The Preparation and Characterization of Highly Purified, Enzymically Active Complex III from Baker’s Yeast*

JAMES N. SIEDOW, SCOTT POWER, FRANCISCO F. DE LA ROBA, AND GRAHAM PALMER

From the Department of Biochemistry, Rice University, Houston, Texas 77001

A soluble enzymically active cytochrome b-\(c_1\) complex has been purified from baker’s yeast mitochondria by a procedure involving solubilization in cholate, differential fractionation with ammonium sulfate, and ultracentrifugation. The resulting particle is free of both cytochrome \(c\) oxidase and succinate dehydrogenase activities. The complex contains cytochromes \(b\) and \(c_1\) in a ratio of 2.1 and quinone and iron-sulfur protein in amounts roughly stoichiometric with cytochrome \(c\). EPR spectroscopy has shown the iron-sulfur protein to be present mainly as the Rieske protein.

EPR spectroscopy also shows a heterogeneity in the cytochrome \(b\) population with resonances appearing at \(g = 3.60\) (cytochrome \(b_0\)) and \(g = 3.76\) (cytochrome \(b_1\)). A third EPR resonance appearing in the region associated with low spin ferric hemes (\(g = 3.49\)) is assigned to cytochrome \(c_1\).

Anaerobic titration of the complex with dithionite confirmed the heterogeneity in the cytochrome \(b\) population and demonstrated that the oxidation-reduction potential of the iron-sulfur protein is approximately 30 mv more positive than cytochrome \(c_1\). An intense EPR signal assigned to the coenzyme Q free radical appeared midway in the reductive titration; this signal disappeared toward the end of the titration.

A conformational change in the iron-sulfur protein attendant on reduction of a low potential species was noted.

Recent years have seen a renewed interest in the study of Complex III of the mitochondrial electron transfer chain (coenzyme Q-cytochrome \(c\) oxidoreductase). Such studies have been concerned with the spectroscopic characterization of the various components of Complex III (1), the nature of the interaction of Complex III with other respiratory chain complexes (2), and its biogenesis (3). As noted by Yu et al. (2), the usefulness of many of the Complex III preparations used in past studies has been limited due to contamination by other respiratory chain components. They reported the purification of a soluble cytochrome \(b-\(c_0\) complex from beef heart mitochondria which was free of any gross contamination by components not associated with the Complex III region of the electron transfer chain. A similar preparation has recently been isolated from baker’s yeast (3).

The application of baker’s yeast is proving extremely valuable in the study of the biogenesis of mitochondria (4, 5), but its use in the study of mechanistic aspects of mitochondrial electron transfer is not as widespread. In light of the potentially useful manipulative aspects associated with this organism, we are currently using baker’s yeast as a source for the purification of mitochondrial complexes. Unfortunately, many of the established procedures generally applied to the purification of beef heart components fail to work with yeast, and this has, therefore, required the development of new preparative procedures (3, 4, 6).

The recent preparations of Complex III have all been performed in the presence of antimycin A, an irreversible inhibitor of this enzyme complex; and, consequently, the complex as prepared is enzymically inactive, severely limiting its usefulness in mechanistic studies.

This paper describes the purification and characterization of a soluble cytochrome \(b-\(c_1\) complex from baker’s yeast which is isolated without the addition of antimycin A; the isolation of the submitochondrial particles is performed in the presence of a serine protease inhibitor to minimize the possibility of proteolytic degradation. The product of the purification is enzymically active and is enriched in cytochromes \(b\) and \(c_1\), to levels that exceed the most highly purified preparations obtained from beef heart. A preliminary account of this work has appeared (1).

**Experimental Procedures**

**Materials**—Cytochrome \(c\) (type III), NADH, DPIP, phenylmethlysulfonyl fluoride, and cholic acid were obtained from Sigma Chemical Co. The cholate was recrystallized as described by Mason and Schatz (6). Enzyme grade ammonium sulfate was from Mann. All other chemicals were of the highest purity available from commercial sources.

**Methods**—Protein determinations were made according to the method of Lowry et al. (8). The concentrations of cytochrome \(b\) and \(c_1\) were determined spectrophotometrically using oxidation-reduction difference spectra (9, 10) as described under "Results." Total iron content was obtained using both atomic absorption spectroscopy and the spectrophotometric assay for total iron (method A) of Brumby and Massey (11). Iron-sulfur protein was measured by double integration of the EPR spectrum using reduced spinach ferredoxin as a reference.

1 The abbreviations used are: DPIP, dichlorophenolindophenol; SDH, succinate dehydrogenase; PMS, phenazine methosulfate; FMSF, phenylmethlysulfonyl fluoride; HPIP, high potential iron protein.

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standard. Total copper was assayed by atomic absorption spectros-
copy. Total and acid-extractable flavin and total ubiquinone were
determined spectrophotometrically according to the methods of Rao et al. (12) and Redderbom (13), respectively. Sodium dodecyl sulfate-polyacrylamide
gel electrophoresis was carried out according to Fairbanks et al. (14).

