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Rice University

Ph.D. 1982

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RICE UNIVERSITY

CHEMICAL AND PHYSICAL CHARACTERIZATION OF COMPLEX III; THE OXIDATIVE REACTION MECHANISM

by

AH-LIM T'SAI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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HOUSTON, TEXAS
DECEMBER 21, 1982
"This thesis is a dedication to

my wife and my parents"
ABSTRACT

(1). A comparison of model heme compounds with isolated and with mitochondrial cytochrome b using MCD and EPR showed that the unusual coordination structure of the cytochrome b heme(s) can be represented by hindered bis-imidazole protoheme.

(2). A simple, high-yield purification procedure for cytochrome b from yeast Complex III has been developed involving solubilisation and chemical modification of the lysine residues with 3,4,5,6-tetrahydrophathalic anhydride followed by hydroxyapatite column chromatography. This product has a heme content of 37 nmol of heme b/mg protein and a molecular weight on SDS gels of 25,000-26,000. Amino acid analysis indicates high hydrophobicity and is comparable to the composition deduced from the gene sequence. The latter data indicate a molecular weight of 44,000 for the polypeptide; our heme analyses thus implies the presence of two hemes per polypeptide chain. Optical and MCD spectra are typical of a low-spin b-type cytochrome. MCD-potentiometric titration indicates a one-electron carrier with a single midpoint potential of -44 mV at pH 7.4 and 25°C. The EPR spectrum of isolated cytochrome b has only one g1 signal at 3.70, indicating that the 'strained' heme structure is still maintained.

(3). Potentiometric measurements have been performed on Complex III. The midpoint potentials for the b and c1 cytochrome were measured using MCD and EPR. A value of 270 mV was obtained for
cytochrome $c_1$, regardless of temperature, while the midpoint potentials found for the two species of cytochrome $b$ varied with temperatures, viz. 62 and -20 mV at 23°C (MCD) compared to 116 and -4 mV at about 10 K (EPR). The midpoint potential of the iron-sulfur center obtained by low-temperature EPR was 286 mV. The potentials of the two half reactions of ubiquinone were measured by following the semiquinone radical signal by EPR at 110 and 296 K. Potentials of 176 and 51 mV were found at the low temperature, while values of 200 and 110 mV were observed at room temperature.

The midpoint potential of cytochrome $c_1$ was found to be pH independent. The potentials of cytochrome $b$ were also independent of pH when titrations were performed in deoxycholate buffers, while a variation of -30 mV per pH unit was observed for both $b$ species in taurocholate buffers. These two detergents also produced different MCD-contributions of the two $b$ hemes.

Antimycin-A appears to induce a ubiquinone-mediated transition of the low potential $b$ heme to a high potential species.

(4). The oxidative reaction mechanism was investigated through the reoxidation of 2-electron, 4-electron (CoQ-depleted) and fully-reduced Complex III by ferricyanide.

The nonlinear first-order plots of the oxidation of $c_1$, were obtained for the reaction of 2-electron reduced sample at all tested ferricyanide concentrations. These data were explained by an extremely rapid intramolecular electron equilibration ($>2000$ s$^{-1}$) between the iron-sulfur center and cytochrome $c_1$. Neither antimycin-A nor CoQ
had a substantial effect on the reoxidation kinetics of $c_{1}$. The oxidation of both $b$ and $c_{1}$ cytochromes of 4-electron reduced and fully-reduced samples monitored by the absorbance changes at 561.5 and 553.5 nm exhibited multiphasic kinetic data. The absorbance change at 553.5 nm was preceded by that at 561.5 nm during the first reaction period, indicating a fast depletion of electrons from cytochrome $b$ through $c_{1}$ (or iron-sulfur center) to ferricyanide. Satisfactory simulation for the kinetic data collected at both wavelengths was achieved by a linear scheme:

$$
slow \quad fast \quad fast \quad k_{ox} \\
{b}_H \rightarrow {b}_L \rightarrow \{2Fe/2S\} \rightarrow c_{1} \rightarrow \text{oxidant}
$$
ACKNOWLEDGEMENTS

I want to thank Dr. Palmer for his constant advice and numerous inspirational ideas on my research during last four and half years. His assistance on the instrumentation and computer systems was a tremendous help.

Special thanks should be given to Dr. Olson who always provides me a pleasant research atmosphere. His brilliant suggestions on my kinetic studies, and his patience and consideration during the experimentations are deeply appreciated.

I will always remember those who helped me when I needed it: Dr. Schroepfer, Dr. Carter; and those who offered me excellent advices and technical assistance: Dr. Porras, Dr. De la Rosa;........etc.
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CHAPTER 1  INTRODUCTION

1. General historical discussion

In 1884 an Irish physician's discovery opened a new scientific field now known as "Bioenergetics". Using a light microscope, MacMunn observed histohematins and myohematins (i.e., cytochromes) in five classes of chordata (1). However, MacMunn's misinterpretation of his discovery led to Keilin's receiving the major credit for characterizing the function of cytochromes (2).

The extensive investigation of the oxidation-reduction properties of cytochromes and other nonheme components in the early 20th century resulted in the concept of substrate-oxidation by the respiratory chain. The theory was first formulated in the 1920's after much contention between Warburg and Keilin concerning the functions and interrelationships of dehydrogenase, oxidase and oxygen (2). Ball in 1938, determined the oxidation-reduction potentials of the cytochromes, placing the cytochromes in the order of b (-0.04 V) → c (0.27 V) → a (0.29 V) (3). As the techniques of biochemical and biophysical analysis were gradually improved, the relative positions of cytochrome c₁ (13, 14), cytochrome a₃ (16), succinate dehydrogenase (15) and NADH dehydrogenase (17) in the respiratory chain were confirmed. Crane et al. (18) found Coenzyme Q is necessary for electron transfer and Kroger and Klingenberg determined the redox potential of Coenzyme Q to be close to that of cytochrome b (19). The modern schemes of the electron transport
system were then proposed. A typical example is the one presented by Palmer in 1978 as shown in Scheme 1.

Of equal importance to the respiratory chain is the concept of "energy coupling", the reversible energy transformation between electron transport and oxidative phosphorylation. The energy-coupling phenomenon, i.e. the occurrence of phosphorylation in the respiratory chain, was first recognized in 1930(11). The crossover response from reduction- to oxidation-steady state of specific respiratory components, (e.g. NADH dehydrogenase and cytochrome b) in response to the transition from resting State 4 (deficient in ADP) to the active State 3 (in the presence of abundant ADP) (21) and ATP-linked apparent midpoint potential changes of the cytochromes in mitochondria (22) further evidenced this energy coupling apparatus.

During the last several decades, scientists in the field of bioenergetics faced two major questions along with the discovery and characterization of "new" oxidation-reduction components of the respiratory chain:

(1) How are electrons transferred from one redox component to the other?

(2) What is the mechanism of energy-coupling on a molecular level?

To address the first question, two prevalent hypotheses of electron transfer are currently investigated; namely, "electron tunneling" (23) and "outer-sphere electron transfer" due to orbital overlap (24). These lines of research require prodigious mathematical and physical calculations and are not the intention of my research. In
SCHEMATIC REPRESENTATION OF MITOCHONDRIAL ELECTRON TRANSPORT PATHWAY (1978)
considering the second question, three main theories have evolved since 1930, viz, the chemical, conformational and chemiosmotic theories. According to the chemical hypothesis offered by Slater (25), energy transformation and transmission from substrate oxidation to ATP formation involve the formation of high energy intermediates. The second hypothesis introduced by Boyer (26) stated that energy coupling is completed through a series of high-energy conformations of membrane-bound proteins. The third hypothesis, originally proposed by Mitchell (27), stated that the energy coupling mechanism operates by means of a vectorial proton translocation through an "insulating" membrane. Although the first two possibilities have not been excluded, they are currently less popular due to lack of direct experimental evidence. The chemiosmotic theory is appealing because of a large body of corroborating experimental evidence and has the following attractive features:

(a) The asymmetrical arrangement of the electron-transport components across the membrane. This "sidedness" is essential to the vectorial proton movement.

(b) The independent function mechanism of the respiratory chain and the proton-pumping ATPase. This point is in sharp contrast with the other two hypotheses both of which require direct physical contact between the electron-transport components and energy-coupling device.

The great impact of Mitchell's hypothesis is echoed by the avalanche of research literature trying to prove or disprove his theory. Currently, most of the available evidence supports the two
main features of the chemiosmotic hypothesis. It has been observed that the energy-transducing components in the membrane can reversibly generate a proton gradient and a membrane potential across the membrane; energy transfer between energy-transducing components can also occur through a proton gradient and/or a membrane potential. A clear picture of a coupled electron-transfer system and ATP formation will not emerge until the structure and chemical mechanism of individual energy-transducing components as well as their relationship in the membrane have been elucidated. This is the major goal of my studies of Complex III (bc1 complex or ubiquinol-cytochrome c reductase) associated with the second energy-coupling site in the inner mitochondrial membrane.

2. Molecular properties:

The knowledge on the molecular properties of Complex III was mainly accumulated from studies on the isolated protein complexes. Complex III is a physically homogeneous segment isolated as a functional unit of electron transport. Further degradation of this Complex results in irreversible modification (28). Extensive studies on mammalian Complex III have been carried out for more than twenty years. It was first isolated from beef heart (4) by detergent and salt extraction. Complex III isolated from yeast (5,6) has been available only recently. The mitochondrial structures of yeast are similar to those of mammalian cells, and the possibility of obtaining mutants and isotope-incorporated samples makes yeast a more attractive tool for
bioenergetic studies.

Yeast Complex III was first prepared by Katan et. al (5) using antimycin-A, a strong inhibitor for Complex III, to maintain the integrity of the isolated Complex III; the presence of this inhibitor resulted in an enzymatically inactive preparation. Reed and Hess (6) prepared an active Complex III with 8 nmole total heme per milligram protein. Siedow et. al (7) in this laboratory prepared active Complex III containing 14 nmole heme per milligram protein from both Fleishman and Red Star yeast. The purification procedure involved cell breakage by mechanical milling, detergent solubilization and ammonium sulfate fractionation. An outline of Siedow's procedure is indicated in Fig. 1. The molecular properties of this preparation and comparison with other Complex III preparations will now be discussed.

a. Molecular weight of the subunits of Complex III:

There are ten polypeptide subunits in yeast Complex III as estimated by SDS gel electrophoresis. The apparent molecular weight of each subunit using 12.5% acrylamide gel under the electrophoresis conditions of Laemmli(8) are 47.5K (subunit I), 42.7K(II), 32.7K(III), 32.7K(IV), 22.4K(V), 17.6K(VI), 14.5K(VII), 11.5K(VIII), 7.9K(IX) and 5.8K(X). The double bands at the position with an apparent molecular weight equivalent to 32.7K can be resolved to 32.7K and 38.1K by using a 16% gel system. These values are very similar to the data reported by Katan (5) and that of Reed and Hess (6) for their preparations of yeast Complex III. The relative molecular weights of the first four subunits appear to be very consistent compared to the data of
Fig. 1.1

A flow chart of the purification procedure of yeast Complex III.

This purification procedure involves cell-breakage by mechanical milling, detergent solubilization and ammonium sulfate fractionation. The whole purification procedure was carried out at 4-8°C. The critical figures of different steps are enclosed in the parentheses.
Yeast (24 lbs)
  ↓
Cell breakage (7.5-8K psi)
  ↓
 2 Centrifugations (4500 x g)
  ↓
Submitochondrial Particles (~90 g)
  ↓
Cholate Extraction (1.6%)
  ↓
Ammonium Sulfate (20%)
  ↓
First day .................................................................
  ↓
AmSO₄ (35%), 18500 x g, 40 min.
  ↓
Cholate Extract
  ↓
AmSO₄ (45%), 18500 x g, 30 min.
  ↓
(Scrub Complex IV)
  ↓
S₁
  ↓
AmSO₄ (~55%), Spin
  ↓
Crude Complex II & III
  ↓
Resuspension
  ↓
2 AmSO₄ (40% & 45%), Spin
  ↓
P₂
(Scrub Complex II)
  ↓
S₂
  ↓
AmSO₄ (55%), Spin
  ↓
Crude Complex III
  ↓
Second day ...............................................................
  ↓
Change buffer, remove salt
  ↓
2 washes & Spin
  ↓
(100K x g, 3.5 hr)
  ↓
Complex III (4 ~ 5 g)
mammalian system and Neurospora (9). The assignment of these subunits, however, is controversial. The two subunits having largest molecular weights are generally assigned to Core proteins I and II. These two subunits have no known redox functions; core protein I was suggested to be a gel artifact (10), an aggregation of low molecular weight subunits.

All assignments of subunits to electrophoresis bands are ambiguous save the 31-33K band of cytochrome $c_1$, which fluoresces. The iron sulfur protein was determined to have a molecular weight of 25,000 in mammalian systems as well as Neurospora (29,30). The identification of the iron-sulfur protein band in yeast Complex III is uncertain in all three yeast preparations (5,6,7) and is subject to ongoing research.

Cytochrome b exhibits anomalous behavior on SDS gels (56,142). Resistance to protease digestion by cytochrome b indicates that this subunit is band III which exhibits a molecular weight of 32.7K on 12.5% gel and 38K on 16% gel; both values are different from that of the purified protein, i.e. 25-26K (31, and see Chapter 3). The five subunits having molecular weights lower than 20K are unassigned and are of unknown function.

b. Chemical composition and stoichiometry:

The chemical composition of purified yeast Complex III is summarized and compared with other Complex III preparations in Table 1. The cytochrome b:iron-sulfur protein:cytochrome $c_1$ ratio is very close to 2:1:1. Guanine/cytochrome $c_1$ ratios as high as 1.5 have been measured (7). The total heme content is clearly higher than the other
Table 1. Composition of Complex III from yeast and beef heart mitochondria
(Data shown below are the most pure preparations)

<table>
<thead>
<tr>
<th>Components</th>
<th>Siedow et.al. (yeast)</th>
<th>Katan et.al. (yeast)</th>
<th>Hess et.al. (yeast)</th>
<th>Rieske (beef heart)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Composition nmole/mg</td>
<td>Ratio equiv/molecule c₁</td>
<td>Composition nmole/mg</td>
<td>Ratio equiv/molecule c₁</td>
</tr>
<tr>
<td>Cytochrome c₁</td>
<td>4.6</td>
<td>1.0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>9.3</td>
<td>2-2.1</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Total iron</td>
<td>26.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe/S protein</td>
<td>4.6-6.3</td>
<td>0.8-1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Excess iron</td>
<td>3.4</td>
<td></td>
<td>15</td>
<td></td>
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<tr>
<td>CoQ</td>
<td>5.8</td>
<td>1.15-1.25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavin</td>
<td>0.1</td>
<td>SDH contamination</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
yeast preparations (5,6). The ratio of the oxidation-reduction components are comparable to those reported for the beef heart preparation (33). Chemical analysis of iron and sulfur together with double integration of the ascorbate-reduced Complex III EPR signal, with ferredoxin as standard, indicate a two iron, two sulfur cluster in the iron-sulfur protein. The excess amount of non-heme iron is believed to be associated with the g=4.3 rhombic iron resonance. Two cytochromes b, cytochrome c₅₅₃, an iron-sulfur cluster and ubiquinone are the oxidation-reduction centers identified in yeast Complex III and the structure of the relevant prosthetic groups are shown in Fig.2. The stoichiometry of these centers is further supported by a combined optical-EPR reductive titration. As shown in Fig.3, there are 7.5 reducing equivalents consumed per mole of Complex III, this number is only one greater than that expected from 5 redox centers (3 hemes, 1 iron-sulfur cluster and 1.25 equivalent of CoQ). The additional equivalent can be accounted for by the content of extraneous iron.

This Complex III preparation has very little contamination of Complex II (with an succinate dehydrogenase-phenazine methosulfate reductase activity<0.05 units/mg at 25°C) or Complex IV by a cytochrome c oxidation assay (k<0.3 min⁻¹/mg). Eighty percent of the flavin associated with these purified particles is acid-extractable, in contrast to the covalently-linked flavin found in succinate dehydrogenase.

c. Spectral properties:

(a) Optical spectra:

The absolute absorption spectra of the oxidized and reduced forms of
Fig. 1.2

Chemical structures of the oxidation-reduction prosthetic groups found in Complex III. The stoichiometry among these redox centers is:

\[
b: c_1 : [Fe/s]_2 = 2:1:1; \text{ while the amount of ubiquinone varies from 1-1.5 relative to cytochrome } c_1. \text{ Ubiquinone and the iron-sulfur center are displayed in oxidized form.}
\]
CoQ_{10}  

PROTOHEME (HEME B)  

HEME C  

IRON-SULFUR CENTER
Fig. 1.3

Observed fraction of the oxidation-reduction components during the titration of yeast Complex III with dithionite. Complex III was suspended in 6.0 ml of 0.1 M KPi, pH 7.4 containing 0.1% deoxycholate and 0.1% Triton X-100 to a protein concentration of approximately 13 mg/ml and an anaerobic dithionite titration was carried out at 10 C. The fractional amounts of reduced cytochrome c\textsubscript{1} (●) and cytochrome b (■) were derived from the absorbance changes at 553.5 nm and 561.5 nm, respectively, and from the intensities of EPR resonances at 3.49(Δ), 3.60(□) and 3.76(Φ). The amounts of reduced iron-sulfur protein(▲) and free radical(Φ) were obtained from the intensities of the EPR signals at g=1.89 and 2.00, respectively. The radical was quantitated using diphenyl picrylhydrazyl as an intensity standard.
yeast Complex III are shown in Fig. 4a. and 4b. and summarized in Table 2. The oxidized spectrum has absorbance maxima at 415 and 526 nm and a shoulder at 560 nm. In the dithionite-reduced form, the Soret band shifts to 429 nm and a peak appears in the alpha band region at 562 nm (cytochrome b) with a shoulder at 554 to 555 nm (cytochrome c$_1$); the beta bands for the two reduced cytochromes are at 530 and 523 nm, respectively. These visible spectra agree with those previously reported for the Complex III from both beef heart (33) and yeast (5).

The ratio of the intensities at the maxima in the Soret of the reduced protein to the ultraviolet (280 nm) was found to be 0.5, about half the value of 1.0 found for the beef heart preparation (33), but about the same as that obtained from the yeast preparation of Katan et al. (5).

Concentrations of the two cytochromes are calculated by the method described by Vanneste (34) based on changes in absorbance at four wavelengths upon reduction of the oxidized complex with dithionite. The method is:

\[
\text{[cytochrome b]}_{\text{mM}} = \left( \frac{A_{562-575}}{28.5} \right)_{\text{reduced-oxidized}}
\]

\[
\text{[cytochrome c$_1$]}_{\text{mM}} = \left( \frac{A_{553-540}}{5.1} \right)_{\text{reduced-oxidized}} - \text{[cytochrome b]}_{\text{mM}} \times 18.8
\]

The results are presented in Table 1 and the optical parameters are shown in Table 2.

(b) EPR Spectra:

There is no EPR spectrum of iron-sulfur protein observed in fully-
Fig. 1.4

Absolute absorption spectrum of yeast Complex III at various states of oxidation and reduction. The sample was dissolved in 0.1 M KPi, pH 7.4, containing 0.1% both deoxycholate and Triton OS-30 and 1 mM EDTA. Spectra were recorded at 22°C. Dashed line, a sample oxidized with a slight excess of ferricyanide; dotted line, reduced with excess ascorbate; solid line, reduced with excess dithionite.
### Table 2
Optical parameters for yeast Complex III

<table>
<thead>
<tr>
<th>Oxidation-reduction state</th>
<th>(\text{nm})</th>
<th>(\text{mM}^{-1}\text{cm}^{-1})×(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized</td>
<td>280</td>
<td>338</td>
</tr>
<tr>
<td></td>
<td>416</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>524</td>
<td>34.8</td>
</tr>
<tr>
<td></td>
<td>532</td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>553</td>
<td>29.4</td>
</tr>
<tr>
<td></td>
<td>561.5</td>
<td>29.2</td>
</tr>
<tr>
<td>Partially reduced (+ ascorbate)</td>
<td>418</td>
<td>382</td>
</tr>
<tr>
<td></td>
<td>524</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td>532</td>
<td>35.5</td>
</tr>
<tr>
<td></td>
<td>553</td>
<td>45.2</td>
</tr>
<tr>
<td></td>
<td>561.5</td>
<td>24.9</td>
</tr>
<tr>
<td>Fully reduced (+ dithionite)</td>
<td>429</td>
<td>469</td>
</tr>
<tr>
<td></td>
<td>524</td>
<td>42.2</td>
</tr>
<tr>
<td></td>
<td>532</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>553</td>
<td>56.4</td>
</tr>
<tr>
<td></td>
<td>561.5</td>
<td>74.8</td>
</tr>
</tbody>
</table>

\(a\) Calculated on a cytochrome c₁ basis assuming that the mole ratio of cytochrome b to c₁ is 2:1 and that the concentration of cytochrome b is reliably measured by the procedure described in the text.
oxidized Complex III from 3100 to 4000 gauss at 9.24 GHz. The EPR spectrum of ascorbate-reduced Complex III is shown in Fig. 5A. The spectrum exhibits three major resonances with nominal g values of 2.026, 1.89 and 1.79 respectively. These g values are generally associated with the Rieske type iron-sulfur center of beef heart Complex III (35). The EPR spectrum obtained on reduction with ascorbate differs slightly from the dithionite-reduced (Fig. 5C) sample, with the high field trough being somewhat sharper and centered at g= 1.81 and the low field peak moving slightly to higher field at g=2.025. A similar change was noted previously with the beef heart protein (36). The cause of this line shape change is uncertain but has been suggested to be a secondary response to an altered oxidation-reduction state of either cytochrome b or CoQ (7, 36). The only other resonances observed in this region are two minor bands at g=2.08 and g=1.94, respectively the g_x and g_y of a second mitochondrial iron-sulfur center, the so-called "center 5" (37). This iron-sulfur center is a minor component and the EPR signals can be seen only upon reduction with dithionite. In addition, contamination by Complex II, if present, produces resonances at g=2.02, and 1.91 (5). They are related to the HPIP type iron-sulfur center S-3 (38) and centers S-1 and S-2 respectively in the resting enzyme (39). Lack of features of these g values confirms the purity of the enzyme preparation (Fig. 5).

The EPR spectrum of yeast Complex III between 1600 and 2300 Gauss include features attributed to low-spin ferrihemoproteins. The difference spectrum (resting minus ascorbate-reduced sample) exhibits a weak, broad band centered at g=3.49 attributed to the low field
Fig. 1.5

EPR spectra of complex III in the iron-sulfur region. A. Complex III containing 2 electrons/mole of cytochrome c₁. B. Complex III containing 4 electrons/mole cytochrome c₁. C. Complex III containing 7.5 electrons/mole cytochrome c₁. The three spectra were recorded under identical EPR instrumental settings between 3100 and 4000 gauss at 9.24 GHz, but note that the radical signal is heavily saturated under these conditions. The spectra are taken from the experiment of Fig. 3.
resonance of cytochrome c<sub>1</sub>. This resonance is shifted some 90 gauss
downfield from that observed in beef heart (g=3.33) (36). The ascorbate-
reduced complex includes a large asymmetric resonance observed around g
value of 3.7 (Fig.6). From reductive titration (7) and potentiometric
titration (described in Chapter 5), this absorption results from
contributions at g=3.60 and g=3.76. These correspond approximately to the
field positions assigned to the two beef heart mitochondrial b cytochromes
(36) where the high potential cytochrome (b<sub>K</sub> or b<sub>562</sub>) has a low field
g value of 3.44 and the low potential cytochrome (b<sub>T</sub> or b<sub>566/558</sub>)
exhibits a maximum at g=3.78. A small resonance at g=2.95 probably
corresponds to a "modified" form of cytochrome b (40). Broader scans show
an EPR signal at g=4.3 (high spin ferric iron) with an amplitude
comparable to the g=3.70 signal. No obvious signal indicative of high
spin ferric heme is observed in the g=6 region.

(c) MCD and CD spectra:
Magnetic circular dichroism is a most useful probe in determining the
function and structure of biological molecules (41). The spin state
analysis of hemoglobin derivatives by MCD was reviewed by Vickery et
al. (42,43). A combination of MCD, EPR and magnetic susceptibility has
been employed to characterize the electronic state of cytochrome
oxidase hemes (44,45). Few investigators have studied the MCD of
Complex III. Arutjunjan et al. used room-temperature and liquid
nitrogen temperature visible region MCD to resolve c-type and b-type
cytochromes in beef heart submitochondrial particles (46). The same
authors utilized computer simulation of the visible MCD spectra of
Fig. 1.6

EPR spectra of Complex III in low field cytochrome region (between 1600 and 2600 gauss at 9.24 GHz).

