome b stage of purification. The heme moiety was determined to reside in the 30,000 dalton range by SDS column chromatography(58). Two major proteins were identified in this region and they were separated by preparative gel electrophoresis, electroelution, acetone extraction and finally dried in a rotary vacuum. The 29K and the 32K proteins are believed to be the two apocytochrome b forms based upon previous immunological evidence(58). These purified proteins were checked for molecular weight purity by loading samples over a ten-fold range of concentration (10-100ug) on analytical SDS gels. Both purified proteins were visualized by Coomassie blue staining to be free of contamination from proteins of other molecular weights. The molecular weight purity of the 32K and the 29K proteins are seen in figure 2 lanes 2 and 3, respectively.

SDS gels as described by Laemmli(79) contains a ratio of acrylamide to bisacrylamide of 30:1, respectively. By changing the ratio of acrylamide to crosslinker e.g. 15:1, I had been able to split another major protein in the purification into two components. After readjusting the acrylamide concentration with increased crosslinker to optimize

the 30,000 range, I was unable to see more than single bands for either the 29K or 32K proteins. The method of urea gel electrophoresis places emphasis on the charge characteristics of the individual protein, as well as its size. Secondly, not all proteins become totally denatured under urea conditions and any retention of secondary structure will also affect the migration of the protein bands. The results of urea gel electrophoresis seen in figure 4, indicates again that the 32K protein migrates as a single bank at 34,000 and the 29K migrates as a single band at 30,000.

The relative molecular weight(Mr) of each of these purified proteins was determined by SDS gel electrophoresis. The SDS denatured cytochrome b in mitochondrial or in SMP preparations had previously been shown by Beattie et al (104) to migrate abnormally when tested at different acrylamide concentrations. Cytochrome b was found to have a molecular weight from 30,000 to 35,000 depending on the acrylamide concentration used. These purified proteins however migrate normally with a molecular wieght of 32,000 daltons for the larger one and 29,000 daltons for the smaller one. The range of acrylamide concentrations tested were 9%, 11%, 13% and 15%.

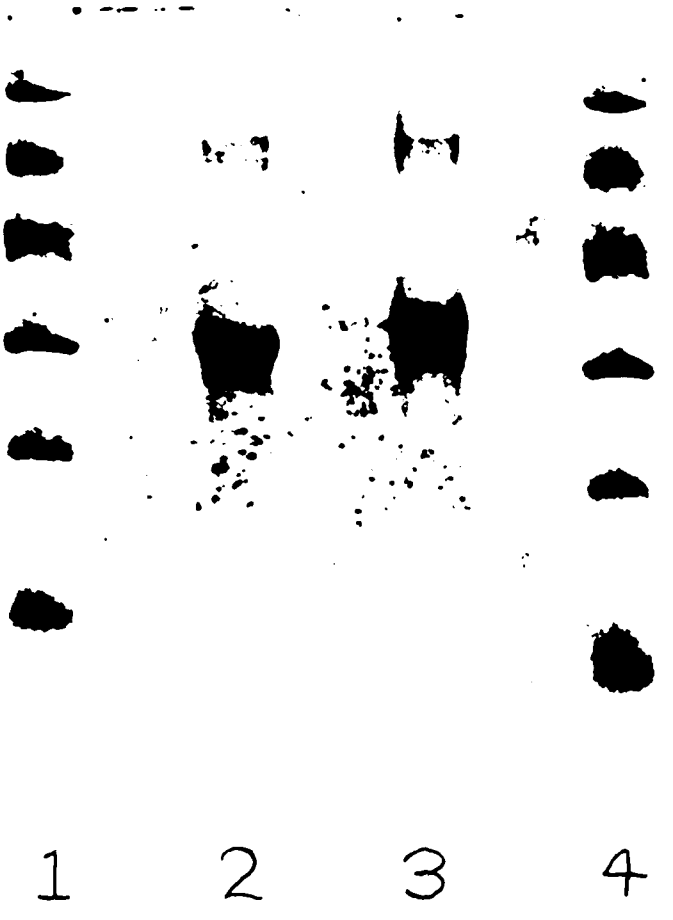


Figure 4 - Urea gel electrophoresis was performed as described in the methods. The molecular weight standards of lanes 1,4 are the same as used in figure 3 . Lane 2 contains the purified 29K protein migrating at 30,000 while lane 3 contains the 32K protein migrating at 34,000 daltons.

Previous immunological evidence had shown that the 29K protein and the 32K protein are antigenically related. The first step used to investigate the homology between these two apoproteins, was to compare their amino acid compositions. In N. crassa, two forms of cytochrome b were isolated with nearly identical amino acid compositions(55). The amino acid analysis was performed a number of times on both of the purified proteins so p-values could be determined for each amino acid. The results are reported in figure 5 as the number of residues of each amino acid. It can be seen that the difference between certain amino acids are found to be highly significant. Also included in this figure are standard error bars to identify the levels of variation between the assays performed. Certain differences in the amino acid composition make it unlikely that the two purified proteins are identical. The 29K protein was found to contain more aspartate and threonine residues per molecule than was found for the 32K protein. From this evidence the 29K protein could not be considered a direct cleavage product of the 32K protein. One possible explanation is that the 29K protein contains residues from a different portion of the cytochrome b gene. It is known that the potentially transcribable portions of the genome are much larger than

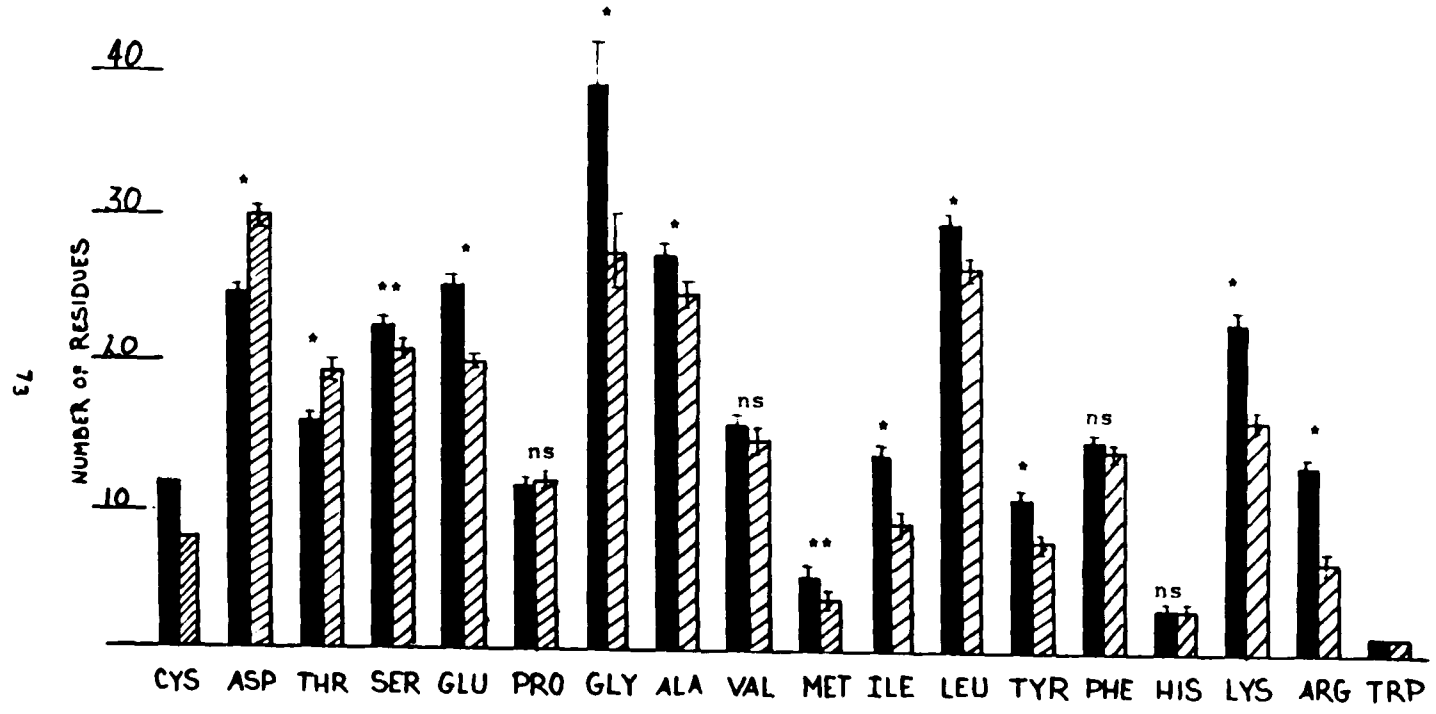


Figure 5 An amino acid analysis comparison between the 32,000 cyt b (black bars) and the 29,000 cyt b (hatched bars). The cysteine and tryptophan values are the result of two assays, while all the other amino acids are the accumulated results of five to seven assays. Error bars represent the S.E. values.

* - p 0.01

** - p 0.05

ns - difference not statistically significant

the size of the observed protein. Another possible explanation could be that one of both of the proteins are contaminated with other unrelated proteins of the same molecular weight.