Cytochrome c oxidase was assayed by the spectrophotometric procedure of Smith and Conrad (15) and SDH was assayed at 25°C
using PMS-DPIP as the electron acceptor (16).

The enzymic activity of Complex III was measured spectrophotometrically at 25°C by measuring the reduction of 50 μM ferricyto-
chrome c by the synthetic Q analog 2,3-methoxy, 5-methyl, 6-pentyl
benzohydroquinone (25 μM). The buffer employed was 0.05 M
potassium phosphate, pH 7.4, containing 50 mM EDTA.

Anodeactive dithionite solutions were prepared and standardized against lumiflavin-3-acetate essentially according to the modifica-
tion of the method of Burleigh et al. (17) described by Lambeth and Palmer (18). The mill effluent is passed through a stainless steel coil buried
in ice to keep the suspension frozen. The cold suspension is centrifuged in the Beckman J10 rotor for 15 min at 10,000 × g at 4°C, the pellet is discarded, and the clear, orange supernatant is adjusted to pH 8.1
with 0.1 M NaOH. At this point and for all future steps, the pH of the supernatant is adjusted from 7.2 to 7.3 with 4 N KOH. The solution is frozen for the first time and stored in liquid nitrogen.

RESULTS

Isolation and Purification of Yeast Cytochrome b\textsubscript{c}, Complex The procedure for the overnight extraction of yeast submitochon-
drial particles with cholate represents a modification of the method of Mason (5). Twenty-four batches of fresh Fleischmann's yeast were
submitted to the purification procedure described in this article. The ratios of the components remain approximately constant from one preparation to the next with the content of cytochrome b varying from 1.9 to 2.05 on a
cytochrome c, basis, while quinone/cytochrome c, ratios as high as
1.5 have been measured. The heme content of the particle
meets or exceeds that found in the most highly purified preparations from beef heart (2) and clearly contains a higher
heme content than the recently reported complex from yeast
(3). The ratios of the individual oxidation-reduction components are comparable to those reported for the beef heart
preparation (2).

The quantity of Rieske iron-sulfur protein present in the preparation is determined by double integration of the EPR
spectrum, using reduced spinach ferredoxin as standard: to
minimize the contribution of the small amounts of other iron-
sulfur proteins present, ascorbatereduced Complex III is used
for these estimations. The integrals are approximately stoichiometric with cytochrome c, (Table I). Assuming that the EPR
signal arises from a two-iron iron-sulfur protein, the total iron
represented by the heme and iron-sulfur content accounts for
90% of the total iron found in the preparation. If the EPR
signal were due to a four-iron species, then we would have
calculated a value for the total iron significantly in excess of

2 If the yeast is not fresh, succinate dehydrogenase may begin to
come down here and Complex III contaminated with succinate
dehydrogenase comes down at the next step. If this occurs, resuspend
the next pellet in approximately 200 ml of potassium phosphate
buffer, add ammonium sulfate to 243 g/liter, centrifuge as before,
run the pilot tests for the optimum ammonium sulfate level, elimi-
nate the succinate dehydrogenase by washing the free ammonium sulfate level, elimi-
nate the succinate dehydrogenase by washing the supernatant with 31 g/liter of ammonium sulfate, and collect the Complex III at 60% saturation (i.e. omit the step using 31 g/liter of ammonium sulfate).
that actually measured (Table I) and it thus seems that in yeast, as well as in beef heart, the Rieske iron-sulfur protein is a two-iron species. The iron unaccounted for amounts to 0.8 g atom/mol of cytochrome c1 and, at least in part, must be responsible for a significant g = 4.3 rhombic iron resonance present in the EPR spectrum of the resting enzyme.

The purified cytochrome b-c1 complex contains no measurable copper and only negligible (<0.1 nmol/mol of cytochrome c1) amounts of flavin, 80% of which is in an acid-extractable form. Only a very small amount of SDH-PMS reductase activity (<0.05 units/mg of protein, 25°) and essentially no measurable cytochrome c oxidase (k < 0.3 min−1/mg of protein) activity were found on the purified particle. These values represent specific activities which are approximately 0.35% and 0.02% of the values associated with purified beef heart succinate dehydrogenase and yeast cytochrome c oxidase, respectively (4, 16). The preparation is not reduced by NADH after incubation under anaerobic conditions for 2 h, but reduction is observed after 48 h.

Enzyme Activity—Under the standard conditions described under "Experimental Procedures," the particle catalyzes the oxidation of the synthetic coenzyme Q analog 2,3-methoxy, 5-methyl, 6-pentyl benzohydroquinone with horse heart ferricytochrome c as electron acceptor. The turnover number under kinetic parameters. Vmax = 200 s−1; Km (quinone) = 50 µM; Km (cytochrome c) = 175 µM. The graphical pattern is representative of the "ping-pong" kinetic mechanism.

