Ubiquinol-Cytochrome c Oxidoreductase of Higher Plants

ISOLATION AND CHARACTERIZATION OF THE bc1 COMPLEX FROM POTATO TUBER MITOCHONDRIA*

(Received for publication, October 29, 1990)

Edward A. Berry‡, Li-shar Huang‡, and Victoria J. DeRose§

From the ‡Cell and Molecular Biology Division and the §Chemical Biodynamics Division, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

A procedure is described for isolation of active ubiquinol-cytochrome c oxidoreductase (bc1 complex) from potato tuber mitochondria using dodecyl maltoside extraction and ion exchange chromatography. The same procedure works well with mitochondria from red beet and sweet potato.

The potato complex has at least 10 subunits resolvable by gel electrophoresis in the presence of dodecyl sulfate. The fifth subunit carries covalently bound heme. The two largest ("core") subunits either show heterogeneity or include a third subunit.

The purified complex contains about 4 μmol of cytochrome c1, 8 μmol of cytochrome b, and 20 μmol of ion/g of protein. The complex is highly diphilated, with 1–6 mol of phospholipid and about 0.2 mol of ubiquinone/mole of cytochrome c1. Nonetheless it catalyzes electron transfer from a short chain ubiquinol analog to equine cytochrome c with a turnover number of 50–170 mol of cytochrome c reduced per mol of cytochrome c1 per s, as compared with ~220 in whole mitochondria. The enzymatic activity is stable for weeks at 4°C in phosphate buffer and for months at ~20°C in 50% glycerol.

The activity is inhibited by antimycin, myxothiazol, and funiculosin. The complex is more resistant to funiculosin and diuron than the beef heart enzyme.

The optical difference spectra of the cytochromes were resolved by analysis of full-spectrum redox titrations. The a-band absorption maxima are 552 nm (cytochrome c1), 560 nm (cytochrome b-560), and 557.5 + 565.5 nm (cytochrome b-566, which has a split a-band). Extinction coefficients appropriate for the potato cytochromes are estimated.

Despite the low lipid and ubiquinone content of the purified complex, the midpoint potentials of the cytochromes (257, 51, and ~77 mV for cytochromes c1, b-560, and b-566, respectively) are not very different from values reported for whole mitochondria.

EPR spectroscopy shows the presence of a Rieske-type iron sulfur center, and the absence of centers associated with succinate and NADH dehydrogenases.

The complex shows characteristics associated with a Q-cycle mechanism of redox-driven proton translocation, including two pathways for reduction of cytochromes by quinols and oxidant-induced reduction of cytochromes in the presence of antimycin.

Ubiquinol-cytochrome c oxidoreductase (EC 1.10.2.2, the cytochrome bc1 complex) is a multi-subunit enzyme complex involved in electron transport in energy conserving membranes of mitochondria and bacteria. Homologous complexes are involved in photosynthetic electron transport in chloroplasts and blue-green algae (1).

Traditional methods for purification of the bc1 complex use bile salt solubilization and ammonium sulfate precipitation. Such procedures have proved quite successful for the enzyme from animal (2–4) and yeast (5, 6) mitochondria, but application of such procedures to the complex from plant mitochondria (7–9), Neurospora crassa (10), or bacteria (11) has found limited success.

Ashai’s group (8) purified the complex from sweet potato mitochondria using cholate and ammonium sulfate precipitation, but lost all enzymatic activity in the process. They reported that large spectral changes occurred in the bc cytochromes on solubilization of the mitochondria with cholate, but not on solubilization with Triton X-100. The inactive complex was purified to a high degree, allowing characterization of the constituent polypeptides by SDS-gel electrophoresis.

Degli Esposti et al. (9), using Jerusalem artichoke tuber mitochondria, were able to maintain activity through cholate solubilization and the first steps of purification, but the complex was unstable at high salt concentrations in the presence of cholate, and so could not be fully purified. The partially purified complex showed oxidant-induced reduction of bc cytochromes in the presence of antimycin, indicating a "proton-motive Q-cycle" (12) type of mechanism. The midpoint potential of cytochrome c1 was similar to the value found in mitochondria, but both cytochrome b potentials were more positive by about 55 mV than in mitochondria. The Rieske iron sulfur signal was clearly detectable in EPR spectra but was a minor feature overshadowed by signals from iron sulfur centers in contaminating dehydrogenases (9).

Instability in bile salts at high salt concentrations is also a problem for the fungal complex. Weiss and Juchs (10) reported that the N. crassa bc1 complex would not withstand the high ionic strength involved in the cholate/ammonium sulfate procedures.

With nonionic detergent, various column procedures have

*This investigation was supported by National Institutes of Health Grants S07RR05918-05 and GM36884 and by the Office of Health and Environmental Research, U. S. Department of Energy, under Contract DE-AC03-76SF00098. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; Brij-35, polyoxyethylene 23 lauryl ether; cys., cytochrome; DCIP, 2,6-dichlorophenol-indophenol; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethyleurea; DM, dodecyl maltoside; E-64, trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane; KPi, potassium phosphate buffer; MOPS, morpholinopropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; RMS, root mean square; TMBZ, 3,3',5,5'-tetramethyl benzidine; TMPD, N.N.N',N'-tetramethyl phenylenediamine; UB, 2-methyl-3-undecyl-5,6-dimethoxybenzoquinone; UBH, reduced UB.
been successful for animal (13, 14), fungal (15), and bacterial (16-18) bc complexes.

Hawkesford and Leaver (19) separated plant mitochondrial membrane proteins by ion exchange chromatography after dodecyl maltoside extraction. They identified the cytochrome b, cytochrome c, and core polypeptides of the bc complex by immunological methods. The bc complex eluted near the end of the salt gradient. They did not, however, achieve resolution of the bc complex from other proteins.

While the work reported here was in progress, Peiffer et al. (20) applied ion exchange chromatography to dodecyl maltoside extracts of wheat germ mitochondria. Both the cytochrome oxidase and bc complex were obtained at high purity, and the oxidase was extensively characterized. Activity of the bc complex was not reported.

We report here modification of the procedure used for a bacterial complex (16) to isolate an active bc complex from mitochondria of potato. The resulting complex has polypeptide composition comparable with that of the pure inactive preparation (8) and yet is more active by a factor of 6-10 on a protein basis than the most active preparation previously available (9). In addition this preparation is much more stable than the cholate preparation. We have successfully applied the procedure to mitochondria from red beet and sweet potato as well as potato and believe the stability of this preparation will make it useful for experiments involving the bc complex from a wide variety of plant sources.

**EXPERIMENTAL PROCEDURES**

**Preparation of Mitochondria**—Potato mitochondria were prepared by a modification of the method of Diolez and Moreau (25). Whole potato tubers (30 pounds) were homogenized with a culinary juicer into 2 liters of extraction buffer diluted with 9 volumes of washing medium and then centrifuged at 8500 rpm in the Beckman JA-14 rotor (7000 g).

Polyvinylpyrrolidone 10,000, 0.1 mM PMSF, pH 7.5). The homogenate was filtered through nylon filter cloth, 200-μm mesh (Hygienic Fabrics and Filters, Lanark, IL) and then centrifuged at 3000 rpm in the GS-3 rotor (13000 g) for 20 min. The supernatant was centrifuged at 8500 rpm in the Beckman JA-14 rotor (7000 × g) for 20 min.

To prepare “washed” mitochondria, the 7000 × g pellet was resuspended in washing medium (50 mM KP, 0.3 M Sucrose, 1 mM EDTA, 1 g/liter SSA, 14 mM 2-mercaptoethanol, 6 g/liter polyvinylpyrrolidone 10,000, 0.1 mM PMSF, pH 7.5). The homogenate was centrifuged at 35000 rpm in the SS-34 rotor (9200 × g) for 20 min. The pellet (washed mitochondria) was then resuspended in a small volume of washing medium + 0.1 mM PMSF, giving a final protein concentration of 20-40 g/liter, and stored frozen at −20 °C.

Percoll gradient-purified mitochondria were prepared essentially by the method of Moreau and Romani (26). The 7000 × g pellet was resuspended in 225.5% Percoll, 0.35 M sucrose, 5 mM Tris-MOPS, pH 7.2.