Since these two forms of cytochrome b do not appear homolgous their results were compared to the other published amino acid compositions of cytochrome b. In table 2 the comparison between various preparations of cytochrome b includes; cytochrome b isolated from beef heart, N. crassa, two prior purifications in yeast which were discussed in the introduction and the two isolated forms of cytochrome b from this study. No two sets of data appear homologous and their percent polarity varies from 30.0% to 44.3%. Capaldi and Vanderkoo (105) had categorized 240 proteins and found that 85% of the soluble proteins have an average polarity of 47% ±6 whereas the membrane proteins were spread between 28.6% and 43.4 polarity. All of these proteins classified as membrane proteins require detergents for solubilization. In table 2 are listed two other mitochondrial translation products found in the inner membrane, and a typical soluble protein. The date from table 2 shows a polarity range of 30.0%-44.3% for the membrane proteins whereas the soluble protein has a value of 48.8%.

	CYTOCHROME B							CYTOCHROME OXIDASE				
	Bovine (54)	N.crassa (55)	Yeast (57)	Yeast (41)	Yeast (63)	Yeast (58)	Yeast (58)	Sub 2 (107)	oxi 1 (108)	Sub 3 (106)	oxi 2 (106)	Carbonic Anhydrase (9)
MW	31K	27K	26K	30K	44K	32K	29K	30K	28K	21K	30K	30K
Ala	7.2	7.2	6.5	7.6	5.7	8.9	8.5	7.0	6.0	7.9	8.2	7.3
Arg	2.2	4.1	4.0	3.9	2.8	4.2	2.4	2.5	2.0	2.8	2.6	2.7
Asx	7.5	8.6	7.9	8.6	7.4	8.3	11.4	8.2	8.8	5.9	5.2	11.9
Cys	0.9	1.2	-	-	1.0	3.7	2.8	1.9	1.6	-	0.7	0.4
Glx	3.5	7.4	4.0	6.6	3.0	8.3	7.6	9.2	10.1	5.7	4.1	8.5
Gly	6.6	7.1	7.9	6.5	6.5	12.9	10.9	14.8	4.8	6.3	7.1	6.1
His	3.1	2.4	3.4	2.9	3.2	1.2	1.3	2.1	2.0	4.3	5.6	4.2
Ile	9.4	8.0	9.4	8.1	10.6	4.4	3.4	6.5	11.6	6.8	7.1	3.8
Leu	15.7	12.0	11.1	11.5	11.2	9.7	10.2	8.5	11.6	13.0	13.4	7.7
Lys	2.7	3.8	3.1	4.4	1.8	7.4	6.2	7.2	3.2	2.6	6.7	6.9
Met	3.8	2.1	3.9	2.6	4.1	1.6	1.3	1.7	2.0	3.2	4.5	0.8
Phe	6.2	5.5	8.8	8.4	9.3	4.8	5.3	3.8	4.8	5.4	5.9	4.2
Pro	5.9	4.7	5.0	4.9	4.9	3.7	4.4	4.3	5.2	3.0	3.3	6.5
Ser	5.9	7.9	5.3	5.7	7.3	7.4	8.0	8.1	5.2	11.2	5.9	11.5
Thr	7.6	4.7	2.8	5.3	4.1	5.1	7.4	4.9	6.4	8.5	7.8	3.1
Trp	3.0	2.7	1.8	-	1.8	0.3	0.4	1.5	2.0	-	3.3	2.3
Tyr	4.0	4.0	5.4	3.4	5.4	3.4	2.9	2.4	5.6	5.2	5.6	3.1
Val	4.7	6.6	9.6	9.4	9.3	5.0	5.5	5.4	7.6	7.9	8.9	6.5
Polarity %	32.5	38.9	30.0	37.4	30.0	41.9	44.3	42.2	37.7	41.0	31.9	48.8

Table 2 - A comparison of the various cytochrome b preparations, mitochondrial translation products of cytochrome oxidase and a typical soluble protein. Amino acid values are expressed in mol% and polarity values were calculated according to the method of Capaldi and Vanderkovi(105).

A review of the technique used in assaying the two forms of cytochrome b discloses the probability of erroneous values for a glycine and isoleucine. When the purified proteins were subjected to automated Edman degradation (see results discussed below), a steady peak of glycine was seen throughout the several cycles tested. Glycine had been added exogenously during the purification but was supposedly removed at a later dialysis step. The standard error bars of figure 5 are broad for glycine unlike any of the other amino acids tested, indicative of variations in the glycine concentration from sample to sample preparation. This should result in an overestimation of glycine. From the DNA sequence peptide bonds like Ile-Ile or leu-Ile appear often. Under the hydrolyate conditions used, 12-40 hrs. at 110°C these bonds would remain largely uncleaved. It has been reported that it takes nearly 120 hrs. at 110°C to completely cleave these types of bonds. Values of isoleucine and to a lesser extent leucine may be underestimated. Even with these potential limitations in the procedure, it appears that the two forms of cytochrome b are not identical.

The pure 32 K and 29K proteins were subjected to N-terminal analysis. Dansylation and polyamide TLC was tried

first, to locate the N-terminal. When the 29K and 32K proteins were examined by this procedure only the dansylated ϵ-lysine plus asparagine and glutamine were identified on the chromatographic plate. As a control bovine insulin was tested by this procedure and both of its N-terminals were identified. Previous studies done on cytochrome b from beef heart mitochondria and on N. crassa had shown the N-terminal to be formyl methionine which is non-reactive with the dansyl reagent. All attempts to deformylate the proteins under 0.1NHCl methanol conditions were unsuccessful and a prolonged deformylation would result in hydrolysis of the peptide. After deformylation and subsequent 6NHCl hydrolysis the results were no different than had been observed in the absence of deformylation. This procedure was either incapable of identifying the N-terminal amino acid of these membrane proteins or the N-terminals are blocked by a group unreactive toward deformylation.

A second approach was tried to locate the N-terminal of these proteins, namely by automated Edman degradation. The 32K protein was subjected to automated Edman degradation and for the several cycles tested no cleavage products were found to occur. This data is indicative of a protein with a blocked N-terminal. When the 29K protein was subjected

to Edman degradation, multiple amino acid peaks were observed through all the cycles tested. No clear sequence pattern could be recognized from these results. This data is indicative of the presence of multiple N-terminals in the sample. Perhaps the multiple N-terminals are the product of non-specific changes during purification or that a number of proteins of the same molecular weight exist in the purified fraction.

The purified 29K and 32K proteins were subjected to isoelectric focusing (IEF) to investigate the homogeneity of these purified proteins. A commercially prepared set of acetylated cytochrome c markers were run as an IEF control with the purified proteins. The PH gradient had been established within 4½ hours as viewed from the control markers and the gel was allowed to run for an additional hour to ensure focusing. Under these conditions, the PH gradient , seen in figure 6, shows a deviation from the linear portion of the graph near both electrodes. The ampholines are in the 3-10 range and the control value of PI 9.2 which is near the upper limit is not in good agreement with the assayed value of PI 9.7. The 32K protein was found to contain a major band at PI 8.3 and was surrounded by two minor bands at PI 8.2, 8.4 plus 2 faint bands. The bands are represented by the arrows pointing