Disc Gel Electrophoresis—Electrophoresis of the purified yeast cytochrome b-c1 complex showed seven major bands running at or behind the dye front, together with an additional two bands which run ahead of the dye. This pattern is observed on denaturation in 2% sodium dodecyl sulfate alone, plus 2% mercaptoethanol, or plus 2% mercaptoethanol. No difference was observed when denaturation was carried out for 2 h at 37° or at 2 min at 100°. The approximate molecular weights of the first seven bands are 47,000, 39,000, 31,000, 23,000, 14,000, 13,000, and 11,000. This distribution generally follows that described by Katan et al. (3) except that the discrete band we observe with a molecular weight of 23,000 appears to have split into several less intense components in Katan's preparation; this may be a consequence of proteolysis. We also observe an intrinsic fluorescence associated with the band of molecular weight 31,000; this has been ascribed to the covalently bound heme of cytochrome c1. The two minor bands which run faster than the dye front are believed to be aggregated forms of protoheme.

Spectral Properties—The absolute absorption spectra of the oxidized and reduced forms of the yeast cytochrome b-c1 complex are shown in Fig. 1 and further summarized in Table II. The oxidized form has absorption 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 α-band region at 562 nm (cytochrome b) with a shoulder at 554 to 555 nm (cytochrome c). The α-bands for the reduced cytochromes are at 530 nm and 523 nm, respectively. These visible spectra compare well with those previously reported for the cytochrome b-c1 complex from both beef heart (2) and yeast (3). 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 which is about one-half the value of 1.0 found for the beef heart preparation (2) and yeast (3).

Quantification of the two cytochromes is achieved using the oxidation-reduction difference spectra shown in Fig. 2; these

![Fig. 1. Absolute absorption spectrum of yeast Complex III at various states of oxidation and reduction. The sample was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1% of both deoxycholate and Triton X-100 and 1 mM EDTA. The concentration of cytochrome b was 6.6 µM. Spectra were recorded at 18°. Dashed line, a sample oxidized with a slight excess of ferricyanide; solid line, reduced with excess ascorbate (ASC); dotted line, reduced with excess dithionite. The path length was 1.0 cm.](http://www.jbc.org/)

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**Table I**

| Component     | Composition | Ratio |
|---------------|-------------|-------|
|               | nanomol     | range |
| Cytochrome c1 | 4.6         | 1.0   |
| Cytochrome b  | 9.3         | 2-2.1 |
| Total iron    | 26.5        |       |
| Iron-sulfur protein: | By EPR | 4.6 | 0.8-1.0 |
|               | By difference (total iron) | 6.3 |
|               | - heme iron |       |
| Excess iron   | 3.4         |       |
| Quinone       | 5.8         | 1.15-1.25 |

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Iron-sulfur protein: total iron, 26.5. Computed as described under "Methods." The concentration of iron-sulfur protein was derivatized both indirectly, assuming all the non-heme iron is present as a two-iron iron-sulfur protein (total iron), and directly, by double integration of the EPR spectrum using reduced spinach ferredoxin as a standard.
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have been performed in several ways, using the methods of Yu et al. (2), Berden and Slater (9), and Vanneste (10). The method of Yu et al. (2) quantifies the content of cytochrome c1 from the change in absorbance at 553 after reduction by ascorbate using a $\Delta \varepsilon_{553}$ of 17.1, while the two other methods employ more complicated formulas based on changes in absorbance at four wavelengths on reduction of the oxidized complex with dithionite. The values obtained with these latter methods agree closely but are approximately 10% higher than that obtained with the former method, suggesting that perhaps 10% of the cytochrome c1 cannot be reduced by ascorbate. Interestingly, after prolonged incubation with excess ascorbate partial reduction of cytochrome b can be observed.

The methods of Yu et al. (2) and that of Vanneste (10) give identical values for the content of cytochrome b, while the method of Berden and Slater (9) yields values which are some 10% higher. Because of these inconsistencies we have standardized on the method of Vanneste for measuring both cytochromes; we believe this procedure gives more reliable and (for cytochrome b) more conservative results. The methods is (10):

\[
\text{[cytochrome b]}_{\text{red}} = \frac{\Delta A_{418-470} \text{ (reduced-oxidized)}}{28.5}
\]

\[
\text{[cytochrome c1]}_{\text{red}} = \frac{\Delta A_{553-559} - \text{[cytochrome b]}_{\text{red}} \times 5.1}{18.8}
\]

The results are presented in Table I and the optical properties of the two cytochrome components are summarized in Table II.

