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# Purification of highly active cytochrome $bc_1$ complexes from phylogenetically diverse species by a single chromatographic procedure

Per O. Ljungdahl [a], Jeffrey D. Pennoyer [a], Dan E. Robertson [b] and Bernard L. Trumpower [a]

[a] *Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03756, and* [b] *Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104 (U.S.A.)*

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**A method has been developed for purification of highly active ubiquinol-cytochrome *c* oxidoreductase (cytochrome $bc_1$) complexes from wild-type *Rhodobacter sphaeroides, Rhodobacter capsulatus* MT1131, bovine heart and yeast mitochondria. This is the first report of the isolation of cytochrome $bc_1$ complex from a wild-type strain of *Rb. sphaeroides* and from any strain of *Rb. capsulatus*. The purification involves extraction of membranes with dodecyl maltoside and two successive DEAE column chromatography steps. All of the resulting $bc_1$ complexes are free of succinate dehydrogenase and cytochrome *c* oxidase activities. The purified $bc_1$ complexes from both photosynthetic bacteria contain four polypeptide subunits, although the molecular weights of some of their subunits differ. They are also free of reaction center and light-harvesting pigments and polypeptides. The turnover number of the *Rb. sphaeroides* complex is 128 $s^{-1}$, and that of the *Rb. capsulatus* complex is 64 $s^{-1}$. The $bc_1$ complex from bovine heart contains eight polypeptides and has a turnover number of 1152 $s^{-1}$, while the yeast complex contains nine polypeptides and has a turnover number of 219 $s^{-1}$. The activities of these complexes are equal to or better than those commonly obtained by previously reported methods. This method of purification is relatively simple, reproducible, and yields cytochrome $bc_1$ complexes which largely retain the turnover number of the starting material and are pure on the basis of optical spectra, enzymatic activities and polypeptide composition. The purification of cytochrome $bc_1$ complexes from energy-transducing membranes which differ markedly in their lipid and protein composition makes it likely that with minor modifications this method could be applied to species other than those described here.**

## Introduction

Ubiquinol-cytochrome *c* oxidoreductase (cytochrome $bc_1$) complexes are constituents of energy-transducing systems in both photosynthetic and non-photosynthetic organisms, and are the most nearly universal of the electron-transfer complexes participating in energy transduction. This enzyme complex has been isolated from bovine heart [1–5], and yeast mitochondria [6,7], mutant strains of *Rhodobacter sphaeroides* [8–10], *Paracoccus denitrificans* [11], as well as several other sources (see Ref. 12 for a review). All of the cytochrome $bc_1$ complexes contain four prosthetic groups: one 2 Fe-2 S cluster, one *c*-type and two *b*-type hemes, which participate in transfer of elec-

Correspondence: B.L. Trumpower, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03756, U.S.A.

trons from dihydroquinol to cytochrome *c*. Available evidence indicates that electron flow through the $bc_1$ complex occurs by a protonmotive Q-cycle pathway [13].

The majority of published procedures for purification of cytochrome $bc_1$ complexes utilize bile salts for extraction of the complexes from their membrane environment and subsequent salt precipitation steps. These manipulations are frequently difficult to reproduce, and optimum conditions for each species and each membrane preparation must be established. This variability is probably partly due to differing membrane-lipid compositions. Even within one species, the optimum detergent and salt parameters for purification may vary as the diet of the animal or growth medium of the microorganism is changed. In addition, the bile salts frequently used to extract mitochondrial cytochrome $bc_1$ complexes are ineffective at extracting bacterial membranes [11]. More importantly, these procedures often yield preparations with low enzymatic activity. Consequently, it is difficult to assess species-specific properties, such as subunit composition and optical properties of the cytochromes, when an uncertainty exists as to whether the low activity may result from partial denaturation of the complex during purification.

Cytochrome $bc_1$ complexes have also been purified by extraction with Triton X-100 followed by chromatography [4,5,14]. Though easily reproduced, these preparations have low enzymatic activity due to the deleterious effects of Triton X-100. One well-documented effect of this detergent is to destabilize the structure of the cytochrome $bc_1$ complex, resulting in the dissociation of the iron-sulfur protein [15–17].

The synthetic nonionic detergent dodecyl maltoside has proven to be an effective dispersing agent of a multitude of energy-transducing membranes, and seems to be well suited for extracting and fractionating a variety of integral membrane protein complexes [18,19]. Dodecyl maltoside has been used to isolate highly active and stable cytochrome *c* oxidase [20], rhodopsin [21], and photoreaction center [22].

In a recent study, dodecyl maltoside and various maltosides with alkyl chains of differing lengths were compared to other homogeneous nonionic and zwitterionic detergents in terms of their effects on the activity of cytochrome *c* oxidase [23]. The alkyl maltoside detergents supported the highest enzymatic activities, and dodecyl maltoside was nearly optimal in terms of alkyl chain length. It was also noted that commercial preparations of Triton X-100 and poly(oxyethylene)alkyl ethers are heterogeneous mixtures, contain numerous unidentified impurities, and spontaneously form peroxides and free radicals. The enzymatic activity of cytochrome *c* oxidase in Triton X-100 was only 1% of that in dodecyl maltoside [23].

Cytochrome *c* oxidase purified in the presence of dodecyl maltoside has an extremely low phospholipid content, while maintaining high enzymatic activity [20]. Highly active cytochrome $bc_1$ and cytochrome oxidase complexes are monomeric in the presence of dodecyl maltoside [24–26], even though the complexes were initially purified in other detergents, whereas these complexes dispersed in Triton X-100 are consistently dimeric [27,28]. Since the formation of dimeric aggregates is detergent dependent, the inferred existence of active dimers within energy-transducing membranes seems questionable, as the physical properties of the complexes may be an artifact of the isolation procedure.

We have developed a method which uses extraction of membranes with dodecyl maltoside and anion-exchange chromatography in this same detergent to purify highly active cytochrome $bc_1$ complexes from phylogenetically diverse organisms. This is the first report of the purification of cytochrome $bc_1$ complexes from phototrophically grown wild-type *Rb. sphaeroides* and microaerophilically grown *Rb. capsulatus*. Previously, the cytochrome $bc_1$ complex from photosynthetic bacteria has been isolated only from mutant strains of *Rb. sphaeroides* [8–10], owing to the difficulty of removing the extensive quantities of bacteriochlorophyll and carotenoids present in wild-type strains, and those preparations exhibit low enzymatic activity. We have also purified cytochrome $bc_1$ complexes from yeast and bovine heart mitochondria with enzymatic activities that are equal to or better than those obtained by previously reported methods.

## Experimental procedures

Serum albumin (essentially fatty acid free), horse heart cytochrome *c*, and DEAE-Sepharose CL-6B were obtained from Sigma. DEAE-BioGel A and sodium dodecyl sulfate were obtained from Biorad. Dodecyl maltoside was obtained from Boehringer Mannheim as a fine white powder. This detergent was initially purchased from Calbiochem; however, we switched sources when subsequent batches contained a yellow tinted impurity, possibly lauryl alcohol, which adversely affected enzymatic activity.