The mitochondria were separated on a self-generating Percoll gradient by centrifuging at 18000 rpm in the SS-34 rotor (30,000 × g) for 30 min. The brown mitochondrial band was collected and diluted with 5 volumes of washing medium and then centrifuged at 6500 rpm in the RSA rotor (5600 × g) for 15 min. The pellet (Percoll-purified mitochondria) was resuspended in a small volume of washing medium + 0.1 mM PMSF, giving a final protein concentration of 20-40 g/liter, and stored frozen at −20 °C.

The mitochondria used in all subsequent experiments were washed and Percoll-purified mitochondria.

**Purification of the bc Complex**—All steps were carried out at about 4 °C. Mitochondria (1 g of protein or less) were thawed and diluted with 50 mM KP, pH 7.5. Sodium chloride, dodecyl maltoside (DM), and PMSF were added to give final concentrations of 10 g/liter protein, 260 mM NaCl, 15 g/liter DM, and 0.1 mM PMSF (solubilization mixture). The DM was added as a freshly prepared solution in 50 mM KP, pH 7.5. PMSF was added from a 0.1 M ethanolic solution. The material was homogenized (glass homogenizer with a Teflon pestle) between the additions and (for small preparations) during addition of the dodecyl maltoside. If the final volume was greater than 55 ml, dodecyl maltoside and PMSF were added while stirring magnetically.

Aliquots of the suspension were taken for assay of protein, activity, and heme content and for gel electrophoretic analysis. The remainder was centrifuged 25 min at 45,000 rpm in a Ti-70 rotor (150,000 × g). Aliquots of the supernatant were taken for assays and gels, and the remainder was applied to a DEAE-Sepharose column (1.5 × 48 cm). After all the sample was loaded, the column was washed with at least 90 ml (actual volume given in text or figure legends) of 50 mM KP, 260 mM NaCl, 0.1 g/liter DM, pH 7.5. This was followed by a linear gradient (145 + 145 ml) of 260-500 mM NaCl in 50 mM KP, 0.1 g/liter DM. In the above-mentioned experiment to test for proteolysis, the solubilization mixture and column buffer were supplemented with 0.1 mM E-64 and 0.1 mM PMSF.

Fractions of about 5 ml were collected during loading, wash, and gradient. The flow rate was 25 ml/h during loading and wash and then was reduced to 16 ml/h for the gradient. The fractions were assayed for protein and for cytochrome c reductase and oxidase activities. Red fractions eluting early in the gradient with cytochrome c reductase activity but with no significant oxidase activity were pooled. Portions were taken for assay, and then the rest was concentrated by adsorption to a small hydroxyapatite column (1 × 1.5 cm) washed with 50 mM KP, 260 mM NaCl, 0.1 g/liter DM, pH 7.5. The bc complex was eluted with 300 mM KP, 0.5 mM Na-EDTA, 1 g/liter Brij-35 detergent, pH 7.0, and collected in a small volume (0.5-1 ml). This was further concentrated by dialyzing against 50 ml of 50 mM K-MOPS (or KP), pH 7.4, 0.5 mM EDTA, and 500 ml/liter glycerol for at least 12 h. The concentrated bc complex was stored at −20 °C until used.

**RESULTS**

**Concentration and Activity of the bc Complex in Potato Mitochondria**

The cytochrome b content of washed potato mitochondria ranged from 0.1 to 0.5 μmol/g protein, estimated from pyridine hemochrome spectra of whole mitochondria (8 preparations). Quinol-cytochrome c oxidoreductase activity varied from 0.25 to 1.7 units/mg protein (10 preparations). A large part of this variation may be due to seasonal differences or state of dormancy of the potatoes, rather than variation in the isolation procedure.

In one experiment washed and Percoll-purified mitochondria were prepared from the same homogenate. Cytochrome reductase activity, cytochrome oxidase activity, and cytochrome b content were enriched 1.8-, 2.5-, and 1.25-fold in the Percoll-purified mitochondria relative to the washed mitochondria. The “enrichment” in activity may be due to removal of contaminants which accelerate inactivation as well as to actual enrichment of mitochondria. Lipid peroxides produced by lipoxygenase have been reported to cause rapid destruction of cytochromes, especially cytochrome aa₃, in plant mitochondria (44).

Unfortunately the Percoll gradient is not effective if loaded very heavily (recommended load 50-60 mg of protein/36 ml of gradient (45)). Thus it is quite expensive to prepare large quantities of Percoll-purified mitochondria. We therefore isolated the bc complex from washed as well as purified mitochondria. As no difference was noted in the purified complex, most of our preparations have been from washed mitochondria.

In order to use activity as an indicator of the native state...
of the isolated protein, it was important to know the turnover number of the bc complex in mitochondria. Because other pigments interfered with the accuracy of the heme assay, and because cytochromes not belonging to the bc complex contribute to total heme of classes b and c, the antimycin titer was measured in one case. Quinol-cytochrome c oxidoreductase activity in the whole mitochondria was largely antimycin-insensitive; however, succinate oxidase activity was sensitive enough to give an accurate endpoint. From this endpoint the washed mitochondria used in the experiment of Fig. 1 contained 0.12 μmol/g protein of antimycin binding sites. The estimated contents of hemes c, b, and a were 0.13, 0.29, and 0.19 μmol/g protein, respectively. The ubiquinol-cytochrome c reductase activity was 1.7 units/mg protein, which if all due to the bc complex gives a turnover number of 220 s⁻¹ based on antimycin binding sites.

Although this is lower than the turnover number of the bc complex in beef heart mitochondria (we find 250-400 s⁻¹ for bc; from beef heart, values above 1000 s⁻¹ have been reported (46)), it seems adequate to account for the reported respiration rates in plant mitochondria. Douce et al. (47) found succinate oxidase rates of 308 nmol O₂/min.mg in gradient-purified potato mitochondria that contained 0.27 nmol/mg cytochrome b. Assuming the bc concentration is 1/3 or 1/2 of cytochrome b gives turnover numbers of 222 or 148 mol of cytochrome c/mol of bc/s.

**Purification of the bc Complex**

**Extraction of the bc Complex from Potato Mitochondria**—Potato mitochondria were extracted with DM at 1.5 g/g protein in the presence of 260 mM NaCl. Under these conditions, essentially all of the cytochrome reductase activity and 70% of the cytochrome oxidase activity was found in the supernatant after extraction (Table 1). This is based on measurement of activity in the mitochondrial suspension after adding detergent, before and after centrifuging down the unsolubilized material. Thus activation by detergent should be the same in both cases, and should not mask loss of enzyme. The lost oxidase activity (Table 1) is due mainly to lability in the extraction mixture, as the activity in the pellet was not high enough to account for the shortfall (not shown).

**DEAE-Sepharose Chromatography of Dodecyl Maltoside Extract of Potato Mitochondria**—Fig. 1 shows the elution profile of protein, cytochrome reductase, and cytochrome oxidase from the DEAE-Sepharose column. Most of the protein is not adsorbed under these conditions and is found in the flow-through (fractions 6-24). The flow-through also contains considerable cytochrome oxidase: a sharp peak in turbid fractions at the void volume, which probably represents incompletely solubilized membranes, and a broader peak later in the flow-through. Because of the lability of the oxidase in the extraction mixture, the activity of these fractions may have decreased in the time between elution and assay. The oxidase activity is substantially reduced if the flow-through fractions are assayed the day after the extraction. A small amount of UBH-dependent cytochrome reductase activity also elutes in the flow-through fractions, but it is not clear that this is due to presence of the bc complex in these fractions. Although spectra indicate the presence of b- and c-type cytochromes, the activity is not inhibited by 10 μM antimycin.

After the sample is loaded, the column is washed with several column volumes of starting buffer. This elutes most of the bound cytochrome oxidase. A salt gradient then elutes the cytochrome reductase activity, usually preceded by another small peak of cytochrome oxidase (around fraction 88 in Fig. 1). The amount of overlap between this last oxidase peak and the reductase peak is somewhat variable. In some cases the overlap is greater, and a significant part of the reductase has to be excluded from the pooled fractions to avoid excessive contamination.

In a typical experiment (Table 1) the pooled fractions from the DEAE-Sepharose column contained 40% of the c-type heme, 60% of the protoporphyrin, and 52% of the cytochrome reductase activity of the starting material.