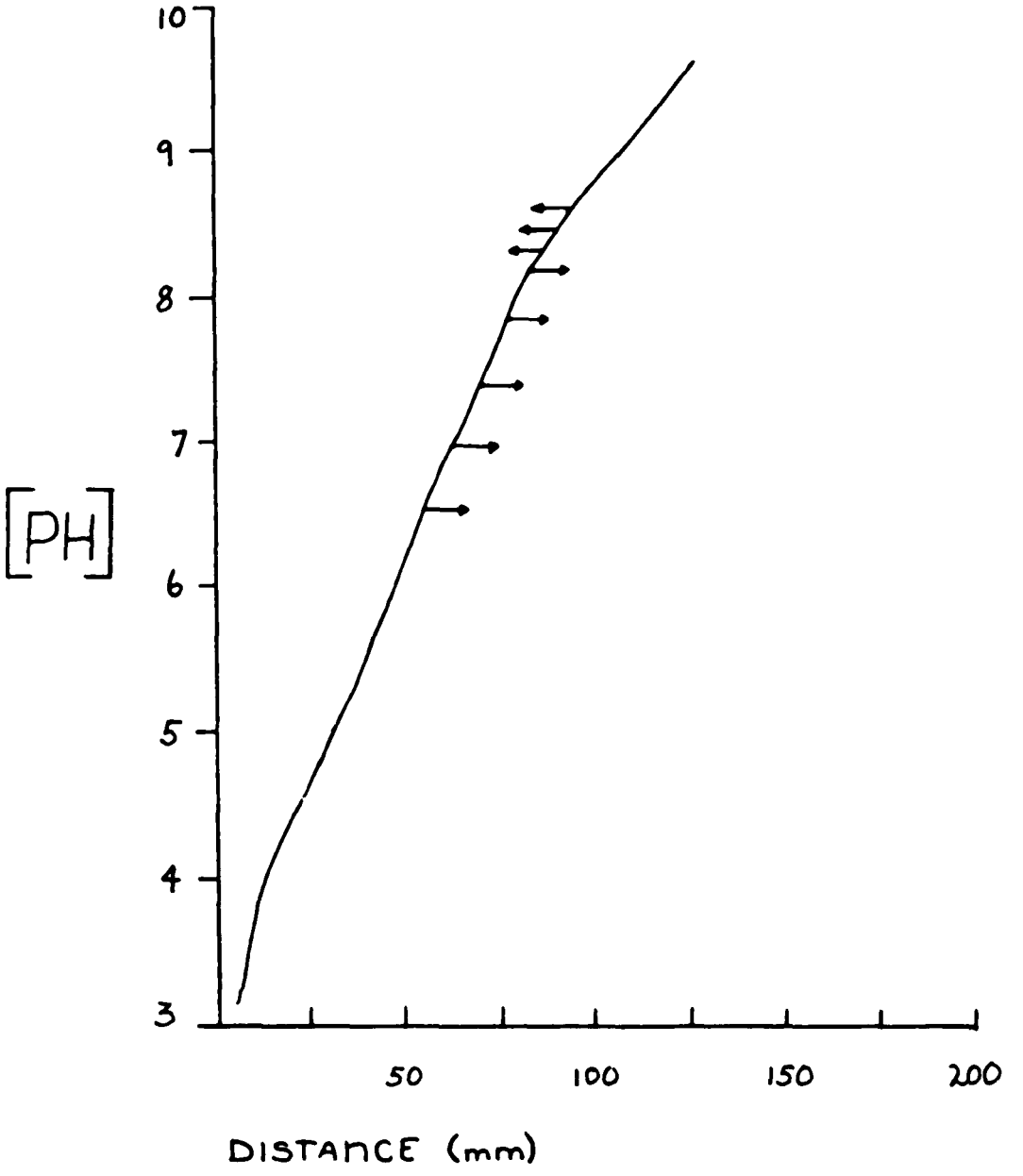


Figure 6 - The isoelectric focusing obtained for pure 29K and pure 32K proteins. The PH gradient values were obtained by cutting a series of 5mm slices on both sides of the gel, eluting the ampholines into distilled water then measuring values on a PH meter. The 5 arrows projecting to the right of the PH gradient represent the 5 bands found for the 29K protein (PI values: 6.7, 6.9, 7.4, 7.6, 7.9). The 3 arrows extended to the left of the gradient represent the bands found for the 32K protein. The middle being more intense (PI values: 8.2, 8.3, 8.4).

left. The 29K protein separates into 5 bands of equal intensity with PI values of 6.7, 6.9, 7.4, 7.6, 7.9. None of these bands coincide with the bands found for the 32K protein. These bands are represented in the figure 6 by the arrows pointing right. A photograph of the Coomassie stained isoelectric focusing gel can be seen in figure 7.

The 32K bands appear in a narrow PI range 8.2-8.4 and this type of variation is consistent with a pure protein. Linnane et al(109) previously reported a PI value of 7.9-8.0 for their cytochrome b apoprotein in vivo translation product of S. cerevisiae with a molecular weight of 32,000. Also Sidhu and Beattie(42) reported a PI of 8.3 for cytochrome b in a purified complex III from yeast mitochondria. Conversely, the five equal intensity 29K bands are spread over a much larger PI range, 6.7-8.0 and the presence of more than one type of polypeptide is therefore indicated. To investigate the possibility of different proteins being present in the 29K protein band, a comparison of cleavage products appeared necessary.

The 32K and 29K proteins were purified in an identical manner but the results obtained by cyanogen bromide cleavage indicate differences in their primary structure. The cyanogen

Figure 7 - A 1.2mm thick, 4.5% acryl-6M urea isoelectric focusing gel, run as described in the methods. The protein bands are visualized by Coomassie blue staining after the ampholines were removed by extensive rinsing. Lane 1 contains 30ug of purified 32K protein and lane 2 contains 30ug of purified 29K protein. The migration is toward the cathode, away from the injection spots. These bands correspond to the PH gradient graph seen in figure 6.

bromide cleavage patterns of these proteins are compared in figure 8. The 32K cleavage peptides, lanes 2-4, range in molecular weights between 6,000 and 32,000 daltons. Thirteen bands could be identified by densitometric scanning. Only 8% of the Coomassie blue stained peptides remained uncleaved as the 32K protein. Several bands in the same molecular weight range were found for the cleaved 29K protein lanes 6-8, which correspond with bands seen for the 32K protein (lane 4). More than 75% of the Coomassie blue stained 29K protein remains uncleaved whereas no such homologous cleavage property occurs for the 32K protein. These results infer that at least 70% of the 29K protein isolated differs from that of the 32K protein. A summary of the evidence obtained on the purified proteins as follows:

1. The results obtained by analytical SDS gel electrophoresis provide evidence that the 29K and 32K proteins are pure with respect to their molecular weights.
2. In figure 5 the number of threonine and aspartate residues are found to be greater in the 29K protein than in the 32K protein. It is logically inconsistent with the idea that the 29K protein is a direct cleavage product of the 32K protein.

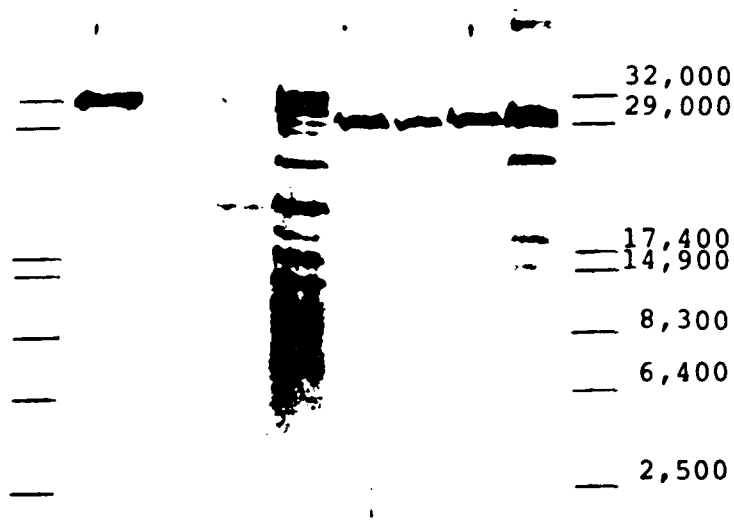


Figure 8 - Cyanogen bromide cleavage patterns for purified 32K and 29K proteins are separated on a 19% acrylamide slab gel as described in the methods. The molecular weights listed are from purified proteins 32K, 29K and the cleavage products of spermwhale myoglobin. Lanes 1 and 5 contain 10ug of uncleaved 32K and 29K, respectively. Lanes 2, 3 and 4 are CNBr cleavage products from 10, 20 and 40ug, respectively of protein 32K while lanes 6, 7 and 8 represent 10, 20 and 40ug of protein 29K cleaved by CNBr.

3. No N-terminal amino acids were detected by dansylation for either the 29K or the 32K proteins even after deformylation treatment. When samples were subjected to automated Edman degradation, no N-terminal amino acid was found for the 32K protein while the 29K protein had many amino acid peaks. These multiple amino acid peaks persisted throughout several cycles and no clear sequence pattern could be determined. Indications are that the 29K protein has multiple N-terminals.
4. When isoelectric focusing was performed, the 32K protein appeared as a major protein band at PI 8.3 with minor bands surrounding it in a narrow PI range. These minor bands could be accounted for by some modifications in the polypeptide caused by the purification procedure e.g. PMSF covalent binding. The 29K prctein has 4 to 5 bands of equal intensity spread over at least one PH unit. These results are indicative of more than one protein at that molecular weight.
5. Cyanogen bromide cleavage of the 29K protein and the 32K protein under identical conditions reveals several cleavage bands of similar molecular weight. The densitometric scans reveal that nearly 70% of the stained 29K polypeptide was not digested by CNBr whereas all

but 8% of the 32K protein was cleaved into smaller peptides.

One can conclude from this characterization of the pure 29K and pure 32K proteins that only the 32K polypeptide is pure. The 29K protein that was previously found to be antigenically related to cytochrome b is not pure and its major polypeptide is unrelated to the 32K cytochrome b apoprotein.

