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The *Rhodospirillum rubrum* cytochrome bc_1 complex: redox properties, inhibitor sensitivity and proton pumping

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A detergent-solubilized, three-subunit-containing cytochrome bc_1 complex, isolated from the photosynthetic bacterium *R. rubrum*, has been shown to be highly sensitive to stigmatellin, myxothiazol, antimycin A and UHDBT, four specific inhibitors of these complexes. Oxidation-reduction titrations have allowed the determination of E_m values for all the electron-carrying prosthetic groups in the complex. Antimycin A has been shown to produce a red shift in the α-band absorbance maximum of one of the cytochrome *b* hemes in the complex and stigmatellin has been shown to alter both the E_m and EPR *g*-values of the Rieske iron-sulfur protein in the complex. Western blots have revealed antigenic similarities between the cytochrome subunits of the *R. rubrum* complex and those of the related photosynthetic bacteria, *Rb. capsulatus* and *Rb. sphaeroides*. The *R. rubrum* complex has been incorporated into liposomes. These liposomes exhibit respiratory control and are able to couple electron transfer from quinol to cytochrome *c* to proton translocation across the liposome membrane in a manner consistent with a Q-cycle mechanism. It can thus be concluded that neither electron transport nor coupled proton translocation by the cytochrome bc_1 complex requires more than three subunits in *R. rubrum*.

Introduction

The cytochrome bc_1 complexes of photosynthetic bacteria catalyze electron flow from ubiquinol to cytochrome c_2 and couple this electron flow to the transfer of two protons (per cytochrome c_2 reduced) from the cytoplasm to the periplasmic space [1–6]. The cytochrome bc_1 complexes of photosynthetic bacteria have proven particularly useful for studying electron transfer and proton translocation in energy-transducing, electron transfer chains. Among the advantages of these complexes for such studies is the possibility of studying the passage of single electrons through the complexes in situ after activation of electron flow by short, single-turnover light flashes and the relatively simple subunit composition of the solubilized bacterial complexes compared to that of the functionally similar complexes found in the membranes of aerobic eukaryotes [1,5–7]. Cytochrome bc_1 complexes have been isolated and characterized from four species of photosynthetic bacteria: *Rhodobacter capsulatus* [8,9], *Rhodobacter sphaeroides* [9–13], *Rhodospirillum rubrum* [13–16] and *Rhodopseudomonas viridis* [14,17]. These complexes contain no more than four peptide subunits and, in the case of *R. rubrum* and *Rps. viridis*, appear to contain only three subunits. The three peptides of the *R. rubrum* and *Rps. viridis* complexes contain the electron-carrying prosthetic groups of the complex: cytochrome *b* contains two inequivalent protohemes; cytochrome c_1 contains a single, covalently bound heme *c* and the Rieske iron-sulfur protein contains a single [2Fe-2S] cluster [1–6,14,15,17]. Cytochrome bc_1 complexes isolated from mitochondria contain the same prosthetic groups as the bacterial complexes but have approximately ten peptide subunits [7].

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EPR, electron paramagnetic resonance; Q_2H_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; UHDBT, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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The detergent-solubilized *R. rubrum* complex has proven to be highly suitable for studying certain aspects of cytochrome bc_1 complex function. As the solubilized *R. rubrum* complex retains a high-affinity binding site for cytochrome c_2, this complex has proven to be particularly useful for studying interactions between the cytochrome bc_1 complex and its electron-accepting substrate [18,19]. The *R. rubrum* complex is also well suited for spectroscopic measurements utilizing techniques such as resonance Raman spectroscopy [20]. There have been two recent independent confirmatory reports [13,16] of the observation, originally made in our laboratory [14,15], that the *R. rubrum* cytochrome bc_1 complex contains only three peptide subunits. In the light of recent evidence [12,13], confirming earlier observations [9,10], that the cytochrome bc_1 complex of the related bacterium *Rb. sphaeroides* contains four rather than three subunits, it seemed of interest to further characterize the solubilized, purified *R. rubrum* cytochrome bc_1 complex to insure that this three subunit complex retains the sensitivity to specific inhibitors, the prosthetic group oxidation-reduction properties and the ability to couple electron flow to proton translocation characteristic of these complexes in situ. Such information would indicate that if a fourth subunit were present in the *R. rubrum* complex and were lost during purification, the putative fourth subunit is not essential for these aspects of cytochrome bc_1 complex function. In this communication, we report the results of further characterization of the *R. rubrum* cytochrome bc_1 complex and provide evidence the *R. rubrum* membranes do not contain a protein antigenically related to the fourth subunit of the *Rb. sphaeroides* cytochrome bc_1 complex.