Protein determinations were made according to the method of Lowry as modified by Markwell et al. [29]. Concentrations of cytochrome *b* in membranes and in detergent solubilized membranes were determined by dithionite minus ascorbate-reduced difference spectra, using the coefficient $\varepsilon_{562-577} = 25.6\ \text{mM}^{-1} \cdot \text{cm}^{-1}$ [30]. After chromatography, which removes contaminating *c*-type cytochromes, the contents of cytochromes *b* and $c_1$ were determined spectrophotometrically from oxidation-reduction difference spectra as described by Vanneste [31]; [cytochrome $c_1$] = $(\Delta A_{553-540})5.365 \cdot 10^{-2} - (\Delta A_{562-577})9.564 \cdot 10^{-3}$ mM; [cytochrome *b*] = $(\Delta A_{562-577})3.539 \cdot 10^{-2} - (\Delta A_{553-540})1.713 \cdot 10^{-3}$ mM. Ubiquinol-cytochrome *c* oxidoreductase and cytochrome *c* oxidase activities were assayed as previously described [32].

Electrophoresis was carried out according to Laemmli [33], except that SDS was omitted from the gel and lower electrode buffer. Gels containing a 13–17% linear gradient of acrylamide, stabilized within a 3–12% sucrose gradient, were made from a stock acrylamide solution of 30% T, 2.7% C [33]. Gels were stained for heme according to Thompson et al. [34].

*Rb. sphaeroides* wild-type strain NCIB 8253 was grown phototrophically in the medium of Cohen-Bazire et al. [35] at 30 °C in sealed 1 l Rous bottles to mid-logarithmic growth phase. Light was provided by 75 W flood lamps at 1800 lx. French-pressure cell extracts were prepared from washed cells, centrifuged at 10000 × *g* for 10 min, and the supernatant centrifuged at 96000 × *g* for 4 h on linear 5–35% (wt/wt) sucrose gradients over a 60% (wt/wt) cushion [36]. The chromatophore fraction was removed with a Pasteur pipette, diluted with an equal volume of 1.0 mM Tris-HCl (pH 7.5), and sedimented at 368000 × *g* for 60 min. The pellet was suspended in 50 mM Tris-HCl (pH 8.0)/1 mM diisopropylfluorophosphate.

*Rb. sphaeroides* strain MT1131 [37] was grown under microaerophilic conditions at 2.5% $O_2$ in RCV medium according to Weaver et al. [38]. Chromatophore membranes were isolated as described above for *Rb. sphaeroides*.

Yeast submitochondrial particles were prepared from commercially available Red Star yeast or laboratory strain DC-5 (Mat $\alpha$, Leu 2-3, Leu 2-112, His 3, Can 1-11) according to Siedow et al. [7], with the following modifications. The concentration of phenylmethylsulfonyl fluoride was increased from 0.5 to 1 mM in all buffers. In addition, 0.5 mM diisopropylfluorophosphate and 4 mM potassium fluoride were added to the suspended cells just prior to cell disruption.

Bovine heart mitochondria were prepared according to Smith [39]. Isolated mitochondria and submitochondrial membranes were stored at a protein concentration of 60 mg/ml in 0.25 M sucrose/10 mM potassium phosphate (pH 7.4)/1 mM EDTA/1 mM phenylmethylsulfonylfluoride at −70 °C.

A flow scheme summarizing the purification procedures is shown in Fig. 1. All steps were performed at 4 °C. Membranes were suspended at 30 mg/ml protein in buffer A (50 mM Tris/HCl (pH 8; at 4 °C)/1 mM $MgSO_4$/1 mM phenylmethylsulfonylfluoride), and the pH of the suspension was adjusted to 8. The suspension was centrifuged at 100000 × *g* for 90 min, and the resulting pellet resuspended to a protein concentration of 10 mg/ml in buffer A containing 1 mM diisopropylfluorophosphate.

Suspended membranes were solubilized with dodecylmaltoside, using an amount of detergent determined to be optimal for each species as described below. In all cases the optimal solubilization conditions were established with regard to detergent/protein ratios vs. amount and stability of the solubilized ubiquinol-cytochrome *c* reductase activity. For the purifications described here the optimal ratio was 0.5–1.5 g of dodecyl maltoside per g of membrane protein. Appropriate volumes of a 100 mg/ml dodecyl maltoside stock

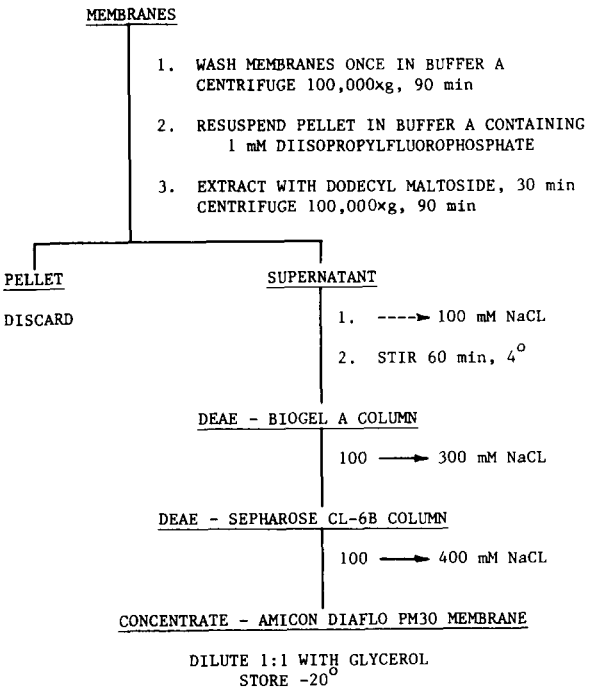


Fig. 1. Flow scheme for the isolation of ubiquinol-cytochrome *c* oxidoreductase.

solution were added to suspended membranes; the mixture was stirred for 30 min and centrifuged at $100\,000 \times g$ for 90 min. To the resulting supernatant a 4 M solution of NaCl was added to obtain a final concentration of 100 mM NaCl, and this mixture was stirred for 60 min.

The mixture was then applied to a DEAE-Bio-Gel A column equilibrated with buffer B (50 mM Tris-HCl (pH 8; at 4°C)/1 mM $MgSO_4$/0.1 mg per ml dodecyl maltoside) containing 100 mM NaCl. The concentration of dodecyl maltoside in buffer B, 0.1 mg/ml, is the critical micellar concentration for this detergent in distilled water. At the salt concentrations used, the critical micellar concentration is undoubtedly lower than this value. The dodecyl maltoside was added to prevent possible aggregation of solubilized protein. The column with adsorbed sample was washed with three column volumes of equilibration buffer, and then eluted with four column volumes of a linear 100–300 mM NaCl gradient in buffer B. Fractions were analyzed for absorption at 415 nm, and for ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase activities. Fractions containing maximal cytochrome *c* reductase activity and minimal cytochrome *c* oxidase activity, typically eluting between 210 and 290 mM NaCl, were pooled.

These pooled fractions were applied directly to a DEAE-Sepharose CL-6B column equilibrated as above. The column was washed with a half column volume of equilibration buffer, and eluted with a linear 100–400 mM NaCl gradient in buffer B. Pure $bc_1$ complex was eluted between 290 and 400 mM NaCl. These fractions were free of succinate dehydrogenase and cytochrome oxidase activities. Fractions containing pure $bc_1$ complex were concentrated on an Amicon Diaflo PM30 membrane and diluted with an equal volume of glycerol. Both DEAE columns were 15 cm in length. The width was varied with the amount of protein applied. The fractions collected were one-tenth the column volume.