D. Anticytochrome b Antibody Studies

Since the 32K protein is the only pure cytochrome b isolated, the subsequent immunogenic studies were performed only on the antibody derived against this purified protein. The anticytochrome b antibody was tested against the pure 29K protein; the pure 32K protein and against SDS solubilized, then Triton diluted mitochondria. It was found by Western blotting that the antibody only reacts against a protein band at 32,000; in the spectrally pure cytochrome b stage samples; in mitochondria and to a lesser extend in SMP (see figure 9). The preimmune serum did not react with any of these antigens. This anticytochrome b antibody was tested for its ability to inhibit electron transfer through complex III of the inner mitochondrial membrane. In the inhibition studies, three different mitochondrial preparations were used, which preferentially exposed different portions of the inner membrane. A hypotonic treatment of the mitochondria will disrupt the outer membrane therefore allowing the outer surface of the inner membrane to become exposed to added antibody. These outer membrane disrupted particles are called mitoplasts (Mp). Sonication of mitochondria causes a pinching off of the cristae therefore making inner membrane vesicles. Electron micrographs(9) of these

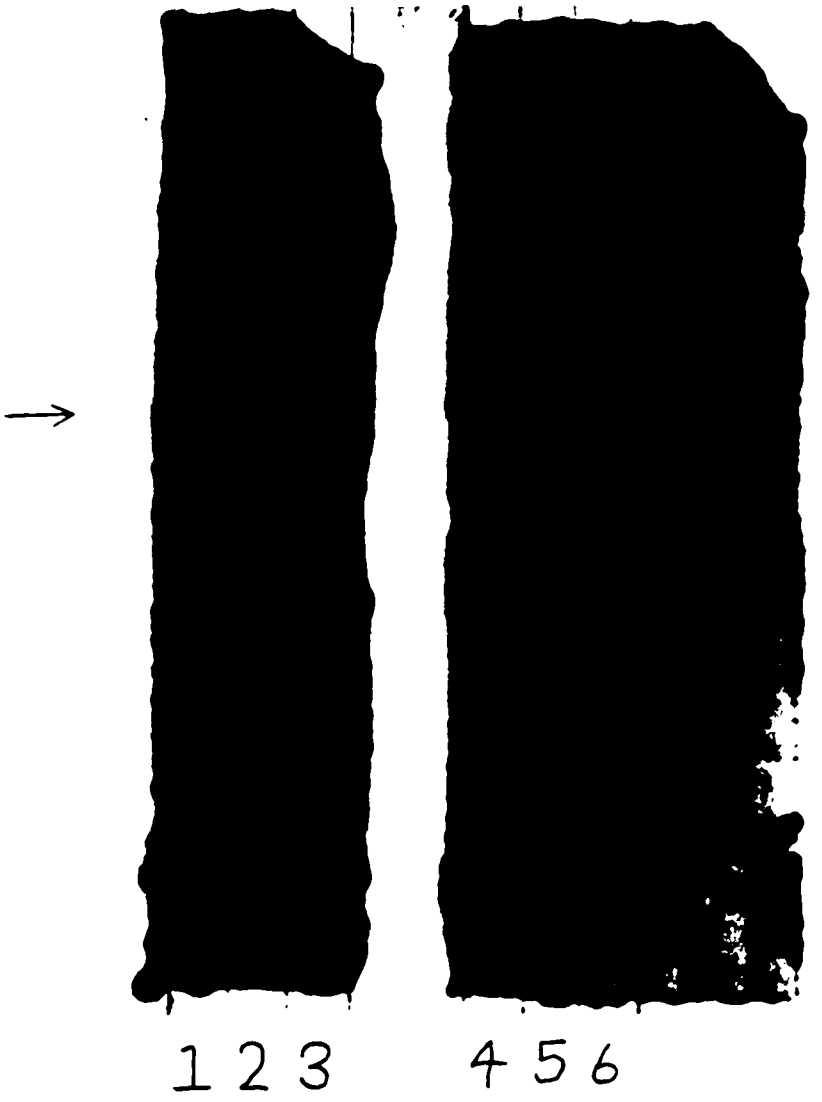


Figure 9 - Preimmune serum and anti-32K antibody were compared for antigen antibody reactivity. Both immunotransfers contain the same antigenic samples. The transfer on the left was incubated with anti 32K antibody while the immunotransfer on the right was incubated with preimmune serum. Lanes 1,4 contain 150ug of protein from the spectrally pure cytochrome b stage; lanes 2,5 contain 300ug of SMP protein; lanes 3,6 contain 300ug of mito protein. The arrow points to a M_r=32.000 band as determined by comparison with the stained portion of the slab gel.

vesicles reveals that they contain inner membrane spheres (F_1ATPase) on their surface. The submitochondrial particles (SMP) are largely "inside out" compared with the intact inner mitochondrial membrane. The third method of mitochondrial preparation was to partially solubilize the membrane by adding cholate which substitutes for the phospholipids in the membrane.

The three inner membrane orientations provide different surfaces for the reaction of antibody and defines the region on the mitochondrial supra structure where the antigenic loci may be found. The results of studies on succinate cytochrome c reductase activity are seen in table 3. The addition of antiserum was found to cause an activation of reductase activity, prior to the addition of substrate, so all inhibition studies were done with the IgG fraction of the antiserum. A maximal inhibition of 20% could be obtained by incubating this antibody with mitoplasts. Under these conditions, the inhibition of activity occurs at the lowest titers of the antibody tested and no dose dependent titration curve was observed. In contrast, submitochondrial particles were not inhibited by incubations with the anticytochrome b IgG fraction showing that no antigenic site for this antibody was found on the inside of the inner membrane. Values higher than the control were observed in the cholate assays.

Unlike the mitoplasts and SMP's, incubations with cholate cause a rapid decrease in activity. As cholate solubilizes the membrane, it disrupts the close association of the membrane components causing a loss of activity. In certain experiments a greater than 50% decrease in activity was observed for the control IgG, after only one hour of cholate incubation. The greater activity observed for cholate solubilization in table 3, may indicate that the IgG complexing to the membrane has a greater effect at maintaining membrane integrity and therefore masking its inhibitory effect.

The results of the inhibition of succinate cytochrome c reductase activity clearly demonstrate that the antigenic site of this anticytochrome b antibody lies on the outer surface of the inner membrane. Complex III contains 7-10 subunits one of them being cytochrome b. 2,3 dimethyl-5-methyl-6-n decyl-1,4-benzoquinone (DBH_2) is a reduced analog of ubiquinone and transfers its reduced equivalents directly into complex III. When succinate was used as the substrate, the presence of the complex II is required to transfer the electrons to complex III. The immunoinhibition with anticytochrome b IgG for the reduction of cytochrome c should still be seen if DBH_2 was used as the substrate instead of succinate since cytochrome b resides in complex III. To test this hypothesis both substrates were compared

% Succ. Cyt c Reductase Activity

ul IgG_{32K} per mg protein	Mp	SMP	Cholate Solub.
10	83	101	115
50	85	86	123
100	78	103	147
200	82	120	136
400	82	102	112
800	78	---	---

Table 3 - Succinate cytochrome c reductase activity is assayed after the IgG fraction of anticytochrome b serum (IgG_{32K}) is incubated with mitoplasts (Mp), submitochondrial particles (SMP) and 2% cholate solubilized mitochondria (cholate sol.). The final conc. of mitochondrial protein is 1 mg/ml with the IgG_{32K} added and after a 45 min. room temperature incubation, the assay is performed. The concomitant assays of IgG control and IgG_{32K} are compared and the % activity is reported.

in an immunoinhibition study with mitoplasts. The results in table 4 show that the 32K IgG inhibits DBH_2 to cytochrome c reductase activity in a dose dependent manner although only 18% inhibition was achieved. This clearly demonstrates that inhibition caused by the antibody must result from a protein localized in the bc_1 complex. The only other protein of similar molecular weight in complex III is cytochrome c_1 and spectral proof has been given that during the purification cytochrome c_1 was completely removed. These results demonstrate that the antibody against the 32K protein is specific for cytochrome b.

Thus far, the anticytochrome b antibody has been found to partially inhibit complex bc_1 activity and it reacts immunogenically against the purified 32K protein. It is necessary to test the antibody for cross reactivity with other proteins which might be found in mitochondrial preparations. Immunotransfer, also called "Western blotting" is the method ideally suited for this purpose. The proteins are separated on SDS gels and transferred to nitrocellulose paper where the incubating antibody will bind specifically to all the antigenic protein bands. This antigen antibody complex is next tagged with $[^{125}I]$ protein a S. aureaus cells which specifically attach to antigen antibody complexes. The results of these transfers are viewed in autoradiograms.

ul IgG_{32K} per mg Mp protein	Succ. Cyt c Activity	DBH_2 Cyt c Activity
10	95%	97%
25	92%	93%
50	90%	90%
75	94%	82%

Table 4 - Mitoplasts are assayed for inhibition of cytochrome c reductase activity using different substrates. 2,3 dimethyl-5-methyl-6-n decyl-1,4-benzoquinone (DBH_2), a reduced analog of ubiquinone, delivers its electrons directly to the bc_1 complex. Succinate donates electrons to complex II which then transfers them to the bc_1 complex. Low levels of IgG_{32K} are tested in parallel for succinate and for DBH_2 inhibition with concomitant assays of control IgG. Results are reported in percent of control activity. For assay conditions, refer to the methods section.