Materials and Methods

The *R. rubrum* cytochrome bc_1 complex samples used for most of the experiments described below were prepared as described previously [15], except that the chromatophore membrane concentration was adjusted to a protein concentration between 10 and 11 mg/ml during the detergent-solubilization step. A modification of this procedure, in which the detergent solubilization step and the first chromatography step were carried out in the presence of 20% (v/v) glycerol, was used in some cases. The inclusion of 20% glycerol in the buffers used for these steps in the purification protocol resulted in an increased yield of the complex but did not affect any of the properties (i.e., subunit composition, absorbance spectrum, prosthetic group content, bacteriochlorophyll content or specific activity) of the purified complex. However, in the presence of 20% glycerol, the cytochrome bc_1 complex was eluted from the first anion exchange column by buffer containing 200 mM NaCl, compared to the 300 mM NaCl required to elute the complex in the absence of glycerol. The purified complex was stored unfrozen at $-20\,^{\circ}C$ in buffer containing 50% (v/v) glycerol. The complex proved to be considerably more stable when stored in this fashion than when stored frozen in 20% (v/v) glycerol at 77 K.

Polyclonal rabbit antibodies against the cytochrome *b* and 12 kDa subunits of the *Rb. sphaeroides* cytochrome bc_1 complex were prepared as described previously [21]. Monoclonal antibodies against *Rb. capsulatus* cytochromes *b* and c_1 [22] were a generous gift from Prof. Fevzi Daldal (Department of Biology, University of Pennsylvania). Western blots, using these antibodies, were performed according to the method of Davidson et al. [23]. Antimycin A, valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and equine cytochrome *c* were purchased from Sigma. Myxothiazol was purchased from Boehringer Mannheim Biochemicals. Stigmatellin was a generous gift from Dr. G. Hofle (Gesellschaft für Biotechnologische Forschung, Braunschweig) and 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) was a generous gift from Prof. B.L. Trumpower (Department of Biochemistry, Darthmouth Medical School). 2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q_2H_2) was prepared according to the procedure of Yu and Yu [24]. Azolectin was obtained from Associate Concentrate. 'Phast' polyacrylamide gels and buffers for electrophoresis in the presence of sodium dodecylsulfate were obtained from Pharmacia LKB Biotechnology. Molecular weight standards for electrophoresis were obtained from Bio-Rad.

Optical absorbance spectra used to determine the cytochrome *b*: cytochrome c_1 ratio of the complex, the bacteriochlorophyll content of the complex and the concentration of the complex were obtained using a Shimadzu Model UV-2100 spectrophotometer. The quinol: cytochrome *c* oxidoreductase activity of the complex was assayed as described previously [15]. The subunit composition of the complex was determined using polyacrylamide gel electrophoresis in the presence of SDS to separate the subunits, as described previously [14,15]. Electrophoresis was performed using a Pharmacia Phast system and the gels were stained for protein with Coomassie brilliant blue.