As noted above, the optimal amounts of dodecyl maltoside used to extract the $bc_1$ complex were established for each species. Bovine heart mitochondria were extracted with 1.0 g dodecyl maltoside per g protein, and the cytochrome $bc_1$ complex was purified as described above.

Yeast submitochondrial membranes were initially depleted of cytochrome *c* [40]. This washing does not deplete the cytochrome *c* content entirely, but does remove significant amounts of extraneous protein. Submitochondrial membranes were extracted with 0.8 g dodecyl maltoside per g protein, and the cytochrome $bc_1$ complex was purified as above.

Chromatophore membranes from *Rb. sphaeroides* were extracted with 0.66 g dodecyl maltoside per g protein. This centrifugation yields a hard lower and a loose upper pellet, unique to these chromatophores. The upper pellet was removed along with the supernatant. This fraction, containing only 46% of the total ubiquinol-cytochrome *c* reductase activity, was brought to 100 mM in NaCl, stirred for 60 min, and centrifuged as before, resulting in a hard pellet. This supernatant contained about 30% of the total units.

Simultaneously, the hard lower portion of the pellet from the initial solubilization was resuspended to 10 mg/ml protein, reextracted with 0.5 g dodecyl maltoside per g protein and centrifuged

as above. The resulting pellet was discarded, while the supernatant was incubated with 100 mM NaCl, as above. This second supernatant contained about 20% of the total ubiquinol-cytochrome *c* reductase activity.

The cytochrome $bc_1$ complex in the combined supernatants, amounting to approx. 50% of the total cytochrome *c* reductase activity, was purified as described above except that the DEAE-BioGel A column was washed with ten column volumes, prior to starting the salt gradient, to remove contaminating pigments, which eluted slowly in the wash.

*Rb. capsulatus* chromatophore membranes were extracted with 1.5 g dodecyl maltoside per g protein, and the cytochrome $bc_1$ complex was purified according to the general procedure described above. As with *Rb. sphaeroides*, the DEAE-BioGel A column was washed with 10 column volumes to remove contaminating pigments.

## Results

Recoveries of activity, cytochromes, and protein during the purification of ubiquinol-cytochrome *c* oxidoreductase from bovine heart and yeast mitochondria are tabulated in Tables I and II, respectively. Representative column profiles for these purifications are shown in Fig. 2. In each case addition of dodecyl maltoside to the membranes caused a significant increase in ubiquinol-cytochrome *c* reductase activity. The degree of activation varied from nine-fold with bovine heart mitochondria to two-fold with yeast submitochondrial particles. Calculations of recovery of activity are based on these detergent dispersed membranes.

The increase in specific activity of the detergent extract, after correction for the initial activation, suggests that dodecyl maltoside selectively solubilized the cytochrome $bc_1$ complex from these membranes. There was also a selective solubilization of the cytochrome oxidase complex (results not shown). Incubation of the dodecyl maltoside extract with 100 mM NaCl prior to chromatography facilitated the subsequent separation of cytochrome oxidase from the cytochrome $bc_1$ complex.

With the $bc_1$ complex from both bovine and yeast, more than 80% of the applied protein washed through the first ion-exchange column (DEAE-BioGel A). With bovine heart this flow-through included the bulk of the cytochrome oxidase activity (Fig. 2). No measurable cytochrome *c* reductase activity was present in the column flow-through or subsequent 100 mM NaCl wash. In yeast, cytochrome oxidase eluted just prior to the peak of cytochrome *c* reductase activity. The cytochrome *c* reductase activity of bovine heart eluted btween 210 and 275 mM NaCl, while that of yeast eluted from 260 to 280 mM NaCl.

DEAE-Sepharose CL-6B proved to be an effective subsequent chromatographic matrix due to its greater binding capacity, allowing direct application of the fractions pooled from the initial column without dilution or dialysis. Contaminating proteins were removed prior to the application of the salt gradient in the column flow-through and wash. Pure $bc_1$ complex eluted between 290–360 mM NaCl for bovine heart and between 320–360 mM NaCl for yeast. In both cases the pure com-

TABLE I

PURIFICATION OF UBIQUINOL-CYTOCHROME *c* OXIDOREDUCTASE FROM BOVINE HEART

Cyt, cytochrome.

| Fraction | Protein (mg) | Activity (units) | Yield (%) | Purification (-fold) | Cyt $c_1$ (nmol) | Cyt *b* (nmol) | *Tn* ($s^{-1}$) |
|---|---|---|---|---|---|---|---|
| SMP | 2000 | 6450 | – | – | – | 1100 | 196 |
| SMP + DM | 2051 | 55303 | 100 | 1.0 | – | 1004 | 1843 |
| DM extract | 1536 | 49178 | 89 | 1.2 | – | 916 | 1790 |
| DEAE-BioGel A | 152 | 24800 | 45 | 6.0 | 315 | 709 | 1312 |
| DEAE-Sepharose 6B | 96 | 13150 | 24 | 5.1 | 240 | 525 | 913 |
| Conc. glycerol diluted | 92 | 16867 | 31 | 6.8 | 244 | 465 | 1152 |