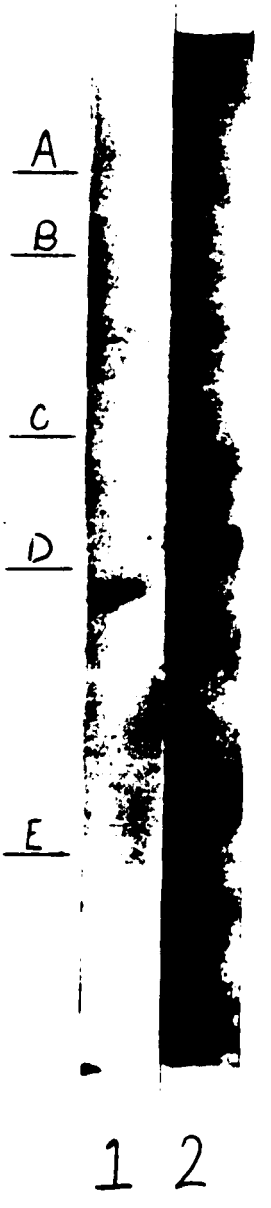
A variety of mitochondrial preparations can be tested for antigenicity by this technique. The results of one of these transfers is seen in figure 10. The anticytochrome b antibody reacts strongly with the purified bc_1 complex in lane 1 and with the pure 32K protein in lane 5 at its molecular weight of 32,000. The mitochondria sample in lane 3 and the spectral cytochrome b stage sample in lane 2 react poorly in this transfer because these samples were frozen and stored for over a month however stronger reactivity has been seen on freshly prepared samples. In figure 9 it had been shown that both of the weakly reacting samples above have a good degree of antigenicity when applied fresh. Refer to figure 11, Lane 2 for an example of another mitochondrial sample transfer. Although the pure 29K protein in figure 10, lane 4 was tested several times in other transfers, it never reacted with this anticytochrome b antibody to form an antigen antibody complex. The immunotransfer results show that whenever strong immunological responses were obtained in the transfers, only a single band at Mr=32,000 was detected using the anticytochrome b antibody raised against a protein of this molecular weight.

Figure 10 - Autoradiogram of an immunotransfer study where the anticytochrome b antibody was reacted against various antigenic preparations. Lane 1 contains purified bc_1 complex, lane 2 has a sample of the spectral cytochrome b stage of purification, lane 3 contains mitochondria, lane 4 has purified 29K protein and lane 5 has purified 32K protein. The molecular weights are obtained from a stained/destained portion of the slab gel. The molecular weights are as follows: A. phosphorylase b, 94,000; B. bovine serum albumin, 67,000; C. ovalbumin, 43,000; D. carbonic anhydrase 30,000; E. soy bean trypsin inhibitor 20,100.

Chen and Beattie had found that the anti-29K antibody was reacted with cytochrome b and caused an inhibition of activity. Under the technique of immunotransfer the older anti-29K antibody was compared to the anti-32K antibody using "spectral cytochrome b stage" samples as the antigen. From figure 11 it can clearly be seen that these antibodies react against their respective molecular weight proteins. In lane 1 the anti-29K antibody showed a single band with a relative migration of 0.549 whereas the anti-32K antibody in lane 2 had a single band with a value of 0.514. These values are significant since both sample lanes came from the same transfer and the only difference was the antibody they were incubated with. This experiment shows that the anti-29K antibody reacts with a protein that the anti-32K antibody does not recognize.

The anticytochrome b antibody was tested for its ability to immunoprecipitate cytochrome b from mitochondria. Yeast cells were grown in [^{3}H] leucine and mitochondria were isolated. The mitochondria were first dissociated with SDS than diluted with Triton buffer before addition of the antibody. The SDS modification was necessary since Triton buffer alone does not dissociate these membrane proteins well. The labelled protein products of the immunoprecipitation are seen in figure 12. Again it is clear that this

Figure 11 - Immunotransfer study of the spectral cytochrome b stage samples using antibodies against the 29K protein and against the 32K protein. The immunotransfer nitrocellulose blots were identical since they were made from the same gel. The only difference is the antibody used to detect the antigenic protein. In lane 1, the anti-29K antibody (#3288) was used. In lane 2, the anti-32K antibody (#3806) was used. Molecular weight standards are A. 96,000; B. 67,000; C. 43,000; D. 30,000; E. 21,000.



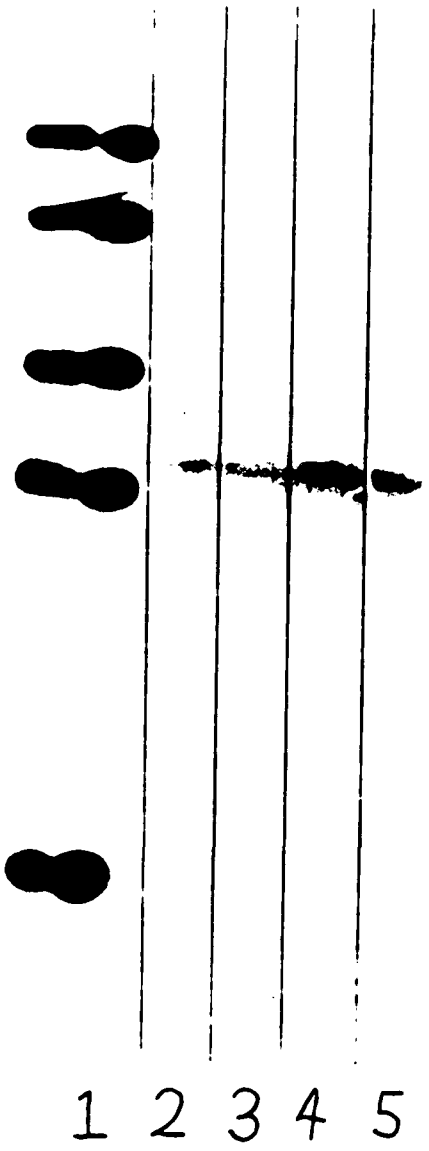


Figure 12 - An autoradiogram was made from mitochondria isolated from [^{3}H] leucine grown cells which were immunoprecipitated with anti-cytochrome b antibody. Lanes 2, 3,4, and 5 contain immunoprecipitates from 20ul, 40ul, 80ul, and 120ul of antibody/mg mito protein respectively. Lane 1 contains the molecular weight standards listed in figure 10 which have been labelled with [^{14}C]. The samples were run on 11% SDS acrylamide gels which were then soaked in 4M sodium salicylate, dried and autoradiographed.

antibody only reacts with the cytochrome b protein. A low concentration of antibody (20ul/mg mito protein) is all that was needed to immunoprecipitate the cytochrome b. These concentrations are comparable to what was used in the inhibition studies (see table 3 and table 4). From all of the studies on the anti-32K antibody it was shown that it has a specificity only for cytochrome b.

E. DEFINING A PROTEIN CLOSELY ASSOCIATED WITH CYTOCHROME b

According to the thesis work of Chen(80), the original antibodies raised against the two forms of cytochrome b, coprecipitate a protein with a molecular weight of about 55,000. This protein was believed to be an aggregated form of cytochrome b. In my studies using analytical SDS gels, the mitochondrial samples are routinely dissociated overnight at room temperature because if they are heated above 70°C aggregation of cytochrome b occurs. In these heated mitochondrial samples or in heated 32K purified protein, a new band appears at 64,000. These observations were verified in a study reported by Clejan and Beattie(110). Concomitantly no change was detected at 55,000 making it unlikely that this protein is an aggregated form of cytochrome b. It was unclear how this protein is related to cytochrome b. Could it be structurally related to cytochrome b e.g. reading into the coded intron regions of the gene? If it is just a tightly associated protein with cytochrome b, than what is its function? In either case its persistent presence warrants further investigation.

At the spectrally pure cytochrome b stage of the purifaction only three proteins were found to be in high concentration in the fraction; the 29K protein, the 32K protein

and a 53K protein, as seen in figure 2. The concentration of all these protein bands became relatively increased as one progresses through the stages of purification from mitochondria, to SMP, the the spectral cytochrome b stage. Since this 53K protein co-purifies with cytochrome b, it is of interest to investigate the possible role of this protein in the electron transport chain of the mitochondria. This protein has been recognized as a contaminant in a recently reported isolation of cytochrome b from yeast(57). The 53K protein was first purified by the same procedure used in the purification of the 29K and 32K proteins. When this 53K purified protein was tested for molecular weight purity by examination over a 10 fold range of concentration on an SDS gel, it was found to contain traces of other molecular weight proteins. Figure 13 is a photograph of an overloaded gel, to demonstrate the number of other proteins still present at this stage of purity. The partially purified 53K protein was again run through preparative gel electrophoresis, electroelution and acetone precipitation. It then appeared pure with respect to molecular weight as determined by analytical SDS gel electrophoresis. This double purified 53K protein was injected into rabbits so antibodies against the 53K protein could be obtained.

Figure 13 - A 9% SDS gel showing the impurities still present after the first passage through preparative gel electrophoresis, electroelution and the purified protein precipitation of the purification procedure. The lane on the left contains the molecular weight standards previously described. Reading from the top down they are: 96,000; 67,000; 43,000; and 30,000 daltons. The two lanes on the right contain 60ug each of this crude purification of the 53K protein.