Oxidation-reduction titrations were performed under anaerobic conditions as described by Dutton [25]. Absorbance spectra, taken at defined E_h values during oxidation-reduction titrations, were obtained using a Biomedical Instrumentation Group (University of Pennsylvania) spectrophotometer interfaced with an IBM personal computer. Low-temperature EPR spectra of the Rieske iron-sulfur center were obtained using a Varian Model E-109 X-band spectrometer equipped with an Air Products Model LTD 3-110 variable temperature, flowing helium cryostat.

The beef-heart mitochondrial cytochrome bc_1 complex was prepared according to the method of Yu and

Yu [26] and incorporated into azolectin phospholipid vesicles (liposomes) using the cholate dialysis method of Racker and Kagawa [27]. The *R. rubrum* cytochrome bc_1 complex was incorporated into phospholipid vesicles in the same manner. In some cases, the *R. rubrum* cytochrome bc_1 complex was precipitated with 60% saturated ammonium sulfate to remove the glycerol in which the complex was stored and the precipitate, after collection by centrifugation, was dissolved directly in azolectin-cholate solution prior to the dialysis step. Proton translocation coupled to electron flow through the complex was measured at 25° C using a Beckman Model 3500 pH meter and Model 39532 combination pH electrode. The reaction mixture contained, in a volume of 1.4 ml, 0.15 M KCl, 2.9 μM equine ferrocytochrome *c*, 18 μM Q_2H_2, 0.26 μM valinomycin and 25 μl of liposomes. Electron flow was initiated by the addition of 5 nmol of potassium ferricyanide, which oxidizes the cytochrome *c* and thus provides an electron acceptor for the complex. Electron flow under conditions where no transmembrane ΔpH is formed was measured in an identical manner except that the protonophore CCCP was present at a concentration of 0.5 μM to make the membrane permeable to protons.

Results

Despite the fact that work performed in our laboratory [14,15] and by two other groups [13,16] had failed to detect a 12–14 kDa subunit in the *R. rubrum* complex, it seemed possible that such a subunit could be present in situ and subsequently be lost during purification of the complex. The observation that an antibody prepared against the 12–14 kDa subunit of the *Rb. sphaeroides* cytochrome bc_1 complex inhibited the quinol : cytochrome *c* oxidoreductase activity of the *Rb. sphaeroides* complex (Yu, L. and Yu, C.-A., unpublished data) suggested that this antibody might be able to detect a related peptide in unfractionated *R. rubrum* membranes. Of course, in order for such an approach to be useful it is necessary that the subunits of the two complexes be sufficiently similar that an antibody raised against a subunit isolated from one bacterial species would be capable of recognizing the corresponding subunit from another bacterial species. In fact, it has been shown that antibodies raised against the cytochrome c_1 and cytochrome *b* peptides of the *Rb. sphaeroides* complex recognize the corresponding subunits of the *R. rubrum* complex (see Ref. 15 for the cytochrome c_1 data; the cytochrome *b* data, not shown, were obtained in this study). These antigenic similarities between two subunits of the two bacterial complexes suggested that a 12–14 kDa subunit of the *R. rubrum* complex, if one existed, might be detected by an antibody against the *Rb. sphaeroides* peptide. Western blots of solubilized *R. rubrum* membranes (chromatophores) failed to detect

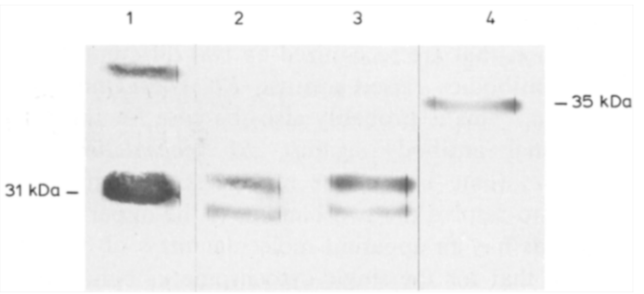

Fig. 1. Recognition of *R. rubrum* cytochrome c_1 and cytochrome *b* peptides by monoclonal antibodies against the *Rb. capsulatus* peptides. Western blots were performed as described in Materials and Methods. 1–4 show the results of separate experiments in which approximately equal amounts (10–15 μg of protein per electrophoresis run) of the *R. rubrum* cytochrome bc_1 complex were separated into constituent subunits by electrophoresis in the presence of sodium dodecyl sulfate, blotted, and treated with monoclonal antibodies raised against *Rb. capsulatus* cytochrome c_1 (1, antibody D42; 2, antibody D3; and 3, antibody D1) or *Rb. capsulatus* cytochrome *b* (4, antibody D50). The antibody designations correspond to those of Ref. 22.