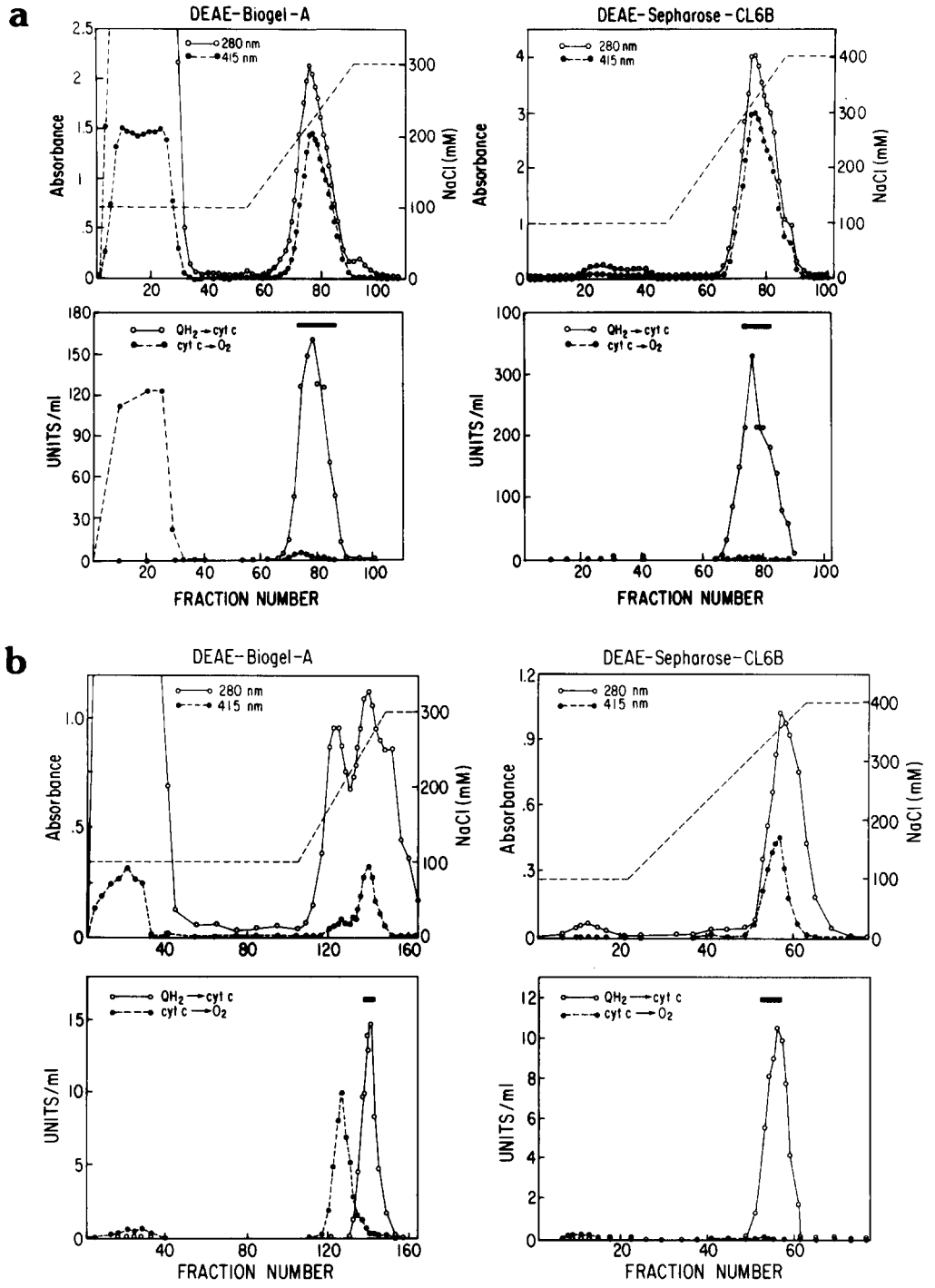


Fig. 2. DEAE column profiles obtained during the purification of the (a) bovine heart and (b) yeast cytochrome $bc_1$ complex. The upper and lower left panels show the DEAE-BioGel A profile and the two right panels show the DEAE-Sepharose CL-6B profile. The upper panels depict the absorbance at 280 nm (protein), 415 nm (heme) as indicated and the NaCl gradient (dashed line). The lower panels show enzymatic activity as indicated: solid bars indicate the ubiquinol-cytochrome *c* oxidoreductase containing fractions that were pooled. Cyt, cytochrome.

TABLE II

PURIFICATION OF UBIQUINOL-CYTOCHROME *c* OXIDOREDUCTASE FROM YEAST

Cyt, cytochrome.

| Fraction | Protein (mg) | Activity (units) | Yield (%) | Purification (-fold) | Cyt $c_1$ (nmol) | Cyt *b* (nmol) | *Tn* ($s^{-1}$) |
|---|---|---|---|---|---|---|---|
| SMP | 3000 | 2016 | – | – | – | 267 | 252 |
| SMP + DM | 2979 | 3456 | 100 | 1.0 | – | 378 | 305 |
| DM extract | 920 | 2824 | 82 | 2.7 | – | 255 | 372 |
| DEAE-BioGel A | 138 | 3266 | 95 | 20.0 | 148 | 302 | 368 |
| DEAE-Sepharose 6B | 22 | 809 | 23 | 32.0 | 87 | 175 | 154 |
| Conc. glycerol diluted | 22 | 1164 | 34 | 46.0 | 89 | 177 | 219 |

plex was free of succinate dehydrogenase and cytochrome oxidase activities. The purification obtained from the second column was immediately apparent from SDS-polyacrylamide gel electrophoresis; however, maximum ubiquinol-cytochrome *c* reductase activity was not observed until the preparation was concentrated and diluted with an equal volume of glycerol (see Tables I and II).

Dilution of the purified mitochondrial complexes for storage in 50% glycerol immediately increased their activity. An additional activity increase occurred upon storage, reaching a maximum during the first 2 weeks. Storage in 50% glycerol also eliminates the detrimental effects of freezing and thawing. When stored in this manner the preparations are stable for at least 1 year at −20 °C [41], and there is no detectable intercomplex aggregation, as preparations apparently remain disperse.

The subunit compositions of purified bovine heart and yeast cytochrome $bc_1$ complexes are shown in Fig. 3. The bovine heart complex has eight subunits and the yeast complex has nine. The apparent molecular masses of the subunits of the bovine heart complex are: core protein 1, 49.2 kDa; core protein 2, 46.0 kDa; comigrating cytochromes *b* and $c_1$, 31.1 kDa; iron-sulfur protein, 24.4 kDa; and three low-molecular-weight subunits, 12.1, 10.6 and 8.2 kDa. The apparent molecular masses of the subunits of the yeast complex are: core protein 1, 47.0 kDa; core protein 2, 41.1 kDa, comigrating cytochromes *b* and $c_1$, 31.3 kDa; iron-sulfur protein, 22.8 kDa; low-molecular-weight subunits, 15.7, 13.5, 11.1 and 8.4 kDa. The functions of the two core proteins and the low-molecular-weight subunits are not known. Nor is it known why the number of low-molecular-weight subunits differs between species.

The dithionite-reduced minus oxidized difference spectra for the purified bovine heart and yeast cytochrome $bc_1$ complexes are shown in Fig. 4. The absence of cytochrome oxidase contamination is evident by the lack of absorption at 605 nm. The cytochrome contents for the bovine heart complex are: cytochrome *b*, 5.05 nmol/mg protein; cytochrome $c_1$, 2.65 nmol/mg protein; with an apparent ratio of cytochrome *b* to $c_1$ of 1.91. The cytochrome contents for the yeast complex are: cytochrome *b*, 8.0 nmol/mg protein; cytochrome $c_1$, 4.0 nmol/mg protein; with a cytochrome *b* to $c_1$ ratio of 2.00.

Recoveries of activity, cytochromes, and protein for the purification of ubiquinol-cytochrome *c* oxidoreductase from *Rb. sphaeroides* and *Rb. capsulatus* are shown in Tables III and IV. Representative column profiles are shown in Fig. 5. As with bovine heart and yeast, addition of dodecyl maltoside to membranes caused a significant increase in ubiquinol-cytochrome *c* reductase activity (4-fold for both bacterial chromatophore membranes). There was specific solubilization of activity for both species, but it was significantly greater for *Rb. sphaeroides*. This difference is apparent in the SDS-polyacrylamide gel electrophoresis gel in Fig. 6 (*Rb. sphaeroides*, lanes a and b; *Rb. capsulatus*, lanes e and f). With *Rb. capsulatus* the gel profiles for membranes and detergent extracts look identical. In contrast, with *Rb. sphaeroides* the reaction center polypeptides (three major bands around the 25.7 kDa standard, reaction center H, L and M) and light-harvesting polypeptides (low molecular weight bands below the