The antigenic response of the anti-53K antibody is seen in the immunotransfer of figure 14. The control serum did not react while the anti-53K antibody shows an antigenic response with the purified 53K protein. The sensitivity of this technique is demonstrated in lane 6 of figure 14 by the fact that the purified 29K protein contains a contaminent of 53,000 which was not seen on the Coomassie blue stained gels. In lane 5 the purified 32K protein clearly does not react with this antibody. On another transfer (data not shown) the anti-cytochrome b antibody did not react with the purified 53K protein. This anti-53K antibody was also tested for its ability to precipitate protein(s) from the mitochondria. Immunoprecipitation experiments were performed on isolated mitochondria from [^{3}H] leucine grown yeast cells. The results in figure 15 show that anti-53K antibody precipitates two proteins from the mitochondria with molecular weights of 53,000 and 32,000. In the range of antibody concentration tested, the 300ul Ab/mg mito protein precipitates an appreciable amount of 53K protein while 50ul Ab/mg mito protein precipitates much less. The results from immunoprecipitation, figure 15, indicates that the anti-53K protein is not specific for the 53,000 protein but

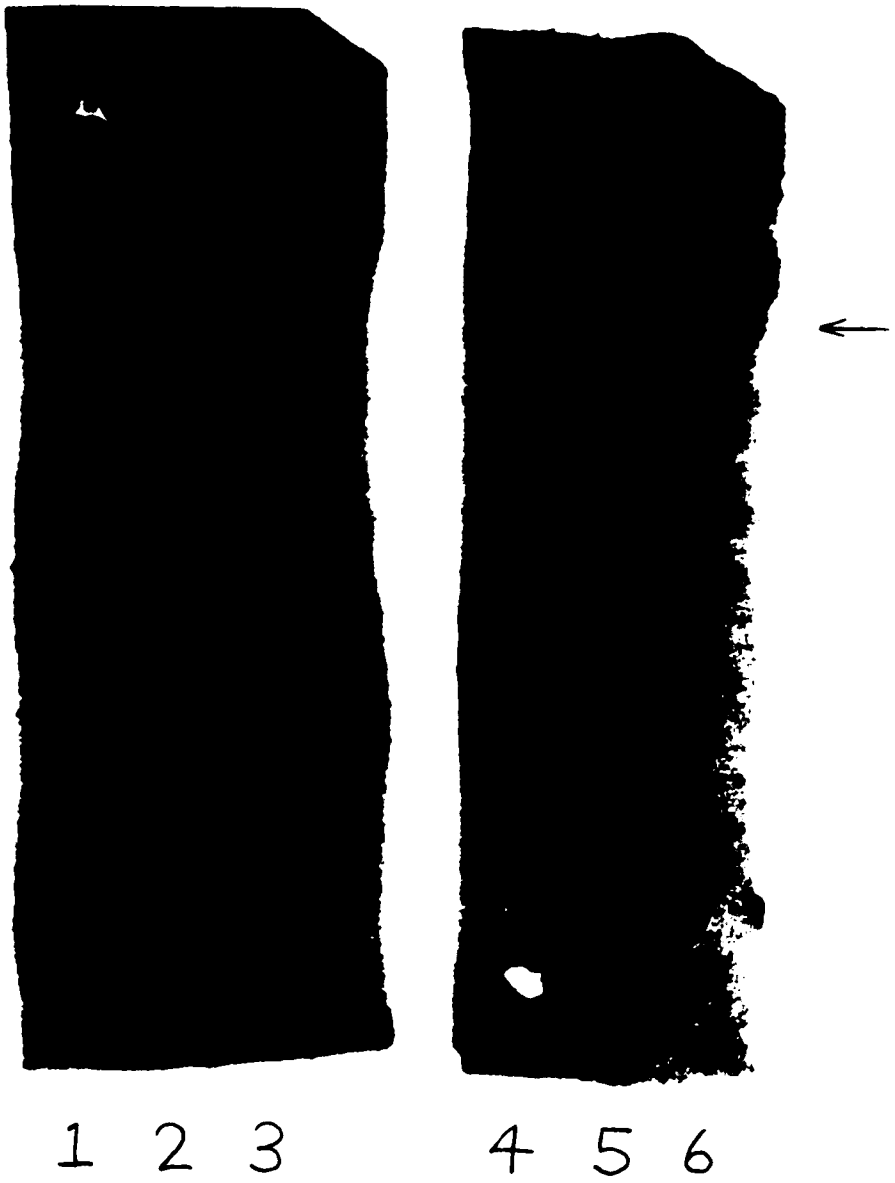
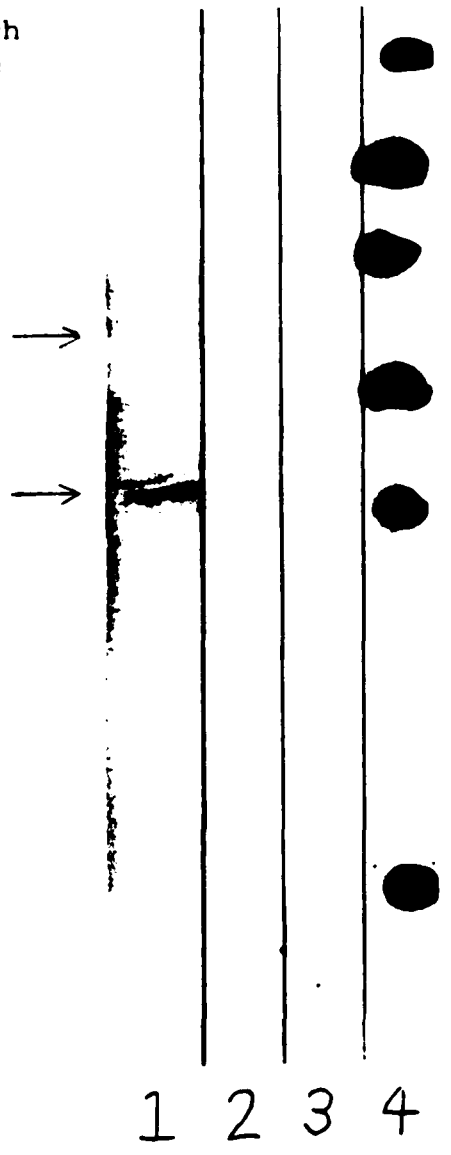


Figure 14 - An immunotransfer autoradiogram comparing preimmune serum against the anti-53K antibody. The left autoradiogram was incubated with preimmune serum while the right autoradiogram was incubated with anti-53K antibody. Lanes 1,4 contain 15ug samples of purified 53K protein. Lanes 2,5 contain 15ug samples of purified 32K protein and lanes 3,6 contain 15ug samples of purified 29K protein. The bands indicated by the arrow have an M_r=53,000.

Figure 15 - The mitochondria were isolated from [^{3}H] leucine grown cells and immunoprecipitated with anti-53K antibody. Lanes 1,2, and 3 contain immunoprecipitates from 50ul, 200ul, 300ul incubations of antibody per mg mito protein respectively. Lane 4 contains the molecular weight standards described in previous figures, which have been labelled with [^{14}C]. The samples were run on 11% SDS acrylamide gels and were then soaked in 4M sodium salicylate, dried and finally autoradiographed.



immunoprecipitates a 32,000 protein as well, possibly cytochrome b. It does not appear likely that this antibody reacts against cytochrome b because results from the immunotransfer study in figure 14 show that the anti-53K antibody does not react with the purified 32K protein. Two plausible explanations are offered. Either the 32K band observed is another protein of similar molecular weight to cytochrome b or that the 32K band is cytochrome b and its tight association with the 53K protein causes it to coprecipitate.

The final studies on the 53K protein were aimed at assigning a functional role to this protein. The anti-53K antibody was tested for the inhibition of succinate cytochrome c reductase activity under the same conditions that had been used in the anticytochrome b antibody studies. The results of these studies are seen in table 5. A dose dependent inhibition of activity was found to occur in SMP and cholate solubilized mitochondria but not in mitoplasts. The minor inhibition seen for mitoplasts must be judged as not significant due to the observed variation in results over the three times the experiment was performed. These results indicate that the 53K antibody reacts with a protein on the inner surface of the inner membrane. Since the anti-53K antibody inhibits succinate cytochrome c reductase activity,

TABLE 5

Inhibition of Succinate Cytochrome C Reductase by Anti-53K IgG

ul IgG_{53K}/mg protein	% Succinate Cyt c reductase activity		
	M_p	SMP	Cholate Sol.
10	90	96	108
50	90	84	107
100	101	81	78
200	95	71	53
400	99	52	38
800	92	--	--

Succinate cytochrome c reductase was assayed after the IgG fraction of anti-53K IgG was incubated against mitoplasts, submitochondrial particles and 2% cholate solubilized mitochondria The final conc. of mitochondrial protein was 1mg/ml with the IgG_{53K} added and after a 45 min room temperature incubation the assay was performed. The concomitant assays of IgG control and IgG_{53K} were compared and the %activity was reported.

the 53K protein is an inner membrane protein intimately involved in electron transport.

Succinate cytochrome c reductase activity spans two of the complexes (II and III) and it is necessary to assign the antigenic 53K protein to one of the complexes. DBH_2 is a reduced analog of ubiquinone which inserts between complex II and complex III. The assay in which the electrons from succinate are donated to the electron acceptor dichlorophenolindophenol(DPIP) measures succinate dehydrogenase activity and is specific for complex II. The results of these assays are seen in table 6. The succinate cytochrome c reductase activity of this experiment was measured and recorded to provide evidence that these SMP tested are similar to the previous studies done. The DBH_2 as substrate was found showing that this antibody has some effect on complex III. In comparison, a steady 50% inhibition of succinate dehydrogenase activity was found over the concentration range tested.