### Table II

**Optical parameters for yeast Complex III**

| Oxidation-reduction state | \(\lambda\) (nm) | \(\varepsilon\) (mM$^{-1}$cm$^{-1}$) |
|---------------------------|-----------------|----------------------------------|
| Oxidized$^b$              | 280$^c$         | 338                              |
|                           | 416             | 350                              |
|                           | 524             | 34.8                             |
|                           | 532             | 36.4                             |
|                           | 553             | 29.4                             |
|                           | 561.5           | 29.2                             |
| Partially reduced (+ascorbate) | 418          | 382 (387)$^d$                   |
|                           | 524             | 38.8 (39.2)                      |
|                           | 532             | 35.5 (35.5)                      |
|                           | 553             | 45.2 (46.5)                      |
|                           | 561.5           | 24.9 (24.5)                      |
| Fully reduced (+dithionite)| 429             | 469                              |
|                           | 524             | 42.2                             |
|                           | 532             | 45.6                             |
|                           | 553             | 56.4                             |
|                           | 561.5           | 74.8                             |

$^a$ Calculated on a cytochrome c1 basis assuming that the mole ratio of cytochrome b to cytochrome c1 is 2:1 and that the concentration of cytochrome b is reliably measured by the procedure described in the text.

$^b$ As observed in freshly prepared complex or older material treated with a minimum amount of potassium ferricyanide.

$^c$ The ultraviolet absorbancy does not vary greatly with purity of the protein, the ratio $A_{280}$ (oxidized)/$A_{260}$ (reduced) decreasing from 0.78 to 0.72 as the content of cytochrome b is raised from 7 to 13 nmol of heme/mg of protein.

$^d$ The values in parentheses are those calculated on the assumption that the cytochrome c1 is only 92% reduced which would only be true if the spectrophotometric procedure of Yu et al. (2) is valid.
tions. If care is not taken in choice of the concentration of deoxycholate in the final buffer, this iron-sulfur signal will be 50 to 100% formed in the resting state complex. However, it is straightforward to adjust the level of detergent so that all spectrally detectable species are oxidized; under these conditions there are no EPR signals in the g = 2 region, but addition of ascorbate or dithionite brings about complete formation of the iron-sulfur signal. The concentration of reduced iron-sulfur protein in the cytochrome b–c₃ complex was obtained by double integration of the EPR spectra. A range of values of 0.83 to 1.0 iron-sulfur center per cytochrome c, has been obtained (Table I). The Rieske iron-sulfur protein is felt to be a two-iron type iron-sulfur protein with a relatively high oxidation-reduction potential (22). The agreement between the EPR integrations and non-heme iron content discussed above (Table I) supports this assumption.

The EPR spectrum produced on reduction with ascorbate differs slightly from that observed in the presence of dithionite, with the high field trough being somewhat sharper and centered at g = 1.81 and the low field peak moving slightly to higher fields with a g value of 2.026. A similar change has been noted previously with the beef heart protein (1).

The only other resonances observed in this region are two very minor bands to the low field side of g = 2.026 and g = 1.89; these are located at g = 2.08 and g = 1.94, respectively, and represent the gₓ and gᵧ values associated with a second mitochondrial iron-sulfur protein, the so-called "Center-5" (23). These signals only appear upon reduction with dithionite and require high sensitivity for observation. This is in marked contrast to the beef heart cytochrome b–c₃ complex purified by the method of Yu et al. (2) in which the Center-5 signal was present in greater concentration than that of the Rieske protein. Ruzicka and Beinert (24) have recently reported that the protein associated with this signal is an iron-sulfur flavoprotein that plays a role linking the fatty acyl dehydrogenase pathway with the electron transfer system. If this signal is due to Center-5, it is present only in extremely small amounts relative to the Rieske protein and, as such, may account for the residual amounts of flavin referred to above.

In the yeast cytochrome b–c₃ complex recently described by Katan et al. (3), several EPR signals were observed in the iron-sulfur region which are not present in our preparation. These include a resonance at g = 2.02 and a free radical signal at g = 2.00 in the oxidized form (in this oxidation-reduction state we see no signal at all) and lines at g = 1.93 and g = 1.91 in the reduced form which are in addition to the Rieske protein signals; as noted by Katan et al. (3), these latter two signals correspond to the gₓ and gᵧ resonances of the SDH iron-sulfur centers S-1 and S-2 (25). In addition, the resonance in the oxidized complex peaking at g = 2.02 is characteristic of the HIPIP type iron-sulfur Center S-3 (26) present in succinate dehydrogenase. Our own finding in purifying the yeast cytochrome b–c₃ complex was that succinate dehydrogenase is the hardest component to eliminate. During the course of developing the purification procedure, however, we observed a direct relationship between the amount of succinate dehydrogenase activity remaining in the particle and the amount of the EPR signal of Center S-3 present. Only when we were able to remove essentially all of the succinate dehydrogenase enzymatic activity did the HIPIP EPR signal disappear from the EPR spectrum of the oxidized preparation. The source of the radical signal observed by Katan et al. (3) is unknown; however, as we see a radical signal at intermediate states of reduction, it may be due to the semiquinone form of coenzyme Q (see below).