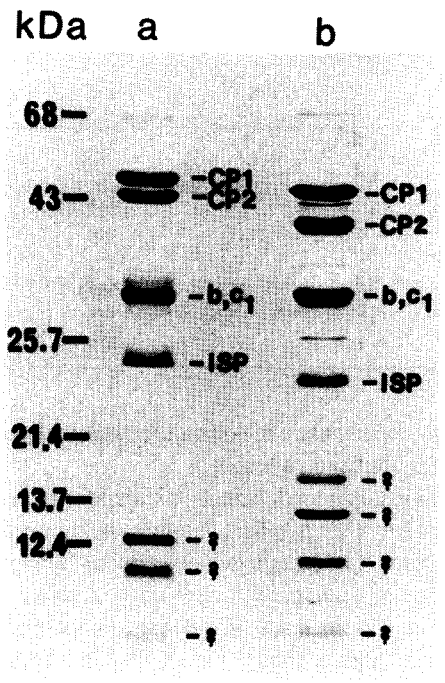


Fig. 3. SDS-polyacrylamide gel electrophoresis of purified cytochrome $bc_1$ complexes from (a) bovine heart and (b) yeast. Samples were first reduced in 2% β-mercaptoethanol and then denatured at 50°C for 10 min. The protein standards utilized were bovine serum albumin (68 kDa), ovalbumin (43 kDa), chymotrypsinogen (25.7 kDa), trypsin inhibitor (21.4 kDa), ribonuclease A (13.7 kDa), and horse heart cytochrome *c* (12.4 kDa).

12.4 kDa standard) are only partially extracted.

Both bacteria possess cytochrome *c* oxidase activity (see Discussion) with exhibited differences in chromatographic behavior during the purification procedures. With *Rb. capsulatus* the bulk of the contaminating proteins including cytochrome *c* oxidase activity flow through the DEAE-BioGel A column. In comparison, half the contaminating proteins of *Rb. sphaeroides* eluted in the flow through and column wash, and the other half just prior to the cytochrome *c* reductase activity in a peak of protein which included the bulk of the cytochrome *c* oxidase activity. No measurable ubiquinol-cytochrome *c* reductase activity was present in the flow-through or column wash. The cytochrome *c* reductase activity of *Rb. sphaeroides* eluted between 250 and 290 mM NaCl, while that of *Rb. capsulatus* eluted between 225 and 260 mM NaCl.

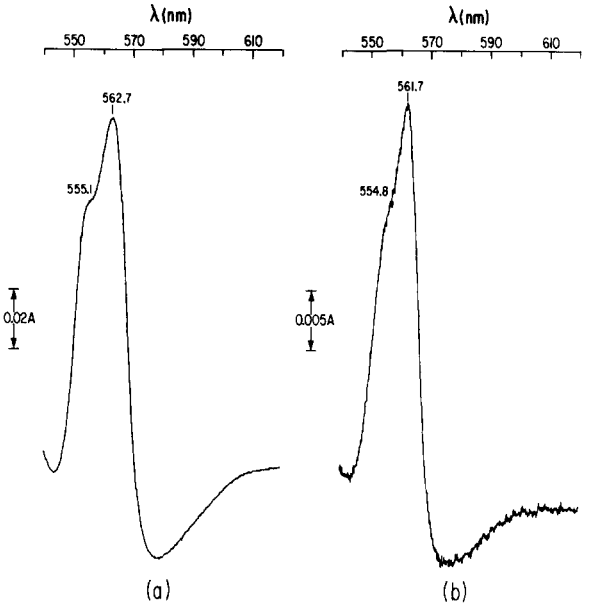


Fig. 4. Dithionite-reduced minus ferricyanide-oxidized absorption-difference spectra of purified cytochrome $bc_1$ complexes from (a) bovine heart suspended at 1.0 mg/ml protein and (b) yeast suspended at 0.17 mg/ml protein.

In a manner analogous to bovine heart and yeast, DEAE-Sepharose CL-6B chromatography proved to be an effective second purification step, and contaminating protein eluted in the flow-through and column wash. Pure *Rb. sphaeroides* $bc_1$ complex eluted between 375 and 400 mM NaCl and pure *Rb. capsulatus* complex eluted between 300 and 375 mM NaCl. Glycerol dilution had little activating effect in either bacterial species (data not shown). Values given for the activity after DEAE-Sepharose CL-6B chromatography in Tables III and IV are for the concentrated, glycerol diluted preparations.

As shown in Fig. 6, the $bc_1$ complexes from both photosynthetic bacteria (lanes d and h) contain four polypeptides. It is evident that the most extensive purification occurs on the first column. The purification achieved on the DEAE-Sepharose CL-6B column is not obvious on the gel for *Rb. capsulatus* due to the lower sample load in lane g. However, for both bacterial complexes, the second chromatography resulted in a doubling of specific activity.

The subunit compositions and heme-staining polypeptides of the purified complexes are com-