In the assaying of succinate cytochrome c reductase, the results are calculated after the reaction has reached a linear reaction rate. In the initial phase of the assay, the rate of cytochrome c reduction is rapid and slows as the linear phase of the reaction is reached. In the previous studies with anticytochrome b IgG, it took about 45 seconds

ul IgG_{53K} per mg SMP protein	% Succ. Cyt c Activity	$\%DBH_2$ Cyt c Activity	% SDH Activity
50	90%	--	48%
100	83%	--	49%
200	67%	--	60%
400	52%	94%	53%
600	33%	90%	--

Table 6 - Three types of electron transfer activities were compared. All assays are described in detail in methods and materials. The anti-53K IgG fraction was incubated for 45 minutes at room temperature using varying amounts of IgG. At a concentration of 1 mg/ml SMP in phosphate buffer, 20ul aliquot of sample were assayed for the various activities. These results are obtained from a single experimental preparation and control assays were performed on the same incubated samples.

to reach the linear reaction rate. When the anti-53K IgG was compared, a lengthening of this initial phase occurred in a dose dependent manner (see the graph in figure 16). At the higher doses, it took greater than 3 minutes to reach linear phase. Since this anti-53 inhibits SDH activity, it is probably a polypeptide of succinate dehydrogenase in yeast. These results are unlike what had been found in beef heart (18) where the SDH portion of complex II contains two subunits, one at 70,000 and the other at 27,000. Others working on the isolation of the bc_1 complex from beef heart and S. cerevisiae have had difficulty removing all of the SDH activity from their preparations, providing evidence for how tightly bound SDH is to the bc_1 complex. In a similar manner, the minor inhibition of electron transport through complex III that was observed in the DBH_2 study can be explained by the tight association SDH has with the bc_1 complex.

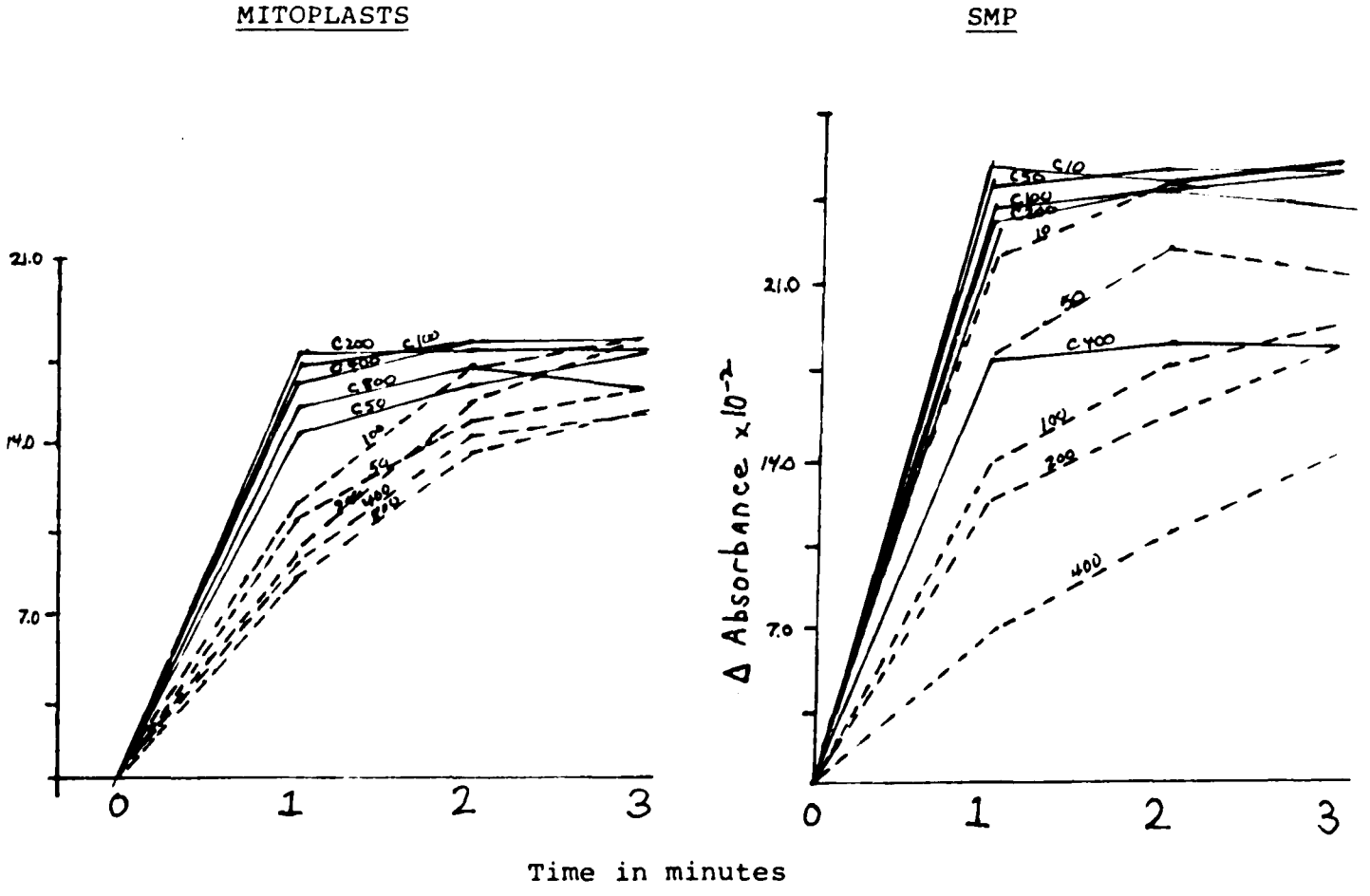


Figure 16 - The initial rates of succinate cytochrome c reductase activity incubated with anti-53K antibody were studied. Mitoplasts and SMP were incubated at 1mg/ml conc. with varying amounts of control IgG and anti-53K IgG.The number above each plotted line (solid for control IgG and dashed for anti-53k IgG) correspond to the amount of IgG used ul/ml in the incubation mixture. the reaction was started by the addition of succinate using 30 ul of incubation mixture in each assay. The absorbance was recorded immediately and graphed as ΔA/min.

DISCUSSION

The four respiratory chain complexes in the inner mitochondrial membra are responsible for the transfer of the reducing equivalents produced during metabolism to molecular oxygen. How these complexes participate in the transfer of hydrogen ions across the membrane, has yet to be determined. It is believed that four protons are released during the electron flow from ubiquinone to cytochrome c (111,112). Ubiquinones and flavoproteins transfer $2H^+$ and $2e^-$ while cytochromes and FeS protein transfers $1e^-$. The transfer of electrons from the two redox equivalent hydrogen ion carrier, namely ubiquinone to the one redox equivalent electron carrier, namely cytochrome b, takes place in complex III.

In this study, emphasis has been placed on the study of the two forms of cytochrome b. Studies by Papa et. al. (113) describe an anisotropic location of the two forms of cytochrome b, b_{565} being nearer the cytosolic side whereas b_{562} is nearer the matrix. He has also reported that b_{565} oxidation is faster than b_{562} but when the ΔPH is collapsed by nigericin, the b_{562} oxidation is faster. The heme moiety of cytochrome b appears buried in

the membrane (112) and DABS labeling studies have shown that cytochrome b transverses the inner membrane (80). In my studies, the inhibition of cytochrome c reductase activity by the anticytochrome b antibody only shows an inhibition from the cytoplasmic side. It has yet to be clearly established if two forms of cytochrome b do exist or if conformational constraints, brought on by different energy states are responsible for the observed two forms of cytochrome b. Although certain authors have claimed to have isolated these two forms, no assignment of function has yet to be made for either of the isolated forms. For this type of a study on function, the absolute purity of the antigenic protein and an absolute specificity of the antibody are a necessity. From my results, it was apparent that a study using the above criteria could not be done on the two forms of cytochrome b isolated by Chen et.al. (58).

The previous antibodies made by Chen were shown not to be specific for the cytochrome b protein. Additional protein bands were seen at 44,000; 58,000; 21,000 as well as its characteristic 31,000 cytochrome b band (80). My initial studies were to define the purity of these cytochrome b antigens and to test the specifity of the antibodies raised against them. It was concluded from this study that the 29K

antigen was not pure, therefore no new antibody was made against it. The 32K antigen was determined to be pure so a new antibody was raised against it. Immunotransfer and immunoprecipitation studies on this new antibody showed that it was reactive only with the 32K protein. During my studies, it was recognized that this 32K antibody does not cross-react with the purified 29K protein, a contradiction to what was observed in the work of Chen. One of the recognized problems with the original procedure occurs during the column chromatography step, when the purification sample sat nearly two weeks in SDS buffers, often at room temperature. One plausible explanation could be that during this period of time, the cytochrome b protein was partially degraded, causing antigenicity to appear in other lower molecular weight protein bands. Additional evidence presented here which favors the 32K protein as being the only cytochrome b is that only a specific increase in the 32K band was observed under growth conditions in lactate.