SDS gel analysis of fractions from DEAE-Sepharose columns such as that of Fig. 1 show ten bands eluting in parallel with ubiquinol-cytochrome c oxidoreductase activity: three in the range 50-60 kDa; 34, 32, 24, and 14 kDa; and three bands in the range 6-10 kDa. Because these bands are present in the same proportion in each fraction, it is likely they are all associated in one complex. Two weaker bands, at about 42 and 85 kDa, also elute in this region. These bands are relatively stronger in the later active fractions and so presumably are not associated with the bc complex. These bands were also present as trace contaminants in the final concentrated product (lane 5, Fig. 2B), indicating they were concentrated on the hydroxyapatite column.

**Concentration of the Active Fractions by Adsorption to Hydroxyapatite and Step Gradient Elution**—The pooled fractions were concentrated by adsorption to a column of hydroxypatite and elution by a step gradient. The color was collected in several column volumes of starting buffer. The color was collected in about 0.5 ml and dialized against 50 mM phosphate or MOPS buffer, pH 7.3, containing 50% glycerol and 0.5 mM EDTA. The dialysis further concentrated the sample about 3-fold and introduced enough glycerol to prevent freezing at -20 °C, at which temperature it was stored. When the MOPS dialysis buffer was used it also eliminated most of the inorganic phosphate, facilitating phospholipid assay.

**Yield of bc Complex**—The recovery is given in Table 1. Essentially all of the heme and activity in the pooled fractions were recovered in the final concentrated product. In some cases more activity was recovered after dialysis than had been present in the pooled fractions, indicating activation by the process of concentrating and dialyzing against glycerol. In the experiment of Table 1, 51% of the cytochrome reduc-
Cytochrome bc Complex of Potato Mitochondria

In the experiment of Fig. 1, fractions 99-108 were pooled and concentrated, yielding 38 nmol of bc complex. This is a recovery of 53 nmol/g mitochondrial protein. Fractions 90-98 were separately pooled, yielding 13 nmol of bc complex, contaminated with some oxidase. Together, this is a recovery of 51 nmol of bc, 71 nmol/g mitochondrial protein, and 57% of the bc complex in the starting material based on antimycin binding titer. This was the most active preparation of washed mitochondria, however, and yields from more typical mitochondria were around 30 nmol of pure bc complex/g of starting protein.

Solubility—The concentrated bc complex can be diluted at least 50-fold into detergent-free buffer to obtain an optically clear solution. Presumably this is due to the presence of tightly bound detergent and to the low cmc of the detergent (Brij-35) present in the concentrated solution.

Stability—We have stored the purified complex at -20 °C. It does not freeze at this temperature because of the glycerol introduced by the dialysis step. Stability has been somewhat variable, with two preparations retaining 62 and 58% of their activity after 6 and 11 months, but a third preparation retaining only 20% of its activity after 9 months. Von Jagow’s group (48) noted proteolysis occurring on prolonged storage at low temperature in glycerol, and it may be that some preparations have more protease present than others. For assay, the concentrated bc complex is diluted 25-fold in 50 mM KP0, 0.5 mM EDTA, pH 7.4. This dilution can be kept at 4 °C for several weeks with little loss in activity.

The Polypeptide Composition of the bc Complex

To compare the polypeptide composition of the plant bc complex with that of vertebrate mitochondria, the complexes from potato, beet, and sweet potato were electrophoresed beside beef and chicken heart bc complex (Fig. 2). To identify the c cytochrome of the plant complexes, the gels were first stained for peroxidase activity. Type c cytochromes retain their covalently bound heme during electrophoresis and can be visualized by the peroxidase activity of the heme (31). As can be seen in Fig. 2, A and C, the 32-kDa band is positive. The vertebrate cytochromes c migrate slightly faster than the plant ones, at 30 kDa.

Fig. 2, B and D, show the same gels stained with Coomassie Blue. The major subunits of the vertebrate enzymes can be identified by comparison with published 15% Laemmli gels (48) or tricine gels (49). Most of the polypeptides of the beef complex have obvious counterparts in the plant complexes, although at slightly higher or lower molecular masses. However, in the 50–60 kDa range, where the beef complex has 2 “core” proteins, the potato and beet complexes have three bands. In the Laemmli system (Fig. 2B) the three core proteins are well resolved in the potato complex. In the beet complex, bands I and II are barely resolved, but in less heavily loaded gels the separation is quite clear. In the Schagger and von Jagow tricine system (30) (Fig. 2D), only two core proteins are visible for potato, but the three bands of beet complex are better resolved than in the Laemmli system (28). In either system, only two core proteins were resolved for the sweet potato complex. Nakajima et al. (8) also resolved only two core proteins for the sweet potato complex. As resolution of the three bands in potato and beet is dependent on the gel system and acrylamide concentration, the possibility remains that the sweet potato complex also has three peptides in this region that simply have not yet been resolved. Fig. 6 of Peiffer and co-workers (20) seems to indicate the presence of three core peptides in the wheat germ bc complex. The possible
significance of a third core protein will be taken up again in the discussion section.

The polypeptides of less than 12 kDa migrate somewhat differently from the vertebrate counterparts, and it would be useless to speculate on the correspondence between them. It seems reasonable to assume, however, that the four polypeptides in the molecular mass range 12–50 kDa migrate in the same order in the different complexes, in which case the 35-kDa band is cytochrome b, the 32-kDa heme-staining band is cytochrome c₁, the 24-kDa band is the iron-sulfur protein, and the 13-kDa band corresponds to the beef 13,389 Da “Q-binding protein.”

The potato bc₁ complex used for Fig. 2 was isolated from washed potato mitochondria. As it is realized that such preparations contain membranes that are not mitochondrial, several preparations were made using Percoll-purified mitochondria. The same pattern of ten bands was observed in these cases (not shown).

To estimate the molecular mass of the potato bc₁ complex subunits, the complex was electrophoresed on SDS gels together with molecular mass standards. These standards included both soluble proteins and beef heart mitochondrial membrane proteins for which the molecular mass is known from the sequence. Molecular masses of the potato bc₁ subunits estimated from such plots at different gel concentrations are given in Table 2, columns 1–5. For most of the polypeptides essentially the same molecular mass was obtained at each different gel concentration. However cytochrome b has a higher apparent molecular mass, and the third core protein has a lower apparent molecular mass, in more concentrated gels.

The anomalous behavior of these two peptides is seen in Ferguson plots of the peptide mobility versus gel concentration. Cytochrome b and subunit III have slopes out of proportion to their mobility, with cytochrome b being more sensitive and subunit III being less sensitive to gel composition than other peptides of about the same mobility. The order of migration reverses for subunits 1 and 3 and for cytochromes b and c₁, between 6 and 12% acrylamide. This was confirmed by running two-dimensional gels, using 15% gels for the first dimension and 6 or 8% gels for the second (not shown).

Table 2, column 6, shows molecular mass based on the slope of the Ferguson plot relative to the slope for the standard proteins. With the exception of subunit 3 and cytochrome b, the values from the Ferguson plot are in fair agreement with those obtained from single gels.

An estimate of the minimum molecular mass of the bc₁ complex can be made by adding the molecular mass of the different subunits. Taking the values from the 15% gels (column 3 of Table 2), this gives a molecular mass of 296 kDa. If some of the subunits are present in multiple copies, or if there are subunits we failed to visualize or resolve, the molecular mass would be higher. On the other hand if subunits 1 and 2 are iso-forms of the same protein and only one of the two is present on a particular molecule of the complex, the molecular mass would be about 238 kDa. Reasons for suggesting this possibility will be mentioned below.

Composition of the Potato bc₁ Complex

The chemical composition of the isolated bc₁ complex is summarized in Table 3. Specific values are based on protein content determined by the Lowry assay (32). As described under “Experimental Procedures,” a modified biuret assay was used to measure protein in three preparations. The ratio of protein determined by the Lowry method to protein determined by the biuret assay was 1.05, 1.02, and 0.94. Although this reproducibility is not very satisfactory, it seems unlikely that the Lowry estimate of protein is systematically in error by more than 6%.

Heme—Fig. 3 (curve 1) shows the spectrum of reduced pyridine hemochromes formed from the bc₁ complex at 0.38 g/liter protein. The spectrum was best fit by the spectra of 1.49 μM heme c, 2.89 μM protoheme, and 0.02 μM heme a. The root mean square fitting error (in absorbance units) over the range 521–621 nm was 3.4 × 10⁻⁴, whereas the root mean square difference between two subsequent spectra of the same sample was 1.2 × 10⁻⁴. The hemochrome spectra of myoglobin and cytochrome c are also shown in Fig. 3, scaled to the concentrations determined for the respective hemes in the bc₁ complex hemochrome spectrum. Trace 4 is the difference between the bc₁ hemochrome spectrum and the sum of the scaled heme c and heme b spectra. It shows the trace of heme a indicated above (absorption peak at 588 nm) and almost no feature around 550–556 nm. The peak in the experimental spectrum at 553.6 nm is accounted for almost perfectly by the linear combination of the pyridine hemochromes formed from myoglobin and cytochrome c.