Solid line, oxidized Complex III; long dash-dotted line, Complex III reduced with excess ascorbate; dotted line, difference spectrum of previous two spectra. The last spectrum has an extremum at $g=3.49$ corresponding to the low spin $g_x$ signal of cytochrome $c_1$. EPR spectrum of ascorbate-reduced Complex III exhibits a spectrum with two peaks at $g=3.60$ and $g=3.76$. Further resolution of this spectrum was complicated by the abnormal line shape and extensive overlap between the EPR spectra attributed to the two $b$ species.
isolated Complex III (47) to conclude that $b_{562}$ and $b_{566/558}$ are identical heme-proteins but are built asymmetrically into the enzyme resulting in a midpoint potential shift of $b_{566/558}$ negatively away from that of $b_{562}$. The coordination structure of cytochrome $b$ heme is further discussed in Chapter 4. The MCD characteristics of $b$ and $c_1$ cytochromes of yeast Complex III are detailed in Chapter 5.

The MCD and CD spectra of well characterized two-iron two-sulfur iron-sulfur proteins were reported by Stephens et al. (48) and Palmer and his colleague (49). No similar feature of CD and MCD that could be attributed to the yeast Complex III iron-sulfur center has been presented mainly due to the huge background of heme absorbance. This objective awaits the isolation of the iron-sulfur protein or the discovery of a specific modifying reagent of the iron-sulfur cluster.

CD and MCD measurement on Rieske's iron-sulfur protein recently isolated by Trumpower and Edwards from beef heart mitochondria (29) will be of great help in a comparative study of iron-sulfur proteins.

d. Enzyme activity:

Purified Complex III catalyzes oxidation of the coenzyme Q analog, 2, 3-methoxy,5-methyl,6-pentyl-1,4-benzoquinone (25 uM) with horse heart ferricytochrome $c$ (50 uM) as electron acceptor. The turnover number at 25°C, pH 7.4 is 70 s$^{-1}$ (moles of cytochrome $c$ reduced/mole of cytochrome $c_1$), essentially the same as submitochondrial particles. This activity is inhibited approximately 95% by concentrations of antimycin-A stoichiometric with cytochrome $c_1$. About ten percent catalytic activity is inhibitable by superoxide
dismutase, implying auto-oxidation of Complex III resulting in superoxide formation (50). Steady state analysis under the same conditions yield these kinetic parameters: $V_{\text{max}}=200 \text{ s}^{-1}$; $K_m(\text{quinone}) = 50 \text{ uM;} K_m(\text{cytochrome c})=175 \text{ uM.}$ The graphic pattern is indicative of a "ping-pong" kinetic mechanism.

e. Redox potentials:

The measurement of the midpoint potentials of each redox center of our Complex III preparation is described in this thesis and will be detailed in Chapter 5; the result is summarized in Fig.7 which displays the midpoint potentials at pH 7.4 using various instrumentation at ambient and low temperatures. The potential values for each redox component are in good agreement with stoichiometric reductive titration data (Fig.3). For both titrations the midpoint potentials were in descending order: $(2\text{Fe}/2\text{S})_c_1Q_\text{H}L_b\_H b\_L$. The midpoint potentials of the iron-sulfur protein and cytochrome $c_1$ were in close proximity in both kinds of titrations. This relationship is discussed when internal electron transfer between $c_1$ and the iron-sulfur center is explored in Chapter 6.

3. Function of the components of Complex III

a. Cytochrome b(s):

Besides its role as an electron carrier, cytochrome b may act as an energy transducer and/or proton translocator (52).

The first additional function of cytochrome b involves its energy-linked reduction. This phenomenon was originally reported by
A summarizing diagram of the results of potentiometric measurements.

Potentiometric titrations were carried out anaerobically with Complex III suspended in 0.1 M KPi, pH 7.4 (at 22°C), containing 0.1% both deoxycholate and Triton QS-30 and 1 mM EDTA in the presence of mediator dyes. Dithionite solution was used as reductant while dry air or ferricyanide solution was used as oxidant in all titrations. The oxidation-reduction change of iron-sulfur center was followed by EPR signal at g=1.89 at liquid helium (LHe) temperature; the redox changes of cytochromes were followed both by liquid helium EPR and room-temperature (R.T) MCD. Cytochrome b was monitored by g=3.60 and 3.76 resonances (EPR) and 562 nm signal change (MCD), and cytochrome c₁ was followed by g=3.49 signal (EPR) and the amplitude difference between 548 and 553 nm (MCD). The redox change of ubiquinone was monitored by the semiquinone radical signal at g=2.00 generated during potentiometric titrations using both liquid nitrogen (LN₂) and room temperature EPR measurements.
$\langle \text{Fe}/\text{S}\rangle_2$: EPR (LHe)

$a_1$: EPR (LHe), MCD (R.T.)

CoQ$^\cdot$: EPR (LN$_2$, R.T.)

$b_H$ & $b_L$: EPR (LHe), MCD (R.T.)
Wilson et al. (51). The $E_{m}$ of cytochrome $b_{566}$ ($b_{T}$) shifts from -30 mV to 245 mV upon addition of ATP to anaerobic, mammalian mitochondria which have coupled electron transport and proton-pumping ATPase. This phenomenon was taken as evidence that cytochrome $b_{566}$ functions as a primary energy transducer in energy coupling site 2; however, this interpretation has been challenged by other investigators. The apparent change of midpoint potential observed by Wilson et al. may result from an ATP-induced electron transfer by changing the steady-state redox level of cytochrome $b$ (53, 54). The assignment of $b_{566}$ as the only energy transducer may be a misconception; under the experimental conditions employed for the higher potential $b$ species, $b_{562}$ is completely reduced before ATP addition. Ereinsckya (143) remeasured the individual reduction of both $b$ cytochromes under different respiratory states of pigeon heart mitochondria. She found that only cytochrome $b_{566}$ exhibited a respiratory-state dependent midpoint potential change; i.e., the $E_{m}$ of $b_{566}$ became 160 mV more negative than that of $b_{562}$ in response to a high [ATP]/[ADP][Pi] ratio. Whether this phenomenon is a direct reflection of energy-transduction or a secondary effect resulting either from cooperative interaction between the $b$ cytochromes (55) or a shift in equilibrium due to local membrane changes (52) is still to be determined.

The suggestion that cytochrome $b$ might play a role as proton translocator originates from its pH dependent redox properties. The midpoint potential(s) of $b$ cytochrome(s) were reported to be
pH-dependent in the physiological pH range 6-9 (52, 57, 58, 203). This cooperative linkage between electron transfer by the heme and proton translocation by an amino acid residue or prosthetic group is sometimes called "redox Bohr effect", analogous to the oxygenation Bohr effect observed for hemoglobin. Direct measurement of scalar proton transfer on isolated Complex III yielded a ratio of 0.88 Bohr protons per cytochrome b (59).

Von Jagow and Engel (60) proposed that the cytochrome b dimer functions as a proton translocator. Protonation on the matrix side and deprotonation on the cytosolic side is presumed to be controlled by the alternating conformational changes induced by the changes in pK of a protonated group of each monomer. This model is very similar to those proposed for cytochrome oxidase (61), H⁺-ATPase (62), and Na⁺/K⁺-ATPase (63). Presently, very little experimental evidence is available to support this model.

b. Cytochrome c₁:

The functions of cytochrome c₁ are more firmly established than those of cytochrome b due to the availability of the method of bioassay and the close similarity of chemical and physical properties of the isolated cytochrome c₁ compared to the parent Complex III or submitochondrial particles. The redox acceptor for cytochrome c₁ has been identified as cytochrome c by potentiometric (14), kinetic investigations (64, 66) protein mapping (65) and complex formation between c₁ and c (67). Characterization of the membrane-bound and water-soluble c₁ fragment from Neurospora (68) and the sequencing of
beef heart cytochrome $c_1$ (69) led to the suggestion that the
hydrophobic amino acid residues at the C-terminus are anchored in the
mitochondrial membrane and possibly form a hair-pin-like bend upon
itself. Both C and N termini would be located on the outer surface of
mitochondrial inner membrane; with this arrangement the heme
prosthetic group of cytochrome $c_1$, located close to the N-terminus,
could react with both the electron acceptor, cytochrome $c$, and the
electron donor(s) inside Complex III. The immediate electron donor to
cytochrome $c_1$ in Complex III is not known. Chance (70) subjected
pigeon heart mitochondria to CO inhibition with an excess amount of
substrate followed by flash photolysis. He found that the rate of $c_1$
oxidation was close to that of semiquinone formation but not to that
of $b$ oxidation or $Q$ formation. His suggestion that $QH_2$ is the
immediate reductant for cytochrome $c_1$ is not convincing for during
observation cytochrome $b$ has turned over several times. The possible
role of cytochrome $b$'s as direct electron donor can be excluded only
after an experiment involving one-turnover of cytochrome $b$ is
performed and a discrepancy in the rates of oxidation of $b$ and
reduction of $c_1$ is observed. In addition, the role of iron-sulfur
center is not considered yet.

c. Rieske's iron-sulfur protein:

The iron-sulfur protein of Complex III was first discovered and
purified by Rieske (71). The succinylation of the protein, necessary
for its solubilization and isolation, precluded the determination of
function. Previous studies of optical-EPR reductive titrations by
Rieske (35) indicated that this protein is in close proximity to cytochrome c₁ and apparently exhibits parallel redox behavior to cytochrome c₁. Studies using antimycin-A (72) suggested the iron-sulfur protein is located on the oxygen side of the antimycin block. Yamashita and Racker (73) prepared a reconstituted succinate-cytochrome c reductase with very low nonheme-iron content (0.2 nmole/mg) which still exhibited nearly full succinate-cytochrome c reductase activity compared to the normal iron-content control. Little emphasis has been placed on iron-sulfur protein function during the past 15 years due to the perception that this protein is not a rate-limiting component in the b-c₁ region of the respiratory chain.

The function of the iron-sulfur protein has attracted renewed interest in the last few years stemming from the isolation of reconstitutively active pure protein from beef heart by Trumpower and Edwards (74). This iron-sulfur protein can now be reconstituted substoichiometrically into the iron-sulfur protein-depleted succinate-cytochrome c reductase obtained by treatment with guanidine and cholate. As much as 30% of the original activity can be regained (75). The following data (76) demonstrated the requirement of iron-sulfur protein for reduction of c₁ but not of cytochrome b. In the presence of antimycin-A, this iron-sulfur protein is required for both c₁ and b reduction. It is also required for the oxidation of cytochrome b, but not c₁, by cytochrome c and oxidase. In the presence of antimycin, this Rieske protein is necessary for the oxidant-induced reduction of b (77). Trumpower proposed the iron-sulfur
protein as the direct electron donor to cytochrome $c_1$ and mediator of electrons from ubiquinol to cytochrome $c_1$. Trumpower's ordering of electron transfer components was supported by experiments using the Q analog UHDBT to inhibit b-$c_1$ activity by interacting with the iron-sulfur protein (78). He concluded that the iron-sulfur protein is a ubiquinol-cytochrome $c_1$/ubisemiquinone-cytochrome b oxidoreductase catalysing two redox reactions. Direct evidence establishing ubiquinone as the immediate electron donor has yet to be reported.

The breakthrough of Trumpower et al. in iron-sulfur protein research may consolidate several seemingly diverse findings. The "oxidation factor" originally isolated by Racker's group (79) is now recognized as a crude preparation of iron-sulfur protein. Slater's BAL(2,3-mercaptoopropanol) labile factor (80) was recently identified as Rieske's iron-sulfur center. The "X" factor described by Baum et al. as a modulator of the redox potential of cytochrome b (81) or the "Y" factor reported to transform kinetically-sluggish b to an active b by Eisenbach and Gutman (82) are apparently different forms of Rieske's iron-sulfur protein (126,127).

d. Ubiquinone (coenzyme Q) and ubiquinone-binding protein (QPs,QPc):

Ubiquinone was first reported by Crane (18) to be a redox component in mitochondrial inner membrane. Later, Szarkowska's Q-depletion-reconstitution using NADH oxidase (83) and Q-stoichiometric incorporation to Q-depleted submitochondrial particles by Ernster et al. (84) established ubiquinone as an essential component of the respiratory chain. This component exists in the membrane in relatively high concentrations and is
not stoichiometric with any other electron transfer redox component.

Kinetic and inhibitor studies (86) led scientists to locate ubiquinone between flavoprotein dehydrogenase and the b-c\textsubscript{1} complex. Ubiquinone is associated with at least four enzyme complexes, NADH-dehydrogenase, succinate-dehydrogenase, flavoprotein quinone reductase (an iron-sulfur flavoprotein accepting electrons from electron transfer flavoprotein (ETF) related to the beta-oxidation of fatty acid and donating to coenzyme Q (85)) (on the reduction side) and Complex III (on the oxidation side). Ubiquinone's role of accepting reducing equivalents and transferring them to Complex III, together with the simple kinetic behavior of nearly all coenzyme Q present in submitochondrial particles (86) led Kroger and Klingenberg to develop the "pool function" concept for ubiquinone (87). They proposed a homogeneous pool of ubiquinone having sufficient rapid lateral mobility that ubiquinone's reduction and oxidation rates are determined by the turnover number of the dehydrogenases and Complex III respectively.

The lateral mobility of respiratory chain complexes has been demonstrated by lipid-phase transition, lipid protein separation (patch formation) and lateral translational diffusion (88). The lateral movement and the related electron transfer capability of mobile ubiquinone were substantiated by Hackenbrock's group. Phospholipid-enrichment of mitochondrial membrane (increasing the distance between Complex I and III) led to 60% loss of succinate cytochrome c reductase activity and 85% loss of NADH-cytochrome c reductase activity (89). Incorporating different phospholipids into the
mitochondrial inner membrane by membrane fusion led to decreased activity in succinate- and NADH-cytochrome c reductase and ubiquinone cytochrome c reductase proportional to the increase in phospholipid area. These activities were substantially restored in phospholipid plus ubiquinone-supplemented membranes. The above experiments imply a diffusion-limited step in the transfer of reducing equivalents from the dehydrogenases to cytochrome bc₁. Hauska's kinetic studies of liposome-trapped ferricyanide reduced by external dithionite mediated by liposome membrane-incorporated ubiquinone demonstrated electron and proton transmembrane movement (91). The mechanism for this translocation is unclear; when ubiquinone is anchored in the lipid bilayer with the isoprenoid side chain aligned perpendicular to the membrane, it is energetically unfavorable for the quinone head group to translocate across the membrane to function in transmembrane reduction. The intramolecular rotation around any single bond of the isoprenoid chain is severely restrained due to the trans double bond and the vicinal methyl residues of each of the ten isoprenoid units (92,93). Despite these restraints, transmembrane hydrogen transfer increases as the number of isoprenoid units increase (94). These model membrane experiments have limited relevance due to the marked differences in liposomal and mitochondrial membrane composition.

In addition to the mobile electron carrier function of coenzyme Q, evidence indicates this compound can act as an immobilized prosthetic group bound to protein (95). At least two thermodynamically stable forms of ubisemiquinone are found in beef heart succinate-cytochrome c
reductase (96). The amount of stable antimycin-A-sensitive semiquinone, 
SQc, found in the b-c1 region poised at suitable redox potentials 
varies from 10 to 50 percent of that of cytochrome c1 (96,97,98). The 
stability constant of this semiquinone radical was estimated to be 
10^{-2} to 10^{-3} (corresponding to Em (Q/Q^-) - Em(Q^-/QH_2) = -78 to -166 
mV (96)); compared to its stability constant, 10^{-10}, in a hydrophobic 
environment (99), this value is strongly indicative of a stabilized form 
of semiquinone. Yu et al. have partially purified a protein with 15,000 
molecular weight (QPs) from beef heart (100). The depleted protein is 
capable of stabilizing the ubisemiquinone and is reconstitutable with 
soluble succinate dehydrogenase to restore succinate-ubiquinone reductase 
(101). Addition of QPs to the bc1-complex upon addition of succinate in 
the presence of a catalytic amount of succinate dehydrogenase generated 
a stable ubisemiquinone radical (Ks = 0.5) (102).

This functional duality is reflected in the original CoQ kinetic 
measurements by Kroger and Klingenberg (86). About 80-90% of the total 
ubiquinone responded homogeneously to changes in respiratory activity. 
First-order kinetics were observed, possibly indicating a 
diffusion-limited rate between mobile and protein-bound ubiquinones. 
Zhu et al. (103) extended the ubiquinone functional investigations of 
Kroger and Klingenberg (87) by inhibiting CoQ oxidase with the Q 
analogue, heptylhydroxyquinoline N-oxide (HQNO); They concluded that 
85-90% of the functional ubiquinone exists as a mobile "Q-pool" with 
10-15% bound to protein for generation of stable semiquinone molecules 
(for further discussion, see section 4, reaction mechanism). Zhu et
also observed pool-function kinetics in substrate oxidation, with free ubiquinone present or depleted in submitochondrial particles, are due both to the pool quinone and to direct collision between Q-loaded enzymes. They emphasized that it is the "relative motion" of ubiquinone and the Q-reducing, and OH$_2$-oxidizing enzymes rather than the "absolute motion" of ubiquinone interacting with "static" proteins which determines the electron-transfer rate, a concept supported by the experiment of Schneider et al. (89,90) mentioned previously.

4. Inhibitors of Complex III

Utilization of inhibitors to study the function and relative position of electron transfer components have provided avenues of valuable research. There are several natural and synthetic inhibitors of the b-c$_1$ segment as shown in Fig.8. The most commonly used are antimycin-A and ubiquinone analogs:

a. Antimycin-A:

Antimycin-A, a fungicide produced by a species of Streptomyces, is a mixture of antimycin-A$_1$, A$_2$, A$_3$, and A$_4$ with small differences in their chemical structures. The A$_1$ species shown in Fig.8 is an acyl- and alkyl-substituted dilactone ring linked via an amide bond to 3-formamidosalicylic acid. The fungicide is a very potent inhibitor (K$_d$ =10$^{-11}$ to 10$^{-13}$ M) of Complex III (104). Antimycin-A affects Complex III in four different ways:

(1) Stoichiometric inhibition of OH$_2$-cytochrome c reductase activity.
(2) Stoichiometric inhibition of cleavage of Complex III by dissociating reagents.

(3) Pronounced changes in visible and EPR spectra: a bathochromic shift of the alpha-absorption of ferrocyanochrome b of antimycin-treated beef or pigeon heart Complex III occurs (105,106) together with a downfield shift of the EPR signal of $b_{562}$ from $g=3.44$ to 3.48.

(4) Altered oxidation-reduction properties of cytochrome b. In the presence of a suitable oxidant (e.g. cytochrome c, oxidase and oxygen, or ferricyanide), the rate and extent of cytochrome b reduction by substrates are enhanced (107) while cytochrome c and oxidase are oxidized; this well-known phenomenon is "the oxidant-induced reduction of cytochrome b".

Despite extensive studies, the binding component of antimycin-A is still uncertain. Both Berden's observation that antimycin's binding strength is governed by the redox state of cytochrome b (108) and von Jagow's antimycin titration of Neurospora mitochondria deficient in cytochrome b (110) indicate cytochrome b as the antimycin binding component. A 1:1 stoichiometry of antimycin:cytochrome $b_{562}$ inhibited mitochondrial respiratory activity of Neurospora. Berden and Slater (113) studied fluorescence quenching of Antimycin-A interacting with a reconstitutively-active cytochrome b-containing fraction and discovered 1:1 stoichiometric binding. Thirdly, Roberts et al. (111) investigated antimycin binding of mitochondria of wild type yeast and b-deficient mutants, and concluded that antimycin-A binds to cytochrome b. Storey (144) suggested that antimycin forms a stable complex with cytochrome $b_{562}$.
similar to cytochrome $a_3/CO$ complex especially when $b_{562}$ is reduced. However, Slater (104) calculated the distance between cytochrome b heme iron and antimycin-A phenoxy ring from fluorescence quenching to be about 20Å. The distance is too far for direct cytochrome b and antimycin-A binding if Storey's suggestion is adopted, because the formation of stabilized complex between antimycin and cytochrome b should yield a distance of just a few angstrom units. Das Gupta (112) covalently linked a N-bromoacetate and deformamido azido antimycin-A to Complex III (photo-affinity labelling) and assigned a molecular weight of 11,500 on SDS polyacrylamide gel to the "antimycin-A binding protein".

The above findings indicate antimycin-A binds to a site very close to cytochrome b; lack of binding to purified cytochrome b implies additional protein component(s) are also responsible for binding. The moieties of antimycin-A, in order of increasing inhibitory contribution, are acyl, alkyl-substituted dilactone, the formamido and the phenolic hydroxyl groups (114) (Fig.8). Of these three moieties only the phenolic hydroxyl group is absolutely necessary for inhibition. Antimycin analogues with strong affinity for the antimycin-binding site, yet devoid of inhibitory activity are unknown suggesting antimycin inhibits by occupation of a "critical space" rather than by ligation of a prosthetic group required for electron transfer.

(b). Ubiquinone analogues: Linnane's group in Australia and Folkers' laboratory at Austin, Texas (115) examined the effects of thirty-three synthetic quinone derivative on mitochondrial electron transfer in yeast. Four potent inhibitors were identified: 5-n-undecyl-6-hydroxy-4,7-
dioxobenzo-quinone (UHDBT), 7-w-cyclohexyloctyl-6-hydroxy-5,8-quinoline-quinone, 7-n-hexadecyl-mercapto-6-hydroxy-5,8-quinolinequinone (HMHQ) and 3-n-dodecylmercapto-2-hydroxy-1,4-naphthoquinone (see Fig. 8). All inhibit the growth of ethanol-fed yeast. Spectroscopic studies indicate these inhibitors block electron transfer between cytochrome b and c$_1$ acting as coenzyme Q antagonists as indicated by reversal of inhibition by added coenzyme Q. The Q analogues have in common two fused aromatic rings, a hydroxyl group ortho to one of the quinone oxygens, and a long aliphatic side chain adjacent to the other oxygen of the analogs. Trumpower and his collaborators (116,117) reported that UHDBT inhibits the activity of Complex III (with an apparent $K_i$ of $10^{-8}$ M at pH 7.0) but not that of succinate-ubiquinone reductase. Inhibition of succinate-cytochrome c reductase requires protonation of the 6-hydroxyl group of UHDBT (117).

Inhibition studies on succinate-cytochrome c reductase and iron-sulfur protein-depleted samples demonstrated UHDBT addition to isolated succinate-cytochrome c reductase mimics iron-sulfur protein depleted Complex III (78, 116). The change of EPR line shape and midpoint potential (+70 mV) of the Rieske's iron-sulfur center (78) concomitant with activity loss in succinate-cytochrome c reductase indicated UHDBT binds to the iron-sulfur protein. Similar inhibition by UHDBT was reported for the Rieske's iron-sulfur protein in the b-c$_2$ region of photosynthetic bacteria (119).

The Q analogue, 2-heptyl-4-hydroxyquinoline-N-oxide(HOQNO) (Fig. 8), causes a crossover between cytochrome b and c$_1$ during steady-state respiration, and similar to antimycin-A, inhibits the energy-linked functions (120). HOQNO specifically binds to b-c$_1$ region and can be
Structure formulas of some important inhibitors of Complex III.

Antimycin-A is an antibiotic; other compounds are chemically synthesized analogs of ubiquinone. The structure of ubiquinone is also given for comparative purposes.
Antimycin-A

![Antimycin-A structure](image)

Antimycin (A 1)

Ubiquinone and Q analogues

![Ubiquinone structures](image)

Ubiquinone

DBMIB
displaced by antymycin-A. HOQNO and UHDBT are competitive for the same inhibition site and their inhibitory effects are additive; therefore, HOQNO exerts a two-fold inhibitory effect on Complex III (121).

2,5-dibromo-3-methyl-6-isopropyl benzoquinone (DBMIB), an antagonist of plastoquinone, inhibits electron transfer between plastoquinol and cytochrome f in chloroplasts (Chain and Malkin, 122) and isolated cytochrome b$_6$f complex (145). At high potentials (E$_h$ = 20 - 100 mV) interaction of oxidized DBMIB's with reduced iron-sulfur converts the original g=1.89 signal to a g=1.94 signal; at low potentials (-200 - -300 mV) as DBMIB is converted to its hydroquinol form, the EPR signal reverts to g=1.90. At intermediate potentials (-20 - -180 mV) none of the g=1.90 or 1.94 signals are observed, leading to speculation of a strong antiferromagnetic coupled semiquinone of DBMIB and reduced iron-sulfur center (123). The potential dependence of these EPR spectral changes indicate the quinone form of DBMIB binds to the iron-sulfur protein 100 times as strongly as its quinol form and that the iron-sulfur protein coupling stabilizes this semiquinone.