any component that cross reacted with this antibody (data not shown). Furthermore, the antibody had no effect on the quinol : cytochrome *c* oxidoreductase activity of the *R. rubrum* complex. These results indicate that either the *R. rubrum* cytochrome bc_1 does not contain a fourth subunit or that, if a fourth subunit is present, it is antigenically unrelated to the 12–14 kDa subunit of the *Rb. sphaeroides* complex.

It had previously been demonstrated that polyclonal antibodies raised against the *Rb. capsulatus* Rieske iron-sulfur protein recognized the corresponding *R. rubrum* protein [15]. To further explore possible antigenic similarities between the other two subunits of the cytochrome bc_1 complexes of these two bacteria, four separate Western blots were run with the *R. rubrum* complex using three monoclonal antibodies raised against *Rb. capsulatus* cytochrome c_1 and one monoclonal antibody raised against *Rb. capsulatus* cytochrome *b*. Fig. 1 (lanes 1–3) shows that three monoclonal antibodies raised against *Rb. capsulatus* cytochrome c_1 recognize *R. rubrum* cytochrome c_1. It is interesting to note that one of these monoclonal antibodies against *Rb. capsulatus* cytochrome c_1 (antibody D42, used in lane 1) does not recognize *Rb. sphaeroides* cytochrome c_1 [29], raising the possibility that greater structural similarity may exist between the *R. rubrum* and *Rb. capsulatus* cytochromes c_1 than between the *Rb. capsulatus* and *Rb. sphaeorides* cytochromes. Monoclonal antibody D42 (Lane 1) also recognizes a band of higher molecular weight than that of monomeric cytochrome c_1 (31 kDa; Refs. 14, 15), indicating the presence of some aggregated material. Such aggregates have been detected in previous studies of the *R. rubrum* complex [13–16]. Examination of lanes 2 and 3 in Fig. 1 indicates the presence of two closely spaced bands,

rather than a single band, in the resolved *R. rubrum* preparation that are recognized by two different monoclonal antibodies raised against *Rb. capsulatus* cytochrome c_1. This is probably also the case for the third monoclonal antibody against *Rb. capsulatus* cytochrome c_1 (lane 1), but the intense staining makes it difficult to resolve the two bands. As the upper of the two bands has an apparent molecular mass of 31 kDa, equal to that for the single cytochrome c_1 band previously observed in our laboratory [14,15], we conclude that some proteolysis of the cytochrome occurred under the conditions used for the current experiments. Fig. 1 (lane 4) also shows that *R. rubrum* cytochrome *b* is recognized by a monoclonal antibody raised against *Rb. capsulatus* cytochrome *b*.