The average heme content measured from such spectra of nine preparations was 4.02, 7.67, and 0.06 μmol/g protein of hemes c, b, and a, respectively. Heme c content ranged from 3.87 to 4.34 μmol/g protein. The ratio of heme b to heme c was between 1.83 and 2.00, averaging 1.91. This may not be significantly different from 2, given the variability in the measurements and the uncertainty in the extinction coefficients.

Adding the molecular mass of the subunits from column 3 of Table 2 gives a molecular mass of 296 kDa for the bc₁ complex if the subunits are present in one copy each. This molecular mass for the complex would allow a maximum heme c content of 3.3 μmol/g protein. If all 10 peptides are present at unit stoichiometry, the observed heme content could be explained by gross underestimation of protein by the Lowry assay, overestimation of polypeptide molecular masses by gel electrophoresis, or partial depletion of some heme-free subunits in the process of isolation.

However if as suggested under the “Discussion” section each complex contains either subunit 1 or subunit 2 but not both, the average molecular mass would be about 238 kDa,
giving 4.2 μmol/g, consistent with our measurements assuming one cytochrome c1 per complex.

**Iron**—The preparations contained 4.9 mol of iron/mol of cytochrome c. As the c and b cytochromes account for 1 and 1.9 of this, about 2 mol non-heme iron are present/mol of cytochrome c. Results from the direct analysis of non-heme iron were consistent with this (1.7 ± 0.7 mol/mol c1).

**Phospholipid and Ubiquinone**—The complex contained from 0.5 to 6.0 mol of lipid phosphate and 0.15 to 0.25 mol of ubiquinone/mol of cytochrome c1. As discussed below, the preparations with lower phospholipid tended to have lower activity, but no such correlation with ubiquinone content was observed.

**Carotenoid**—The crude mitochondrial preparation contained considerable carotenoid as indicated by peaks at 444 and 472 nm in the absorption spectrum of acetone extracts. However the spectrum of purified bc1 complex from crude mitochondria (Fig. 5) gives no indication of carotenoid present. Carotenoid was present in the unadsorbed fractions ("flow-through") of the DEAE-Sepharose column, as indicated by absorption spectra.

**Enzymatic Activity**

Activity of the purified preparations was assayed using 35 μM ubiquinol analog (UBH) as electron donor and 50 μM horse heart cytochrome c as acceptor, without extrapolating to Vₘₐₓ. The activity of the isolated complex, unlike that in membranes, was highly dependent on detergent concentration. Fig. 4 shows the turnover number of two preparations as a function of dodecyl maltoside concentration. Although the maximum turnover numbers differed by a factor of 3-fold for the two preparations, the optimum detergent concentration was about 100 mg/liter for both. We have arbitrarily used 50 mg/liter dodecyl maltoside in our standard assay, which is suboptimal for the purified enzyme.

Although the basis for the variability in turnover of the purified enzyme has not been determined, it may be related to phospholipid content. The more active preparation (0) contains 4.6 mol of phospholipid/mol of cytochrome c1, whereas the less active preparation (Δ) contains only 0.9 mol. As the inset of Fig. 4 shows, there is in fact a good correlation between activity and lipid content of the isolated enzyme.

This does not prove lipid depletion is the cause of the low activity in our case, as the same conditions which lead to lipid depletion during purification may lead to loss of other components required for activity. It should be pointed out that even our most lipid-rich preparations had only about 6 mol of phospholipid/complex. With the beef heart complex, activity was reduced to 25% when phospholipid content was reduced to 12 per complex (50).

A dependence on detergent such as is shown in Fig. 4 has been reported for other bc complexes isolated in the presence of dodecyl maltoside (16, 18). Phospholipid stimulates activity in the absence of added detergent (16), and it seems likely that detergent is fulfilling a requirement that was met by lipid in the original membrane. Yu et al. (51) found that some detergents could substitute for phospholipid in stimulating activity of lipid-depleted cytochrome oxidase. In the bc1 complex, a hydrophobic medium may be required to serve as "solvent" around the quinone binding sites, to allow diffusional exchange of ubiquinone and ubiquinol. Such a general requirement for a hydrophobic phase may explain the difference between the complex in membranes, which does not require detergent for activity, and the purified complex, which does. On the other hand the difference between the two preparations in Fig. 4 at optimum detergent concentration, if due to the difference in lipid content, would seem to indicate a specific lipid requirement which cannot be met by detergent.

Even the most active preparations were less active than the beef complex, isolated by a similar method and assayed the same way (turnover number 250–400 s⁻¹). As mentioned above, our best estimate for the turnover number of the potato complex in situ is only 220 s⁻¹. Although it is quite possible that the enzyme in potato actually turns over more slowly than that in mammals, it must be remembered that a mammalian cytochrome c is being used as substrate in our assay.

When bc1 complexes are purified by the bile-acid/salt precipitation methods, a common contaminant is succinate dehydrogenase. We could detect no succinate dehydrogenase activity in our preparation, using the PMS-linked DPIP reductase assay. As a more sensitive test, addition of succinate did not lead to reduction of any of the cytochromes of the complex. This would have detected even a single turnover of the enzyme. The EPR spectra (Fig. 8) further document the absence of either succinate or NADH dehydrogenases.

As already mentioned, the bc1 preparations were contaminated by a variable amount of cytochrome oxidase. The amount depended on the column washing and elution regime and on the choice of fractions from the DEAE-Sepharose to pool. The ratio of cytochrome oxidase to reductase activities in the purest active preparations was about 1:200. One preparation had no detectable oxidase activity, but the reductase turnover number was only 50 s⁻¹.

**Inhibitor Sensitivity of the Potato bc1 Complex**

A large number of inhibitors have been found to block electron transfer through the bc1 complexes of mammals, yeast, and bacteria. These can be classified into three groups based on the pattern of electron transfer inhibition (52). Four of these inhibitors were tested for inhibition of the isolated potato bc1 complex.

Antimycin and myxothiazol inhibit the potato complex stoichiometrically, implying a dissociation constant well below the enzyme concentration (12 nM). In beef heart submitochondrial particles, the dissociation constant for antimycin is 32 pm (53).

For funiculosin we found 50% inhibition at 0.5–2.0 μM with potato bc1 complex and 35 nM with the beef heart complex.
In beef heart mitochondria, Nelson et al. (54) found 50% inhibition of respiration at a total concentration of 70 nm, and the dependence of the inhibition curve on the amount of protein present suggests the free concentration was somewhat lower. In wild-type yeast 50% inhibition occurs at 300 nm (55) or 80 nm (56). Thus the isolated potato complex seems to have a significantly lower affinity for funiculosin than does the mammalian and yeast complexes.

Diuron inhibits the bc₁ complex in Saccharomyces cerevisiae mitochondria by 90% at 0.5 mM in wild-type, but various mutations in cytochrome b greatly decrease the extent of inhibition (57, 58). In one of these, residue 17 is changed from isoleucine to phenylalanine (58). In beef, the wild-type cytochrome b has phenylalanine in this position, but the plants sequenced to date have leucine (59). It might therefore be expected that the potato would be sensitive to diuron and the beef bc₁ complex would be resistant, but we found the opposite to be the case.

Diuron inhibited the beef complex 50% at 0.1 mM and 85% at 0.5 mM, but the potato complex was inhibited only 25% at 0.5 mM and 35% at 1 mM. These results should be considered qualitative because the diuron used was not pure, but it appears that the potato complex is significantly more resistant than the beef complex. This may be related to the residue at position 31 in the yeast sequence. Changing this from asparagine to lysine makes yeast resistant to diuron (58). Asparagine is present at the corresponding position in mammals, but is replaced by glycine in plants.

**Optical Spectra of the Potato bc₁ Complex**

**Absolute Spectra**—Absolute spectra of the fully oxidized and reduced potato bc₁ complex are shown in Fig. 5. Absorbance extrema and millimolar extinction coefficients estimated from heme content are given in Table 4, parts A and B.