Preliminary EPR results of DBMIB interactions with succinate-cytochrome c reductase indicate that DBMIB exerts a similar effect as in chloroplast (Malkin and Trumpower, quoted in (127)). This result is surprising as DBMIB mimics the inhibitory effect of UHDBT, yet contains none of the critical functional groups: a hydroxyl group, a long aliphatic chain, or a double ring structure previously described by Folkers and coworkers (115). A study of competition between DBMIB and UHDBT should provide valuable information on the structural requirement for inhibition by the Q-analogues.
(e). BAL and DCMU:

A simple compound, 2, 3-dimercaptopropanol or BAL (British Anti-
Lewisite) was found by Slater (124) to inhibit the succinate oxidase
but not the succinate dehydrogenase activity of aerobic Keilin-Hartree
heart muscle preparation. Spectroscopic studies located the site of
inhibition between cytochrome b and c. Recently, Slater and de Vries (80)
using EPR spectroscopy identified the Rieske iron-sulfur protein as the
interaction site of BAL. The iron-chelating ability of BAL offers a
simple explanation; also a disulfide is made which could oxidize protein
disulfide. No similar interaction has been demonstrated on purified
Complex III or iron-sulfur protein, weakening the above supposition.

Diuron, 3(3,4-dichlorophenyl)-1,1-dimethyl urea, or DCMU, a
photosynthesis inhibitor, also inhibits electron transport between
yeast cytochrome b and c₁ (125). It induces an "extra-reduction" of
b cytochromes as does antimycin-A. Kinetic studies indicate two DCMU
sites in yeast submitochondrial particles but no data have been
reported concerning the nature of its binding sites.

5. Reaction mechanism:

The redox mechanism of multicomponent Complex III promises to be
one of the most complicated and interesting in the electron transfer
chain for these reasons:

(a) Complex III is a confluence point of reducing equivalents from
at least four redox-linked enzyme systems. The reactions of ubiquinone
reduction and ubiquinol oxidation correspond to a transition from
two-electron redox centers to one-electron centers.

(b) The second energy coupling site of electron transport chain is located in the b-c1 region. According to Mitchell's chemiosmotic theory, proton translocation couples to electron transfer within Complex III. The stoichiometry of proton translocation for each pair of electrons transferred was calculated to be 4H+/2e- at coupling site 2(59,128). Ubiquinone, a 2-proton and 2-electron carrier, seems to be the only candidate for proton transport. As actual measurements indicate that four protons have to be translocated for each pair of electrons being transferred in the b-c1 region (59,128), a proton translocator other than ubiquinone has been vigorously sought. Cytochrome b has been suggested for the role of proton pump due to its pH-dependent redox behavior.

(c) Substrates added anaerobically to submitochondrial particles do not reduce the b cytochromes to the extent expected from the relative redox potentials of the substrate and cytochrome b couples (130,131). After a pulse of oxygen a transient full reduction of cytochrome b occurs (70,132) concomitant with full oxidation of cytochrome c1 and cytochrome oxidase. This extra reduction, or "oxidant-induced reduction", of cytochrome b, usually seen at low temperature (2–5 C), is stabilized at room temperature upon antimycin-A or HQNO inhibition of cytochrome b (107,135). With appropriate substrates and oxidants, these phenomena occur in submitochondrial particles, isolated succinate-cytochrome c reductase and isolated ubiquinol-cytochrome c reductase (107). This crossover oxidation-
reduction phenomenon of cytochromes b and c₁ has not been explained using a classical linear sequence of electron transfer.

(d) Involvement of coenzyme Q in electron transfer and proton translocation:

The concentration of coenzyme Q is in excess and is not stoichiometric with any other redox component in mitochondria, isolated succinate-cytochrome c reductase or ubiquinone-cytochrome c reductase.

In an attempt to compose a clear picture from the intricate observations mentioned above, Mitchell ingeniously explained electron transfer and coupled proton translocation in Complex III with a flexible "protonmotive Q cycle" (99 and Fig. 9a). Trumpower modified this model by introducing Rieske's iron-sulfur protein into the scheme (92, 127 and Fig. 9b). In this revised model, a ubisemiquinone anion localized on the matrix side of mitochondrial inner membrane, Q-i, accepts one electron from a flavoprotein dehydrogenase and simultaneously abstracts two protons from the matrix space to form ubihydroquinol. Ubiquinol transports reducing equivalents across the membrane and then is oxidized to another kind of localized semiquinone anion, Q-o on the cytoplasmic side by the iron-sulfur protein. The latter becomes the electron donor of cytochrome c₁. Simultaneously, two protons are released to the cytosol. Further oxidation of this semiquinone by cytochrome b produces a diffusible ubiquinone. The cytochrome b's then transport the electrons back across the membrane and reduce ubiquinone which has meanwhile diffused from the cytosolic to the matrix side, regenerating Q-i and completing the cycle. This
Fig. 1.9.

A. General formulation of the protonmotive $Q$ cycle originally proposed by Mitchell in 1975 (99). The main feature of this scheme is that one electron is transferred from succinate to cytochrome $c$, 2 protons are actively translocated from matrix side to the cytosolic side of the mitochondrial inner membrane. The role of coenzyme $Q$ is emphasized by omission of the electron donors and acceptors of ubiquinone.

B. A detailed sequential formulation of protonmotive $Q$ cycle proposed by Trumpower (92). The possible electron donors and acceptors of different redox species of ubiquinone are introduced. It is proposed that antimycin prevents the oxidation-reduction of $b_{562}$ by the $Q/Q^-$ couple.
A.

Cytochrome \( c \leftarrow e^- \) \( \rightarrow \) \( QH_o \)

\( H^+ \leftarrow \) \( \rightarrow \) \( QH_o \)

\( H^+ \leftarrow \) \( \rightarrow \) \( QH_i \)

\( QH_i \leftarrow e^- \) \( \rightarrow \) Succinate

Cytoplasm

Matrix

B.
Q cycle proceeds in "one-electron" transfer steps and satisfies the measured stoichiometry of $4H^+ / 2e^-$. It also successfully explains the "oxidant induced extra-reduction of cytochrome b" requiring no modulators, i.e., "X" factor (81) or "Y" factor (82) for altering the midpoint potential of cytochrome b, by assuming antimycin-A (or HOOONO) inhibits the reduction of ubiquinone by $b_{\text{562}}$. The extra reduction of cytochrome b may simply result from a shift of equilibrium by antimycin-inhibition. Furthermore, this model does not depend upon a stoichiometric relationship between CoQ and $c_1$. The topographical distribution and membrane sidedness of cytochromes b, $c_1$ and iron-sulfur protein agree with available data on chemical dissociation (56) and membrane crystal structure (30,137). The isolation and characterization of the iron-sulfur protein by Trumpower and his collaborators greatly clarified the redox reaction mechanism of Complex III. It was demonstrated by reconstitution experiments that the iron-sulfur center is the immediate electron donor of cytochrome $c_1$ (127). The "proton-motive Q cycle" can explain all the functions of Rieske's iron-sulfur center previously mentioned (see section 3.c).

This Q-cycle is still in its formulative stage. The mechanism of electron transfer of Complex III (or b-$c_1$ region) is still speculative. Several unresolved problems remain:

1. The model absolutely requires separate localized ubisemiquinone species and forbids electron transfer between the two semiquinones. The existence of stabilized semiquinone and Q-binding protein(s) are generally recognised (95-98), but neither the location of these
stabilised semiquinones nor the mechanism of stabilisation is known. De Vries et al. recently reported EPR radical signals in the mitochondrial particles ascribed to Q-in and Q-out (139). This interpretation, although interesting, should be weighed carefully because there is no knowledge about the exact location where these Q radicals were generated under their experimental conditions.

(2) The transmembrane movement of ubiquinone and ubiquinol must be thermodynamically allowed and proceed with sufficient speed to fulfill the electron transfer and proton translocation functions, especially if the functional quinone is stoichiometric with $c_1$ and the iron-sulfur protein. Although it has been demonstrated that liposome-incorporated ubiquinone can transfer reducing equivalents across the membrane (91,94), the physical and chemical characteristics of ubiquinone molecules in the membrane are speculative. Citing the rigidity of the isoprenoid side chain of ubiquinone, Trumpower proposed that ubiquinone may align itself parallel to the membrane with its isoprenoid side chain bound in the region in between the bilayer lipids of the membrane, possibly to a hydrophobic site on a protein. The quinone functional "head group" is therefore allowed to travel between two reaction centers juxtaposed across the membrane by rotation about the long axis parallel to the membrane surfaces (127).

(3) Based on this scheme, any redox linkage existing between cytochrome $b$ and $c_1$ (or iron-sulfur center) is mediated through Coenzyme Q. The $b$ cytochromes play only a passive role in recycling the second reducing equivalent across the membrane, an apparently
futile or redundant function. For instance, if the Q-binding protein can transfer a reducing equivalent across the membrane by a conformational change, cytochrome b is unnecessary.

(4) The immediate acceptor and donor of the half-reaction pairs $\text{QH}_2/\text{QH}^-$ and $\text{Q}^-/\text{Q}$ as depicted in Fig. 8b are still hypothetical, being the product of data from potentiometric and inhibitor studies. No convincing transient kinetic data are available.

(5) The assignment of the inhibition site of antimycin-A in Q cycle is relatively arbitrary. Although the steady-state redox change of the cytochromes and the iron-sulfur protein observed in the presence of antimycin-A is consistent with this assignment, the nature of the antimycin-A binding site is as ill defined as the Q cycle itself.

With all its uncertainties, the "protonmotive Q cycle" still explains many experimental results and remains the best available descriptive model for electron transfer in the $b-c_1$ region; furthermore, it can be subjected to experimentation.

Central to evaluation of the protonmotive Q cycle is characterization of the electron transfer sequence internal to Complex III. Steady-state kinetics are insufficient in this quest. Pre-steady state kinetics involving more than one turnover for individual subunits as in cytochrome b yield complicated kinetic results and interpretation becomes extremely difficult (140). Experiments designed to minimize the turnover numbers of individual redox components but not the whole enzyme complex are required. In photosynthetic systems this is achieved by a time-controlled saturating light flash; definite
numbers of reducing equivalents are pumped into the system under spectroscopic scrutiny. In the mitochondrial electron transfer chain, such a convenient experimental system is not available, although Packham et al. ingeniously prepared an artificial complex between Complex III and bacterial reaction centers to produce light excited electron transfer in the $b-c_1$ region (141).

In this thesis, I investigated the oxidative reaction mechanism by reacting 2 electron-reduced (cytochrome $c_1$ and iron-sulfur protein alone are reduced), 4 electron-reduced (CoQ-depleted) and fully-reduced yeast Complex III with ferricyanide, keeping the turnover number of individual redox components to a minimum. Important information about internal electron transfer rates was obtained as shown in chapter 6 and 7. Also included in this thesis are investigations on the physical and chemical characteristics of cytochrome $b$ both on the protein and the heme structure (Chapter 3 and 4). These investigations were approached either by direct isolation and characterization (Chapter 3) or comparative studies with model compounds (Chapter 4). Relatively complete potentiometric studies on the redox potentials of individual redox component are detailed in Chapter 5. The effects of different parameters, i.e., temperature, pH, dye mediators, detergent systems and antimycin on the redox potentials are also assiduously monitored. The data generated from these studies were used to directly interpret the kinetic data in Chapters 7 and 8.
Chapter 2. MATERIALS AND METHODS

1. Experiments on the coordination environment of mitochondrial cytochrome b:

Hemin chloride was obtained from Calbiochem-Behring Corp. 1-methyl imidazole, 2-methylimidazole, and 1,2-dimethylimidazole were obtained from Aldrich Chemical Co. Solutions of protoheme chloride in dimethyl sulfoxide (DMSO) were made 0.6M in the appropriate imidazole for spectroscopy.

Complex III from yeast mitochondria was isolated by the procedure of Siedow et al. (7). Just prior to MCD spectroscopy, Complex III was oxidized with ferricyanide and passed through a Biogel P-6 column (1.5 cm x 7 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.4 containing 0.1% deoxycholate and 0.1% Triton QS-30. Cytochrome c₄ was isolated from yeast Complex III which had been treated with dithiothreitol and was subsequently purified using DEAE cellulose (Whatman DE52) and Sephadex G-100. Cytochrome b was solubilized from yeast Complex III using tetrahydrophthalic anhydride and purified by chromatography on hydroxylapatite. Cytochrome c₄ was oxidized prior to MCD spectroscopy with ferricyanide and passed through a Biogel P-6 column (1.5 cm x 7 cm) equilibrated with 0.1 M Tris-HCl pH 7.6 containing 0.1% deoxycholate and 0.1% cholate. Cytochrome b was dissolved in 0.1 M potassium phosphate buffer, pH 7.4 containing 0.5%
cholate.

Heme concentration was measured by the pyridine-hemochromogen method (134) or by the use of published extinction coefficients (7). EPR spectroscopy was performed on a Varian E-6 EPR spectrometer under the following conditions: 4 mW power at 6.9 K and 9.243 GHz with 10 gauss modulation. The scan rate was 1000 gauss/min with a time constant of 0.3 s.

MCD spectra were recorded on a Jasco J-500C spectropolarimeter equipped with an electromagnet (1.4 Tesla) and a Jasco DP-500 data processor for data accumulation, signal averaging, and manipulation.

Both instruments were interfaced to the laboratory data system for storage, manipulation, and presentation of the data.

2. Experiments on purification and characterization of cytochrome b:

The Complex III was isolated from yeast (Red Star) according to the procedure of Siedow et al. (7) and had a purity of 9 mmole cytochrome b per milligram protein. Cholic acid was obtained from Sigma and recrystallized from ethanol. 3,4,5,6-Tetrahydrophthalic anhydride was purchased from Aldrich and dissolved in a minimum volume of peroxide-free p-dioxane immediately prior to use. Hydroxylapatite was prepared using the procedure of Jenner (152). The Antimycin-A (Sigma Chemical Co.) used for fluorescence quenching experiments was prepared as 1 or 10 mM solutions in dimethyl-sulfoxide. Acrylamide and bis-acrylamide were BioRad products. All other chemicals were reagent grade.
Purification procedure:

All steps were carried out at 4°C. Complex III in 0.1 M potassium phosphate buffer, pH 7.4, 0.5% cholate was centrifuged at 100,000 x g for 2.5 h. The pellet was resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 0.5% cholate to 3 mg/ml, and tetra-hydrophthalic anhydride was added dropwise to a concentration of 2 mg per mg protein. The solution pH was maintained close to 8.0 by addition of 1M KOH and the reaction was complete in approximately 50 min as judged by the absence of any further pH change. The clear red solution was then applied to a hydroxylapatite column (3x10cm). The column was first washed with 80 ml 50mM Tris, pH 8.0, containing 0.5% cholate and subsequently eluted with a linear gradient constructed using 200 ml volumes of 50 mM KPi, pH 7.9, and 0.4 M KPi, pH 7.9; each buffer contained 0.5% cholate. Alternatively, following collection of the first orange band which eluted at about 150 mM KPi during the phosphate gradient step, the major cytochrome b fraction was eluted with 0.3 M KPi, pH 7.9, containing 0.5% cholate. Fractions with a constant ratio of A_{415}/A_{280} were pooled and, when necessary, concentrated using a Micro-ProDicon concentrator (Bio-molecular Dynamics, Beaverton, OR) with a vertical dialysis membrane.

The purity of cytochrome b was routinely verified by electrophoresis on a 12% gel or a 12.5 - 20% gradient gel. SDS polyacrylamide slab gel electrophoresis was performed as described by Laemmli(8). Heme concentration and extinction coefficients were
determined via formation of pyridine hemochrome (154) and protein concentration was determined by Biuret method (155) or according to Lowry et al. (156). Enzyme assay for Complex III was the same as described by Siedow et al. (7) by measuring the reduction of 50 μM ferricytochrome c by 25 μM 2,3-dimethoxy,5-methyl,6-pentyl benzohydroquinone (PBQ) at 25°C in pH 7.4 buffer. A similar procedure was carried out on purified cytochrome b. The reduction of cytochrome b was followed at 561.5 nm; 50 μM reduced PBQ or 1 mM succinate was used to reduce 10 μM cytochrome b in the presence of 5-10 μg Complex II isolated from yeast in 0.1 M KPi, pH 7.4, containing 0.5% cholate. The amino acid composition of purified cytochrome b was determined by hydrolysis of 0.4-0.7 mg samples in 6 M HCl at 110°C for 48, 96, 120 h and quantitation on a Beckman 120-C amino acid analyser. Tryptophan was released by hydrolysis in 4 M methane sulfonic acid plus 0.2% 3-(2-aminoethyl-indole) and analyzed as above (153).

MCD spectra were obtained using a Jasco J-500C spectropolarimeter equipped with a Jasco MCD-1B electromagnet. Spectra were accumulated using a Jasco DP-500 data processor and transferred to the laboratory data system for storage, manipulation, and plotting. The temperature dependence of the MCD spectrum of oxidized cytochrome b was determined in a thin-walled cuvette with a 1.6 mm lightpath. A glass dewar with flat, unsilvered windows was fitted between the electromagnet pole-pieces and the sample in the dewar cooled by liquid N₂ boil-off gas. The temperature was monitored by a platinum resistance temperature sensor placed adjacent to the cuvette. Solvent contraction
due to temperature change was corrected for by recording the Soret band absorbance at each temperature setting.

Potentiometric titrations monitored by MCD were performed as previously described (157).

Electron paramagnetic resonance (EPR) spectra were recorded with a Varian E6 EPR spectrometer equipped with a liquid-helium transfer system.

The antimycin binding experiments were carried out essentially as described by Berden and Slater (113) using an SLM-400 polarization fluorometer. The excitation wavelength was 355 nm and the emission wavelength was 410 nm; 2nm spectral band width was used at both wavelengths. Antimycin binding was observed by two methods, direct fluorescence and a binding-precipitation method. The direct fluorescence procedure consisted of titrating a known quantity of Complex III or cytochrome b with aliquots of antimycin and monitoring the antimycin fluorescence after each addition during the titration. Before and after each addition, samples of the reaction mixture were assayed for enzyme activity. This procedure was repeated with successive additions until the amount of antimycin in the reaction mixture substantially exceeded that needed for a complete loss of activity. The data obtained with Complex III were used as a control to ensure that inner filter effects were not obscuring the stoichiometric binding of antimycin to cytochrome b. The precipitation method involved incubating 7.5 nmole of Complex III or 20 nmole cytochrome b with increasing quantities of antimycin at 4°C. After 10 min., the
reaction mixture was centrifuged at 48,000g, at 4°C, for 3 h and the supernatant used for fluorescence determination of free antimycin.

3. Experiments on the potentiometric properties of Complex III:

Complex III was purified from Baker's yeast (Red Star) according to the procedure of Siedow et al. (7). Preparations used for this work contained 9 to 11 n mole cytochrome b/mg protein and reduced 70 n mole cytochrome c/sec/nmole cytochrome c1 in the activity assay described below. Coenzyme Q-depleted Complex III was prepared essentially as described by Szarkowska (83). An aqueous sample of Complex III was extracted five times with n-hexane, lyophilized to remove residual solvent, and redissolved in an appropriate buffer. Samples partially depleted of CoQ were prepared following the same procedure by removal of an aliquot after each extraction cycle. The CoQ remaining in each sample was determined by subtracting the amount of coenzyme measured in the extracting solvent from the total coenzyme Q present in the original intact Complex III. The coenzyme Q concentration was determined as described by Szarkowska (83) using a difference extinction coefficient between the oxidized and reduced quinone species of 12.25 cm\(^{-1}\).mM\(^{-1}\) at 275 nm.

Cytochrome b was purified from Complex III by a procedure involving treatment with 3,4,5,6-tetrahydro-pthalic anhydride followed by chromato- graphy on hydroxylapatite (45). The purified cytochrome b had a heme content of 37 n mole b/mg protein.

The switterionic buffers, MES (2[N-morpholino]-ethane sulfonic
acid), ADA (N-(2-acetamido)-iminodiacetic acid), TAPS (3(3-tris(hydroxymethyl)methyl)amino propane sulfonic acid), CHES (2-(N-cyclohexylamino)ethane sulfonic acid), BICINE (N,N-bis(2-hydroxyethyl)glycine) were obtained from Sigma. Taurocholate and cholic acid were purchased from Sigma; the latter was recrystallized from ethanol before use. Triton QX-30 was obtained from Rohm & Haas. Antimycin-A (Sigma) was prepared as a 1mM or 10mM solution in dimethyl sulfoxide. Other chemicals were reagent grade.

Enzyme activity was obtained by mixing 50 μM ferricytochrome c, 10 μM 2,3-dimethoxy,5-methyl,6-pentyl benzoquinolone in 0.1M KPi, pH 7.4, containing 1 mM EDTA and following the reduction of cytochrome c spectrophotometrically after the addition of Complex III at 25°C.

Room temperature MCD-potentiometric titrations were performed either in a Brinkmann 5-ml glass titration vessel provided with a side arm to which a 1 cm light-path quartz cuvette was attached, or alternatively, in a custom-made glass titrator with a 1 cm light-path pyrex cuvette attached directly to the base, such that the potential could be monitored continuously during spectroscopic measurements. The Brinkmann titration vessel was fitted with a hard-plastic top with standard taper holes for insertion of (i) the electrode, (ii) a stopcock for degassing, and (iii) syringes containing either oxidant or reductant. The custom-made titrator has similar standard taper joints to mate with these accessories. A platinum silver/silver chloride electrode (Metrohm, type EA 234) was used together with a digital voltmeter to indicate solution potential. This electrode was
calibrated using saturated aqueous quinhydrone solutions at several pH values. The reaction mixture contained 5 μM Complex III or coenzyme Q-depleted Complex III and, unless otherwise stated, 20 μM of the following dyes: p-aminophenol sulfate, N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD), 2, dichlorophenolindolphenol (DCPIP), 1,2-naphtho-quinone (1,2-NQ), phenazine methosulfate (PMS), methylene blue (MB), indigotetrasulfonic acid (ITS), 2-hydroxy-1,4-naphthoquinone (2-OH-1,4 NQ). This reaction mixture was made anaerobic by using techniques previously described by Beinert et al. (176); repeated cycles of evacuation and equilibration with an atmosphere of argon were conducted before a titration was commenced. The potential was adjusted using dithionite as reductant and dry air or ferricyanide as oxidant. Equilibration of the system usually required about 5 minutes after each addition. The equilibrated solution was then transferred to the quartz cuvette via the side arm on the Brinkmann titration vessel or used in place in the custom made vessel.

MCD spectra were obtained using a Jasco-500C spectropolarimeter equipped with an electromagnet and a Jasco DP-500 data processor for data accumulation and manipulation. The instruments were interfaced with a minicomputer for further data processing and presentation. A blank titration in the absence of enzyme indicated that the mediator dyes did not make any contribution to the MCD signal in the wavelength range of interest.

Liquid helium temperature EPR-potentiometric titrations were performed according to a procedure very similar to that of Dutton (177).
A 1 ml Brinkman glass titration vessel was used and the titration was carried out with the vessel directly connected to the anaerobic train (176). The reaction mixture contained 50 \( \mu \text{M} \) Complex III and 20 \( \mu \text{M} \) of each of the eight dyes described above. Before initiating the anaerobiosis, the solution was exposed to air and 50-100 \( \mu \text{g} \) beef heart cytochrome oxidase was added in order to raise the starting potential above 350 mV; the apparatus was then closed and subjected to several cycles of evacuation and gas-replacement. The titration was subsequently carried out exactly as described by Dutton (177) using a bent stainless steel needle for initially flushing the EPR tubes with argon, transferring sample aliquots from the titrator to EPR tubes and flushing the top of EPR tubes before freezing the EPR sample in liquid nitrogen. EPR spectra were obtained using a Varian E6 EPR which was interfaced with the laboratory data acquisition system for data storage and manipulation. The temperature was controlled by an Air Products' transfer system and a heater.

The liquid nitrogen temperature EPR-potentiometric titration for coenzyme Q radical was carried out in much the same fashion except that cytochrome oxidase, p-aminophenol sulfate and TMPD were omitted and the other dyes were present at a concentration of 20 \( \mu \text{M} \) except for methylene blue which was reduced to 5 \( \mu \text{M} \).

Room temperature EPR-potentiometric titration on Q radical was conducted in a 1 ml Brinkmann titration vessel, from which a standard taper side arm projected horizontally near the bottom. A sample transfer tube connected the side arm to a quartz flat cell positioned
in the EPR cavity. The transfer tube was constructed of a 0.042 in.
i.d. polyethylene tubing with standard taper joints cemented to both
ends for connection to the titration vessel and the flat cell. The
polyethylene tubing was enclosed by a 3/16 in. i.d. piece of tygon
tubing with 1/16 in. wall thickness. The annular space between the two
tubes was constantly purged with nitrogen to minimize oxygen
contamination of the sample. The whole titration system was previously
flushed with N\textsubscript{2} for at least 20 minutes. The reaction mixture, which
was the same as that described for liquid nitrogen temperature EPR,
was transferred between the titration vessel and the EPR flat cell by
positive nitrogen pressure either on the end of the flat cell opposite
the sample transfer tube or on the titration vessel.