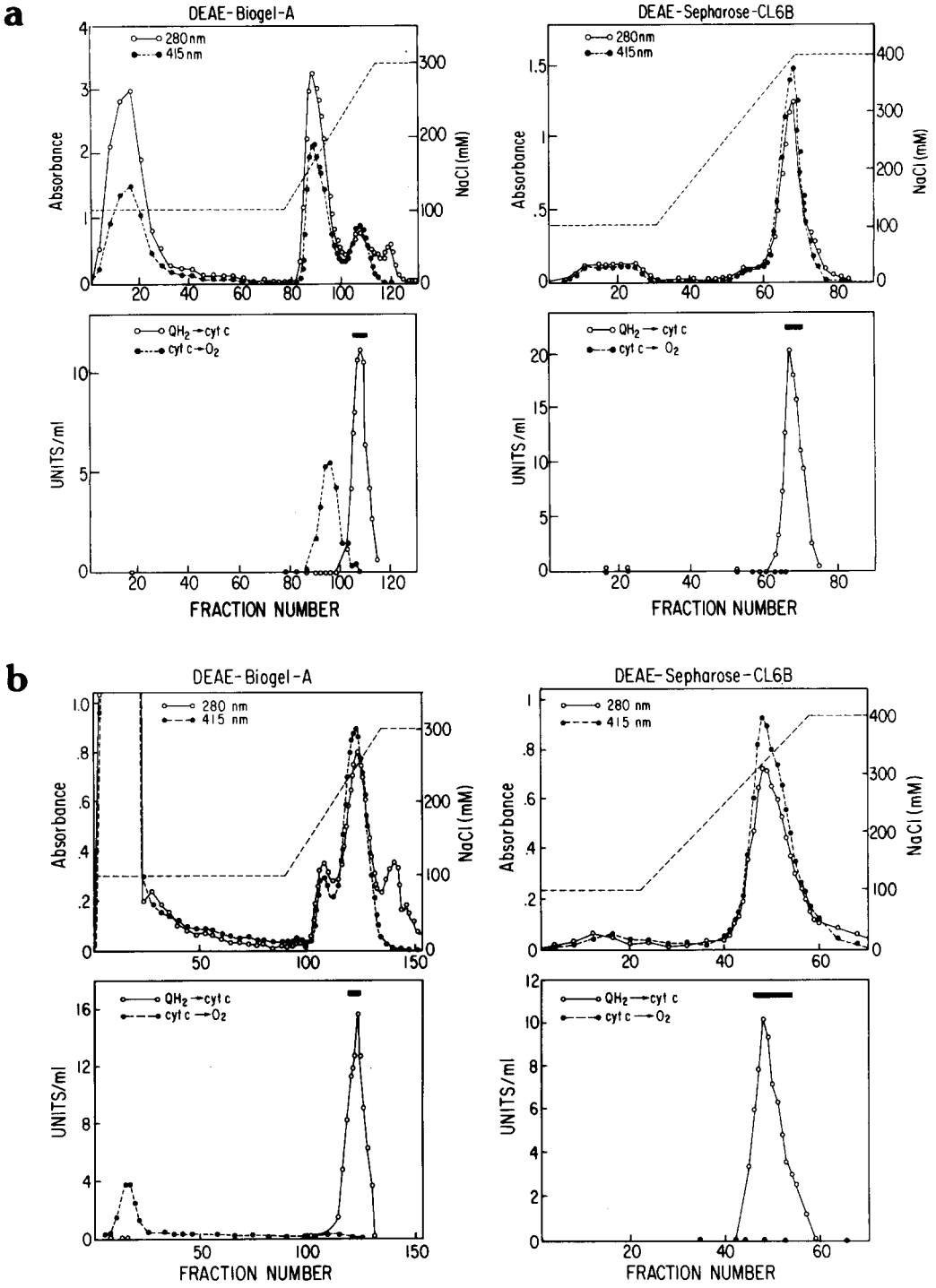


Fig. 5. DEAE column chromatography profiles obtained during the purification of the (a) *Rhodobacter sphaeroides* and (b) *Rhodobacter capsulatus* cytochrome $bc_1$ complex. The upper and lower left panels show the DEAE-Biogel A profile and the two right panels show the DEAE-Sepharose CL-6B profile. The upper panels depict the absorbance at 280 nm (protein), 415 nm (heme) as indicated and the NaCl gradient (dashed line). The lower panels show enzymatic activity as indicated: solid bars indicate the ubiquinol-cytochrome *c* oxidoreductase containing fractions that were pooled. Cyt, cytochrome.

TABLE III

PURIFICATION OF UBIQUINOL-CYTOCHROME *c* OXIDOREDUCTASE FROM *RHODOBACTER SPHAEROIDES*

Cyt, cytochrome.

| Fraction | Protein (mg) | Activity (units) | Yield (%) | Purification (-fold) | Cyt $c_1$ (nmol) | Cyt *b* (nmol) | *Tn* ($s^{-1}$) |
|---|---|---|---|---|---|---|---|
| Chromatophores | 233 | 296 | – | – | – | 249 | 40 |
| Chromatophores + DM | 236 | 1207 | 100 | 1.0 | – | 224 | 180 |
| DM extract | 65 | 561 | 47 | 1.7 | – | 141 | 133 |
| DEAE-BioGel A | 8 | 200 | 17 | 4.9 | 21.9 | 38.4 | 152 |
| DEAE-Sepharose 6B | 1.4 | 67 | 6 | 9.4 | 8.7 | 15.3 | 128 |

pared in Fig. 7. The apparent subunit molecular weights for *Rb. sphaeroides* are: cytochrome *b*, 42.8 kDa; cytochrome $c_1$, 32.7 kDa; iron-sulfur protein, 20.0 kDa; and a 13 kDa polypeptide. For *Rb. capsulatus* the apparent molecular weights of the subunits are: cytochrome *b*, 42.1 kDa; cytochrome $c_1$, 31.2 kDa; iron-sulfur protein, 22.5 kDa; and a 19.8 kDa polypeptide.

The cytochrome $c_1$ polypeptides have been identified by staining of the covalently bound heme, as shown in lanes c and d. The cytochrome *b* polypeptides have been identified by purification by extraction into Triton X-114 [42]. The iron-sulfur proteins of the photosynthetic and mitochondrial complexes have been identified by their cross reactivity to polyclonal antibodies raised against the iron-sulfur protein from *Paracoccus denitrificans* [11]. A common property of the iron-sulfur proteins in both bacterial complexes is that they exhibit low affinity for Coomassie stain (Fig. 7).

The most obvious difference between the two photosynthetic bacterial $bc_1$ complexes is that the low-molecular-weight polypeptides differ significantly in their apparent molecular weights on SDS-polyacrylamide gel electrophoresis (13 kDa vs. 19.8 kDa). In addition both heme-containing polypeptides in *Rb. sphaeroides* are larger than the analogous polypeptides in *Rb. capsulatus*, and the iron-sulfur protein of *Rb. sphaeroides* has a slightly smaller apparent molecular weight than that of *Rb. capsulatus* (20 kDa vs. 22.5 kDa). Whether these apparent differences in molecular weights are real, or merely reflect different mobilities on electrophoresis, remains to be established as the genes for these proteins are isolated and sequenced.

Spectra of the isolated bacterial complexes are shown in Fig. 8. The reduced minus oxidized difference spectra are shown in panels a and c. The heme contents for *Rb. sphaeroides* are: cytochrome *b*, 10.9 nmol/mg protein; cytochrome $c_1$, 6.2 nmol/mg protein; with a *b*-to-$c_1$ ratio of 1.76. The heme contents for the *Rb. capsulatus* complex are: cytochrome *b*, 20.8 nmol/mg protein; cytochrome $c_1$, 12.3 nmol/mg protein; with a *b* to $c_1$ ratio of 1.69. Spectra of the dithionite reduced complexes (Fig. 8b and d) of both species show that they are free of contaminating bacteriochlorophyll and carotenoid pigments. The absence of

TABLE IV

PURIFICATION OF UBIQUINOL-CYTOCHROME *c* OXIDOREDUCTASE FROM *RHODOBACTER CAPSULATUS*

Cyt, cytochrome.

| Fraction | Protein (mg) | Activity (units) | Yield (%) | Purification (-fold) | Cyt $c_1$ (nmol) | Cyt *b* (nmol) | *Tn* ($s^{-1}$) |
|---|---|---|---|---|---|---|---|
| Chromatophores | 150 | 121 | – | – | – | 187 | 19 |
| Chromatophores + DM | 152 | 506 | 100 | 1.0 | – | 212 | 70 |
| DM extract | 109 | 412 | 81 | 1.14 | – | 130 | 93 |
| DEAE-BioGel A | 4.3 | 115 | 23 | 8.03 | 27 | 47 | 71 |
| DEAE-Sepharose 6B | 1.3 | 60 | 12 | 13.4 | 16 | 27 | 64 |

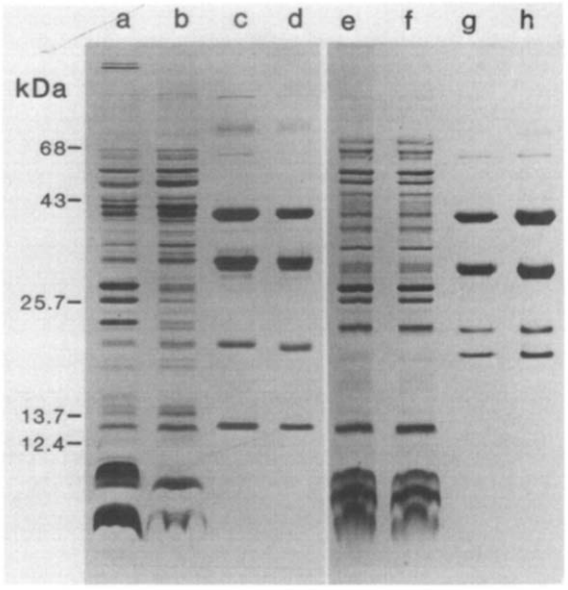


Fig. 6. SDS-polyacrylamide gel electrophoresis demonstrating the purification of the cytochrome $bc_1$ complexes from *Rhodobacter sphaeroides* (a–d) and *Rhodobacter capsulatus* (e–h): chromatophores, a and e; DM extract, b and f; DEAE-BioGel A column pooled fractions, c and g; and pure cytochrome $bc_1$ complex from the DEAE-Sepharose CL-6B column, d and h. Samples were prepared and denatured as in Fig. 3, except that the cytochrome $bc_1$ complex samples were denatured at room temperature.