As already reported for the partially purified complex (9), the α-band appearance is quite different from that of the more familiar complexes from beef heart, yeast, or bacteria. The α-band of the plant complexes is broader and more symmetrical than that of the beef heart complex. The absorption maximum is at 557 nm, as compared with 560 nm for the Neurospora complex (15), 560-561 nm in bacterial bc₁ complexes (60, 18), and 562 nm in the yeast (5) and beef heart (4) enzymes. The β-bands of the reduced complex have maxima at 525 and 530 nm and a shoulder at 537 nm. The Soret band peaks at 413.6 nm in the oxidized form and 427.4 nm in the reduced form.

There is a δ-band at 358 nm in the oxidized form, as observed for purified cytochrome c₁ from beef heart (61). The UV band due to protein peaks at 276 nm (Fig. 5), as compared with 278 for the beef heart enzyme (4) or 280 for that of yeast (5). The differences may not be significant, as light scattering would result in a slanted baseline which could shift the peak slightly. The shallow trough between the 276 nm peak and UV end absorption suggests that turbidity may contribute appreciably. The ratio of the oxidized Soret peak height to the UV peak is 0.80, as it is in the beef complex (4). In the yeast complex the ratio of the reduced Soret to UV peak is 0.5 (5); this ratio is about 1.0 for the potato or beef enzymes.

**Difference Spectra**—Fig. 6 shows difference spectra of the potato cytochrome reductase complex. By titrating the enzyme with hydroquinone, substituted hydroquinones, and dithionite in the presence of menadione, 51 spectra of the enzyme in different oxidation-reduction states were obtained. In Fig. 6, selected spectra are divided into three groups showing the titration of each of the three cytochromes.

In Fig. 6, A and B, showing titration of cytochromes c₁ and b-560, isobestic points are conserved through most of the spectra. This indicates that only a single component is titrating in this range and so the difference spectra are characteristic of that component. C shows the titration of cytochrome b-566, together with some more of b-560 in the lower spectra. The four most reduced spectra, including the one that was subtracted from all, seem to show isobestic points and may be accurate representations of the difference spectrum of b-566.

**Difference Spectra of the Individual Cytochromes**—The spectra of Fig. 6, together with data from the potentiometric titration of Fig. 7, were analyzed to derive difference spectra for the individual cytochromes. The procedure used is described in the “Appendix.” The resulting spectra were qualitatively what would be expected from the experiment of Fig. 6 and so are not shown. Selected absorption extrema and inflection points of the spectra are tabulated in Table 4, part C. Extrema were located as zero-crossings of the first derivative, and inflection points as zero crossings of the second derivative of the spectrum.

**Difference Extinction Coefficients of the Individual Cytochromes**—To estimate difference extinction coefficients of the cytochromes, the fully reduced minus oxidized spectra of different preparations of potato bc₁ complex were fit with a linear combination of the three resolved spectra to determine the contribution of each cytochrome to the observed spectrum. The results, together with the heme content from pyridine hemochrome spectra, were used to normalize the cytochrome spectra assuming the protoheme was derived from equal amounts of b-560 and b-566.

The proportion of cytochromes c₁ and b-560 were fairly constant in the different preparations, as was the ratio of cytochrome spectrum to heme content. The average of the normalization constants from 11 difference spectra, involving 7 different preparations, were used to normalize the spectra of these two cytochromes. The standard deviation of the normalization factors calculated from the 11 spectra was 6% for these 2 cytochromes.

On the other hand, the proportion of b-566 to cytochrome c₁ or to heme b was quite variable. This seemed to be due to incomplete reduction of b-566 in some of the spectra, so instead of averaging the normalization constants obtained from all the different spectra, the average of four giving the largest b-566 absorbance change was used.

These normalization constants allowed assignment of an
extinction coefficient scale to the resolved difference spectra. Difference extinction coefficients at wavelength pairs useful for quantifying the individual cytochrome changes are given in Table 4, part D.

Cytochrome c1.—The spectrum of cytochrome c1 is quite similar to that of cytochrome c1 in other bc complexes, including the prominent shoulder on the β-band at 531 nm.

The α-band maximum is at 552.4 nm, a shorter wavelength than we find for beef c1 by 0.7 nm. The difference extinction coefficient at 552.4–540 nm is 25.2, somewhat larger than the corresponding value for isolated beef heart cytochrome c1 (21.0, Ref. 62). However we find almost the same value in beef (25.0), suggesting that this is probably not a species difference. Perhaps the cytochrome c1 α-band becomes broader and less intense when this cytochrome is split from the complex.

Cytochrome b-566—Cytochrome b-566 has a split α-band, with maxima at 557.6 and 565.6 nm. This is quite similar to the corresponding cytochrome of animals or bacteria. The extinction coefficients for this cytochrome are less certain than those for cytochromes c1 and b-560, due to the above-mentioned difficulty in obtaining full reduction.

Cytochrome b-560—The spectrum of cytochrome b-560 is qualitatively similar to that of the corresponding cytochrome in beef heart or bacteria. However the α-band is broader (7.9 nm between inflection points) than in the beef enzyme (6.6 nm from preliminary results), and the α peak is markedly less intense, being only slightly higher than that of cytochrome c1. With the beef heart complex, we find an extinction coefficient (from the α peak to 577 nm) 38% greater than reported here for potato. Presumably it is this difference that results in the dramatic difference in the absolute spectrum of the reduced complex, with α absorption maximum at 557 in the potato complex instead of at the maximum of the high potential cytochrome b as in beef. Apparently the α peaks of cytochrome c1 (552 nm), b-560, and b-566 form a broad plateau on which the 557.6-nm shoulder of b-566 is supported to give the absorption maximum.

Antimycin causes a red shift in the spectrum of reduced cytochrome b-562 in the bc complex of beef heart (Ref. 52,

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**Fig. 6.** Difference spectra of the cytochromes of the potato bc complex. A, B, and C show differences between spectra obtained during a nonpotentiometric titration of the potato bc complex. The complex was diluted to 2.6 μM in 50 mM KP, 100 mM NaCl, pH 7.5, and fully oxidized with a trace of potassium ferricyanide. After the fully oxidized spectrum was taken, duroquinone and menadione (20 μM each) were added, and the complex was sequentially reduced with small aliquots of unsubstituted, methyl-, 2,6-dimethyl-, and trimethylhydroquinone and dithionite, taking spectra after each addition. The spectra were divided into three groups covering the reduction of cytochrome c1, b-560, and b-566. One spectrum in each group was selected as a base line and subtracted from the whole group to demonstrate changes taking place within that group. A, reduction of cytochrome c1. The base line is the fully oxidized spectrum. B, reduction of b-560. C, reduction of b-566.

**Fig. 7.** Potentiometric titration of potato bc complex. Potato bc complex was diluted to approximately 0.95 μM in 50 mM KP, 100 mM NaCl, pH 7.5. Mediators and redox buffers were 20 μM each of PMS, duroquinone, menadione, and 2-hydroxy-1,4-napthoquinone and 0.4 μM resorufin added at the beginning and 4 μM each of TMPD and pyocyanin added at different points in the reductive titration. The redox potential was varied with ferricyanide, hydroquinones, NADH, and dithionite and was recorded as described under "Experimental Procedures." Spectra were recorded at different potentials and analyzed by generalized matrix inversion for content of TMPD, pyocyanine, hydroxynaphthoquinone, resorufin, and each of the three derived cytochrome difference spectra. The cytochrome content was fit to a sum of Nernstian terms (lines drawn through the points) using the midpoint potentials 249, 48, and −78 mV versus the normal hydrogen electrode (indicated by vertical dotted lines). A, Δ, cytochrome c1; ■, □, b-560; and △, ▽, b-566. Open symbols are points obtained in a reductive titration, and closed symbols were obtained while re-oxidizing the same sample.

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3 E. A. Berry, and L-S. Huang, unpublished results.
and references therein). Such an effect also occurs in the potato complex (not shown), but the magnitude of the shift is smaller. The amplitude of the antimycin difference spectrum (positive peak versus negative peak; nm"cm") is 2.8 for the a-band and 18 in the Soret region, as compared with about 9.5 and 39 for the beef complex. Adding the antimycin difference spectrum to the resolved cytochrome b-560 difference spectrum resulted in a shift of about 0.3 nm; for the beef complex this gave about 1.0 nm. The small size of the shift in the potato complex may be related to the low ubiquinone content. In yeast mutants deficient in ubiquinone, no antimycin induced red shift was observed (63).