4. Experiments on the oxidative reaction mechanism:

Complex III was isolated from yeast mitochondria (7); it contained
9 nmoles cytochrome b/mg and exhibited an activity of 60-70 nmoles c
reduced/sec/nmole c\textsubscript{1} using the Q-analog, 2,3-methoxy,5-methyl,
6-pentyl benzohydroquinone (PBQ) as substrate (7). Coenzyme Q-depleted
samples were prepared using hexane extraction of the lyophilized
complex as described by Sarkanowska (83). The residual coenzyme Q was
less than 5% and the CoQ-depleted sample had the same optical and EPR
properties as the original Complex III. The result of reductive
stoichiometric titration indicates that 4 reducing equivalents were
consumed by one mole of the CoQ-depleted enzyme complex (unpublished
data by F.F. de la Rosa). The potentiometric properties of b and c\textsubscript{1}
cytochromes are essentially the same as its parent Complex III (203).

Purified cytochrome \( c_1 \) was obtained according to a procedure
modified from Ross and Schatz (201) and had a purity of 28 mmole
heme/mg protein; its concentration was determined spectrophotometrically
using an extinction coefficient of \( 17.1 \text{ (mM.cm)}^{-1} \) (reduced minus
oxidized) at 553.5 nm. Potassium ferricyanide was obtained from J. T.
Baker and standardized spectrophotometrically using a millimolar
extinction of 1.02 at 420 nm. Antimycin-A was purchased from Sigma and
dissolved in DMSO.

Two-electron reduced Complex III in which only cytochrome \( c_1 \) and
iron-sulfur center are reduced, was prepared either by titrating with
50 mM ascorbate (pH 7.4) or 5 mM dithionite solution (pH 8.3) or,
alternatively, by reduction with a small excess of ascorbate followed
by passage through a Bio-gel P6 column preequilibrated with 0.1 M KPi,
pH 7.4 containing 1 mM EDTA, 0.1% deoxycholate and 0.1% Triton Q5-30.
This partially-reduced Complex III is not significantly auto-oxidizable
and consequently, the kinetic experiments were carried out aerobically.
Both Complex III and ferricyanide were prepared in the buffer just
described. The concentrations of all reactants are expressed as the
final concentration after mixing unless mentioned otherwise.

Stopped-flow experiments were carried out at \( 6 \pm 1^\circ C \) in a
Gibson-Durrum stopped-flow apparatus using a 2cm lightpath for the
observation cell. Kinetic data were collected at 553.5 nm using an
OLIS model 3820 data acquisition system. Each data set is an average
of 2-3 replicates and was stored on disc memory for later analysis and
presentation.

Fully-reduced Complex III or CoQ-depleted Complex III were prepared in one of two ways: either by stoichiometric titration with standardized dithionite solution or by a photoreduction procedure described by Massey and Hemmerick (20). A catalytic amount of deazariboflavin (0.8% of Complex III) was introduced in the sample solution prepared in a tonometer with an attached observation cuvette. The sample solution, thermostated at 10-15°C, was illuminated with strong incandescent light generated by two 150W photographic lights for 5-7 hours. The completion of the photoreduction was identified by the absorbance at 561.5 nm indicating the formation of ferrocyanochrome b. The stopped-flow reaction between fully-reduced sample and various concentrations of ferricyanide were conducted exactly the same way as for partially-reduced sample at 4±1°C. Kinetic observations were carried out at 553.5 and 561.5 nm corresponding to the absorption maxima of ferro-cytochrome c₁ and b respectively.

Stoichiometric reductive titration on CoQ-depleted Complex III was carried out by de la Rosa in this laboratory as described by Siedow et al. (7) using a combined optical-EPR titration technique designed by Palmer (118). All the optical measurements were made on a Cary model 17 spectrophotometer and the EPR spectra recorded on a Varian E6 spectrometer. Both instruments were interfaced with an Interdata minicomputer for data acquisition and manipulation.
Chapter 3. The Coordination Environment of Mitochondrial Cytochromes b

1. Introduction

It is generally believed that the cytochromes b of the mitochondrial electron transport chain are structurally related to the more thoroughly characterized b-type hemes such as that present in microsomal cytochrome b₅. Consequently, the amino acids which function as heme ligands are presumed to be a pair of histidine residues.

However, the electron paramagnetic resonance (EPR) spectra of cytochromes b₅₆₂ and b₅₆₆/5₅₈ for both yeast (7) and heart (36) mitochondria are unusual in that the g values (gₓ) associated with the low-field absorption feature are atypically large, with values ranging from 3.4 to 3.8 in specific cases. By contrast, the gₓ in cytochrome b₅ (12) and other bis-imidazole species (72) has a value of approximately 3.0.

Based on this difference and the demonstration of comparably large g-values in amine derivatives of protoheme (109), it might be expected that the lysine-histidine pair and/or bis-lysine exist as the heme ligands in the mitochondrial b-type cytochromes. However, this proposal undermines the conclusion of Beychok (129), where a detailed comparison of the amino acid sequence of cytochromes b of both mitochondrial and other origin showed homology among these proteins,
especially in the neighborhood of the heme binding domain.

In this chapter we present the results of some EPR and magnetic circular dichroism (MCD) measurements on several pertinent model compounds which make it clear that the unusual EPR features of the mitochondrial cytochromes b can be explained by the traditional bis-imidazole coordination subjected to steric strain.

2. Results and Discussion

Fig. 1 and 2 present the EPR spectra of ferric protoheme coordinated with several imidazole derivatives prepared either in DMSO (Fig. 1) or dichloromethane (Fig. 2). With 1-methylimidazole a typical rhombic low spin heme spectrum is obtained (Fig. 1A and 2a) with $q_x = 1.51$, $q_y = 2.27$, and $q_z = 2.97$. These values are quite typical of those found in well-characterized proteins such as cytochrome b$_5$ (12).

With imidazoles alkylated at the 2-position, quite different spectra were obtained (Fig. 1B and 2b). The most homogeneous spectrum was obtained with 1,2-dimethyl-imidazole which exhibited the low-spin $g_z$ peak at 3.48 (1900 gauss) with a feature reasonably interpreted as $g_y$ at 2.067; $g_x$ is not observed. The spectrum also exhibited a contribution from an axially symmetric high-spin heme with $g = 6.0$ and $g$ clearly visible at 2.0. There was also a signal from iron at $g = 4.3$. The compound prepared in dichloro-methane has less high spin features compared to that prepared in DMSO. When DMSO is at a much higher concentration than 1,2-dimethylimidazole, a direct ligation
Fig. 3.1

EPR spectra of ferric protoheme-imidazole complexes in DMSO. The protoheme concentration was 440 μM.
Fig. 3.2

EPR spectra of ferric protoheme-imidazole complexes in dichloro-methane. The protoheme concentration was 440 uM.
(a) 1-METHYL-INIDAZOLE (XII) IN DICHLOROMETHANE

(b) 1,2-DIMETHYL-INIDAZOLE (XIII) IN DICHLOROMETHANE
between the weak field ligand, DMSO, and heme iron can occur.

The same features are present in the spectrum of the 2-methylimidazole complex. However, with this derivative the contribution of the high-spin species is much larger, and a second minority low-spin species with $g_x = 2.96$ and $g_y = 2.26$ was clearly evident.

A crude attempt to quantitate the low-spin species using Albracht's approximate method (136) showed that the same amount of heme was present in each case; that is, the $g = 3.48$ resonance observed in the complexes with the imidazoles methylated at position 2 are not minority species. It should be noted that it is a characteristic of the $g = 6$ high-spin resonance to dominate an EPR spectrum even when it represents a minor component in solution. The most important conclusion to be drawn from the results is that the unusual EPR of mitochondrial b cytochromes can be reproduced using sterically hindered imidazoles.

The question then arises whether these derivatives are still low-spin at room temperature or whether this low-spin behavior is a consequence of the low temperature of the EPR measurements. This question is addressed in Fig. 3 which shows the Soret and visible MCD spectra of the model compounds. The analogous MCD spectra of Complex III and its component cytochromes are shown in Fig. 4.

It can be seen from Fig. 3 that the low-spin character of the 2-methylimidazole protoheme complex substantially persists at room temperature, whereas the 1,2-dimethylimidazole protoheme derivative
Fig. 3.3

MCD spectra of ferric protoheme complexes with DMSO and imidazoles taken at 20°C. The protoheme concentration was 5.0 μM in a 1 cm pathlength cell for the Soret region and 80 μM in a 0.2 cm pathlength cell for the visible region. Eight passes were averaged per spectrum for the Soret region and 16 passes for the visible region with a time constant of 0.5 s and a scan speed of 50 nm/min.
Fig. 3.4

MCD spectra of oxidized Complex III and isolated cytochromes b and c₁ taken at 10°C. The heme concentrations for Complex III, cytochrome b, and cytochrome c₁ were: for the Soret region, 7.9 uM, 8.2 uM, and 6.9 uM, respectively; for the visible region, 63 uM, 38 uM, and 41 uM, respectively, all in a 1-cm pathlength cell. Other conditions were as in Fig. 2.
has much more high-spin character. In drawing these conclusions, the 1-methylimidazole derivative was used as representative of a low-spin standard and the trough at 418 nm was used as the benchmark. The high-spin reference was provided by the bis-DMSO protoheme complex with its characteristic trough in the near-infrared at 633 nm. Note that this trough is absent in the 1-methylimidazole derivative and slightly and substantially developed in the 2-methylimidazole and 1,2-dimethylimidazole complexes, respectively. The converse behavior is observed in the Soret region MCD spectra.

The heme proteins of Complex III appear to be completely low-spin at room temperature as gauged by the substantial Soret amplitudes and by the absence of any high-spin feature in the near-infrared region. The shape and intensity of the MCD spectrum of cytochrome b in the visible region are very similar to those of other bis-imidazole heme proteins (43).

From these data, it is concluded that the unusual magnetic resonance properties of mitochondrial cytochromes b can be approached in "strained" complexes of heme with sterically hindered imidazoles. These structures are on the borderline of stability and the equilibrium between the high-spin and low-spin configurations can be modulated by temperature, a circumstance which, by analogy, may provide an explanation for the relative ease with which the mitochondrial b-type cytochromes acquire reactivity with CO (148).

Finally, it is concluded that the available EPR data do not contradict the deductions of Beychok (129) drawn from sequence
homologies between mitochondrial and other cytochromes b.
Chapter 4. A facile purification for cytochrome b from Complex III of Baker's yeast

1. Introduction

The cytochrome b(s) of the mitochondrial electron transport chain have long been known for peculiar oxidation-reduction behavior which is assumed to have a close relationship with energy transduction (52, 56). This redox center appears heterogeneous when studied by spectroscopy, potentiometry, and kinetic measurements on mitochondria and on isolated protein complexes. For example, two distinct species of cytochromes are observed in Complex III; cytochrome b-562 (b_\text{K}) has a higher midpoint potential and a lower g value by EPR measurement while cytochrome b-566/558 (b_\text{T}) has a lower midpoint potential and a higher g value. This heterogeneity has led to more confusion than clarification concerning the function of this component. The specific genetic origin of cytochrome b has recently become the focus of intensive investigation (147) and the gene sequence from both yeast (160) and mammalian (138) mitochondria has been established. The isolation of this cytochrome is a prerequisite for the further understanding of its chemical and physical properties and its biosynthesis. Many purification methods for cytochrome b have been reported (57,147) including two procedures for the protein from yeast (5,150). The procedure of Katan et al. (5) yields a product with significant contamination by other proteins while the protein isolated
by Lin and Beattie (150) appears to have lost a substantial amount of heme during isolation.

Purification of this mitochondrial cytochrome has routinely relied on high concentrations of detergents to release the cytochrome b from its extremely hydrophobic membrane environment. Howlett and Wardrop (151) have used 3,4,5,6-tetra-hydrophthalic anhydride to reversibly dissociate and reconstitute the protein components of the human erythrocyte membrane. This lysine-modifying reagent, which has a relatively effective membrane penetrating capability, has been tested as a tool for the isolation of cytochrome b. In this chapter I introduce a simple, rapid and effective procedure for purifying cytochrome b from Baker's yeast by using 3,4,5,6-tetra-hydrophthalic anhydride treatment. The cytochrome b prepared by this method has a heme content of about 37 nmoles/mg protein with the yield of heme averaging 60%, implying that both heme centers are represented in the final product. EPR and MCD spectra of mitochondrial cytochrome b are presented for the first time.

3. Results:

Fig.1 illustrates the elution profile obtained after phthaloylated Complex III has been applied to a hydroxylapatite column; it shows three major peaks with absorbance at 280 nm. The first peak is predominantly excess unreacted anhydride, the second peak contains cytochrome c₁ and uncleaved Complex III and the third peak, which contained most of the 415 nm absorbing material, is the cytochrome b
Fig. 4.1

Hydroxylapatite chromatography purification of cytochrome b. 46 mg of 3,4,5,6-tetrahydrophathalic anhydride-treated Complex III were applied to a hydroxylapatite column (3.5 x 10 cm) which was first washed with 80 ml 50 mM Tris-HCl, pH 8.0, 0.5% cholate, and then developed by a 400 ml linear phosphate gradient from 50 mM to 0.3 M. The major cytochrome b fraction was eluted by 0.3 M phosphate after the first red band was completely eluted at about 150 mM phosphate concentration. The flow rate was maintained at 0.4 ml/min.
fraction. It takes approximately 14 hours to obtain the pure cytochrome b.

Table 1 summarizes the purity and yield of a representative preparation of cytochrome b prepared by this procedure. The average yield for six preparations was 61%, varying from 46% to 79%. This high yield implies that this preparation of cytochrome b includes a contribution from both species of cytochrome b present in the Complex III assuming that the two cytochrome b species exist in a 1:1 ratio (7).

The purity of cytochrome b varied from 36.6 to 37.8 nmole b/mg protein as determined by the pyridine hemochromogen method. This value which is comparable to that obtained with the best preparations of the heart protein (158) and is equivalent to a minimum molecular mass of 27,000 Da. Attempts at further purification by gel filtration or density-gradient centrifugation resulted in no significant improvement in purity.

After 42 hours of dialysis against 0.1 M MES, pH 6.0, containing 2.0% taurocholate to remove the chemically bound 3,4,5,6-tetra-hydrophthalic anhydride (151); the purified cytochrome b precipitated from solution and the resultant highly aggregated product was extremely difficult to redissolve, even when the solvent contained high concentrations of ionic or nonionic detergents.

A typical polyacrylamide slab gel pattern for purified cytochrome b is shown in Fig. 2a. A band on the top of the separating gel in the rightmost column is the super-aggregate formed by treating the cytochrome b sample at 100°C for 5 min. The other gels correspond to
<table>
<thead>
<tr>
<th>Step of fraction</th>
<th>Protein (mg)</th>
<th>Heme b (nmole/mg)</th>
<th>Heme b/prot. (nmole/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCl-complex</td>
<td>46.2</td>
<td>415</td>
<td>9.0</td>
<td>100</td>
</tr>
<tr>
<td>Anhydride treatment and hydroxylapatite column chromatography</td>
<td>6.9</td>
<td>253</td>
<td>36.7</td>
<td>61</td>
</tr>
</tbody>
</table>

Table 1: Yield and purity of the preparation procedures.
varying amounts of cytochrome b treated at 25°C overnight before applying to the gel. A dark protein band in each column can be clearly seen with a second visible band of higher molecular weight can be observed in the 3th gel to which 25 ug protein were applied. These two bands are also conspicuous when Complex III is treated and electrophoresed by the same procedure. Due to different conditions for sample treatment, this result is not the same as that previously reported (7). An estimate of the molecular weight was obtained by electrophoresis of the cytochrome b sample together with four protein markers on a 12% acrylamide analytical slab gel. The molecular weight of cytochrome b as judged by this method is approximately 25,000-26,000 daltons, in good agreement with the heme content. Isolated cytochrome b apparently exists in an aggregated form since it is eluted in the void volume on gel filtration through Biogel P-60. A small and variable amount of a species of molecular weight 50,000-52,000 is often observed. Although the possibility cannot be ruled out that this is a true contaminant, this value suggests that it might be a dimer of cytochrome b. This high molecular weight species does not correspond to any component observed in Complex III when run in the same electrophoresis system. I found no evidence for two species of closely related molecular weight in the main band even when examined in highly resolved gradient gels; thus, the two forms of cytochrome b recently reported by Chen and Beattie (157) are apparently not present in our preparation.

The amino acid composition of cytochrome b is given in Table 2.
Fig. 4.2

SDS polyacrylamide gel electrophoresis of purified cytochrome b. A 12.5%-20% gradient acrylamide gel with 5% stacking gel was used. 10 μg cytochrome b treated under 100°C, 5 min, was applied on the rightmost column. Samples applied on the other columns were, from right to left, 5, 10, 2, 25, 2, 15 μg. These samples were previously treated with 80 mM Tris-HCl, pH 6.8, containing 2% SDS, 0.1 M dithiothreitol, 10% glycerol, 0.2% bromothymol blue at 25°C overnight.
Table 2:
Amino acid composition of purified cytochrome b from yeast

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mole percentage</th>
<th>Data from gene sequence (14)</th>
<th>This work.</th>
<th>Katan et al. (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid (Asx)</td>
<td></td>
<td>7.3-7.5</td>
<td>7.9</td>
<td>8.6</td>
</tr>
<tr>
<td>Threonine *</td>
<td></td>
<td>4.1</td>
<td>2.8</td>
<td>5.3</td>
</tr>
<tr>
<td>Serine *</td>
<td></td>
<td>7.3</td>
<td>5.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Glutamic acid (Glx)</td>
<td></td>
<td>2.8-3.1</td>
<td>4.0</td>
<td>6.6</td>
</tr>
<tr>
<td>Proline</td>
<td></td>
<td>4.9</td>
<td>5.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td>6.5</td>
<td>7.9</td>
<td>6.5</td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td>5.7</td>
<td>6.5</td>
<td>7.6</td>
</tr>
<tr>
<td>Cysteine</td>
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<td>1.0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Valine @</td>
<td></td>
<td>9.3</td>
<td>9.6</td>
<td>9.4</td>
</tr>
<tr>
<td>Methionine</td>
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<td>3.9</td>
<td>2.6</td>
</tr>
<tr>
<td>Isoleucine @</td>
<td></td>
<td>10.6</td>
<td>9.4</td>
<td>8.1</td>
</tr>
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<td>Leucine @</td>
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<td>11.2</td>
<td>11.1</td>
<td>11.5</td>
</tr>
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<td>Tyrosine</td>
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<td>5.4</td>
<td>5.4</td>
<td>3.4</td>
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<td>Phenylalanine</td>
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<td>8.4</td>
</tr>
<tr>
<td>Tryptophan</td>
<td></td>
<td>1.8</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td>Lysine</td>
<td></td>
<td>1.8</td>
<td>3.1</td>
<td>4.4</td>
</tr>
<tr>
<td>Histidine</td>
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<td>3.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
<td>2.8</td>
<td>4.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Polarity</td>
<td></td>
<td>29.2-30.0</td>
<td>30.5</td>
<td>37.4</td>
</tr>
</tbody>
</table>

* Corrected for destruction by zero-time extrapolation
@ From 120-hour hydrolysis
together with the composition reported by Katan et al. (5) for their
preparation of cytochrome b isolated from yeast mitochondria and that
deduced by Nobrega and Tzagoloff (160) from gene sequence of this
protein. Except for a few polar amino acids, i.e., arginine, lysine,
threonine and glutamic acid, the amino acid composition obtained
directly in this work and indirectly by Nobrega and Tzagoloff (160) are
very similar, especially with respect to the content of nonionic amino
acids (the phthaloyl group attached on lysine residues does not affect
the amino acid analysis, since the amide bond formed between phthalic
anhydride and the free amino group of lysine is completely hydrolyzed
by the 6N HCl). This highly hydrophobic amino acid composition is
consistent with location of cytochrome b as an integral protein in the
mitochondrial inner membrane. The amino acid composition of the
cytochrome b preparation of Katan et al. indicates a greater polarity
for this preparation which may well be due to the impurities which can
be observed in the gel pattern reported (5).

Enzyme activity:

Enzyme activity was tested by following absorption change at 561.5
nm after mixing 5 or 10 fold excess amount of substrate with oxidized
cytochrome b at pH 7.4 in 0.1 M KPi at room temperature. This
preparation of cytochrome b is not reduced by succinate in the
presence of a catalytic amount of complex II, nor does it catalyse
electron transfer from the reduced coenzyme Q analog, 2,3-methoxy,
5-methyl,6-pentyl benzohydroquinol (PBQ), to oxidized cytochrome c.
Preincubation of the purified cytochrome b with excess amount of CoQ₁₀ extracted from yeast Complex III and asolectin suspension did not restore activity; furthermore, at the completion of the derivatization reaction the Complex III had no measurable activity in this assay.

Optical spectra:

The optical spectra of oxidized and reduced cytochrome b in the alpha and Soret region are presented in Fig.3; selected parameters are summarised in Table 3. The absorption maxima of oxidized cytochrome b occur at 567, 530, and 415 nm, and those for the reduced protein are at 561.5, 532, and 428 nm. These spectra have exactly the same extrema as those found for the cytochrome b component(s) in the intact Complex III as judged from the difference spectra between dithionite reduced and ascorbate-reduced Complex III (7).

Carbon monoxide reactivity was investigated by incubating reduced cytochrome b under an atmosphere of CO in a stoppered cuvette with frequent mixing. There was an approximately 60% decrease in the absorbance at 561.5 nm during a 40 min. period. Addition of 1% sodium lauryl sarcosinate to the incubation mixture led to a further large decrease in the peak at 561.5 nm, suggesting that some of the cytochrome b was originally in a form unavailable for reaction with CO.

MCD spectra and the temperature dependence at Soret region:
Fig. 4.3

Optical spectra of purified cytochrome b. The spectrum in the alpha region was taken with 23.0 uM cytochrome b; 4.88 uM cytochrome b was used for the Soret region. A meaningful spectrum in the ultraviolet region could not be obtained due to the absorption of phthaloyl substituents. Samples were dissolved in 0.1 M KPi, pH 7.4 containing 0.5% cholate. The temperature was 24°C. (---), oxidized sample; (----), dithionite-reduced sample.
×4.7
The MCD spectra of ferro- and ferricytochrome b in the Soret region are given in Fig. 4. The spectrum of the reduced sample has extrema at 431, 418 nm with the crossover point at 427 nm, close to the maximum (428 nm) in the corresponding optical spectrum. The oxidized cytochrome b has more conspicuous extrema at 407 nm and 420 nm, the crossover is 413 nm, close to the absorbance maximum. These spectra closely resemble corresponding spectra of cytochrome c and cytochrome b₅ (43) and are typical of low-spin ferric hemes. The intensity of the Soret signal (Fig. 4, Inset) exhibits a linear dependence on the reciprocal of the temperature at both 420 and 407 nm. This linear dependence demonstrates that this derivative-shaped feature is composed primarily MCD C-terms similar to other low-spin ferric heme proteins (42, 43).

Fig 5 presents the MCD spectra for both oxidized and reduced cytochrome b in the alpha region. The spectrum of the oxidized sample has extrema at 551, 569 nm and a crossover point at 558 nm, while the reduced sample displays a spectrum with a peak at 555nm, a trough at 561 nm and a crossover at 558 nm. The very large intensity in the alpha region for reduced cytochrome b (Fig. 5a), $350(M\cdot cm\cdot T)^{-1}$ (peak to trough amplitude), is due to an MCD $A$ term which arises through the removal of the double degeneracy of the excited state for the $\pi-\pi^*$ electronic transition by the external magnetic field (47); this $A$-term is similar to that observed for reduced cytochrome b₅ and myoglobin-imidazole (43) and is typical of $D_{4h}$ hemes. The visible MCD of the oxidized protein (Fig. 5b) shows great similarity to other bis-
Fig. 4.4

MCD in the Soret region of purified cytochrome b and the temperature dependence of the signal at 420 nm and 407 nm (Inset). The sample for the temperature study was prepared by mixing 3 parts 127 uM cytochrome b solution in 0.1 M KPi, 0.5% cholate, pH 7.4 with 7 parts glycerol. The temperature was cycled between 292 K and 141 K, the open and solid symbols represent data obtained with decreasing and increasing temperature, respectively. DITH: dithionite.
Fig. 4.5

MCD spectra of purified cytochrome b. (a) Oxidized and dithionite-reduced cytochrome b spectra. (b) enlarged MCD spectrum of oxidized cytochrome b. Condition for these spectra were as in Fig. 3.
Table 3. Parameters of optical absorption and magnetic circular dichroism of the purified cytochrome b

<table>
<thead>
<tr>
<th>Oxidation-reduction state</th>
<th>Absorption</th>
<th>MCD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda$</td>
<td>$\varepsilon$</td>
</tr>
<tr>
<td></td>
<td>(nm)</td>
<td>(cm mM)$^{-1}$</td>
</tr>
<tr>
<td>Oxidized</td>
<td>567</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>530</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>569</td>
<td></td>
</tr>
<tr>
<td></td>
<td>415</td>
<td>142.6</td>
</tr>
<tr>
<td></td>
<td>413</td>
<td></td>
</tr>
<tr>
<td></td>
<td>420</td>
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<tr>
<td>Dithionite reduced</td>
<td>561.5</td>
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<tr>
<td></td>
<td>532</td>
<td>18.1</td>
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</tr>
<tr>
<td></td>
<td>418</td>
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</table>
imidazole hemes proteins (43) and the bis(1-methylimidazole) protoheme model compound (161). There is no trace of the characteristic charge-transfer transition (Fig. 5b) which is usually observed around 620-640 nm in high-spin protoheme proteins (42).