The EPR spectra of the yeast b–c₃ complex between 1600 and 2300 gauss (9.24 GHz) can be used to characterize the low spin ferrihemoproteins. The simplest spectrum is obtained with the ascorbate-reduced complex in which cytochrome c₃ is reduced (Fig. 4B). A large asymmetric resonance is observed with an approximate g value of 3.7. From reductive titration experiments described later we believe this absorption to have two contributions, at g = 3.60 and g = 3.76. These correspond approximately to the field positions assigned to the two b cytochromes present in beef heart mitochondria (1) where the high potential cytochrome (b₃₉) has a low field g value of 3.44 and the low potential cytochrome (b₇) exhibits a maximum at g = 3.78. (The use of the nomenclature, cytochrome b₉ and b₇, to distinguish the two b type cytochromes will be continued in the remainder of this paper as a matter of convenience.) A small resonance is observed at g = 2.95, a signal generally regarded as due to a "modified" form of cytochrome b (27). Reduction with dithionite completely eliminates all of the signals in this region.

The EPR of resting state complex in the g = 3 region is quite similar to the ascorbate-reduced protein (Fig. 4) though optical measurements show that cytochrome c₃ is approximately 100% oxidized. Close examination of the EPR spectrum reveals a shoulder to the high field side of cytochrome b₉ (g = 3.60) that is absent in the ascorbate-reduced sample, and the computer-derived difference spectrum, resting minus ascorbate reduced, exhibits a weak, broad band centered at g = 3.49 which we attribute to the low field

**Fig. 4.** EPR spectra of Complex III in low field cytochrome region. A, oxidized Complex III; B, Complex III plus 1.8 electrons/mol of cytochrome c; C, Complex III plus 7 electrons/mol of cytochrome c; D, A minus B; E, B minus sample containing 6.5 electrons/mol of cytochrome c; F, C minus dithionite-reduced complex. The spectra are taken from the experiment of Fig. 5.

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3 J. Siedow, unpublished results.
resonance of cytochrome c₁. This resonance is shifted some 90 gauss downfield from that observed in beef heart (g = 3.33) (1), but its position corresponds closely to the value of 3.51 obtained with a purified sample of yeast cytochrome c₁. Broader scans show an EPR signal at g = 4.3 (high spin ferric iron) with an amplitude comparable to the g = 3.70 signal. At these instrumental sensitivities no significant signal indicative of high spin ferric heme is observed in the g = 6 region of the EPR spectrum.

Reductive Titrations — In recent years, the study of electron transfer systems has seen a great emphasis placed on the measurement of reduction potentials of individual components (28). These studies have been extremely useful in helping to elucidate the mitochondrial electron transfer scheme. Such studies are generally done in the presence of a large number of mediators, and, while they can be used to measure individual reduction potentials very accurately, they do not allow the calculation of total electron uptake by a particle and they give no information about the possible role of a component which cannot be directly monitored (e.g. few studies have measured the reduction potential of quinone (29, 30)). A reductive titration can be used to calculate relative reduction potentials and also monitor total electron uptake in the absence of any added mediators. The latter value can be useful in determining the presence or absence of electron acceptors in addition to those which can be directly monitored. It is also useful to be able to compare the relative reduction potentials obtained by potentiometric studies with those obtained by direct reductive titration.

Many of the above points were noted by Boveris et al. (21) in their study using pulses of durohydroquinone to reduce pigeon heart mitochondria. They showed a correlation between the amount of durohydroquinone added and the reduction of various mitochondrial components although most of the added electrons served to reduce ubiquinone. No attempt was made to fit the data to a set of reduction potentials, but a specific order of electron uptake was noted.

In a pioneering study Orme-Johnson et al. (1) also attempted a quantitative evaluation in their studies of the EPR signals associated with the beef heart mitochondrial system. With Complex III they calculated that if the quinone were not reduced they should observe the uptake of two electrons per cytochrome b. They found that 3.5 electrons were taken up per cytochrome b and concluded that species in addition to quinone must be involved in the uptake of electrons by Complex III. Unfortunately, the quantity of quinone present in the preparation was not determined. Furthermore (as judged by the presence of characteristic EPR signals in the iron-sulfur region), the preparation of beef heart Complex III employed was apparently contaminated with succinate dehydrogenase. Nonetheless, the results were most encouraging and indicated that further work along these lines was justified. The present availability of preparations of Complex III free of succinate dehydrogenase is the basis for reinitiating the following studies.

In these experiments Complex III was reduced incrementally with dithionite and the sample incubated in the spectrophotometer at 10° until all changes on absorbances were complete. The optical spectrum was recorded, digitized, and stored on magnetic tape. A sample of the reaction mixture was then transferred to an EPR tube, frozen, and examined by EPR at several pertinent temperatures. The EPR spectra were also digitized and stored on tape. The species which could be monitored were: cytochrome c₁, bₓ, and b₇ (EPR, optical), reduced iron-sulfur protein (EPR), and radical (EPR). The intensities of each of these species as a function of the degree of reduction are presented in Fig. 5. In preparing the figure it was assumed that each of the three cytochromes, c₁, bₓ, and b₇, and the iron-sulfur protein were present in unit stoichiometry. The intensity of the radical was that determined by double integrations of the EPR spectra.