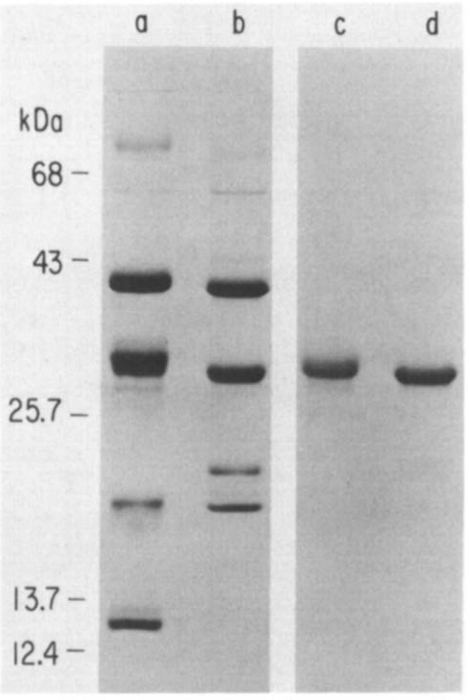


Fig. 7. SDS-polyacrylamide gel electrophoresis comparing the subunit composition and heme staining characteristics of the purified cytochrome $bc_1$ complexes from *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. Lanes a and b show the Coomassie stained complexes of *Rb. sphaeroides* (15 μg protein) and *Rb. capsulatus* (10 μg protein), respectively. Lanes c and d show heme stained complexes (30 μg protein each) for *Rb. sphaeroides* and *Rb. capsulatus*, respectively.

contaminating light-harvesting and reaction-center polypeptides is evident upon SDS-polyacrylamide gel electrophoresis of both purified cytochrome $bc_1$ complexes (Fig. 6), and is confirmed by the lack of absorption from these pigments in the spectra.

## Discussion

We have developed a new method for purification of cytochrome $bc_1$ complexes, and have used this method to purify cytochrome $bc_1$ complexes from bovine heart and yeast mitochondria, and from the photosynthetic bacteria *Rb. sphaeroides* and *Rb. capsulatus*. This is the first report of the purification of this complex from the wild-type strain of *Rb. sphaeroides* and from any strain of *Rb. capsulatus*. The similar specific solubilization by dodecyl maltoside and chromatographic behavior of the various ubiquinol-cytochrome *c* oxidoreductases, from such a wide evolutionary range of organisms, implies a high degree of structural conservation.

With all of these species ubiquinol-cytochrome *c* reductase activity increased upon addition of dodecyl maltoside to membranes. This is presumably due to detergent-induced disruption of sealed membrane vesicles, increasing the accessibility of the substrates ubiquinol and cytochrome *c*. The cytochrome $bc_1$ complex is held together by interactions which are maintained in the presence of dodecyl maltoside, but which are disrupted with detergents like Triton X-100. All of the complexes must have negative surface charge in the presence of dodecyl maltoside in order to adsorb tightly to the DEAE chromatographic matrices. This high negative-charge density is surprising for an in-

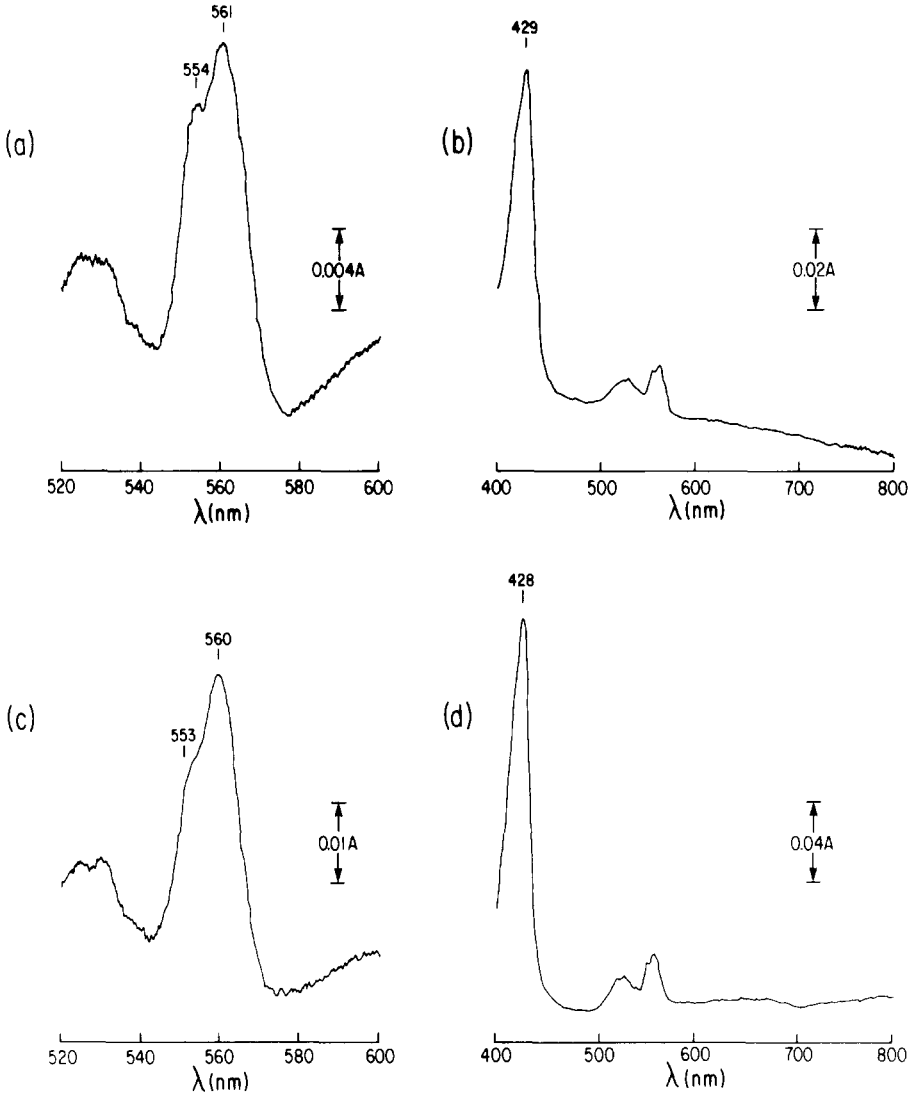


Fig. 8. Absorption difference spectra (dithionite-reduced minus ferricyanide-oxidized, a and c) and dithionite reduced absolute spectra (b and d) of purified cytochrome $bc_1$ complexes from *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. Protein concentrations were: 70 mg/ml for both *Rb. sphaeroides* spectra a and b; 65 mg/ml of *Rb. capsulatus* spectrum c; 51 mg/ml for *Rb. capsulatus* spectrum d.

tegral enzyme complex, and may be significant for the interaction of the $bc_1$ complex with other respiratory components and the response of this enzyme complex to the protonmotive force generated across the energy-transducing membrane.