**Total Cytochrome b**—Because of the common use of measuring “total cytochrome b” at a single wavelength pair (62, 64, 65), the mean extinction coefficients for b-560 and b-566 are also given in Table 4. It must be emphasized, however, that these are applicable only when equimolar amounts of the two cytochromes contribute to the difference spectrum, and they do not take into account other cytochromes present in plant mitochondria (b-556 and “dithionite-reducible b” (66)).

Differences in the absorption band shape of b cytochromes from different species have been noted by Degli Esposti and co-workers (9, 48), and they have questioned the use of extinction coefficients derived from the beef heart complex for b cytochromes from other sources (48). We find an extinction coefficient at 562-577 nm of 23.5 for potato, versus 29.5 for beef. Although the latter value is within error of the literature values from pyridine hemochrome measurement (28.5 (Ref. 67), 28.0 (Ref. 68)), antimycin binding gives a lower extinction coefficient of 25.6 for beef (68). The difference between the values for potato and beef thus are not much greater than the uncertainty in the extinction coefficient for beef.

**Useful Wavelength Pairs—** Redox changes of cytochrome b-560 can be monitored with little interference from cytochrome c1 at 560-540 nm and with little interference from b-566 at 560-570 nm. At 560-577 nm b-560 contributes 68%, and b-566 32%, of the total change due to b cytochrome. Cytochrome b-566 can be monitored at 566-553 nm and c1 at 552-530 nm with little interference from cytochrome b-560. Extinction coefficients for use at these wavelength pairs are given in Table 4.

**Midpoint Potentials of the Cytochromes of the Potato bc1 Complex—** Fig. 7 shows results of a potentiometric titration. The experimental spectra at each potential were analyzed by generalized matrix inversion in the region 500-600 nm using the derived potato cytochrome difference spectra and difference spectra of TMPD, pyocyanine, and hydroxynaphthoquinone. The amount of each potato cytochrome reduced is plotted against redox potential, and best-fitting Nernstian curves are drawn through the points. In addition to the titration of Fig. 7, five other potentiometric titrations were performed. At pH 7.5 the midpoint potentials of cytochrome c1, b-560, and b-566 were 257 ± 4 mV, 51 ± 7 mV, and −77 ± 11 mV (mean ± S.D.), respectively.

In whole mitochondria at pH 7.2, the midpoint potential of cytochrome c1 is 235 mV (69), that of b-560 is 42 (69) or 40-80 mV (68), and that of b-566 is −77 (69) or −75 (66) mV. The midpoint potentials of both b cytochromes in the animal complex become more negative with increasing pH in the range pH 7-8, by less than 60 mV/pH unit (70). Thus to adjust our values to pH 7.2, we may need to add something less than 18 mV to the potentials of the b cytochromes, perhaps 10-15 mV. With this adjustment the potentials of all three cytochromes are slightly more positive than in mitochondria, by 15-25 mV. This may be simply an electrostatic effect due to replacing the negatively charged membrane with neutral detergent. There is no evidence for a drastic change such as reported for the b cytochromes of beef heart mitochondrial succinate cytochrome c reductase on delipidation by repeated ammonium sulfate precipitation in the presence of cholate (71). In those experiments the midpoint potentials became more negative, by 150 mV for b-562 and 60 mV for b-566, and the spectrum of b-566 was made indistinguishable from that of b-562. For the cholate solubilized complex from Jerusalem artichoke mitochondria, midpoint potentials at pH 7.0 of 240, 100, and −25 mV were reported for cytochromes c1, b-560, and b-566, respectively (9).

It has often been reported that b-560 (or b-562) in isolated bc1 complexes or in whole membranes titrates as two Nernstian components, one with a midpoint potential around 50 mV and the other around 150 mV (70, 72-74). In each of our titrations the potato b-560 could be fit with a single component, although parallel titrations of the beef heart complex isolated by the same procedure revealed two components of b-562. The high potential component was eliminated in the presence of antimycin, as reported by Rich and co-workers (70).

**Heterogeneity in the titration of the high-potential cytochrome b** has been attributed to redox interaction between this b heme and the quinone at center i (70, 75). From such models it would be predicted that the heterogeneity would disappear if there is no quinone at the center i site. As the isolated potato complex has only 0.15-0.25 mol of ubiquinone/complex, this site must be largely unoccupied. On the other hand, no high-potential component of b-560 has been reported in whole mitochondria from plants. However this may be because it is masked by (or contributes to) the b-566 component detected in potentiometric titrations.

**EPR Spectra of the Rieske Iron Sulfur Center**

Fig. 8 shows EPR spectra of the purified potato bc1 complex under conditions that allow measuring the signal of Rieske iron sulfur proteins. For spectrum 1, the complex was reduced with hydroquinone, which reduces the iron sulfur protein and most of cytochrome c1, but doesn’t reduce ubiquinol significantly. For spectrum 2, the complex was fully reduced by dithionite. Both traces show the rhombic signal of the Rieske

![Fig. 8. EPR spectra of the Rieske iron sulfur center of the potato bc1 complex. Two portions were taken from a solution of potato bc1 complex, 40 µM in cytochrome c1, in 50 mM KP, pH 7.4, 0.5 mM EDTA, and 50% (v/v) glycerol. One was reduced with a small amount of solid hydroquinone (1) and the other with sodium dithionite (2). EPR spectra were recorded at 15 K and 9.2 GHz with 10 milliwatt power, 10 G modulation amplitude, 100 kHz modulation frequency, and at 16,000 gain.](image-url)
iron sulfur protein. In the hydroquinone-reduced sample, the $g$ values are 1.76, 1.90, and 2.03 for $g(x)$, $g(y)$, and $g(z)$. These signals are essentially the same in the two spectra.

In bc, complexes from other sources (5, 76, 77) the shape of the reduced Rieske signal is dependent on the redox potential. The $g(x)$ signal is sharper and shifted to higher $g$ values at potentials such that ubiquinone is still oxidized, as compared with dithionite reduced. This has been attributed to an effect of oxidized ubiquinone (5) on the lineshape of the iron sulfur protein. The absence of this effect with the potato complex (Fig. 8) may be due to the low quinone content of the preparation. The line width and position of the $g(x)$ signal in either the dithionite or hydroquinone reduced sample is comparable with that in other complexes when the ubiquinone is reduced or displaced by inhibitors.

Another small signal is seen at 2.07 in the hydroquinone reduced sample, but not in the dithionite-reduced sample. This signal has not yet been assigned, but may be the $g(y)$ signal of a cytochrome. The simplicity of the dithionite-reduced spectrum attests to the absence of the succinate and NADH dehydrogenases, as these enzymes have multiple iron-sulfur signals that would be observed under these conditions.

**Electron Pathways through the bc, Complex**

Observations of a number of anomalous reactions of the mitochondrial bc, complex have led to formulation of the Q-cycle mechanism for electron transport and proton translocation by the complex (12). Similar phenomena were observed in the complex from *Paracoccus denitrificans* (60), so apparently a similar mechanism operates in bacteria. One of these phenomena is the oxidant-induced extra-reduction of $b$ cytochromes in the presence of antimycin. Degli Esposti et al. (9) demonstrated that this phenomenon occurs also in the plant complex, implying a mechanism similar to that of animals and bacteria. In Fig. 9 we further extend these observations with the purified plant complex.

Fig. 9A demonstrates the two pathways for reduction of the $b$ cytochromes by quinol, and the redundant controlled reduction of cytochrome $b$ in the presence of antimycin. Menadion was used as the reductant, as it has a midpoint potential more negative than ubiquinol ($E_{m,n} = -5$ mV, (78)) and can reduce most of $b$-560. As seen in trace 1, cytochrome $b$ reduction by menadion occurs very rapidly, probably within the mixing time, and is not resolved here. This reaction does not occur in the presence of antimycin plus myxothiazol (trace 4). It does occur in the presence of myxothiazol (trace 2) or antimycin (trace 3) alone, although it is somewhat slowed in the latter case.

According to the Q-cycle hypothesis, there are two pathways by which quinols can reduce the $b$ cytochromes, and the quinol binding sites involved are labeled center o and center i. Myxothiazol blocks the reaction at center o, and antimycin blocks that at center i. As long as either of these pathways is available, cytochrome $b$ can be reduced, but blocking both prevents cytochrome $b$ reduction.