EPR spectra of purified cytochrome b:

Two $g_z$ signals are observed for cytochrome b in yeast Complex III, with values of 3.60 and 3.76 (7). Slightly different values are found with Complex III from beef heart mitochondrial (162,163). These different $g$ values correspond to cytochrome b species having different environments and apparently midpoint potentials (7). The $g = 3.7$ resonance has an unusual line shape as was previously noted for the mammalian counterpart (163); no explanation has been advanced for this line shape. The purified cytochrome b, however, exhibits a single EPR signal at $g=3.70$ (Fig. 6) midway between the two values found in Complex III. This change in EPR, which is already complete at the end of the derivatization reaction, is indicative of a slight modification of the immediate coordination environment of the metal iron in both heme centers. Nevertheless, this $g$ value is much larger than that found with cytochrome $b_5$ or bis-imidazole protoheme. Thus it appears that this preparation of purified cytochrome b still maintains its proposed "strained" coordination structure (161). Treatment of the protein with a strong denaturant such as SDS (greater than 1%) causes the $g=3.70$ signal to be shifted upfield to $g=2.90$, the normal $g_z$ position for bis-imidazole heme (109). Sonication also produces this
Fig. 4.6

EPR spectra of oxidized Complex III (172 uM cytochrome b) (—) and oxidized cytochrome b (110 uM) (—). The spectrum of reduced cytochrome b is also shown (——). The EPR spectra were recorded under the following conditions: modulation amplitude, 20 gauss; microwave power, 40 mW; microwave frequency, 9.238 GHz; temperature, 8.7°K; scan rate, 250 gauss/min.; time constant, 1 sec.
transition. Cytochrome b purified from aged or less active Complex III shows both g=3.70 and 2.90 signals.

Midpoint potential:
The midpoint potential of purified cytochrome b was determined by an MCD-potentiometric titration in a manner similar to that described by Wilson and Dutton (164). The Nernst log-plot (Fig. 7) strongly resembles that of a simple one-electron carrier and yields a value of -44 mV for the midpoint potential. The same titration procedure performed on Complex III treated with 3,4,5,6-tetrahydrophthalic anhydride resulted in the same midpoint value. However, two potentiometrically different cytochrome b species (Em,v = -20 mV, and +62 mV, respectively) are found in intact yeast Complex III (203). This result combined with the above EPR studies, and with the demonstration of a single species by both optical (Fig. 3) and MCD (Fig. 5) spectroscopy, demonstrates that 3,4,5,6-tetrahydrophthalic anhydride treatment removes the apparent environmental difference between the two heme b's in Complex III and results in a single species.

Antimycin-A binding:
Antimycin-A binding was studied by the fluorescence quenching method described by Berden and Slater (104, 113). The results of direct fluorimetric measurement and determination by the precipitation method of antimycin-A binding to Complex III are presented in Fig. 8. Slope
MCD-potentiometric titration of purified cytochrome b and anhydride-treated Complex III. Titrations were conducted at 25°C, in 0.1 M KPi, pH 7.4, containing 0.1% deoxycholate and 0.1% Triton QS-30, with 9.5 μM cytochrome b and 4.0 μM anhydride-treated Complex III. 20 μM each of the following dyes were introduced, N,N,N',N'-tetramethyl-p-phenylene diamine dihydrochloride (TMPD), 2,6-dichlorophenol indophenol (DCPIP), 1,2-naphthoquinone (1,2-NQ), phenazine methosulfate (PMS), methylene blue (MB), indigo tetrasulfonate, sodium salt (ITS), 2-hydroxy-1,4-naphthoquinone (2-OH-1,4 NQ). Dithionite and ferricyanide solution were used to adjust the potential.
Cytochrome b
Tetrahydrophthalic Anhydride Treated bc₁₀

Log₁₀(REDUCED) / [O₂] × 10⁷

Measured Potential, mV

0
-100
changes are readily observable in data obtained for Complex III
(Fig. 8a) by both methods; however, no break point can be found in
comparable data for cytochrome b (Fig. 8b). The fluorescence end point
and the elimination of enzyme activity observed with Complex III
correspond to the 1:1 binding stoichiometry of antimycin-A. These
results clearly show that antimycin-A binds to yeast Complex III
stoichiometrically and totally inhibits electron transfer as was
previously reported by Slater (104) for the beef heart mitochondrial
Complex. However, the purified cytochrome b did not show any binding
with antimycin-A by either the fluorescence or precipitation method
(Fig. 8b). Binding with antimycin-A also could not be demonstrated with
the second fraction (Fig. 1) eluted from a hydroxylapatite column
(Fig. 8b) nor with Complex III at the completion of the reaction with
phthaloyl anhydride.
antimycin-A binding measurement performed on (a) Complex III and (b) purified cytochrome b. Direct fluorescence method (□---□) was implemented with 7.5 nmole bc$_1$ or 20 nmole purified cytochrome b in 0.1 M KPi, pH 7.4 containing 0.5% cholate. The same amount of bc$_1$ and b were used for the precipitation method (○---○). Centrifugation was done at 4°C and 48,000xg for 3 h. The enzyme activity (▲---▲) was measured as described in(7) using a reduced CoQ analog, PBO, and oxidized cytochrome C as substrates at 25°C (▲---▲ in (b)) represents the direct fluorescence measurement on the pooled second fraction from the hydroxylapatite column. This sample contained 8 μM cytochrome c$_1$. 
4. Discussion:

A convenient high-yield procedure for purified cytochrome b from yeast has been developed. This cytochrome preparation is homogeneous and has a high heme content which has facilitated the study of the heme coordination environment by spectroscopic methods (161). The major step in the procedure is the dissociation of the cytochrome b subunit from Complex III by chemically modifying the ε-amino group of lysine residues. The hydrophobic substituted benzene ring of the tetra-hydrophthalic anhydride appears to be necessary for effective penetration of the hydrophobic environment around cytochrome b, since other acid anhydrides, such as 2,3-dimethyl-maleic anhydride in a 3:1 (w/w) ratio to protein were not sufficient to dissociate cytochrome b from its native complex using the same procedure. Although 3,4,5,6-tetra-hydrophthalic anhydride is a potent dissociating reagent, the immediate heme environment remained relatively unperturbed. Thus the optical and MCD spectra of this purified cytochrome b closely resemble the difference spectra of Complex III which isolate the spectral contributions of the b cytochromes while the EPR spectrum showed only a slight change in the low field g value compared to Complex III.

A wide range of estimates for the molecular weight of yeast cytochrome b has been reported. Katan et al. (165) obtained a value of about 32,000 daltons both with purified cytochrome b and radioactively
labeled Complex III while Lin and Beattie (150) obtained 28,000 for the
purified protein. Tsagoloff and Nobrega (166) deduced a value of 44,000
from the gene sequence. I find a molecular weight of 25,000-26,000 for
cytochrome b isolated by our procedure. This is the third instance in
which the size of a hydrophobic polypeptide determined by SDS gel
techniques is found to be substantially smaller than that anticipated
from the polypeptide sequence inferred from the gene sequence. Thus,
polypeptides I and III of mammalian cytochrome oxidase exhibit $M_r$
values on SDS gels of 36,000 and 21,000, respectively (167), while the
respective genes specify polypeptides of size 57,000 and 30,000 (138).
The value of 57,000 for subunit I is supported by results from chemical
labelling of the single cysteine residue present in the polypeptide
(168). Clearly, size estimates obtained by SDS gel electrophoresis must
be viewed with considerable caution although this ambiguity will not be
resolved until a complete amino acid sequence on the post-translational
purified cytochrome b species is obtained.

Assuming that the results from the gene sequence are in fact
correct, then the heme content of 38 nmol per mg (equivalent to a
minimum molecular weight of 26,000) implies that there are two heme
centers per polypeptide; our preparation would thus contain about
1.5-1.6 mol of heme per mol protein. It is then plausible to equate
one of these with cytochrome b$_{562}$ and the other with b$_{566}$ as I have
no persuasive evidence from either absorbance or MCD measurements of
the presence of more than two heme centers in our preparation of yeast
Complex III.
The MCD and EPR characteristics of the isolated protein are characteristic of a low-spin hemoprotein in both redox states. The visible MCD of the ferric protein is quite similar to authentic bis-imidazole systems, suggesting that this coordination also exists in mitochondrial cytochrome b. The value of $g_2$ in the EPR spectrum is dramatically larger than that found in bis-imidazole protoheme and cytochrome b$_5$ but values approaching this have recently been demonstrated in low-spin protoheme derivatives containing imidazole analogs which are sterically hindered by virtue of methylation at C$_2$(161). It thus seems plausible that the unusual $g$ values of the mitochondrial cytochromes b arise by virtue of structural tension at the iron. The strained structure appears to be substantially maintained in this preparation (Fig.6).

The precise number of cytochrome b species associated with mammalian Complex III is unclear. For example, Berden et al. (169) have obtained optically derived potentiometric data from beef heart mitochondria which demonstrate that all species of cytochrome b are heterogeneous in their response to the addition of ATP. They correlated the behavior of the 558 nm spectrophotometric feature with the low-potential cytochrome b species. In a different approach, De Vries et al. (165) have analyzed the lineshapes of the low-field EPR of the b cytochromes and concluded that the peak at $g$ 3.76 was composed of separate broad and narrow resonances, each contributing 0.5 equivalents of a b heme which correlated with the 558 and 566 nm optical species although specific assignments could not be made. However, the
response of inhibited rat liver mitochondria to succinate led Higuti et al. (170) to conclude that the optical absorption at 558 nm is unrelated to cytochrome to b-566 and may in fact be due to a species unrelated to the mitochondrial respiratory chain. Insofar as I have not obtained any evidence for more than two species of cytochrome b in the yeast system, the simplest interpretation of these facts assumes that cytochrome b$_{566}$ has a highly malleable structure and the exact chemical and physical characteristics of this species depend sensitively on the history of the enzyme preparation.

Potentiometrically, the purified cytochrome b behaves like a single one-electron carrier as do most other purified cytochromes b. However, data from the potentiometric titration of yeast Complex III obtained under the same condition exhibit the traditional biphasic character (171), implying the presence of two different redox-active cytochrome b species; Apparently, this benchmark of multiple cytochromes b was removed by the derivative-formation procedure. Notably, Von Jagow et al. (172) purified a cytochrome b from beef heart which retained the two-cytochrome b character demonstrated by potentiometric titration. Although the midpoint potentials of this purified cytochrome b preparation are quite different from those values obtained with intact Complex III, this multiplicity in potentiometric properties was removed by urea treatment. EPR characterization of this beef heart cytochrome b would provide valuable confirmation of the presence of distinct cytochrome species.

Recently, Roberts et al. (111) tested the antimycin-A binding
capabilities of several cytochrome b-deficient yeast mutants and concluded that cytochrome b is the antimycin-A-binding component of Complex III. However, this conclusion has yet to be substantiated using purified cytochrome b (Refs. 113 and 172 and this work). In our hands, stoichiometric binding of antimycin-A to Complex III was readily demonstrated whereas I could obtain no evidence for antimycin-A binding to the purified cytochrome. If cytochrome b is indeed required for antimycin-A binding, then either the binding site for antimycin-A was modified by the derivative-formation procedure or the binding of antimycin-A results from the cooperative action of the protein and an additional component of Complex III. The latter possibility is supported by the observation by Berden and Slater (113) that partially purified cytochrome b (73) still maintained the capacity to bind antimycin-A, although the stoichiometry is much higher than 2:1; this was attributed to partial denaturation. However, the absence of antimycin-A binding in derivatized Complex III could be taken as evidence in support of the former alternative.

Antimycin-A is known to destabilize the coenzyme Q radical signal (96) in Complex III and also competes with the same binding site for HQNO (2-heptyl-4-hydroxy quinoline-N-oxide), a coenzyme Q analog and a cytochrome bc1 inhibitor (107). In contrast, the Q-binding protein, QPs, is found to stabilize semiquinone radicals in succinate cytochrome c reductase (169). A second Q-binding protein reportedly exists in the Complex III, QPc (102), playing a role similar to QPs. This protein may be the antimycin-A binding protein
reported by Das Gupta and Rieske (171).
Chapter 5. Potentiometric studies on yeast Complex III

1. Introduction

The potentiometric behavior of Complex III has been characterized while present in mitochondria and as purified preparations isolated from beef heart (56,174), pigeon heart (175) and other sources (see review 52,56). A highly purified, enzymatically active Complex III preparation from yeast mitochondria was obtained (7), and the chemical composition, spectral properties and redox characteristics reported (7). In particular, optical and EPR measurements were used to monitor the changes in cytochrome b, cytochrome c₁, iron-sulfur protein and ubiquinone radical during stoichiometric reductive titrations. 7.5 electron equivalents were required to completely reduce one equivalent of Complex III. This stoichiometry is only slightly larger than the value of 6.5 expected on the basis of the presumed complement of redox centers, and the extra equivalent was assumed to reduce the small amount of adventitious iron detected in the preparation. This result implies that there are no other important oxidation-reduction centers present in this yeast preparation.

The redox centers in Complex III can be divided into two groups: components of high potential, namely cytochrome c₁ and the iron-sulfur center, and components of low potential, the cytochrome
b(s) and coenzyme Q. The relative redox potentials of these components estimated from the stoichiometric titration fell within the range of the literature values previously reported for the enzyme from beef heart and other sources.

As part of our program to characterize the internal electron transfer sequence in Complex III, the absolute potentials of each redox component are needed. These are not simply obtained from a stoichiometric titration. Consequently, we have undertaken a potentiometric study of yeast Complex III and its isolated components. Our data includes room temperature MCD potentiometric titrations on cytochrome b and c\(_1\); liquid helium EPR-potentiometric titrations on cytochromes b, c\(_1\) and the iron-sulfur center and liquid nitrogen and room temperature EPR-potentiometric titration of ubiquinone. A number of parameters which might influence the potentiometric properties of each redox component were also investigated. These include the effect of temperature of observation, variation in pH, detergent and mediator dyes and the presence and absence of the inhibitor, antimycin-A.
Data analysis:

In MCD titrations, the redox state of cytochrome c₁ was followed using the change in amplitude of the peak at 548.5 nm relative to the 553.5 nm trough, while the redox state of cytochrome b was monitored using the amplitude of 561.5 nm trough. The data for c₁ yielded a linear Nernst log-plot and the midpoint potential was obtained directly from the graph. For cytochrome b, the Nernst plots were biphasic, and the midpoint potentials of the two components were obtained by extrapolation of the theoretical n=1 line from each arm of the titration curve to the horizontal line drawn at 50% reduction (as illustrated in Fig. 2).

The reduction of the iron-sulfur center was monitored by the intensity of g=1.89 EPR signal recorded at liquid helium temperatures. The reduction of each cytochrome was quantitated by locating empirically the experimental spectrum which led to the best lineshape of each species as judged by visual examination of difference spectra displayed on a graphics monitor. Difference EPR spectra calculated relative to this reference were required to exhibit a systematic decrease in EPR amplitude as the potential was reduced. This procedure facilitated correction of the EPR contribution of each cytochrome for overlap from the other components present. The midpoint potential of each cytochrome was obtained by the same method as described in MCD-potentiometric titration. Oxidized cytochrome c₁ was quantitated
using the amplitude of the \( g=3.49 \) EPR signal, and the high and low potential species of oxidized cytochrome \( b \) were quantitated using the EPR intensity at \( g=3.60 \) and \( g=3.76 \) respectively.

The quantitation of coenzyme \( Q \) radical at both ambient and liquid nitrogen temperature was done using the \( g=2.0 \) radical signal after correction for the contribution from the mediator dyes; this contribution was obtained in a separate experiment from a reaction mixture containing dyes alone at the same potential. The midpoint potential values for the two half-reactions: \( Q/\text{OH}^- \) and \( \text{OH}^-/\text{OH}_2 \) were obtained by fitting the data to the following equation:

\[
[SG] = \left( 1 + 10^{(E-E_1)/59} + 10^{(E-E_2)/59} \right)^{-1}
\]

where \([SG]\) indicates the fraction of the maximum radical signal relative to the total coenzyme \( Q \) present in the original Complex III. \( E_1 \) and \( E_2 \) are the midpoint potential values for the two half reaction, \( Q/\text{OH}^- \) and \( \text{OH}^-/\text{OH}_2 \) respectively. The absolute spin concentration was determined from double integral which was then compared with that of a cupric sulfate standard at liquid nitrogen temperature or a standard solution of potassium nitroso-disulfonate at room temperature.
3. Results:

The MCD characteristics of Complex III and purified cytochromes b and c₁ are presented in Table 1. The MCD spectra of Complex III between 530-590 nm at different redox states are shown in Fig 1. The spectrum of the oxidized enzyme is extremely weak with a peak at 550 nm, a trough at 570 nm and a crossover at 559 nm. Ascorbate-reduced Complex III has intense extrema at 548.5 and 553 nm and a crossover at 551 nm. The difference spectrum of ascorbate-reduced Complex III minus oxidized Complex III is essentially that of reduced cytochrome c₁ (data not shown) and because of the small intensity of the oxidized complex it is very similar to the absolute spectrum of the ascorbate-reduced sample. The MCD spectrum of the dithionite reduced complex exhibits additional intense features due to the formation of reduced cytochrome b. The difference spectrum of dithionite-reduced minus ascorbate-reduced Complex III has a peak at 556.5 nm, a trough at 562 nm and a crossover at 559 nm. These extrema are very close to the corresponding values for purified reduced cytochrome b (31). The calculated MCD intensities per protoheme are 197 (M.cm.T⁻¹) at 556 nm and -262 (M.cm.T⁻¹) at 562 nm for the complexed cytochrome b; these are higher than the corresponding values of 160 and -190 (M.cm.T⁻¹) for purified cytochrome b. The shape and the large intensities of these spectra arise from A-terms and are typical of low-spin reduced protohemes (43). The MCD spectra of cytochrome b closely resemble those
Fig. 5.1

Absolute (a) and difference (b) MCD spectra of Complex III both in alpha and soret regions.

The sample contained 3.9 uM c₁, 8.3 uM b and was solubilized in 0.1 M KPi, pH 7.4, containing 0.1% deoxycholate, 0.1% Triton GS-30. The absolute spectra of ascorbate-reduced and dithionite-reduced sample were recorded after the addition of solid sodium ascorbate and sodium dithionite respectively. The difference spectra were obtained from their absolute spectra by arithmetic subtraction using the laboratory data system.
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** cytochrome c₁ was prepared according to a procedure modified from Ross and Schatz (44).
of submitochondrial particles isolated from beef heart (11). The
difference spectra of the dithionite-reduced minus ascorbate-reduced
samples and the dithionite-reduced minus oxidized Complex III are
identical above 557.5 nm. Importantly, the trough at 562 nm has the
same amplitude in these two derivatives; this feature is attributable
to cytochrome b alone. The Soret MCD of oxidized, ascorbate-reduced
and dithionite-reduced Complex III do not have the apparent exclusive
feature individual to b and c₁ cytochromes; instead, the Soret MCD
spectra contributed by cytochrome b and c₁ always have significant
overlap with each other (Fig.1).

Because of the large MCD intensities for both cytochrome b and
c₁ and the well-separated redox potentials of these species, I was
able to perform MCD-potentiometric titrations with a relatively small
amount of material. However, the intense dye absorbance precludes a
direct comparison of the MCD and absorbance techniques. I therefore
evaluated the MCD approach by comparing MCD and optical data during a
stoichiometric titration following the MCD intensities at 562 nm and
548-553 nm and the absorbance changes at 561.5 nm and 553.5 nm for the
b and c₁ cytochromes respectively. The two sets of measurements were
in excellent agreement. Room temperature MCD potentiometric
titrations:

Since the highly colored mediator dyes make no contribution to the
MCD spectrum in this wavelength range, it was possible to use a
relatively large number of dyes to satisfactorily cover the wide
potential span necessary to observe the oxidation-reduction of both
Fig. 5.2

Room temperature MCD-potentiometric titration curves of Complex III.

The titrations were performed at 23°C in 0.1 M KPi, pH 7.4, containing 1 mM EDTA, 0.1% Triton GS-30 and either 0.1% taurocholate (□) or deoxycholate (△) buffer. 5 uM Complex III and 20 uM dyes were present in the reaction mixture. The reduction of cytochrome c₁ (data at left) was monitored by the change in the difference between the MCD intensities at 548.5 and 553 nm; and the concentration of reduced cytochrome b obtained from the MCD intensity at 562 nm. The midpoint potential was obtained by determining the intersection of the theoretical n=1 lines of the two side arms of the titration curve with the horizontal line drawn at 50% reduction (log[reduced form]/[oxidized form]=0).
cytochromes (178). In these experiments, eight mediator dyes were used, including the three classes of dyes described by Walz (179). It should also be noted that, in contrast to the absorbance properties, the MCD characteristics of the b and c cytochromes do not overlap.

A typical log-plot of data from an MCD-potentiometric titration of Complex III is shown in Fig.2. Cytochrome c₁ behaves as a simple one-electron carrier with a mid-point potential at room temperature of 270mV; however the MCD parameter characteristic of the b cytochrome(s) exhibits the well-known non-linear behavior which can, in this instance, be analyzed as the sum of two one-electron redox processes. This non-linearity can be seen clearly when titrations are carried out in either taurocholate-containing or deoxycholate-containing buffer. The derived mid-point potentials for the two b cytochromes are 62 and -20 mV in deoxycholate buffers; however, in taurocholate both potentials are significantly more positive, 98 and 40 mV respectively.

MCD-potentiometric titrations were carried out at pH 7.4 using ratios of mediator dye/enzyme complex which varied from 2 to 25. When this ratio was less than 10 the mid-point potential of cytochrome c₁ was found to be 270 mV. However on increasing the ratio above 10 the mid-point potential decreased markedly and at a ratio of 25 the potential of cytochrome c₁ was found to be -40 mV. This amounts to a decrease of over 300 mV (Fig. 3) in response to a modest variation in relative dye concentration. Consecutive additions of each of the eight dyes at a dye:c₁ ratio of 20:1 to a solution containing ascorbate-reduced Complex III led to a stepwise disappearance of the MCD signal
of reduced cytochrome \( c_1 \) implying oxidation of reduced cytochrome
\( c_1 \) by the dye additions. Subsequent addition of excess ascorbate did
not restore the original reduced \( c_1 \) MCD signal, consistent with the
midpoint potential of \( c_1 \) being more negative in the presence of
concentrated dyes. This dye effect does not seem to be specific for any
particular dye used, a simple additive effect being apparent. The b
cytochromes, however, do not exhibit this sensitivity to the mediator
dyes, at least in the concentration range that we have studied.
Consequently, all titrations were performed using a ratio of dye: \( c_1 \)
less than 10, a condition which results in a traditional value for the
\( c_1 \) potential (52). As an additional control, a titration was
performed using the following alternative set of dyes (dye: \( c_1 \) = 7:1):
quinhodrine, duroquinone, 1,2-naphthoquinone, thionine, methylene blue,
2,5-dihydroxy 1,4-benzoquinone and indigocarmine. The midpoint
potentials for both \( c_1 \) and b cytochromes obtained using this
alternative system were similar to the values obtained using the original
dye combination at the low dye:protein ratios.