The analysis presented in Fig. 5 is qualitatively that anticipated from the properties of the beef heart system (1). The first additions of dithionite produce reduction of both the iron-sulfur center and cytochrome c₁ with the iron-sulfur resonance appearing faster than both the appearance of the characteristic absorption band at 552 nm and the disappearance of EPR intensity at approximately g = 3.4. These data are qualitatively consistent with the established properties of these two species and establish that the potential of the iron-sulfur center is some 30 to 40 mv more positive than the cytochrome. Complete reduction of cytochrome c₁ and the iron-sulfur protein requires 1.6 to 1.8 reducing equivalents, slightly less than the anticipated value of 2.0. This small discrepancy is consistent with the observation of a small amount of reduction of both species even before any reductant is added.

Only when early changes are complete do the b cytochromes become reduced. This is seen as an increase in the optical absorbance between 555 and 570 nm and an additional decrease in the EPR intensity at g = 3.6 to 3.7. These changes take place in two stages: first of all the increase in absorbance is centered at 562 nm and corresponds to the disappearance of EPR intensity at g = 3.6. Toward the end of the titration the optical characteristics of the spectra change, the absorption band becoming broader with a shoulder to longer wavelength. This is exemplified in Fig. 5 which compares the difference spectra computed during the early phase of b reduction with that apparent toward the end of cytochrome b reduction. These spectra correspond to cytochromes bₓ and b₇, respectively.

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Fig. 6. Comparison of the difference spectra observed during the early and late phases of reduction of the cytochromes b. 

- High potential cytochrome b \( b_{h} \), (Sample B (Fig. 5)) minus Sample A (Fig. 5); 
- Low potential cytochrome b \( b_{l} \), (Sample D (Fig. 5)) minus Sample C (Fig. 5). The absolute spectra are those from the appropriate reduction of Fig. 5, and the difference spectra were obtained numerically from the digitized data and reploted directly by computer.

During this phase of the titration an intense free radical signal is present in the EPR spectrum. The intensity of the radical signal grows to a maximum value of approximately 10% of the total coenzyme Q content of the preparation. The size of the radical signal decreases toward the end of the titration and is immeasurably small at the completion of the experiment. The change in shape of the iron-sulfur resonance referred to above occurs during the titration of the b cytochromes. The horizontal bar in Fig. 5 delineates the range of titrant over which the transition occurs.

There are two species in the preparation which we believe to be impurities, an iron species exhibiting a \( g = 4.3 \) EPR signal and some denatured cytochrome b \( b_{d} \) which is responsible for the small EPR peak at \( g = 2.95 \). The amplitude of this cytochrome b \( b_{s} \) signal decreases early during the titration of cytochrome \( b_{l} \); the \( g = 4.3 \) signal titrates throughout the \( b \) region, suggesting that it is heterogenous.

The total number of electrons consumed in this experiment is approximately 7.5 per \( b-c \) complex. From the composition of the material a total electron uptake approximately 6.5 electrons was anticipated, thus leaving one electron unaccounted for. The probable fate of this electron is reduction of the \( g = 4.3 \) iron component visible in the EPR spectrum.

**Discussion**

The preparative procedure described here consistently yields enzymatically active Complex III with a cytochrome b content approaching 10 nmol of cytochrome b/mg of protein and a \( b:b_{c} \) ratio close to 2.0. The method is simple and can be carried to completion in 3 days. There are two aspects of the preparative procedure which merit comment. The first is the inclusion of the serine protease inhibitor phenylmethylsulfonyl fluoride in all media until the closing steps of the preparation. The presence of proteolytic enzymes in yeast extracts is a problem also encountered by others (e.g., 6) and indeed we find that the properties of the \( b-c_{1} \) complex and of cytochromes \( b_{s} \) and \( c_{1} \), isolated from the complex are modified if the PMSF is omitted.

Second, the use of ultracentrifugation to sediment the Complex III particle achieves a substantial (approximately 3-fold) purification in the closing stages of the procedure. This is a simple and effective step which we have also found useful in concentrating both this complex and cytochrome oxidase.

The analytical data provided demonstrate the high enrichment in cytochromes and iron-sulfur protein that has been achieved: the values that are routinely obtained are significantly larger than other preparations, both from yeast and beef heart. Furthermore, the preparation is stable and particularly well suited for physicochemical experiments although after storage cytochrome \( c_{1} \) and the iron-sulfur protein become spontaneously reduced so that we would recommend the use of freshly prepared material for most spectroscopic experiments.

The turnover number of the preparation with the coenzyme Q analog 2,3-methoxy, 5-methyl, 6-pentyl benzoquinone is high: approximately 200 s\(^{-1}\) under conditions of saturating 2,3-methoxy, 5-methyl, 6-pentyl benzoquinone and cytochrome \( c_{1} \). This value is about an order of magnitude greater than the rate of electron flux through mitochondria during State 3 respiration (31) and suggests that the catalytic efficiency of the preparation has not been severely degraded by the purification procedure. We find, however, that we can only inhibit approximately 95% of this activity with antimycin A, a potent and stoichiometric inhibitor of this complex. Furthermore, a small fraction of the cytochrome reduction proceeds via superoxide, as diagnosed by a reduction in rate produced by superoxide dismutase. (We suspect that this activity may be related to the small amount of denatured cytochrome \( b_{d} \) diagnosed to be present in the preparation by the EPR absorption at \( g = 2.9 \) for this species may well be autooxidizable.)