In parallel experiments with the beef heart complex, the reaction was greatly slowed by the combination of the two inhibitors, but significant reduction occurred in the 120 s allowed. It may be that the $b$ hemes in the potato complex are better insulated against nonenzymatic reduction by aqueous reductants than those in the beef complex. Indeed even reduction by dithionite is greatly inhibited by the combination of inhibitors (trace 4) and does not reach completion by the end of the trace.

For trace 5, antimycin was present and ascorbate was added to pre-reduce cytochrome $c_1$ and the iron sulfur protein. A spectrum taken just before starting the recording confirmed that cytochrome $c_1$ was reduced and the $b$ cytochromes were oxidized. Again no reduction of cytochrome $b$ by menadion was observed.

Fig. 9B explores the oxidant-induced reduction phenomenon. When ubiquinol at site "o" is oxidized, one electron reduces the $b$ cytochromes and the other reduces the iron sulfur protein. Thus reduced iron sulfur protein is a product of the reaction that reduces the $b$ cytochromes, and oxidized iron sulfur protein is a reactant. Having the iron sulfur protein highly oxidized pulls the reaction forward, causing reduction of cytochromes $b$. Having the iron sulfur highly reduced prevents the reaction from going forward, preventing cytochrome $b$ reduction (trace 5 of Fig. 9A). Because the iron sulfur protein is not readily monitored at visible wavelengths, we have followed cytochrome $c_1$, which is in rapid equilibrium with it (79).

The redox state of cytochrome $c_1$ was monitored at the wavelength pair 552.5-530 nm (trace 1), and 560-540 nm (truces 2-4). Traces 1 and 2 of Fig. 9A demonstrate the oxidant-induced reduction. The reduced ubiquinone analog UBH is added at the indicated time, and it reduces cytochrome $c_1$ and part of $b$-560. Adding antimycin has no effect or causes a slight re-oxidation of $b$-560 (trace 2), perhaps due to an effect on the midpoint potential of this cytochrome.

Now adding ferricyanide causes transient oxidation of cytochrome $c_1$ and reduction of the $b$ cytochromes. This state is transient, because the non-enzymatic reduction of ferricyanide by UBH quickly exhausts the ferricyanide, at which point $c_1$ starts to be re-reduced and the $b$ cytochromes return to their redox state before ferricyanide. Finally addition of dithionite causes slow reduction of all the cytochromes, providing the 100% reduced reference level.

For trace 3, the antimycin addition was omitted. Ferricyanide addition causes slight oxidation of cytochrome $b$-560, instead of reduction. According to the Q-cycle model, this is because cytochromes $b$ can be oxidized by a quinone at the center i site as rapidly as they are reduced by quinol at the center o site. Antimycin prevents this re-oxidation, thus allowing the extra reduction to be expressed.

For trace 4, myxothiazol was added before the recording was started. The ferricyanide-induced reduction of $b$ cytochromes is almost completely abolished. This is because myxothiazol prevents reduction of cytochrome $b$ by the quinol at the center o site.

**Discussion**

**Purification Procedure—Potato bc, complex, like that from other sources, has a very high affinity for anion exchange resins. This allows it to be isolated by applying a crude extract to a column at ionic strength so high that few other proteins are adsorbed. Cytochrome oxidase is also partly retained under these conditions, even though part of the oxidase is unadsorbed even at lower ionic strength (not shown). Cytochrome oxidase represents the main contamination present in the bc, complex isolated by this method, but can be reduced to undetectable levels by careful choice of the fractions to be pooled.**

Both bc, and cytochrome oxidase (aox) from a wide phylogenetic range of sources have high affinity for anion exchange columns. This may be due to a concentration of negative charges required for interaction with the lysine residues surrounding the heme cleft of cytochrome c. Although the isoelectric point of cytochromes c of Ambler's class I varies from strongly basic to acidic, they all have a concentration of positive charge around the heme cleft (80). In the bc, complex,
cytochrome c1 and the 9.2 kDa "hinge protein" are involved in binding cytochrome c (81). Purified cytochrome c1 also has high affinity for anion exchange columns, on which it is often purified (Ref. 82 and references therein). It seems likely that cytochrome c1 is mainly responsible for the affinity of the bc1 complex for ion exchange columns.

Cytochrome oxidase eluted in two main peaks in most experiments, part in the flow-through and part later in the wash. The reason for this is not clear. Spectra show cytochrome c and the domain which has high affinity for the anion exchanger. Wash. The reason for this is not clear. Spectra show cytochrome c1 is mainly responsible for the affinity of the bc1 complex for ion exchange columns.

Cytochrome oxidase eluted in two main peaks in most experiments, part in the flow-through and part later in the wash. The reason for this is not clear. Spectra show cytochrome c1 is mainly responsible for the affinity of the bc1 complex for ion exchange columns.

FIG. 9. Reactions indicative of a "Q-cycle" mechanism. Potato bc1 complex was diluted to 0.5 μM in 50 mM KP, 0.5 mM EDTA, pH 7.4. Portions were placed in a stirred cuvette in a dual wavelength spectrophotometer, and absorbance changes accompanying additions were recorded at wavelength pairs selective for cytochrome c1 or b-560. A, dual sites for cytochrome b reduction and reductant-controlled reduction of cytochrome b. Potato bc1 complex was diluted as above, and in some cases a trace of K3Fe(CN)6 was added to completely oxidize cytochrome c1. Where indicated 51 μM menadion or a few grains of dithionite were added. Reduction of b cytochromes was monitored at 560-540 nm. Trace 1 is the control. For trace 2, 3.1 μM myxothiazol was present. For trace 3, 3.1 μM antimycin was present, and in trace 4 antimycin and myxothiazol were both present at 3.1 μM. For trace 5, 3.1 μM antimycin and 300 μM ascorbate were present, and complete reduction of cytochrome c was monitored at 552.5-530 nm before starting the recording. B, oxidant-induced reduction of cytochrome b. The complex was diluted as above and sequentially treated with 25 μM UBH (a ubiquinol analog), 3.1 μM antimycin, 11.2 μM K3Fe(CN)6, and dithionite. Trace 1 was monitored at 552.5-530 nm for cytochrome c1, and traces 2-4 at 560-540 nm for b-560. For trace 3, antimycin was omitted. In trace 4, myxothiazol was added (6 μM) before the experiment was started.

Yang and Trumpower (16) found that incubating at higher salt before applying to the DEAE-cellulose column improved separation of oxidase and bc1 from Paracoccus. This also seemed to be the case with the potato complex. Using DEAE-Sephrose instead of DEAE-cellulose allowed retention of the bc1 complex at higher salt concentrations. The concentration used here (260 mM NaCl) is the highest that could be used with good retention of the bc1 complex.

Subunit Composition—As reported previously by Nakajima et al. (8) for sweet potato bc1 complex, the subunit composition of the plant bc1 complex is similar to animal mitochondrial bc1 complexes. In addition to the three subunits bearing redox centers, there are two or three higher molecular mass (core) proteins and at least four small subunits. This is in contrast to bacterial complexes, which have only the three subunits bearing redox centers (16, 17) or perhaps one additional subunit (18, 85).

One significant difference in subunit composition between plant and animal bc1 complexes is the appearance of three core subunits in potato and beet. We have not yet ruled out the obvious possibility that this is due to partial proteolysis of one of the subunits, but preliminary experiments make this unlikely. The proportions of the three subunits are relatively constant from preparation to preparation. If one of the subunits was formed by proteolysis of another, the extent of this proteolysis would be expected to vary somewhat.

To minimize proteolysis, we routinely include PMSF (an inhibitor of serine proteases) and EDTA (which inhibits metal-requiring proteases) in the buffers for preparation of mitochondria and PMSF in the solubilization buffers. For one mitochondrial preparation, we used four times the concentration of PMSF, and also included E-64 and iodoacetamide (inhibitors of sulfhydryl proteases). In the extraction of these mitochondria, the PMSF concentration was doubled and E-64 was included. PMSF and E-64 were also included in the column buffers. These precautions had no effect on the mobility or proportions of the three core subunits of the purified bc1 complex as visualized on SDS gels. Thus we suspect proteolysis is not responsible for the appearance of the three bands.