Low temperature EPR potentiometric titrations:
Unfortunately, we were not able to identify any feature in the CD
spectrum of Complex III that we could attribute to the \( [2Fe/2S] \)
cluster, even though such iron-sulfur clusters typically have intense
and characteristic CD (48, 49). Consequently we were unable to obtain
data on this component at room temperature. Thus a series of
potentiometric titrations were evaluated at liquid helium temperature
Fig. 5.3

The effect of ratio of dye to protein on the midpoint potentials of the components of Complex III: All titrations were carried out at pH 7.4, in 0.1 M KPi, 0.1% deoxycholate, 0.1% Triton QS-30 and 1 mM EDTA at 23°C, 2.5 μM or 5.0 μM Complex III was used in each titration.
using EPR spectroscopy to follow the signals of the reduced iron-sulfur center and of the three oxidized cytochromes; these latter data provide a connection with the results obtained at room temperature. This redox center exhibits a potentiometric behavior typical of a one-electron carrier, and the midpoint potential of the iron-sulfur center was found to be 286 mV (Fig. 4). The midpoint potential of cytochrome c1 deduced from the low temperature data, 270 mV, is precisely that obtained at room temperature (Fig. 2), and the difference of 16 mV between the midpoint potentials of iron-sulfur cluster and cytochrome c1 agrees reasonably well with the estimate of 30–40 mV obtained from combined optical-EPR stoichiometric titrations (7). However, the midpoint potentials of the b cytochromes obtained at 12°C are clearly different from the values obtained at 296°C; the value of 62 mV found for the high potential cytochrome b in deoxycholate buffer increases to 116 mV at cryogenic temperatures while the value of the low potential b is increased only slightly, from -20 to -4 mV. This difference in potential measurements obtained at the two temperatures can be explained in part by the difficulty in unambiguously determining the EPR contribution of the two b cytochromes from their highly overlapped EPR signals; however, the potential shift of the high potential b cytochrome is too large to attribute to analytical error and demonstrates that the temperature differential of the spectroscopic measurements leads to electron redistribution.

The conformational change of the iron-sulfur center reported previously (7) was observed to occur in a potential range of
approximately 210-220 mV, immediately following the reduction of
cytochrome c₁ and the iron-sulfur cluster. It thus appears that this
change of the iron-sulfur cluster is not a secondary result of changes
in the redox state of the b cytochromes as previously suggested (7,36),
since these components are still oxidized at the completion of the EPR
transition. De Vries et al (163) found this shape change of Rieske
iron-sulfur center of beef heart Complex III was complete at 40-60 mV
(pH 7.2), yet they have also demonstrated that this change in lineshape
is unrelated to the redox behavior of cytochrome b₅₆₂. In our
experiment the change in spectral shape occurs over a potential span
of less than 30 mV, this abrupt behavior is inconsistent with the
presence of two iron-sulfur centers with different midpoint
potentials (181).

Potentiometric properties of coenzyme Q:

The determination of potentiometric behavior of CoQ was
complicated by the presence of an EPR signal generated by the
semiquinone forms of the mediator dyes which have EPR parameters
similar to those of the Q radical. The EPR spectrum of the CoQ radical
obtained at liquid nitrogen temperature exhibits a linewidth of 9
gauss (peak to trough) (Fig. 6a) and exhibits nonhomogeneous power
saturation at around 0.1 mW. The contribution of the mediators was
minimized by judicious selection of dyes and by keeping the dye
concentration as low as was consistent with acceptable equilibration.
By these means the total contribution of the dye signal was kept to a
Fig. 5.4

Liquid-helium EPR-potentiometric titration at pH 7.4.

The measurements were made in 0.1 M KPi, 0.1% deoxycholate, 0.1% Triton QS-30 and 1 mM EDTA. 60 uM Complex III and 20 uM dyes were present in the reaction mixture. The midpoint potential values of the cytochromes and the iron-sulfur center were obtained by following the amplitude change of the corresponding EPR signals as a function of applied potential. The g values of the EPR signals arising from different redox centers are: iron-sulfur center, 1.89(○); cytochrome c₁, 3.49(■); high potential cytochrome b, 3.60(△); low potential cytochrome b, 3.76(▲). The EPR conditions were: modulation amplitude, 20 gauss; microwave power, 0.1 mW for iron-sulfur center and 40 mW for the cytochromes; microwave frequency, 9.238 GHz; temperature, 11.8°K for iron-sulfur center and 8.7°K for cytochromes.
few percent of the size of the CoQ radical at all but the lowest
corcentration of this semiquinone. Fig. 5a shows a titration profile of
Co semiquinone measured at liquid nitrogen temperature. The maximum
radical concentration was found to be only 5% of the total coenzyme Q
present; this corresponds to a stability constant ($[\text{OH}^-]^2/[\text{Q}\text{I}<\text{OH}_2^-]$)
of $1.1 \times 10^{-2}$ assuming that the CoQ present in Complex III is
homogeneous. However, if the Q radicals arise from a pool of Q which
is equimolar with cytochrome c$_1$, then the stability constant is
$2.6 \times 10^{-2}$. Both values are very close to those found for beef heart
succinate cytochrome c reductase by Ohnishi and Trumper (96). The
midpoint potentials estimated by fitting the data to equation (1) are
176 mV and 51 mV respectively.

Similar experiments were carried out at room temperature at pH 7.4
and 8.4 (Fig. 5b). At this temperature, the stable semiquinone in
Complex III exhibits an EPR spectrum with a line width of 15 gauss
(Fig. 6b), almost double that found in beef heart mitochondria (97) and
succinate-cytochrome c reductase (96); this Q radical displays
nonhomogeneous saturation at 10 mW, similar to that reported for beef
heart (98). The maximum amount of stable semiquinone radical observed
is around 8 percent of the total coenzyme Q present at both pH values;
this value is comparable to that obtained at liquid nitrogen
temperature. However, the derived midpoint potentials for the two half
reactions, 200 mV and 110 mV at both pH values, are somewhat larger
than those obtained at liquid nitrogen temperature. As the increase of
pH from 7.4 to 8.4 does not produce any change of the potentiometric
Fig. 5.5

Liquid nitrogen temperature (a) and room temperature (b) EPR-potentiometric titration of coenzyme Q radical.

(a) 40 μM Complex III and 20 μM of DPPI, 1,2-NQ, PMS, ITS and 5 μM MB were present in 0.1 M KPi, pH 7.4, containing 0.1% deoxycholate, 0.1% Triton GS-30. The EPR spectra of coenzyme Q radical were recorded through the whole titration after each addition of reductant or oxidant. The g=2.0 signal amplitude was corrected for dye contribution and normalized against total coenzyme Q concentration. This correction assumes that the presence of the enzyme did not modify the potentials of the dyes present. The midpoint potentials for the two half reactions were calculated according to a least-squares fit to equation (1) as described in "Data analysis". The EPR conditions were: modulation amplitude, 10 gauss; microwave power, 0.1 mW; microwave frequency, 9.238 GHz; temperature, 110°C.

(b) The reaction mixture was the same as in (a) except that 0.1M 3(tris-(hydroxymethyl)-methyl)-amino)propane sulfonic acid was used in the experiments performed at pH 8.5. The EPR conditions were: modulation amplitude, 10 gauss; microwave power, 20mW; microwave frequency, 9.505 GHz; temperature, 296°C.
Fig. 5.4

Typical EPR spectra of stable CoQ radical of Complex III:

(a) EPR spectrum at a redox potential of 149 mV, pH 7.4 and 100°K. The EPR condition and the reaction mixture is the same as in Fig. 5a. The signal contributed from dye molecules was corrected by a blank titration on dyes only.

(b) EPR spectrum of semiquinone at pH 8.4 at 23°C. The spectrum was obtained described in Fig 5b. The redox potential for this spectrum was 151 mV.
characteristics, it would appear that the pK values of the two half reactions are either greater than 8.4 for the oxidized species or smaller than 7.4 for the reduced species.

Effect of pH on the midpoint potentials:

The influence of pH on the midpoint potentials of cytochromes b and c₁ was examined at room temperature using MCD. In a preliminary experiment, a series of assays were performed to find the optimum pH for enzyme activity and to establish the dependence of enzyme stability on pH. The pH optimum was determined in two different ways:

a) Complex III was incubated in 0.1 M KPi, pH 7.4, containing 0.1% deoxycholate and 0.1% Triton Q5-30 and assayed at various pH's using assay mixtures of appropriate buffers; b) Complex III was incubated in buffers of various pH values and assayed in those same buffer solutions, each containing 0.1% deoxycholate and 0.1% Triton Q5-30. Both methods indicate an optimum pH value for enzyme activity of around pH 7.5-8.0.

To study its stability, Complex III was incubated at 4°C in a series of buffers having pH values ranging from 5.0 to 10.0. Samples were removed at intervals up to 48 hours and assayed in the standard system. In the pH range of 5.5-9.0, about 90% activity remained after 4 hrs incubation, 70 to 85% after 24 hrs, and 50 to 80% after two days. Enzyme incubated at pH values greater than 9.0 or lower than 5.5 lost activity much faster; 50 to 60% loss of activity was observed in the first 4 hrs and another 10 to 20% activity loss occurred during
The effect of pH on the midpoint potentials of cytochrome b and c₁.

All titrations were conducted at 23°C in buffers of different pH values: (pyrophosphate (pH 6.5); 2[N-morpholino]-ethane sulfonic acid (pH 6.0, 6.5); N-[acetamido]-iminodiacetic acid (pH 7.0, 7.5); KPi (pH 7.5); Tris (pH 8.0); N,N-bis-[hydroxymethyl] glycine (pH 8.5); 2[N-cyclo-hexylamino] ethane sulfonic acid (pH 9.0)). The closed symbols represent data taken in 0.1% Triton QS-30 and 0.1% deoxycholate. The open symbols correspond to values obtained using 0.1% Triton QS-30 and 0.1% taurocholate. (0-----0) cytochrome c₁; (□-----□) high potential cytochrome b; (Δ-----Δ) low-potential cytochrome-b. The verticals represent the range of values obtained in the oxidative and reductive directions of a single titration.
the next twenty hours. Less than 30% of the original activity remained after two days.

Consequently, titrations were conducted at selected pH values in the range of pH 5.5-9.0. The variation of the midpoint potentials of cytochromes b and c₁ were measured and the variation of these values with pH is presented in Fig.7. It is clear that cytochrome c₁ has a midpoint potential around 260-270 mV which does not change in this pH range. A similar result was observed for both b cytochromes when the titrations were carried out in buffers containing 0.1% deoxycholate and 0.1% Triton QS-30 as detergents.

However when 0.1% taurocholate was substituted for 0.1% deoxycholate, the b cytochromes exhibited pH dependent midpoint potentials between 6.0-9.0. This pH dependency does not appear to be a result of variability in the data, since the value of the midpoint potential of cytochrome c₁ shows little variation in the same experiments. The pH dependence of both b cytochromes was approximately -30 mV/pH. The pK value of the oxidized form is around 6.0-6.5 as judged from the lack of variation of Em with pH at low pH values; the pK value of the reduced form was not established due to the increasing instability of the enzyme at pH values above 9.0. There was no obvious difference in the MCD or EPR spectra of ascorbate-reduced Complex III solubilized in a buffer containing either deoxycholate or taurocholate at both pH 7.5 or 8.5 (data not shown).

The change in detergent also produced a change in the fraction of cytochrome b that titrated at each of the two midpoint potentials
The individual contributions of the high and low potential cytochrome's b were calculated from the fractional change in MCD intensity associated with the inflection point of Nernst plots of the cytochrome b signal (see Fig. 2). The relative contributions of the high-potential b to the low potential b is 0.9:1 in taurocholate containing buffers and 0.2:1 in deoxycholate buffers. This phenomenon can be seen clearly when titrations performed at pH 7.5 in deoxycholate and taurocholate-containing buffer solutions are juxtaposed (Fig. 2). Thus the relative contribution of the two cytochrome b species to the MCD spectrum at 562 nm can be affected markedly by the nature of the detergents even though the total MCD intensity is unaffected by such a change. Changing the detergent had no obvious effect on the envelope of the low-field EPR spectrum recorded at 12°C. However it should be noted that, unlike the beef heart system, the individual contribution of the two b components are difficult to resolve (7).

Effect of Q-depletion:

The presence or absence of coenzyme Q does not seem to have any effect on the midpoint potential values for the cytochromes as indicated in Table 2. The midpoint potential values for cytochromes b and c₁ obtained from native Complex III and Q-depleted sample are essentially the same for the cytochromes within experimental error.

Effect of antimycin-A on midpoint potentials:

As I reported previously (31), antimycin does bind to Complex III
Fig. 5.8

Effect of the nature of the detergent on the ratio of the MCD absorbance contributions of the b cytochromes.

(a) The difference between the MCD spectrum corresponding to the titration end point and the spectrum associated with the inflection point of the titration curve is assigned to the spectrum of low potential b (b_L); and the difference between the inflection point and the started titration of the b cytochrome is assigned to high potential b (b_H). The ratio of signal amplitude at 562 nm between each pair of spectra assigned to b_H and b_L was calculated for titrations carried out at different pH's and plotted opt.

(b) MCD spectra contributed by two cytochrome b species with different mid point potentials as described in (a) in 0.1 M KPi, pH 7.4 and 7.5, containing either taurocholate or deoxycholate.
with a 1:1 stoichiometry and is a potent inhibitor of catalytic activity. However, it should be noted that, in marked contrast to Complex III from heart, antimycin has no effect on the absorbance or MCD characteristics of yeast Complex III. The influence of antimycin-A on the midpoint potentials of the cytochromes was tested by including a 5 fold excess of antimycin-A with Complex III and CoQ-depleted Complex III during MCD-potentiometric titrations. A summary of the titrations involving antimycin are presented in Table 2. Clearly, the presence of antimycin-A at this concentration had little effect on the midpoint potential of cytochrome $c_1$. However, an increase in the midpoint potential of both species of cytochrome $b$ was observed. After each addition of reductant or oxidant, the cytochrome $b$ MCD signal increased and then relaxed to the original value(s) over 20 minutes; these changes are not due to sluggish equilibration but reflect some internal transition. As each MCD-potentiometric measurement took 5 to 15 minutes after each addition of reductant or oxidant, this transition could be incomplete.

Antimycin-A appears to cause an increase in the ratio of high potential cytochrome $b$ to low potential cytochrome $b$. Furthermore, this ratio is largest for intact Complex III and smallest for the sample from which coenzyme Q was almost completely removed.
Table 2
Antimycin-A effect on the midpoint potentials of cytochromes

<table>
<thead>
<tr>
<th></th>
<th>Em (c₁)</th>
<th>Em (b_H)</th>
<th>Em (b_L)</th>
<th>b_H/b_L</th>
</tr>
</thead>
<tbody>
<tr>
<td>bc₁</td>
<td>268 ± 5</td>
<td>62 ± 5</td>
<td>-20 ± 5</td>
<td>0.24 ± .01</td>
</tr>
<tr>
<td>CoQ-depleted bc₁</td>
<td>256 ± 10</td>
<td>64 ± 8</td>
<td>-12 ± 8</td>
<td>0.16 ± .02</td>
</tr>
<tr>
<td>CoQ-depleted bc₁ + Antimycin-A</td>
<td>260 ± 5</td>
<td>95 ± 5</td>
<td>8 ± 5</td>
<td>0.32 ± .02</td>
</tr>
<tr>
<td>bc₁ - 70% CoQ + Antimycin-A</td>
<td>--</td>
<td>100 ± 10</td>
<td>16 ± 5</td>
<td>0.50 ± .01</td>
</tr>
<tr>
<td>bc₁ - 30% CoQ + Antimycin-A</td>
<td>256 ± 5</td>
<td>92 ± 5</td>
<td>16 ± 8</td>
<td>0.76 ± .01</td>
</tr>
<tr>
<td>bc₁ + Antimycin-A</td>
<td>260 ± 10</td>
<td>120 ± 15</td>
<td>26 ± 8</td>
<td>0.82 ± .02</td>
</tr>
</tbody>
</table>
4. Discussion:

Table 3 summarizes the results of a number of potentiometric studies on the components of Complex III. Comparison of data obtained using submitochondrial particles, Complex III and purified b or c₁ cytochromes from a variety of sources documents the close resemblance for the midpoint potentials of the individual redox components. All data for the [2Fe/2S] center and c₁ are in close agreement while the values for cytochromes b and coenzyme Q exhibit only a modest variation (Table 3). Furthermore, the difference reported in the number of species of cytochrome b and the variation in the midpoint potentials observed in the different systems points to a marked sensitivity of the structure of the cytochrome b'(s) to their environment. I have recently obtained highly purified preparations of yeast cytochrome b (31). These preparations typically contain about 37 nmols of protoheme per milligram protein which corresponds to a minimum molecular weight of 27,000. However, Nobrega and Tragooloff have concluded that the three exons of the cytochrome b gene code for a polypeptide consisting of 385 amino acid residues (160). This polypeptide would have a molecular weight of 44,000. Taken together, these facts imply that yeast mitochondrial cytochrome b contains two moles of heme and that our b preparation is about 80% pure (or has lost part of the protoheme during purification). The presence of two heme centers in this protein subunit provides an obvious explanation for our observation of two different species of cytochrome b in yeast
Table 3
Midpoint potential values of the redox components in the $b$-$c_1$ region of the mitochondrial electron transport chain

<table>
<thead>
<tr>
<th>Materials</th>
<th>Component(s)</th>
<th>$E_m$</th>
<th>Temperature</th>
<th>pH</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate Cytochrome c Reductase, Pigeon heart</td>
<td>$c_1$</td>
<td>245</td>
<td>R.T.</td>
<td>7.0</td>
<td>44</td>
</tr>
<tr>
<td>Complex III, Beef heart</td>
<td>$c_1$</td>
<td>232</td>
<td>R.T.</td>
<td>7.2</td>
<td>1</td>
</tr>
<tr>
<td>Complex III, Yeast</td>
<td>$c_1$</td>
<td>268</td>
<td>297°K</td>
<td>7.4</td>
<td>this work</td>
</tr>
<tr>
<td>Complex III, Yeast</td>
<td>$c_1$</td>
<td>270</td>
<td>9°K</td>
<td>7.4</td>
<td>this work</td>
</tr>
<tr>
<td>Purif. $c_1$, Beef heart</td>
<td>$c_1$</td>
<td>225</td>
<td>296°K</td>
<td>7.2</td>
<td>39</td>
</tr>
<tr>
<td>Purif. $c_1$, Beef heart</td>
<td>$c_1$</td>
<td>250</td>
<td>R.T.</td>
<td>7.0</td>
<td>36</td>
</tr>
<tr>
<td>Yeast Mitochondria</td>
<td>$b_3$</td>
<td>-65 ± 15</td>
<td>R.T.</td>
<td>7.0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 ± 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-50 ± 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex III, Beef heart</td>
<td>$b_2$</td>
<td>105</td>
<td>298°K</td>
<td>7.0</td>
<td>36</td>
</tr>
<tr>
<td>Complex III, Beef heart</td>
<td>$b_3$</td>
<td>85</td>
<td>R.T.</td>
<td>7.4</td>
<td>37</td>
</tr>
<tr>
<td>Complex III, Yeast</td>
<td>$b_2$</td>
<td>62</td>
<td>297°K</td>
<td>7.4</td>
<td>this work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex III, Yeast</td>
<td>$b_2$</td>
<td>116</td>
<td>9°K</td>
<td>7.4</td>
<td>this work</td>
</tr>
<tr>
<td>Complex III, Neurospora crassa</td>
<td>$b_2$</td>
<td>60</td>
<td>R.T.</td>
<td>7.0</td>
<td>40</td>
</tr>
<tr>
<td>Purified $b$, Yeast</td>
<td>$b_1$</td>
<td>-44</td>
<td>297°K</td>
<td>7.4</td>
<td>45</td>
</tr>
<tr>
<td>Purified $b$, Neurospora crassa</td>
<td>$b_1$</td>
<td>-70</td>
<td>R.T.</td>
<td>7.0</td>
<td>40</td>
</tr>
<tr>
<td>Purified $b$, Beef heart</td>
<td>$b_2$</td>
<td>-5</td>
<td>R.T.</td>
<td>7.0</td>
<td>36</td>
</tr>
<tr>
<td>Submitochondrial particles, Beef heart</td>
<td>CoQ</td>
<td>204</td>
<td>293°K</td>
<td>7.0</td>
<td>17</td>
</tr>
<tr>
<td>Submitochondrial particles, Beef heart</td>
<td>CoQ</td>
<td>184</td>
<td>77°K</td>
<td>7.0</td>
<td>17</td>
</tr>
<tr>
<td>Complex III, Beef heart</td>
<td>CoQ</td>
<td>83</td>
<td>296°K</td>
<td>8.0</td>
<td>18</td>
</tr>
<tr>
<td>Complex III, Yeast</td>
<td>CoQ</td>
<td>200</td>
<td>297°K</td>
<td>7.4 &amp; 8.4</td>
<td>this work</td>
</tr>
<tr>
<td>Complex III, Yeast</td>
<td>CoQ</td>
<td>110</td>
<td>110°K</td>
<td>7.4</td>
<td>this work</td>
</tr>
<tr>
<td>Mitochondria, Pigeon heart</td>
<td>Fe/S</td>
<td>280</td>
<td>11°K</td>
<td>7.0</td>
<td>42</td>
</tr>
<tr>
<td>Complex III, Beef heart</td>
<td>Fe/S</td>
<td>180</td>
<td>100°K</td>
<td>7.4</td>
<td>43</td>
</tr>
<tr>
<td>Complex III, Yeast</td>
<td>Fe/S</td>
<td>286</td>
<td>9°K</td>
<td>7.4</td>
<td>this work</td>
</tr>
<tr>
<td>Succinate Cytochrome c Reductase, Pigeon heart</td>
<td>Fe/S</td>
<td>280</td>
<td>?</td>
<td>7.4</td>
<td>a</td>
</tr>
</tbody>
</table>

R.T., room temperature, exact data unknown.

a* unpublished data by Bowyer, J., Ohnishi, T. and Trumper, B.
Complex III with one heme center now being identified as $b_{566}$ and the second as $b_{562}$. A competing interpretation is based on the finding from low resolution X-ray diffraction measurement (137,191) that Complex III exists as a dimer in semi-crystalline membrane preparations. In this latter interpretation, one subunit of this dimer provides the first species of cytochrome b and the other subunit provides the second species. However, as this dimer possesses apparent two-fold symmetry, it is not obvious what structural feature could lead to the discrimination between the two b subunits. As the polypeptide sequence of cytochrome b has no obvious internal symmetry, the differentiation of the two heme centers occurs naturally and we consequently favor the former interpretation. The variations in enzyme preparation and the nature of the detergents in the titration mixture appear to control significantly the redox behavior of the b cytochrome(s). In particular, the choice of mediator dyes for this very hydrophobic protein could well have a critical influence on the equilibration between heme centers and the measuring electrode (178). Similar reasoning may explain the variation of the midpoint potentials of coenzyme Q. Prince et al. (22) have assessed the physical and chemical properties of a variety of mediator dyes and based on their conclusions, several shortcomings of our dye system are to be noted. These include the instability of TMPD, the alkaline degradation and possible photodecomposition of PMS by the measuring beam, and the anticipated strong binding of some rather hydrophobic quinone dyes to Complex III.
In this chapter, we present a dramatic example of an experimental artefact generated by mediator dyes with a shift of midpoint potential by more than 300 mV was observed for cytochrome c₁. This large change of the midpoint potential is most simply explained if the binding of dye molecules to oxidized c₁ is much stronger than to the reduced form. However, the dye dependence cannot be fitted by assuming that a single dye species is bound; rather it appears that binding sites for several dye species are present. This assumption is substantiated by the demonstration of the oxidation of ascorbate-reduced Complex III by consecutive addition of the components of the dye mixture. Therefore, in order to avoid this problem, it is absolutely an obligation to perform the potentiometric titration by using different concentrations and dye mixtures. However, at low dye concentrations, I was able to obtain plausible values for the midpoint potential for all redox components of the Complex III. The consistency between the data from stoichiometric titration (7) and potentiometric titrations using two different dye systems and low dye concentration imply that the values for the midpoint potentials I report are reliable. Unfortunately, the interference of high concentration of mediator dyes on the midpoint potential of cytochrome c₁ has limited the versatility of MCD-potentiometric titrations with this component.

Substituting deoxycholate by taurocholate in the reaction mixture not only changed the pH-dependency of the midpoint potentials of cytochrome b but also modified the relative contribution of the two b species to the total MCD signal. There is, however, no observable
difference in the absolute MCD spectra of fully-reduced samples containing one or other detergent. These two observations imply that the high potential b center can be modified to a form with substantially reduced potential but with unchanged MCD characteristics at 562 nm. Earlier, Leigh and Erecinska observed that cytochrome b₅₆₆ present in succinate cytochrome c reductase exhibited a different pH-dependence when titrated as a phospholipid-supplemented sample as compared to a triton-deoxycholate solubilized sample (175), and an effect of phospholipid on the redox behavior of b cytochromes was also found by Yu et al. (192) when studying beef heart Complex III. It seems clear that the potentiometric behavior of cytochrome b is extremely sensitive to the detergent used to solubilize the sample.

Because the entropy changes of the redox centers need not be the same, varying temperature dependencies of the redox potentials are to be expected. Data obtained using room-temperature EPR and MCD and low temperature EPR are in reasonable agreement. Thus the temperature artifacts discussed previously (182,183) do not appear to be a serious complication in this experimental system. Although significant differences are seen for high potential cytochrome b and coenzyme Q, the midpoint potential of cytochrome c₁ is apparently temperature insensitive and the midpoint potentials for the two half reactions of coenzyme Q measured at liquid nitrogen temperature and room temperature fall in between the high potential components (iron-sulfur cluster and cytochrome c₁) and low-potential b-cytochromes. By contrast, the midpoint potential values of both the b and c₁
cytochromes of pigeon heart succinate cytochrome c reductase appear to be independent of measuring temperature (Table 3 and ref. 175).