This modified purification led to the isolation of a pure cytochrome b determined by the following criteria; it was pure in molecular weight; it has a narrow P_I range with a value comparable to other published reports on cytochrome b from yeast and to it was raised a specific antibody which only immunoprecipitated a protein of the same molecular weight.

Other work performed in this study was geared toward identifying the N-terminals of these two forms of cytochrome b for comparison purposes. From this study, it was recognized that the mature cytochrome b at 32K was blocked at the N-terminal. One of the problems recognized with the deformylation technique used was that the cytochrome b apoprotein precipitated from solution, therefore making this procedure highly ineffective. Perhaps a future study using an alternate procedure e.g. 0.1M piperidine (114) may be more effective with this cytochrome b apoprotein if its solubility can be maintained. The amino acid composition data and the Edman degradation data indicates that varying amounts of glycine were found in different preparations of the purified protein. Any future work on the primary structure would require the use of phosphate buffer or barbital buffer, rather than the tris-glycine buffering system used during the electrophoresis and electroelution steps in the purification. Cyanogen bromide is generally believed to be specific for the cleavage at the methionine residues however, it will also cleave at cysteine residues at a slower rate. In this study, CNBr cleavage was used as an analytical tool to compare two polypeptides with supposed sequence homology. This procedure could possibly be optimized to fragment the polypeptide by changing the PH and/or by adding N-ethylmaleimide

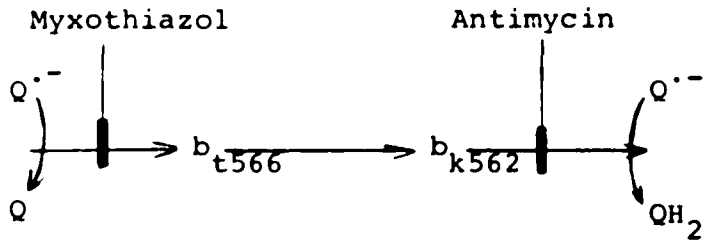
to block the available sulfhydryl groups of cysteine. With some of these modifications, I believe an attempted sequencing of parts of the purified cytochrome b protein could be accomplished.

In cardiac mitochondria, it was found that the bc_1 complex requires the addition of succinate dehydrogenase, a ubiquinone binding protein and phospholipids for reconstitution of the succinate cytochrome c reductase activity (115). In beef heart, succinate dehydrogenase consists of two subunits, one with a molecular weight of 70,000 and the other of 27,000. In yeast, a protein of 53,000 daltons was shown to be a succinate dehydrogenase subunit on the basis of immunoinhibition studies. The tight association of cytochrome b and this 53K protein was established by the copurification of both proteins and by the recent isolation of a pure cytochrome b with a 53,000 contaminant (57). When an antibody was raised against this purified 53K antigen, two proteins were immunoprecipitated, the 53K protein and the 32K protein. This coprecipitation is believed to be due to the tight association between these two proteins rather than as an immunogenic response because the antibody to the 53K protein does not react with purified 32K protein. It appears that *in vivo* cytochrome b must be closely associated with this 53K succinate dehydrogenase subunit.

The succinate cytochrome c reductase complex isolated from bovine heart mitochondria manifests a prominent g=2.00 EPR signal attributable to ubisemiquinone (15,21). The ubisemiquinone appears to be heterogeneous, consisting of at least two populations of stable semiquinone which can be distinguished on the basis of their different thermodynamic and EPR characteristics and their differential response to antimycin. In addition to the ubisemiquinone in proximity to succinate dehydrogenase, it was proposed that a second site exists for binding of ubisemiquinone, which gives rise to a more slowly relaxing EPR signal being abolished by antimycin binding (116). By using mutants of cytochrome b and antimycin resistant mutants, it was shown that binding is significantly less in cytochrome b mutants and is proportional to the amount of spectral cytochrome b observed. Antimycin is therefore believed to bind to the apoprotein cytochrome b (117). Wikstrom and Berden (118) proposed that the oxidation-reduction poise of the ubisemiquinone couples ($Q/QH^{\cdot}$ and $QH^{\cdot}/QH_2$) may determine the apparent potential of the b cytochromes.

The formation of semiquinone is a prerequisite for the transfer of electrons through QH_2 cytochrome c reductase. DeVries et.al. (119) describes a specific binding site for the antimycin sensitive semiquinone anion $QH^{\cdot}$ with a bound

E_m=84mV. Mucidin resistant mutants show an extra reduction of cytochrome $\underline{b}_{565}$ when induced by mucidin (120). These mutations have been localized to three different exon regions of the cytochrome $\underline{b}$ gene. A red shift maximum of 568 nm has been seen when the inhibitor myxothiazol is used whereas antimycin causes a shift to 564 nm. Myxothiazol seems to bind directly or in the close vicinity of the b_t center (b_{566}) whereas antimycin binds directly or near to the b_k center (b_{562}) (121). A proposed model linking the semiquinones and the two forms of cytochrome $\underline{b}$ would be as below:



The resolution and reconstitution of the iron sulfur protein has established unequivocally that it is required for electron transfer within the bc_1 complex (122). The iron sulfur protein is required for reduction of cytochrome $\underline{c}$, by succinate and, specifically in the presence of antimycin, is required for reduction of both cytochrome $\underline{b}$ and cytochrome $\underline{c}_1$. If cytochrome $\underline{c}_1$ is reduced before the addition of succinate and antimycin is present, succinate is unable to reduce cyto-

chrome b. Upon oxidation of cytochrome c_1, cytochrome b then can be reduced. The use of 5-n-undecyl-6-hydroxy-4, 7 dioxobenzothiazole (UHDBT) inhibited this oxidant induced reduction of cytochrome b by preventing the electron transfer from the iron sulfur cluster to cytochrome c_1 (122). In another study, 2,3 dimercaptopropanol (BAL), which blocks FeS reduction, was used with similar results to the above (123). The role of the oxidized FeS protein is to produce the reductant for cytochrome b by oxidation of QH_2 and by the fact that when QH_2 is bound to it, the reduced FeS protein cannot be oxidized by its natural oxidant, cytochrome c_1.

Complex III in the inner membrane is capable of regulating electron flow in the electron transport chain. One state of semiquinone appears to be controlled by the FeS protein while the other semiquinone form is reacting in the proximity of the SDH and cytochrome b association region and implicated in the presence of the SDH iron sulfur cluster S_3. The two forms of cytochrome b respond at different potentials, each being activated by the electron flow through the membrane. In this study, no proof has been offered for identifying the physical differences in the two forms of cytochrome b. It is recognized that these two forms of cytochrome b do occupy different locations. Perhaps my specific anticytochrome b antibody may show a preference for one of the locations.

A study using this antibody and the bc_1 site inhibitors of electron transport may offer a better understanding of the different sites of cytochrome b. The close association between the SDH-53K subunit and cytochrome b make a similar study using anti-53K antibody another possibility. This region is important because the region of association between SDH and cytochrome b has been postulated to be the site where one of the semiquinone forms transfers an electron to cytochrome b.

Cytochrome b appears to be a major protein involved in mitochondrial regulation. It has been linked to the proton transfer of complex III. It collects electrons from complex I substrates, complex II substrate and from fatty acids through ubiquinone. As the first one electron carrier in the respiratory chain, it regulates the electron flow through the chain to molecular oxygen. Regulation is also seen at the biogenesis level since the proper assembly of the bc_1 complex requires the presence of cytochrome b. It is the only mitochondrial translation product in the bc_1 complex and its apoprotein assembles in the membrane, even in the absence of heme (124). As described earlier, the FeS protein requires the presence of cytochrome b in the membrane as a prerequisite for the assembly of the FeS cluster (15). A continuing study of the cytochrome b structure and its associations with other proteins will help

to develop a clearer understanding of its function in the electron transfer chain.

ABBREVIATIONS

ATP	adenosine-5-triphosphate
Ag.Ab	antigen antibody complex
BSA	bovine serum albumin
bis-acryl	N,N'methylene-bis-acrylamide
CCCP	carbonyl cyanide m-chlorophenylhydrazone
Cyt ox	cytochrome oxidase
DBH_2	2,3 dimethyl-5-methyl-6-n-decyl-1,4-benzoquinone
EDTA	ethylenediaminetetraacetate
HEPES	N'-2-hydroxyethylpiperazine N'-2-ethane sulfonate
IEF	isoelectric focusing
M_p	mitoplast
M_r	relative molecular weight
mt DNA	mitochondrial DNA
NBCS	new born calf serum
NEM	N-ethylmaleimide
NAD^+	oxidized nicotinamide adenine nucleotide
PMSF	phenyl methyl sulfonyl flouride
PITC	phenylisothiocyanate
PTH	phenylthiohydrazone
SDS	sodium lauryl (dodecyl) sulfate
SMP	submitochondrial particles
TEMED	N,N,N',N',tetramethylethylenediamine
tris	hydroxymethyl aminomethane

Abbreviations contd...

TLC	thin layer chromatography
TCA	trichloroacetic acid
TLCK	Nα-p-tosyl-l-lysine chloromethyl ketone
TPCK	1-1-tosylamide-2-phenylethyl chloromethyl ketone
URF	unidentified reading frame

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