The cytochrome *c* oxidases from yeast and *Rb. sphaeroides* have similar chromatographic properties on DEAE-BioGel A (i.e., higher affinity) as compared to those from bovine and *Rb. capsulatus* (see Figs. 2 and 5). Cytochrome *c* oxidase activity in anaerobically grown *Rb. sphaeroides* at the levels we observed indicates that the genes for this enzyme complex are constitutively expressed. The oxidase activity was not altered by dark adaptation of samples and was abolished with cyanide, and thus exhibits characteristics in common with

mitochondrial cytochrome *c* oxidases.

In bovine and yeast the activation of ubiquinol-cytochrome *c* reductase activity with glycerol is probably due to partitioning of excess detergent away from the complex. An additional advantage of dilution and storage in glycerol is that samples can be stored at −20 °C and used directly without freeze-thaw cycling.

Purified *Rb. sphaeroides* and *Rb. capsulatus* chromatophore membranes were utilized for isolation of cytochrome $bc_1$ complex, as these membrane fractions represent the mature photosynthetic intracytoplasmic membrane. Crude chromatophore membranes are heterogeneous in that they contain both mature and incomplete energy-transducing complexes [43]. Chromatophore membranes from *Rb. sphaeroides* were difficult to solubilize in comparison to the other membranes utilized in this study, and the ubiquinol-cytochrome *c* reductase activity was more sensitive to higher detergent concentrations.

The finding that dodecyl maltoside almost quantitatively solubilizes the constitutive proteins of *Rb. capsulatus* chromatophores could be useful in purifying the photoreaction center and light-harvesting complexes, as it is likely that this detergent would leave these complexes intact. We did not analyze the levels of reaction center and light-harvesting complexes in this study, but it is possible that these complexes could also be purified using DEAE column chromatography in dodecyl maltoside.

The turnover numbers for *Rb. sphaeroides* reported here are five times higher than previously reported [9]. This is probably due to the use of dodecyl maltoside. The turnover rates reported by Gabellini et al. [8] are very low, which may be due to the combined effects of using Triton X-100 and ubiquinol-9, instead of the more soluble ubiquinol derivative decylbenzodihydroquinol ($DBH_2$). Their resulting purified cytochrome $bc_1$ complex had a turnover number of 0.03 $s^{-1}$, which is 277-times less than that of the washed chromatophores [8]. We observed only a slight decrease in turnover number, and the turnover numbers of 128 $s^{-1}$ in *Rb. sphaeroides* and 64 $s^{-1}$ in *Rb. capsulatus* are significantly greater than those previously reported [8,9]. The theoretical turnover number, based on flash kinetic studies [43], is thought to be greater than 500 $s^{-1}$.

The ratios of cytochrome *b* to $c_1$ in the $bc_1$ complexes of *Rb. sphaeroides* and *Rb. capsulatus*, are 1.76 : 1 and 1.69 : 1, respectively. This discrepancy from the expected value of two, as is typical of eukaryotic cytochrome $bc_1$ complexes, may be partly due to the loss of non-covalently bound *b* heme during purification, and partly due to the use of extinction coefficients for cytochromes *b* and $c_1$ based on those of the cytochromes of the isolated bovine-heart complex.

The minimum molecular weights of the photosynthetic bacterial $bc_1$ complexes calculated from the contents of heme *b* and $c_1$ also differ from the molecular weights estimated from these complexes by summing the apparent molecular weights of the individual polypeptides. The molecular weights calculated for the *Rb. sphaeroides* complex from the heme *b* and $c_1$ contents are 184 and 161 kDa, respectively. The combined subunit molecular weight of this complex, assuming one copy of each polypeptide, is 108 kDa.

The molecular weights calculated for the *Rb. capsulatus* complex from the heme *b* and $c_1$ contents are 96 and 81 kDa, respectively, while the molecular weight estimated from the subunits is 116 kDa. Thus, in one instance (*Rb. sphaeroides*) the heme contents appear to be significantly underestimated, in comparison to the sum of the subunit molecular weights, while in the other (*Rb. capsulatus*) the heme contents appear to be slightly overestimated. In order to make this calculation in a reliable manner, it will be necessary to know the true molecular weights of the constituent polypeptides, as may be deduced from sequencing the genes, and to obtain reliable values for the extinction coefficients of the bacterial cytochromes.

It is of interest that the isolated cytochrome $bc_1$ complexes from *Rb. sphaeroides* and *Rb. capsulatus* consist of four subunits, but Gabellini et al. [44] isolated an operon containing only the genes encoding the three prosthetic-group-containing polypeptides. The molecular weights of the three prosthetic-group-containing polypeptides are very similar in the two bacteria (see Fig. 6). The apparent molecular weights of the fourth, non-prosthetic group containing polypeptides are 13 kDa and 19.8 kDa for *Rb. sphaeroides* and *Rb. capsulatus*, respectively. Whether these polypep-

tides have a function in electron transfer is not known, and it is not known whether the difference in size of these two polypeptides in these two bacteria has any functional significance. It has been speculated [45], based on data obtained with mammalian ubiquinol-cytochrome *c* oxidoreductase [46–48], that these polypeptides may be involved with ubiquinone/ubiquinol binding. In general, the function of the non-prosthetic group containing polypeptides present in all isolated $bc_1$ complexes, except *Paracoccus denitrificans* which has only three subunits [11], remains to be elucidated.

The significance of purifying an active cytochrome $bc_1$ complex from a wild-type strain of *Rb. sphaeroides* is that most of the mutants previously used are not well characterized and could have pleiotrophic mutations that affect the integrity of this enzyme complex. Purification of this complex without contaminating pigments (see Fig. 8) obviates the need to use mutants that lack (R26, R26.1) or have blue-shifted carotenoids (GA, $G_1C$), or that lack light-harvesting complex (R26, R26.1). Previously reported preparations of cytochrome $bc_1$ complex from *Rb. sphaeroides* GA contained contaminating pigments with absorption peaks at 458 nm and 490 nm (GA carotenoids), at 590 nm (bacteriochlorophyll $Q_x$), and at 689 nm (bacteriopheophytin) [8]. When a different procedure was used to purify a cytochrome $bc_1$ complex from the carotenoidless mutant R26, pigment contamination (bacteriochlorophyll $Q_x$) was apparent as an absorption peak at 590 nm [9].

The purification procedure described here is simple, reproducible, and yields preparations with activities which are equal to or better than those previously obtained. This method has allowed the isolation of pure cytochrome $bc_1$ complexes from membranes which differ markedly in lipid and protein composition, and the elution behavior of the $bc_1$ complexes on the DEAE matrixes are very similar. It thus seems likely that with minor modifications this method could be applied to species other than those described here.

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