I consistently find a stability constant around $10^{-2}$ for the semiquinone radical at pH 7.4 regardless of the temperature of measurement. This value, which corresponds to 0.1–0.2 spin/c_1, is similar to that obtained by Ohnishi and Trumpower with succinate-cytochrome c reductase from beef heart (96), but is smaller than the value of 0.5 reported by De Vries et al. with beef heart mitochondria (97) at pH 7.4. Nagaoka et al. (98) also found a maximum semiquinone concentration of 0.1 spin/c_1 with beef heart Complex III using room temperature-EPR at pH 8.0. However, in contrast to our observation, Nagaoka found the concentration of semiquinone to be pH dependent and maximal at pH 9.0. This pH dependence was also observed with the beef heart succinate cytochrome c reductase (96) and submitochondrial particles (97, 184); pH values of 6.4 and 8.0 were obtained with the former and 6.4 and $\geq$ 9.0 with the latter system. It appears that in our enzyme preparation the pH values of the reduced and oxidized forms of Q lie outside the range of pH 7.4–8.4. It is not known whether or not this property is inherent to yeast mitochondria or whether the lack of pH dependence of the midpoint potential I observe is a consequence of the preparative procedure. Measurements on yeast submitochondrial particles should answer this question.

The stoichiometry of proton translocation accompanying electron transfer at Complex III is not established. Recent data from two different groups (59, 128) indicate a stoichiometry of $4 H^+/2e^-$. 

with Coenzyme Q assumed responsible for the translocation of 2 protons; however, the mechanism of translocation of the remaining two protons has not been defined, and there is no indication that cytochrome $c_1$ plays a role in proton translocation (Fig.7). Prince and Dutton (188) find the midpoint potential of the iron-sulfur center of pigeon breast mitochondria is only dependent on pH in a nonphysiological pH range. In contrast, the pH dependence of the redox behavior of cytochrome b is well established and of significant magnitude (175,189,190). In this study, we found a dependency of $-30$ mV/pH for both b cytochromes between pH 6.5 and 9.0 in buffers containing taurocholate. It must be emphasized however that no dependence on pH was observed when titrations were carried out in the buffers that used deoxycholate as detergent. Apparently, taurocholate induces a structural change exposing an ionizable group which on deprotonation affects the midpoint potentials of the b heme. The pH-dependency of the midpoint potentials of the two b cytochromes, $-30$ mV/pH, imply that there are 2 equivalents of electrons involved in oxidation-reduction for each equivalent of proton.

Antimycin-A does not have an obvious effect on the midpoint potentials of cytochrome $c_1$, while the measured increase in the midpoint potential of the b cytochromes might simply be a consequence of the retarded equilibration observed for this species when antimycin is present. Even though we increased the equilibration period substantially, absence of complete equilibration cannot be ruled out. Antimycin-A clearly influences the relative contribution of the two
species of b to the total MCD signal at 562 nm. This seems to be mediated through coenzyme Q and provides another example of the transition between different species of b being affected by a chemical reagent. The regulation of the redox potential of cytochrome b by coenzyme Q was suggested by Kok and Slater from a comparative study of the oxidant-induced reduction of b in wild type and coenzyme Q deficient strains of yeast (193). However, replenishing Q-depleted Complex III with coenzyme Q has no influence on the midpoint potential of the b cytochromes (192) and the effects we describe are fully demonstrable in Complex III with its regular complement of Q. It appears that coenzyme Q only modulates the redox potential of b cytochromes in the presence of antimycin-A and thus the extra reduction of cytochrome b observed by Kok and Slater (193) may well arise from a shift in the equilibrium between the two cytochrome b species which is induced by antimycin-A and mediated by coenzyme Q.
Chapter 6.

The oxidation of yeast Complex III: Evidence for a very rapid equilibrium between cytochrome c₁ and the iron-sulfur center

I. Introduction:

Although there is a substantial amount of steady-state kinetic data available for Complex III (7,197,198), the presteady state measurements needed to define the kinetic reactivity of the individual redox centers have not been satisfactorily implemented. The most notable data include flash-activated cyclic electron transfer in an artificial complex formed between Complex III and bacterial reaction centers (141), reaction of cytochrome c with cytochrome c₁ (64,66) and with antimycin-A treated Complex III (99); rapid kinetic observations on the reaction of reduced duroquinone (used as an analog of CoQ) with succinate-cytochrome c reductase and submitochondrial particles (126, 140). However, little definitive data on the kinetic reactivity of the several iron and quinone centers have been reported, nor have the rates of intramolecular electron transfer been established.

As a prelude to our efforts to provide a thorough characterization of the electron transfer capabilities of this complex, we have compared the rates of oxidation of reduced cytochrome c₁ and two-electron reduced Complex III with potassium ferricyanide. Our data show that the reactivity of cytochrome c₁ is not significantly modified by isolation from the complex and that there is an extremely rapid intramolecular electron equilibration between the iron-sulfur center and cytochrome c₁.
Neither the addition of antimycin-A nor the removal of CoQ had a substantial effect on the reoxidation kinetics.
II. Results and discussion:

The reaction between purified yeast cytochrome c\textsubscript{1} and excess ferricyanide is accurately first-order (Fig.1, left) for 90% of the reaction. The observed rate constant varies linearly with ferricyanide concentration (Fig.1 right); little if any rate saturation was observed even when the ferricyanide concentration was raised to 15 mM (Fig.5). The second-order rate constant for the reaction is $4.4 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}$ ($6^\circ\text{C}$). This value is comparable to that recently reported by Konig et al. for the reaction of beef heart cytochrome c\textsubscript{1} with ferricyanide at $10^\circ\text{C}$ and somewhat different ionic strength (202), but is very much smaller than the value of $3 \times 10^7 \text{ M}^{-1}\text{sec}^{-1}$ found for reaction of beef heart cytochrome c\textsubscript{1} with cytochrome c (66, 200).

When ferricyanide is reacted with two-electron reduced Complex III, clearly non-linear first-order plots are obtained (Fig.1, center); this non-linearity is observed at all ferricyanide concentrations tested. However the overall reactivity of cytochrome c\textsubscript{1} is very similar in the two experiments (Fig.1, left, center). The possibility that this nonlinearity might due to the heterogeneity of cytochrome c\textsubscript{1} in the particle seems unlikely because no heterogeneity was observed in both stoichiometric (7) and potentiometric titrations (203) using optical, EPR and MCD measurements.

The simplest explanation for this result supposes a rapid electron
Fig. 4.1

Kinetic data for the reaction of isolated cytochrome $c_1$ and two-electron reduced Complex III with potassium ferricyanide. First order plot of the reaction of purified $c_1$ (left) and two-electron reduced Complex III (center) with a tenfold excess of ferricyanide. The reaction was followed in the stopped spectrophotometer at 553.5 nm. All reactants were prepared in 0.1 M KPi, pH 7.4 containing 1 mM EDTA, 0.1% deoxycholate and 0.1% Triton QS-30. The final concentration of cytochrome $c_1$ and Complex III were 4 and 4.5 uM respectively. The points are the logarithm of the normalized absorbance change and the solid lines are theoretical curves obtained from fitting of the kinetic trace to a single exponential process. The concentration dependence of the observed pseudo first-order rate of oxidation of isolated cytochrome $c_1$ (◻-◻-◻) and the intrinsic rate of $c_1$ in its parent Complex III obtained from simulation(■-■-■) (right panel). The solid lines in right panel are fits to a simple linear dependence. The data points for Complex III are the pseudo first-order rate constants used in fitting the data to Scheme 1.
equilibration between the iron-sulfur center and cytochrome c_{1}. The validity of this idea was tested by simulation of the kinetic results using the following scheme:

Scheme 1

\[ \text{Scheme 1} \]

\[ \begin{align*}
    c_{1}^{+2} + \text{Fe(CN)}_{6}^{-3} & \xrightarrow{k_{ox}} c_{1}^{+3} + \text{Fe(CN)}_{6}^{-4} \quad (1) \\
    c_{1}^{+2} + \text{Fe/S}^{+} & \xleftarrow{k_{1}} c_{1}^{+3} + \text{Fe/S} \quad (2)
\end{align*} \]

To quantitatively evaluate the data, it was necessary to confirm that the relative potentials of these two centers as observed kinetically were consistent with available potentiometric data and to confirm that the partially-reduced Complex III contained only two reducing equivalents. This was established from the absorbance changes observed when partially-reduced Complex III was reacted with one and two equivalents of ferricyanide (Fig. 2). Reaction of one equivalent of ferricyanide produced a 55% change in absorbance at 553.5 nm implying an equilibrium constant \( K_{eq} \) of 0.67 for reaction (2). The measured potentials are 286 mV \( ((2\text{Fe/2S})) \) and 270 mV \( (c_{1}) \) (203), these values correspond to an equilibrium constant of 0.54, in reasonable agreement with the kinetic result. Reaction of the partially reduced complex with two equivalents of ferricyanide led to complete oxidation of cytochrome c_{1} (Fig. 2) indicating that the complex contained no more than two reducing equivalents.

In simulating the above scheme, the ratio \( k_{2}/k_{1} \) fixed at 0.67.
Fig. 6.2

Reaction of partially-reduced Complex III with one (▲) and two (▲) equivalents of oxidant. The dashed line and solid line represent their simulation using 2.2:1 and 1.0:1 ratio of reactants. The final concentration of Complex III was 6 μM.
and \( k_{\text{ox}} \) fixed at \( 1.2 \times 10^5 \, \text{M}^{-1} \, \text{sec}^{-1} \) (Fig.1, right); \( k_1 \) was varied so that the computed change in \( c_1^{+2} \) matched the kinetic data. Fig.3 shows that equilibration is fast and \( k_{\text{ox}} \) \( 2000 \, \text{s}^{-1} \).

Examples of fitted data are provided in Figs 2, 3 and 4 for a variety of experimental conditions. The available data were satisfactorily fit by these minimal requirements provided that \( k_1 \) was made sufficiently large so that the intramolecular electron equilibration between the iron-sulfur center and \( c_1 \) was maintained at equilibrium. Thus, this scheme I provides a satisfactory explanation of our kinetic results with only two parameters, one of which \( K_{eq} \) is independently established while the other is valid over a range of ferricyanide concentrations (Fig.1, right). Interestingly, use of the potentiometric value for \( K_{eq} \) \( 0.54 \) does not affect the simulations significantly.

It is noteworthy that values of \( k_1 \) too small to establish equilibrium between the iron-sulfur center and \( c_1 \) lead to an apparent increase in the rate of oxidation of the heme center (Fig.3). Under these conditions, reactions 1 and 2 are uncoupled and the rate-limiting intramolecular step only contributes towards the end of the reaction; i.e., the redox state of \( c_1 \) is not buffered under these conditions. Our results make it clear that intramolecular electron transfer is instantaneous by stopped-flow criteria with a minimum value of \( k_1 \) of \( 2000 \, \text{s}^{-1} \). Data obtained with the ferricyanide concentration raised to \( 15 \, \text{mM} \) (so that the oxidation reaction is complete within 10 ms) is still satisfactorily fit by
Fig. 6.3

Reaction of partially-reduced and CoQ-depleted Complex III with 1000 fold excess of ferricyanide.

The concentration was 3 uM after mixing; other conditions were the same as in Fig.1. Only the simulated behavior of c₄ is shown. The dependence of the simulated time courses on the rate of intramolecular electron transport is illustrated.
Fractional Change

\[ k_1 = 1000 \]
\[ k_1 > 2000 \]
\[ k_1 = 500 \]
\[ k_1 = 100 \]
\[ k_1 = 4 \]
\[ k_1 = 10 \]

1000:1
\[ k_{o-x} = 8.9 \times 10^4 \]
Q-depleted bcl
Fig. 6.4

Reaction of unmodified and chemically treated Complex III by ferricyanide.

Two-electron reduced Complex III (middle panel), CoQ-depleted sample (upper panel), or the sample containing antimycin-A (with a molar ratio 5:1 between antimycin-A and Complex III) (bottom panel) were mixed with 20 fold excess of ferricyanide using the conditions of Fig.1. The simulated time courses for the oxidation of $c_1$ (———) and the iron-sulfur center (———) are shown. The data are the normalized absorbance change at 553.5 nm.
Scheme 1 (Fig. 5).

Preincubation of Complex III with antimycin-A has only a small effect on the kinetic data (Fig. 4, bottom) with \( k_{\text{ox}} \) being reduced to about 60% of its uninhibited value. This lack of effect of antimycin-A is strikingly different to the report of a marked inhibition of electron transfer between the iron-sulfur center and cytochrome \( c_1 \) observed during reduction (140).

Reaction of ferricyanide with partially reduced CoQ-depleted Complex III are also not strikingly different from the untreated complex (Fig. 4, top), \( k_{\text{ox}} = 8.9 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1} \). Clearly neither antimycin-A nor CoQ have an important role in these reactions.

**Appendix**

The alternative way to analyze the kinetic data is presented in Scheme 2:

![Scheme 2 Diagram](image-url)

or

\[
\begin{align*}
\text{Fe/S, } c_1^{+2} & \underset{k_1'}{\rightarrow} \text{Fe/S, } c_1^{+3} \\
\text{Fe/S, } c_1^{+2} & \underset{k_2'}{\rightarrow} \text{Fe/S, } c_1^{+3}
\end{align*}
\]
Fig. 6.5

Reaction of partially-reduced and CoQ-depleted Complex III with 5000 fold excess of ferricyanide. The reaction conditions were exactly the same as in Fig.3. except the ferricyanide concentration, which was increased to 14.8 mM. Simulations were carried out by giving more emphasis on the fitness of the initial part of the reaction traces corresponding to the most reliable data.
5000:1

\[ k_1 = 60000 \]

\[ k_{ox} = 1.2 \times 10^5 \]

Red-Fe/S

5000:1

\[ k_1 = 60000 \]

\[ k_{ox} = 8.9 \times 10^4 \]

Red-Fe/S

Q-depleted bc1
\( E(2) \), \( E(1) \), \( E(0) \) are the redox species corresponding to 2-electron, 1-electron reduced and fully oxidized state, respectively. \( \kappa' \) is the same as \( k \), the intrinsic reaction rate of cytochrome \( c_1 \);

\[
\kappa' = f_c \cdot k
\]

\[
f_c = \left( \frac{\text{Fe}^+/S^+,c_1^{+2}}{[\text{Fe}^+/S^+,c_1^{+2}] + [\text{Fe}/S^+,c_1^{+3}]} \right).
\]

The numerical solutions of the above three redox species are:

\[
E(2) = E_0 \cdot e^{-kt}
\]

\[
E(1) = E_0 / (1-f_c) \cdot [-e^{-kt} + e^{-f_c kt}]
\]

\[
E(0) = E_0 \left[ 1 + f_c / (1-f_c) \cdot e^{-kt} - 1/(1-f_c) \cdot e^{-f_c kt} \right]
\]

where \( E_0 \) represents the total concentration of all redox species.

Therefore,

\[
\text{AOD}_{553.5\text{nm}} = \text{AOD}_0 / (1-f_c) \cdot [(1-2f_c) e^{-kt} + e^{-f_c kt}]
\]

Theoretically, all the reaction traces should be fitted as two-exponential curves. By practicing two-exponential fitting on the data of Complex III and the CoQ-depleted sample, a similar set of rate
Table 1. Pseudo first-order rate constants obtained by simulation and two-exponential fitting:

<table>
<thead>
<tr>
<th>Sample</th>
<th>(Ferricyanide) mM</th>
<th>$k_3(k) , s^{-1}$</th>
<th>$k_1'(k) , s^{-1}$</th>
<th>$k_2' , s^{-1}$</th>
<th>$f_c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 e\textsuperscript{-}-reduced bc\textsubscript{1}, 2.5uM</td>
<td>0.025</td>
<td>3.0</td>
<td>3.1</td>
<td>1.1</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.050</td>
<td>6.5</td>
<td>6.7</td>
<td>1.9</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>16.0</td>
<td>14.7</td>
<td>3.9</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>0.250</td>
<td>31.0</td>
<td>24.9</td>
<td>5.5</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>2.96</td>
<td>240</td>
<td>273.4</td>
<td>74.0</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>14.80</td>
<td>899</td>
<td>818.3</td>
<td>157.3</td>
<td>0.19</td>
</tr>
<tr>
<td>2 e\textsuperscript{+}-reduced Q-depleted bc\textsubscript{1}, 3.6uM</td>
<td>0.036</td>
<td>3.5</td>
<td>3.3</td>
<td>1.4</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>0.072</td>
<td>7.0</td>
<td>5.9</td>
<td>2.4</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>0.180</td>
<td>18.5</td>
<td>13.0</td>
<td>3.0</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.360</td>
<td>33.0</td>
<td>26.9</td>
<td>9.3</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>3.60</td>
<td>225.0</td>
<td>218.9</td>
<td>96.8</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>18.00</td>
<td>1150</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 e\textsuperscript{-}-reduced bc\textsubscript{1}+antimycin-A 2.5uM</td>
<td>0.025</td>
<td>2.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>8.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
constants were obtained for the intrinsic rate of cytochrome $c_1$ (Table 1). $k_{1'}$. However, the ratio of $k_{2'}/k_{1'}$ does not appear to be consistent. The theoretical value is 0.42 for $f_c$. This is because the data scattering of the slow phase is much more serious than the first fast phase and the two-exponential fitting becomes less effective when the two exponential components have very close reaction rates.
Chapter 7.
Oxidative reaction mechanism of Complex III: the kinetic behavior of cytochrome b

1. Introduction:

As an extension of our kinetic observation on the partially-reduced Complex III (Chapt. 6), further investigations on the kinetic behavior of other lower potential components, viz. cytochrome b's and coenzyme Q, were followed using fully-reduced Complex III and a four-electron reduced sample (CoQ-depleted).

The kinetic data I obtained for the reaction between the reduced complexes and ferricyanide strongly indicate a reaction mechanism involving a fast irreversible electron transfer from low potential b to either cytochrome c₁ or iron-sulfur center. Coenzyme Q appears to be unnecessary for the oxidative reactions and antimycin exerts no effect on the oxidation of CoQ-depleted sample, while a significant effect was found on parent Complex III.

2. Results and data analysis:

(a) Reductive titration of CoQ-depleted Complex III (unpublished data of de la Rosa in this laboratory)

Fig.1 displays a typical stoichiometric titration data of Coenzyme Q-depleted Complex III. This Q-depleted sample was completely reduced by 4.5 equivalents of reductant. Four of these can be accounted for by cytochromes b₅₆₂ and b₅₅₈/₅₆₆', cytochrome c₁ and iron-sulfur
Fig. 7.1

Observed fractions of the oxidation-reduction components during a reductive titration of yeast CoQ-depleted Complex III with dithionite.

CoQ-depleted Complex III (containing 10% residual CoQ) was suspended in 6.0 ml of 0.1 M KPi, pH 7.4, containing 0.1% deoxycholate and Triton QS-30, to a protein concentration of 11 mg/ml and anaerobic dithionite titration was performed at 10°C as described under "Methods". The fractional amounts of reduced cytochrome c₅ (●) and cytochrome b (■) were derived from the absorbance changes at 553.5 nm and 561.5 nm, respectively, and from the intensities of the EPR resonances at 3.49(▲), 3.60(□) and 3.76(●). The amounts of reduced iron-sulfur protein(▲) and free radical(●) were obtained from the intensities of the EPR signals at g=1.89 and 2.00 respectively. The radical was quantitated using diphenyl picryl hydrazyl as an intensity standard and was normalized based on 10% total CoQ content.
center; and the remainder is principally consumed by the 0.1 moles of coenzyme Q left in the sample after Q-extraction. This residual CoQ is clearly seen by the semiquinone formation during the titration (Fig.1). Comparing these data with the reductive titration performed on intact Complex III (Chapter 1, Fig.3), we found essentially the same redox behavior for cytochrome $c_1$ and iron-sulfur center in both cases. Cytochrome b's and coenzyme Q were titrated in the same order with the parent Complex III having larger separation between the titration curves of the two b species due to the intervention of coenzyme Q. These data together with the data of potentiometric titrations indicate the spectral and redox properties of individual redox component remained unchanged after the coenzyme Q extraction. Therefore, a comparative kinetic study between Complex III and its Q-depleted sample should provide valuable information on the reaction mechanism.

(b) Reaction between fully-reduced (4e$^-$-reduced) Q-depleted Complex III and ferricyanide

A representative kinetic experiment at 561.5 nm and 553.5 nm for CoQ-depleted sample is shown in Fig.2. Similar kinetic results were obtained at several ferricyanide concentrations. The rate of decrease of absorbance change at 561.5 nm is consistently faster than that of 553.5 nm in the first period of reaction. The absorbance change at 553.5 nm then decreases in a rate faster than that at 561.5 nm in the rest of the reaction and a crossover of these two kinetic traces is observed (Fig.2). As absorbance at 561.5 nm is mainly a reflection of the $b$ cytochromes (the contribution of cytochrome $c_1$ at this
Fig. 7.2

Kinetic data for the reaction between fully reduced CoQ-depleted Complex III and ferricyanide.

The reduced complex was reacted with twenty-fold excess ferricyanide and the absorbance changes at both 561.5(———) and 553.5 nm (———) were followed spectrophotometrically. All reactants were prepared in 0.1 M KPi, pH 7.4 containing 0.1% deoxycholate and 0.1% Triton OS-30. The final concentration of the CoQ-depleted Complex III was 7.4 uM.
wavelength is only 8.6% (Table 1), the phenomenon we observed as shown in Fig. 2 can simply be interpreted by Scheme 1. This scheme involves an initial irreversible fast electron transfer from cytochrome b to a high potential species, namely $c_1$ or the iron-sulfur center, followed by the oxidation of $c_1$ and iron-sulfur center. The last portion of slow phase of the 561.5 nm absorbance change and a small percentage of 553.5 nm change are contributed by the slow oxidation reaction of cytochrome b.

Theoretically, computer simulation can be performed by solving all the rate equations according to scheme 1 using finite differences as demonstrated in Chapt. 6 on the data of the partially-reduced sample. By so doing, the simulations involving at least 6 rate constants and 4 redox species become very time-consuming because the time-increment which must be used to do the numerical integration is strictly limited by the fast internal electron transfer rate between $c_1$ and iron-sulfur center. Therefore, an alternative scheme was used for the data simulation as shown by Scheme 2, which is another presentation of Scheme 1. The redox species containing four to zero reducing equivalents ($E(4)\cdots E(0)$) are sequentially arranged in a linear irreversible reaction scheme. $k$ is the pseudo first-order rate constant of the oxidation of ferrocytochrome $c_1$ by ferricyanide, and $k'$ is the rate constant of the slow phase oxidation of cytochrome b ($b_m^r$). $f$ is defined as the fraction of ferrocytochrome $c_1$ in $E(2)$ species similar to the definition in Chapter 6. $f_1$ and $f_b$ are the contributions of low potential b, $b_L$, to 561.5 nm and 553.5 nm.
absorbance change, respectively (Fig. 3). The rate constant for each oxidation step was assigned assuming that only cytochrome $c_1$ interacts directly with ferricyanide and the fast equilibrium described in Chapter 6 between $c_1$ and iron-sulfur center was involved. The numerical solutions for the time dependence of the five redox species ($E(4), E(3), \ldots, E(0)$) are shown in Fig. 3. The normalized absorbance changes at 561.5 nm and 553.5 nm are formulated using the extinction values given in Table 1 (refer to (7)). Simulations were then conducted by introducing the equations relating to each redox species (as shown in Fig. 3) in the simulating program.

The experimental data and simulation of the reaction between Q-depleted Complex III and 10 fold excess ferricyanide are shown in Fig. 4 with data shown in both short and long reaction periods. The pseudo first-order rate constants ($k, k'$) and the value $f$ used for obtaining the best simulations are listed in Table 2. Acceptable simulations were obtained for both the 561.5 nm and 553.5 nm data (Fig. 4). The same simulation procedures were carried out on the data of CoQ-depleted sample preincubated with five-fold excess of antimycin-A. The simulation for the reaction with 20-fold excess ferricyanide is given in Fig. 5. All the simulation parameters of reactions involving various ferricyanide concentrations are listed in Table 2. Direct comparison in Figures 4 and 5 indicates that there is no effect of antimycin-A on the oxidation of at least cytochromes b and $c_1$ for the CoQ-depleted sample. In both experiments, the best simulations were obtained by assigning value of 0.3 for $f_1$ and 0.15
Table 1. Difference Molar Absorption Coefficients ($\Delta \varepsilon$) For Simulations

<table>
<thead>
<tr>
<th>$\lambda$ (nm)</th>
<th>Treatments</th>
<th>$\Delta \varepsilon$ (mM$^{-1}$ cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>561.5</td>
<td>Dithionite reduced - Oxidized</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>Dithionite reduced - Ascorbate reduced</td>
<td>49.9</td>
</tr>
<tr>
<td></td>
<td>Ascorbate reduced - Oxidized</td>
<td>-4.3</td>
</tr>
<tr>
<td>553.5</td>
<td>Dithionite reduced - Oxidized</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>Dithionite reduced - Ascorbate reduced</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>Ascorbate reduced - Oxidized</td>
<td>15.8</td>
</tr>
</tbody>
</table>
Fig. 7.3

Mathematical expressions of normalized absorbance changes and numerical solutions of the redox species of CoQ-depleted Complex III generated in the oxidation reaction with ferricyanide.