If the three bands actually represent three different pep-
tides present in the potato and beet bc complex, it still remains to be decided whether each molecule of complex contains three core peptides or each contains two peptides. Considering first the possibility of three core proteins in each complex, it has been suggested that the beef heart enzyme contains two copies of the smaller core protein (86). The data on which this was based, however, have been explained (87) as due to use of a modified Lowry protein assay to quantitate subunits and the greater content of tyrosine in the second core protein. Schagger et al. (49) state that all the subunits of the beef heart complex are present in equimolar ratio.

The heme content supports heterogeneity of one polypeptide from complexes because, as was explained under heme content, there is not enough protein for each of the 10 subunits observed to be present in a stoichiometry of 1 per heme c. Heterogeneity has been reported for core protein 2 of yeast bc, complex (88). Heterogeneity could result from heterozygosity, from tissue or developmental stage specificity, or from heterogeneity of post-translational processing.

Cultivated potato varieties are tetraploid and highly heterozygous (89). Monoploid and homozygous diploid potato varieties have been constructed (90), and isolation of the complex from one of these strains should tell whether heterozygosity is responsible. In our usual mitochondrial preparation the entire tuber is extracted, so more than one tissue is involved. However one preparation from peeled potatoes gave the same polypeptide banding pattern. No difference in the banding pattern was observed between complex from Russet Burbank potatoes and supermarket potatoes, but it would not be surprising if the supermarket potatoes were also Russet Burbank potatoes as this variety is the major commercial russet potato.

If two of the core subunits are different allelic or tissue specific forms of the same protein, it seems likely that subunits 1 and 2 are the pair involved. Subunits 1 and 2 appear slightly less intense than subunit 3 on gels. Subunit 3 seems quite different from subunits 1 and 2 in hydrodynamic properties and in allergenicity. While subunits 1 and 2 give nearly parallel lines on the Ferguson plot, subunit 3 is less sensitive to gel concentration than subunits 1 and 2. We have raised antibodies against subunit 3 in two rabbits, one injected with whole bc, complex and the other with a mixture of the first three subunits. In neither case was an antibody against subunit 1 or 2 produced. If subunit 1 or 2 was an iso-form of subunit 3, it might be expected to cross-react with subunit 3 antibodies. The antibodies did cross-react with the third peptide of the beef bc, and the second band (possibly two unresolved peptides) of the sweet potato complex. They do not react with any beef bc, polypeptides. Interestingly, they do react with subunit 3 of the bc, complex from the trypanosome Leishmania tarentolae, which also has three bands in the core protein region. 4

Lipid and Quinone Dependence — The complex isolated from potato as described here is highly delipidated and depleted of ubiquinone, with 1–5 phospholipid molecules and 0.15–0.25 ubiquinone molecules/molecule of complex. The beef bc, complex isolated by the same procedure (one trial) or a similar procedure but with a lower detergent:protein ratio in the extraction and lower salt concentration on the column (two experiments) had 1.8–2.2 nmol ubiquinone and 30–50 mol of phospholipid/mol of cytochrome c. The complexes from P. denitrificans, Rhodobacter sphaeroides, and Rhodospirillum rubrum, isolated by procedures similar to that used here, contained 18 mol of phospholipid and 3.5 mol of ubiquinone (16), only 1 mol of phospholipid but 4 mol of ubiquinone (18), or 1.1 mol of ubiquinone (phospholipid not reported) (17)/mol of cytochrome c. This suggests that ubiquinone is bound less tightly by the potato than beef or bacterial complex.

It has been reported that the beef or yeast bc, complex is inactivated by ubiquinone depletion and that activity can be restored by incubation with ubiquinone and phospholipids in the right order under appropriate conditions (50, 91). This has led to the suggestion (91) that short chain ubiquinone analogs cannot be oxidized directly by the bc complex but act through endogenous ubiquinone. Lipid and ubiquinone were depleted by repeated ammonium sulfate precipitation in the presence of bile salts, and ubiquinone was depleted without removing phospholipids by hexanone extraction (91). In either case, activity could not be restored by adding back ubiquinone alone, but required phospholipids as well.

On the other hand, yeast mitochondria from strains that cannot produce ubiquinone have ubiquinonone:cytochrome c oxidoreductase activity immediately upon adding the ubiquinone analog as substrate (92). Although the activity was low compared with that of complexes with typical lipids, the turnover number was similarly reduced. This was attributed to incomplete catabolite derepression (92) and not to a reduced turnover number of the bc, complex. Another study using ubiquinone-depleted beef heart bc, complex also concluded that endogenous ubiquinone was not required for activity with short chain analogs (93). The bc, complex isolated by hydroxyapatite chromatography in Triton X-100 has no detectable ubiquinone but is active with short-chain ubiquinol analogs (13).

We have found a turnover number of 172 s⁻¹, approaching that in whole mitochondria, with a potato bc₁ preparation having less than 0.2 ubiquinone molecules/complex. There seemed to be no correlation between the amount of endogenous ubiquinone and activity. This supports the conclusion that ubiquinol is not required to mediate between short-chain quinols used as substrate and the bc, complex.

General Observations — On the whole, the potato bc, complex seems quite similar to that of vertebrates. The differences that were noted seem mainly to involve cytochrome b-560 and the center i quinone binding site. The spectrum of reduced b-560 is broader, and the extinction coefficient at the peak lower, than in the beef heart enzyme. The antimycin-induced red shift is significantly smaller, the binding of fusicoccin is weaker, and the extent of inhibition by diuron lower than in the beef heart complex. Potentiometric titrations give no indication of the high-potential component of cytochrome b-560 (b-562) that has been attributed to an interaction between b-560 and the quinone at center i. The affinity of this site for ubiquinone seems lower, as the complex is depleted of ubiquinone by the isolation procedure.

Acknowledgments — We wish to thank Dr. R. M. Glaser for support and encouragement and Dr. A. J. Bearden for making his spectrophotometer available to us. Dr. Gordon Parry of Lawrence Berkeley Laboratory helped with the preparation of antibodies. Dr. Herman Timm of the vegetable crops department, University of California at Davis, and Dr. Harry Carlson of the Tulelake experiment station generously provided potatoes of defined variety for isolation of the bc, complex.

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Cytochrome b, Complex of Potato Mitochondria

**Supplemental Material to Ubiq~in~l-Cyt~~hr~me of Higher Plants: Isolation and Characterization of the bc, Complex from Potato Tuber Mitochondria**

Edward A. Berry, L. U. Hwang, and W. Van Volkenburg

### Table 1. Recovery of Protein, Activity, and Homogeneity at Different Stages in the Purification of the bc, Complex

| Stage                          | Protein (mg) | Activity (nmol/min/mg) | Homogeneity |
|-------------------------------|--------------|------------------------|-------------|
| Step 1                        | 250          | 7.1                    | 0.8         |
| Step 2                        | 180          | 42.9                   | 0.85        |
| Step 3                        | 100          | 172                    | 0.95        |
| Step 4                        | 50           | 411                    | 0.98        |

### Table 2. Molecular Mass of the bc, Complex Estimated by SDS Gel Electrophoresis

| Protein | Molecular Mass (kDa) |
|---------|----------------------|
| Complex | 150                  |

### Appendix

**Procedure for resolving the differences of the individual cytochromes**

**Outline of procedure**

To resolve the differences of the cytochromes, extraction-reduction-elution technique was used. In order to use this technique to extract cytochrome the solution was added to dry cells, and the classes of cytochromes were resolved in a single step. The differences of the cytochromes are then characterized by the difference spectrum of each cytochrome, which is the difference spectrum of each cytochrome after their respective differences have been measured.

The crude extract was mixed with a buffer containing 5% trichloroacetic acid, 0.2% mercaptoethanol, and 2% sodium dodecyl sulfate (SDS). The extract was then centrifuged at 100,000 x g for 30 min to remove any insoluble material. The supernatant was then applied to a column of Sepharose 6B equilibrated with the same buffer, and the column was washed with a buffer containing 5% trichloroacetic acid, 0.2% mercaptoethanol, and 2% SDS. The cytochrome c fractions were then recovered by elution with a buffer containing 5% trichloroacetic acid, 0.2% mercaptoethanol, and 2% SDS.

**Curve-fitting of the difference spectrum**

The difference spectrum of each cytochrome was analyzed by a program which uses the least squares method to minimize the number of terms in a series of natural terms.

Where $A_{x}$ is the value of the $x$ th component and $B_{x}$ is the value of the $x$ th component. The curve can be obtained by ordinary integration of the above terms.

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