The optical properties of the yeast Complex III are very similar to those of the preparation from beef heart although there are clear differences in EPR spectra, primarily in the details of the low field \( g \) values associated with the three cytochromes. The low field \( g \) values for yeast \( c_{1}, b_{h}, \) and \( b_{l} \) are 3.51, 3.60, and 3.74; these compare with the corresponding \( g \) values in the heart complex of 3.3, 3.44, and 3.78, respectively (1). The low field cytochrome \( g \) values in yeast Complex III are clustered more closely together (Fig. 4) with the consequence that the contributions of the three individual species can only be established by computer subtraction of EPR spectra, recorded at different oxidation-reduction states (Fig. 4, lower panel). Because the low field \( g \) values of ferrithionoproteins are extremely sensitive to ligand field parameters, this may be taken as evidence for small differences in the coordination parameters of cytochromes \( b \) and \( c_{1} \) from yeast and heart mitochondria.

Although the reductive titration of the complex proceeds qualitatively according to expectation, there is one significant complication in the data; thus, it seems that the assignment of the oxidized 5 cytochromes as judged by EPR and by optical spectroscopy is different. This is most clearly seen during the early phase of reduction of cytochrome \( b_{h} \); the intensity of the EPR signal at \( g = 3.60 \) attributed to this cytochrome decreases rapidly to a plateau at approximately 75% reduction. In contrast, the optical band at 561 nm only changes by 20% during this phase of the titration. A number of explanations exists for this contradiction. Trivially, one might infer that the combined optical EPR procedure is inapplicable to this system and that EPR data recorded at 8 K cannot be correlated with room temperature absorbance measurements. Although this alternative cannot be unequivocally rejected, the good agreement between optical and EPR measurements for cytochrome \( c_{1} \) (Fig. 5) would indicate that the method is in fact valid. A more intriguing explanation requires that cytochrome \( b_{h} \) and a second species, e.g., coenzyme Q, form an intimate
complex and during this portion of the titration curve the oxidation-reduction process is the production of the paramagnetic, QH'. This then forms an exchange-coupled complex with the low-spin ferric iron of the cytochrome, yielding a diamagnet and consequently eliminating the EPR spectra of both the cytochrome and the Q radical. This interpretation thus requires two pools of QH: one bound to cytochrome b₅₆₇, which is undetectable by EPR, and a second pool which is isolated from any other paramagnetic and exhibits the strong free radical signal. An interaction between S-3, the NADPH-like species of succinate dehydrogenase, and QH' has been considered in explaining anomalies in the EPR spectrum of partially reduced Complex II (32), and iron-ubiquinone complexes have been implicated in the primary photoexcitation centers of bacterial photosynthesis (33). (The hypothesis of a cytochrome b·Q radical complex can be tested by reductive titrations on coenzyme Q-depleted particles: these are underway.)

An intriguing phenomenon is the variation of the EPR line shape of the iron-sulfur center which occurs discretely in the later stages of the titration (Fig. 5). This change must reflect a conformational change at the Fe₃S₄ coordination site brought about by a change in oxidation-reduction state of either cytochrome b₅₆₇ or coenzyme Q. The data of Fig. 5 suggest that the conversion of coenzyme Q from the semiquinone to the fully reduced state is the responsible process. The alternative of a dipolar interaction between the Fe₃S₄ and a second paramagnetic can be excluded because the broader spectrum is observed in the fully reduced complex when all of the other oxidation-reduction carriers are presumably diamagnetic and thus not available for magnetic interactions.

Several reports have appeared demonstrating the presence of previously unidentified electron carriers in the Complex III region of the mitochondrial electron transfer chain. These include: "component X" of Rieske (34), "control factor Y" of Eisenbach and Gutman (35-37), and the "oxidation factor" originally isolated by Yamashita et al. (38) and most recently shown by Trumpower and Katki (39) to be functionally similar to "control factor Y." All of the above factors, however, have been shown to act on the oxygen side of the antimycin block and, therefore, would be expected to have a relatively high oxidation-reduction potential. Indeed, Eisenbach and Gutman (37) have reported the oxidation-reduction potential of "control factor Y" to be roughly equal to that of cytochrome c (250 mV). We have no evidence for an additional electron acceptor with a high potential, our data being explained satisfactorily by the iron-sulfur and c₉ content of our preparation. We do observe the need for additional reducing equivalents toward the end of the titration. However, these are not significantly in excess of the extraneous iron present in the preparation, and the elimination of the g = 4.3 resonance during the titration establishes that rhombic iron is one of the species responsible for the requirement of additional reductant.

A similar conclusion has been reached by Erecinska et al. (40) from the titration of pigeon breast muscle Complex III by NADH.