The solutions were obtained by solving the differential equations related to each redox species based on Scheme 2. $E_0$ represents the total concentration of Complex III. The normalized absorbance changes were calculated out using the difference molar absorption coefficient listed in Table 1 at 561.5 and 553.5 nm.
Numerical Solutions of Fully-reduced CoQ-depleted Complex III

\[ E(4) = E_0 e^{-kt} \]

\[ E(3) = k E_0 t e^{-kt} \]

\[ E(2) = \frac{k E_0}{(f-1)} \left( \left( t - \frac{1}{(f-1)k} \right) e^{-kt} + \frac{1}{(f-1)k} e^{-fk t} \right) \]

\[ E(1) = \frac{f k E_0}{(f-1)(k'-k)} \left( \frac{kt}{(k'-k)} - \frac{k}{(k'-k)} - \frac{1}{(f-1)} \right) e^{-kt} + \]

\[ \frac{f k E_0}{(f-1)(k'-k)} \left( \frac{k}{(k'-k)} - \frac{k'}{((f-1)(k'-fk))} + \frac{1}{(f-1)} \right) e^{-k' t} \]

\[ + \frac{f k E_0}{(f-1) (k'-fk)} e^{-fk t} \]

\[ E(0) = E_0 - E(4) - E(3) - E(2) - E(1) \]

Normalized Absorbance Change:

\[ \Delta \text{OD}_{561.5} = (45.6 \ E(4) + (49.9f_1 - 4.3 \ E(3) + (49.9f_1 + 4.3) \ E(2) + (49.9f_1 - 4.3 \ E(1))/45.6 \]

\[ \Delta \text{OD}_{553.5} = (27 \ E(4) + (15.8 + 11.2 \ f_b ) \ E(3) + (15.8f + 11.2 f_b ) \ E(2) + 11.2 \ f_b \ E(1))/27.0 \]

\[ f_1 : \text{Fraction of cytochrome b contributing to the slow phase} \]

\[ f_b : \text{Contribution of slow phase b to \ OD}_{553.5} \]

\[ f : \text{Fraction of } c_1^{+2} \text{ in } E(2) \text{ species} \]
Fig. 7.4

Kinetic data and simulations of CoQ-depleted Complex III.

CoQ-depleted complex was reacted with 20-fold excess ferricyanide. Reaction conditions were same as described in Fig.2. The symbols represent true data (△, 561.5 nm; △, 553.5 nm) and the simulations (—, —) indicated by lines relating to the data of 561.5 and 553.5 nm respectively. Other curves are the theoretical lines of fractions of different redox species. Kinetic data are given in both short (a) and long reaction periods (b and c).
a

Q-depleted bc

20:1

k = 1.09 \times 10^5

k' = 0.6

553.5 nm

561.5 nm

Fractional Change

Time, (sec)

b

Q-depleted bc

20:1

K = 1.09 \times 10^5

K' = 0.4

561.5 nm

Fractional Change

Time, (sec)
Kinetic data and simulations of CoQ-depleted Complex III pretreated with antimycin-A.

CoQ-depleted Complex III pretreated with five-fold excess antimycin was reacted with 20-fold excess ferricyanide under same conditions as described in Fig.2. The graphic representations are the same as in Fig.4 and is subjected to direct comparison with Fig.4.
for $f_b$. The second-order rate constants for the reaction between $c_1$ and ferricyanide are similar in both sets of data, viz. $1.09 \times 10^5$ M$^{-1}$s$^{-1}$ in the absence of antimycin and $1.32 \times 10^5$ M$^{-1}$s$^{-1}$ in its presence (Fig. 8). With one exception, the slow phase of the two sets of data exhibit a concentration-independent reaction rate at both wavelengths. The reaction of the antimycin-treated sample observed at 561.5 nm exhibits a concentration dependence and has a corresponding second-order rate constant of $274$ M$^{-1}$s$^{-1}$.

(c) Reoxidation of fully-reduced Complex III by ferricyanide

Kinetic traces obtained under the same conditions with the parent-complex (CoQ-rich preparation) show very similar patterns of absorbance changes at both 561.5 and 553.5 nm. Interestingly, I obtained satisfactory simulation at both wavelengths using the same scheme (Scheme 2) previously employed for the simulation of CoQ-depleted sample (Fig. 6 a, b). The rate constants used to simulate these data (Table 2) yield a second-order rate constant of $1.05 \times 10^5$ M$^{-1}$s$^{-1}$ (Fig. 8) for the fast phase at both wavelengths. This value is essentially the same as we obtained using partially-reduced sample (Chapter 6). The rate constants of the slow phase obtained by simulation do not show obvious concentration dependence similar to that observed on CoQ-depleted sample, although the reaction rate is about an order of magnitude smaller in the former case. The values of $f_1$ and $f_b$ used to obtain the most satisfactory simulations are very similar to that of the CoQ-depleted sample, namely 0.32 and 0.1 for the former and 0.3 and 0.15 for the latter (Table 2).
**Fig. 7.6**

Kinetic data and simulations of parent Complex III.

Complex III was reacted with 50-fold excess ferricyanide under the conditions as described in Fig. 2. The final concentration of Complex III was 5 uM. Simulations were conducted according to Scheme 2 exactly as applied to CoQ-depleted samples. Kinetic representations are the same as indicated in Fig. 4.
Figure a shows the fractional change with time for a 50:1 solution with $k = 1.05 \times 10^5$ and $k' = 0.2$. The wavelengths are 553.5 nm and 561.5 nm.

Figure b also shows the fractional change with time for a 50:1 solution with $k = 1.05 \times 10^5$ and $k' = 0.2$. The wavelength is 561.5 nm.
(d) Effects of antimycin-A on the reoxidation of fully-reduced Complex III

It was somewhat surprising to me that the kinetic data obtained with antimycin-treated Complex III can be simulated by Scheme 2 without any additional modification although the kinetic trace exhibited a quite different appearance compared with that of Complex III only (Fig. 7). Simulations were accomplished using almost the same rate constants for reactions involving various ferricyanide concentrations. A second order rate constant of \(1.19 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) was obtained (Fig. 8), very close to the intrinsic rate of cytochrome c\(_1\) obtained using partially-reduced sample. The \(k'\) values used to simulate the slow phase of 561.5 nm absorbance change display a dependence on ferricyanide concentration (having a corresponding second order rate of \(415 \text{ M}^{-1} \text{s}^{-1}\), similar to CoQ-depleted sample pretreated with antimycin, while the concentration dependence of absorbance change at 553.5 nm is not so obvious.

A major difference was observed between CoQ-depleted and intact Complex III in the presence of antimycin-A; the values of \(f_{1}^b\) and \(f_{b}^\text{H}\) used in the simulation for antimycin-treated Complex III are significantly larger than the CoQ-depleted sample. As high as 60% and 47% slow phase \(b\) (\(b_{\text{H}}\)) had to be used for the 561.5 and 553.5 nm data respectively for antimycin-treated sample, while 32 and 10% were found at the corresponding wavelengths for the untreated complex.

3. Discussion:
Fig. 7.7

Kinetic data and simulation of antimycin-treated Complex III.

Complex III pretreated with 5-fold antimycin-A was reacted with 50-fold excess ferricyanide. The reaction conditions and simulation procedures are exactly the same as in Fig.6 but much larger values of $f_1$ and $f_b$ were used as indicated in Table 2. The slow phase kinetic data at 561.5 nm and the simulation are also given(b).
Fig. 7.8

Second-order rate constants of different treatments of Complex III.

The pseudo first-order rate constants ($k, s^{-1}$) of four different samples were obtained from the optimum computer simulations according to Scheme 2 and were described individually as in Fig. 4, 5, 6, and 7. at various ferricyanide concentrations. The second-order rate constants for each sample were then obtained as the slope by a line-fitting between $k$ values and ferricyanide concentrations. The representations are indicated in the figure.
Closed: Q-depleted bc₁

Open: Intact bc₁

Observed Rate Constant, sec⁻¹

[Ferricyanide], mM

k=1.05 \times 10^5

k=1.09 \times 10^5

k=1.32 \times 10^5

k=1.19 \times 10^5

+Antimycin-A
Table 2. Parameters obtained from simulations:

<table>
<thead>
<tr>
<th>[Ferricyanide], mM</th>
<th>$561.5$nm $k$ (s$^{-1}$)</th>
<th>$553.5$nm $k'$ (s$^{-1}$)</th>
<th>$f_1$</th>
<th>$f_b$</th>
<th>2nd-order rate const.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-depleted Complex III</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.074</td>
<td>5.0</td>
<td>6.0</td>
<td>0.3</td>
<td>0.15</td>
<td>$1.09 \times 10^5$</td>
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<td>0.3</td>
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</tr>
<tr>
<td>0.37</td>
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<td>22.0</td>
<td>0.6</td>
<td>0.3</td>
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<tr>
<td>0.74</td>
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<td>41.0</td>
<td>0.6</td>
<td>0.3</td>
<td>0.15</td>
</tr>
<tr>
<td>Q-depleted Complex III +Antimycin-A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.074</td>
<td>6.0</td>
<td>3.0</td>
<td>0.6</td>
<td>0.3</td>
<td>0.15</td>
</tr>
<tr>
<td>0.148</td>
<td>13.0</td>
<td>10.0</td>
<td>0.6</td>
<td>3/28*</td>
<td>0.15</td>
</tr>
<tr>
<td>0.37</td>
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<td>0.6</td>
<td>0.28</td>
<td>0.15</td>
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<td>0.74</td>
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<td>45.0</td>
<td>1.0</td>
<td>0.29</td>
<td>0.15</td>
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<td>Complex III</td>
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</tr>
<tr>
<td>0.05</td>
<td>3.0</td>
<td>0.02</td>
<td>3.0</td>
<td>0.02</td>
<td>$0.34/0.33$</td>
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<td>0.06</td>
<td>6.0</td>
<td>0.6</td>
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<td>1.2</td>
<td>0.25</td>
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</tr>
<tr>
<td>0.50</td>
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<td>26.2</td>
<td>0.06</td>
<td>0.41</td>
<td>$0.28$</td>
</tr>
<tr>
<td>Complex III+Antimycin</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>3.5</td>
<td>0.02</td>
<td>4.5</td>
<td>0.6</td>
<td>0.55</td>
</tr>
<tr>
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<td>0.025</td>
<td>6.5</td>
<td>0.6</td>
<td>$0.61/0.55$</td>
</tr>
<tr>
<td>0.25</td>
<td>16.0</td>
<td>0.05</td>
<td>14.0</td>
<td>1.0</td>
<td>$0.71/0.6$</td>
</tr>
<tr>
<td>0.50</td>
<td>30.0</td>
<td>0.12</td>
<td>27.0</td>
<td>1.2</td>
<td>$0.65/0.57$</td>
</tr>
</tbody>
</table>

* The value on the left side of slash was used to simulate data of short time period and the value on the right side was used to simulate data of the whole reaction period.
Reaction scheme 2 satisfactorily simulates all four sets of kinetic data. Similar schemes have been used to interpret the kinetic data involving enzyme complexes containing multiple redox centers, e.g. xanthine oxidase (133, 204). This general scheme has proved to be useful for the interpretation of electron transfer sequence when the substrate specifically interacts with only one (in this case, the terminal) redox component of the multicomponent system.

A somewhat larger second-order rate constant had to be used to simulate the kinetic data of fully-reduced CoQ-depleted Complex III (Fig. 7) compared to its partially-reduced form (i.e. $1.1 \times 10^5$ vs. $8.9 \times 10^4$ M$^{-1}$s$^{-1}$) (see Chapter 6). This raises the possibility of direct interaction between cytochrome b and ferricyanide. Although this possibility can not be excluded, it is unlikely for the following reasons:

First, cytochrome b is very hydrophobic transmembrane protein demonstrated by surface-labeling and crosslinking experiments (57). The b hemes in isolated yeast Complex III are not accessible to carbon monoxide (unpublished data) in contrast to the purified cytochrome b which reacts readily with CO (31). The poor equilibration between heme b and the measuring electrode in potentiometric titrations lacking hydrophobic mediators is well-known (178, 179).

Second, ferricyanide is a highly negatively-charged hydrophilic molecule at pH 7.4 and reacts as a simple first-order process with negatively-charged ferrocytochrome c$^-$ in conditions of ionic strength similar to those of my experiments (202).
Third, the "oxidant-induced extra-reduction of cytochrome b" was demonstrated with our Complex III preparation in the presence of antimycin (data not shown). Utilisation of cytochrome c as oxidant in the presence of cytochrome oxidase and oxygen may resolve the question whether ferricyanide interact directly with cytochrome b of the Complex III.

From my simulation results, we can barely detect a Coenzyme Q effect on the oxidative reaction of Complex III. Its obvious contribution is a slowing down of the oxidation rate of the slow phase cytochrome b. Apparently, CoQ acts as an electron buffer on the reductive side of the cytochromes and the iron-sulfur center. However, this buffering is only reflected in the slow phase b (b_H^) suggesting the presence of a kinetic barrier of electron transfer between the two b cytochromes. This kinetic barrier is indicated by the slow oxidation rate of reduced b_H by ferricyanide and the concentration independence of this b species.

According to Mitchell's protonmotive Q-cycle (99), and Fig.9 in Chapt.1) CoQ acts as obligatory mediator between cytochrome b and c_1. Thus, in the absence of CoQ, no communication should be observed between b and c_1 cytochromes. A possible explanation for my kinetic data with CoQ-depleted sample resides in an evaluation of the function of the iron-sulfur protein. Trumpower suggests that this iron-sulfur protein acts as a ubiquinol-cytochrome c_1/ubisemiquinone-cytochrome b oxidoreductase (127) and is required for the oxidation of cytochrome b but not c_1 (127). Consequently, if cytochrome b can communicate
with iron-sulfur center even in the absence of coenzyme Q a plausible electron transfer sequence would be:

\[ \text{b} \rightarrow \text{Fe/S} \rightarrow \text{c} \rightarrow \text{oxidant} \]

and no contradiction between my scheme and the Q-cycle remains.

The values of \( f_1 \) used to obtain best simulations (30% for Q-depleted samples either with or without antimycin-A and 32% for Complex III), coincide relatively well with the data of potentiometric titrations (see Chapter 5, section 4). In the same buffer system the contribution of \( b_L \) and \( b_H \) to the total MCD signal are about 75% and 25% respectively while a combined optical-MCD stoichiometric titration yield essentially identical titration curves by both MCD and absorbance measurements.

Antimycin-A was observed to increase the MCD contribution of \( b_H \) to the total signal change in potentiometric experiments. This influence of antimycin on b cytochromes is again manifested in the kinetic data. Antimycin-treated Complex III exhibits a kinetic feature with significantly greater contribution of slow phase (see Table 2), while CoQ-depleted sample does not, even in the presence of antimycin-A. This experimental result suggests a cooperative effect of antimycin and coenzyme Q on the transition between the two heme b species as suggested in the experiments of potentiometric titrations. The mechanistic origin of this phenomenon is unknown and awaits
further knowledge concerning the nature of antimycin-binding site.

The relevance of the kinetic simulation of Complex III using
Scheme 2 will not be addressed at this moment. The real test of the
validity of the electron transfer sequence implied by the proton motive
Q cycle requires kinetic data of CoQ and iron-sulfur center and
especially CoQ. The combined freeze-quenching and EPR measurements may
be the method of choice for this approach.
Chapter 8. Conclusions

Four different approaches described in this thesis were employed to investigate the structure and reaction mechanism of Complex III from yeast:

1. Direct isolation of cytochrome b and characterization of its physical and chemical properties (Chapter 3).

2. Spectroscopic comparison of the heme structures of synthetic model compounds with those of Complex III and isolated cytochromes using MCD and EPR measurements (Chapter 4).

3. Determination of midpoint potentials of the individual redox centers and observations on the influence of various parameters on these midpoint potentials (Chapter 5).

4. Kinetic measurements of oxidative reactions with ferricyanide using different Complex III treatments and proposal of reaction mechanism through model simulations (Chapters 6 and 7).

Detailed discussions of the experimental results were given in each of the chapters corresponding to each of the above four approaches. In this concluding chapter I will try to provide a composite picture of the structure and reaction mechanism of Complex III. Initial emphasis will be on the structure and function of cytochrome b and thereafter the electron transfer sequence in Complex III.

Judging from its amino acid composition, cytochrome b is a very
hydrophobic protein and is believed to be integral to the mitochondrial inner membrane. The protoheme prosthetic group has an uncommon coordination structure and can be simulated by bis-imidazole coordination subjected to steric strain. The iron of the heme has a low-spin electronic configuration both in the isolated cytochrome b and in Complex III.

Several lines of experimental data from this thesis and from the literature suggest that the multiple b characteristics observed in spectroscopic, potentiometric and kinetic studies are results of different microenvironments experienced by the same kind of heme. A strong indication that one apocytochrome b contains more than one heme moiety was also manifested.

1. Although there is much data on b cytochromes isolated from different sources, none of the preparations contained more than one heme b-containing polypeptide (52, 57, 145) with the exception of a cytochrome b preparation from Neurospora (147) which contained two polypeptides with very similar amino acid compositions. In my preparation, only one major b-containing polypeptide was isolated with a yield of heme b as high as 60%. EPR and potentiometric studies indicate one b species in this isolated sample. Interestingly, von Jagow isolated one heme b-containing polypeptide from beef heart exhibiting a redox behavior as a composite of two redox species (148).

2. The amino acid sequence of yeast cytochrome b has been deduced from a recently completed gene sequence (160). A selection of many rho- mutants involving deletion of large segments of mitochondrial
DNA combined with the technique on amplification of restriction fragments in bacteria was performed. The DNA responsible for the transcription of apocytochrome b was determined in different yeast strains by comparing the homology with bovine apocytochrome b gene with known DNA sequence (138). The mosaic gene responsible for the transcription of the b apoprotein has a sequence corresponding to a protein with 385 amino acids. The molecular weight based on this sequence is about 44,000, a value much larger than that reported for isolated yeast b cytochromes (5, 31, 150, 166) estimated by using SDS-gel electrophoresis. This anomalous electrophoretic behavior of cytochrome b was repeatedly observed. Apparently, the extreme hydrophobic character of cytochrome b confers resistance to unfolding reagents like SDS. The partially-denatured protein which still maintains part of its high-order structure would exhibit a greater mobility on the SDS gel than the full-reduced protein, resulting in an underestimation of its molecular weight.

Cytochrome b isolated according to my procedure has an apparent molecular weight of 26,000. The close similarity of the amino acid composition of this preparation with that deduced from the gene sequence, suggests that the difference in the molecular weight (i.e., 26,000 vs. 44,000) is not due to post-translational modification of the apocytochrome b. Therefore, disagreement on the molecular weight is attributed to deceptive gel electrophoretic results.

If we accept the gene sequence data, my cytochrome b preparation, with a purity of 37 nmole b/mg protein, implies the presence of 1.6
mole heme b per polypeptide. Assuming either that a small percentage
of heme b is lost in the preparation procedure or that the purity is
around 80%, would imply 2 mole heme b per polypeptide.

3. Further evidence implicating multiple b heme moieties
associated with a single apoprotein is reflected in the potentiometric
data (Chapter 5). The two b species distinguished by differences in
midpoint potentials exhibit identical pH dependence of their redox
potentials in different detergent systems. The variation of -30 mV/pH
found in the taurocholate buffer systems implies that two reducing
equivalents are involved in one protonation step; in other words, the
two b hemes are in close proximity and respond to the same protonated
group.

4. Cytochrome b heme(s) are very sensitive to the immediate
environment. A shift in detergent from deoxycholate to taurocholate
varies the pH dependency of midpoint potentials as well as the
contribution of each b species to the total MCD signal change (Chapter
5). Implication of mutual conversion between the two b species was
also seen in the presence of antimycin and coenzyme Q.

5. Arunjunjan and his colleague (47), using MCD and computer
simulation, demonstrated that the occurrence of different b species,
b$^{562}$ or b$^{566/558}$ in beef heart complex III can be explained by
assuming that the absorption maxima at 558 and 566 nm are due to
rhombic distortion splitting the Q$_{00}$ ($\pi\rightarrow\pi^*$) transition, which is
centered at 562 nm, rather than a separate conformational state or
vibronic transition of Q$_{00}$.
Based on these data, I suggest that the different cytochrome b species identified by physical observations are simply different signatures of the same heme b conformation responding to perturbations in its immediate environment. The perturbations could originate internally from the protein backbone or from chemical treatment during preparation procedure. Moreover, the two hemes contained in each bc$_1$ complex may well be located in close proximity within one polypeptide; transitions between these two species can occur under the influence of certain effectors. The N and C terminal sequences of the b protein were recently determined to agree with the gene sequence data (personal communication with Tragoloff); this result appears to be a proof of the suggestion that two hemes are associated with one cytochrome b apoprotein.

Whether cytochrome b acts as a passive electron carrier as proposed in the protonmotive Q-cycle or plays an active role as proton translocator is not resolved. The malleable structure of cytochrome b described above bears consideration in further exploration of its functional role. An overemphasis on the multiple species of cytochrome b and the rigid assignment of their relative stoichiometry may be misleading.

Kinetic measurements using the nonphysiological substrate ferricyanide have yielded important information on the internal electron transfer sequence and rate. A very rapid equilibration between cytochrome c$_1$ and the iron-sulfur center was observed during reoxidation of two electron-reduced Complex III. This fast
equilibration is favored by the proximity of their midpoint potentials (Chapter 5). The forward and reverse electron transfer rate constants appear to be greater than 10,000 s⁻¹.

The oxidation of four electron-reduced sample can be simulated by the linear electron transfer scheme:

\[ b_H \rightarrow b_L \rightarrow Fe/S \rightarrow c_1 \rightarrow \text{oxidant} \]

The irreversibility of the electron transfer from \( b_L \) to iron-sulfur center observed by the absorbance changes at both 553.5 and 561.5 nm is due to the large potential gap between these two redox centers. Rapid equilibration similar to that between the iron-sulfur center and \( c_1 \) was not observed with the two \( b \) species although they vary in midpoint potential by less than 90 mV. Any transition occurring between these two \( b \) hemes will simultaneously result in an orientation in which electron transfer is kinetically unfavorable. This idea is supported by the observation of Malviya et al. (149) of the independent reduction and oxidation of \( b_K (b_{562}) \) and \( b_T (b_{566/558}) \) of beef heart submitochondrial particles and cytochrome c deficient mitochondria. This conclusion contradicts the proposed function of \( b \) in the protonmotive \( F \)-cycle (see Fig. 9, Chapter 1), as this involves a direct electron transfer between the two \( b \) hemes.

Although I was able to simulate the data of the oxidation of both intact Complex and antimycin-A treated sample, the presence of CoQ may further complicate the mechanism. Another disturbing problem connected
with employing ferricyanide as oxidant concerns the possibility of direct oxidation of ubiquinol by ferricyanide. This side reaction might explain why I observed a second-order rate constant (k) of complex III similar to that of CoQ-depleted sample. Antimycin exerts definitive influence on the kinetic behavior of b cytochrome(s). This influence must be transmitted through coenzyme Q. A similar cooperative effect of antimycin and CoQ on cytochrome b was observed using potentiometric approaches as indicated in Chapter 5.

The elucidation of redox reaction mechanism of complex III is vital to understanding its function in both the electron transfer chain and energy coupling. The immediate effort as an extension of the work detailed in this thesis is the study of the kinetic behavior of coenzyme Q oxidative reactions. Comparative studies on the oxidative reaction mechanism should be performed using alternative oxidants having different chemical properties, especially cytochrome c. As cytochrome c has a midpoint potential near that of $c_1$ and iron-sulfur protein, cytochrome oxidase and oxygen must be introduced to drive oxidation to completion. A plausible oxidative reaction mechanism, once constructed, can be compared with the reductive reaction mechanism, which is not yet seriously studied. An exogenous reductant that transfers only one electron specifically to coenzyme Q of Complex III should be sought. Only this reaction system can offer an absolutely clear picture about the destiny of this reducing equivalent at every moment of the reaction and thus illuminate the electron transfer sequence.
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