It was not our intention in this paper to provide a formal analysis of the titration data. Rather we have used the spectra obtained during this experiment to document the various species which can be observed in the preparation. A systematic analysis of these data and of experiments conducted on Q-free and inhibited Complex III is in progress.

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REFERENCES

1. Orme-Johnson, N. R., Hansen, R. E., and Beinert, H. (1974) J. Biol. Chem. 249, 1928-1939
2. Yu, C. A., Yu, L., and King, T. E. (1974) J. Biol. Chem. 249, 4905-4910
3. Katan, M. B., Pool, L., and Groot, G. S. P. (1976) Eur. J. Biochem. 65, 95-105
4. Mason, T. L., Poyton, R. O., Wharton, D. C., and Schatz, G. (1973) J. Biol. Chem. 248, 1346-1354
5. Mason, T. L. and Schatz, G. (1973) J. Biol. Chem. 248, 1355-1360
6. Bess, E., and Schatz, G. (1975) J. Biol. Chem. 251, 1991-1996
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
8. Bader, J. A., and Slater, R. C. (1970) Biochim. Biophys. Acta 216, 237-249
9. Vanneste, W. H. (1966) Biochim. Biophys. Acta 113, 175-178
10. Brumby, P. F., and Massey, V. (1967) Methods Enzymol. 10, 463-474
11. Rao, N. A., Felton, S. P., and Huennekens, F. M. (1967) Methods Enzymol. 10, 494-499
12. Reddien, E. R. (1967) Methods Enzymol. 10, 381-384
13. Fairbanks, G., Steck, T. L., and Wallach, D. H. F. (1971) Biochemistry 10, 2606-2617
14. Smith, L., and Conrad, H. (1956) Arch. Biochem. Biophys. 83, 403-413
15. King, T. E. (1967) Methods Enzymol. 10, 322-331
16. Burleigh, B. D., Foist, G. P., and Williams, C. H. (1969) Anal. Biochem. 27, 536-544
17. Lambeth, D. O., and Palmer, G. (1973) J. Biol. Chem. 248, 6095-6103
18. Palmer, G. (1977) Anal. Biochem. in press
19. Orii, Y., and Ohnuki, K. (1969) Annu. Rev. Biochem. 38, 1-14
20. Boeveris, A., Oshino, R., Erecinska, E., and Chance, B. (1971) Biochem. Biophys. Acta 245, 1-16
21. Rieske, J. S., MacLennan, D. H., and Coleman, R. (1964) Biochem. Biophys. Res. Commun. 15, 338-344
22. Ohnishi, T., Wilson, D. F., Asakura, T., and Chance, B. (1972) Biochem. Biophys. Res. Commun. 46, 1631-1638
23. Ruzicka, F. J., and Beinert, H. (1975) Biochem. Biophys. Res. Commun. 56, 302-303
24. Ohnishi, T., Lim, J., Winter, D. B., and King, T. E. (1976) J. Biol. Chem. 251, 2105-2109
25. Ohnishi, T., Solerno, J. C., Winter, D. B., Lim, J., Yu, C. A., Yu, L., and King, T. E. (1976) J. Biol. Chem. 251, 2094-2104
26. DerVartanian, D. V., Albrocht, S. P. J., Berden, J. A., van Gelder, B. F., and Slater, E. C. (1975) Biochem. Biophys. Acta 392, 496-501
27. Dutton, P. L., and Wilson, D. F. (1974) Biochim. Biophys. Acta 346, 105-122
28. Wilson, D. F., and Erecinska, M. (1975) Arch. Biochem. Biophys. 193, 265-275
29. Urban, P. F., and Kingenberg, M. (1969) Eur. J. Biochem. 3, 519-525
30. Chance, B., Erecinska, M., and Chance, E. (1973) in Oxidases and Related Redox Systems (King, T., Mason, H. S., and Morrison, M., eds) Vol. 2, pp. 851-874, University Press Park, Baltimore
31. Ruzicka, F. J., Beinert, H., Scheper, K. L., Dunham, W. K., and Sands, R. H. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2888-2890
32. Okamura, M. Y., Isaacson, R. A., and Feher, G. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3491-3495
33. Rieske, J. S. (1971) Arch. Biochem. Biophys. 145, 179-193
34. Eisenbach, M., and Gutman, M. (1975) Eur. J. Biochem. 5, 107-115
35. Eisenbach, M., and Gutman, M. (1976) FEBS Lett. 46, 388-371
36. Eisenbach, M., and Gutman, M. (1976) FEBS Lett. 61, 247-250
37. Nishiyama-Yamashita, H., Cunningham, C., and Racker, E. (1972) J. Biol. Chem. 247, 698-704
38. Trumpower, B. L., and Katki, A. (1975) Biochim. Biophys. Res. Commun. 65, 16-25
39. Erecinska, M., Wilson, D. F., and Miyata, Y. (1976) Arch. Biochem. Biophys. 177, 133-143

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