Recent characterizations of *Rb. capsulatus* mutants that are resistant to myxothiazol and stigmatellin, two specific inhibitors of cytochrome bc_1 complexes, indicate that the binding sites for these inhibitors are probably located on the cytochrome *b* subunit of the complex [30]. Mutations that confer resistance to antimycin A in aerobic eukaryotes indicate that the binding site for this specific inhibitor of the complex is also likely to be on the cytochrome *b* peptide [31–36]. These three inhibitors of cytochrome bc_1 complexes have structures that suggest that they can function as quinone or quinol analogs and may inhibit the complex by competing with the normal quinone substrates for their binding sites [2,37]. As the 12–14 kDa subunit of the *Rb. sphaeroides* complex has been implicated in quinone binding [38], it seemed important to determine the sensitivity of the three subunit *R. rubrum* complex to these inhibitors. A fourth inhibitor of these complexes, UHDBT, has been shown to affect the properties of the Rieske iron-sulfur protein in *Rb. sphaeroides* [12,39]. As UHDBT is likely to act as a quinone analog [39], it also seemed advisable to determine the sensitivity of the three subunit *R. rubrum* complex to this inhibitor. Fig. 2 shows inhibition curves for all four of these inhibitors of the quinol : cytochrome *c* oxidoreductase activity of the *R. rubrum* complex. It is clear from these results that this solubilized, three subunit complex retains high sensitivity to these four specific inhibitors of cytochrome bc_1 complexes.

Inhibition of the ubiquinol : cytochrome *c* oxidoreductase activity of the *R. rubrum* cytochrome bc_1 complex by myxothiazol and antimycin A had previously been demonstrated [14,15], but the concentration dependence of the inhibition had not been reported. Another effect of antimycin A observed with cytochrome bc_1 complexes from other organisms, including detergent-solubilized complexes from the photosynthetic bacteria *Rb. capsulatus* and *Rb. sphaeroides,* is the shift to longer wavelength the inhibitor produces in the α-band maximum of reduced cytochrome b_H, the higher potential protoheme [8,12,40]. This band-shift

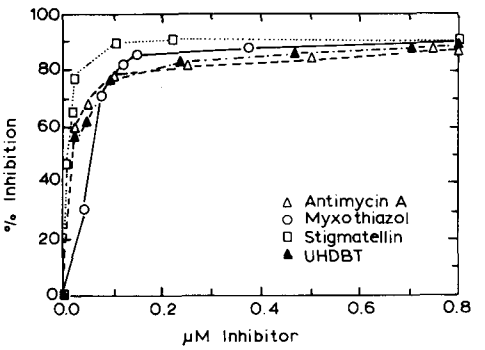

Fig. 2. The effect of inhibitors on the quinol : cytochrome *c* oxidoreductase activity of the *R. rubrum* cytochrome bc_1 complex. The electron transfer activity of the complex was assayed as described in Materials and Methods, using an assay mixture that contained 10 nM cytochrome bc_1 complex. The rates were corrected, by subtraction, for the small amount of direct electron transfer from the quinol to equine cytochrome *c* that was observed in the absence of the complex. Inhibitors, present at the concentrations indicated, were added as small aliquots of concentrated ethanol stock solutions.

had not been previously investigated with the solubilized *R. rubrum* complex. We have now observed such a band-shift with the detergent-solubilized *R. rubrum* complex. Difference spectra (+antimycin A *minus* control) of the dithionite-reduced *R. rubrum* complex show a maximum at 564 nm and a minimum at 554 nm (data not shown), similar to those reported previously for the detergent-solubilized *Rb. capsulatus* [8] and *Rb. sphaeroides* [12,40] complexes. Thus, in so far as the antimycin-A-induced red-shift in the α-band absorbance maximum of cytochrome b_H provides a measure of the interaction of antimycin A with the complex, the presence of a fourth 12–14 kDa subunit does not appear to be required for 'normal' binding of the inhibitor.

Oxidation-reduction titrations, using absorbance changes in the cytochrome α-band region to monitor oxidation state, had previously been performed on cytochrome c_1 and the two hemes of cytochrome *b* in the solubilized *R. rubrum* complex [15]. The EPR spectrum of the reduced Rieske protein in the *R. rubrum* complex had also been reported [14]. However, no measurements of the EPR spectrum at defined redox potentials, which would have allowed a determination of the E_m value of this component, were made. Fig. 3A shows an oxidation-reduction titration of the [2Fe-2S] cluster of the Rieske protein in the solubilized *R. rubrum* cytochrome bc_1 complex, using the amplitude of the $g = 1.90$ feature in the EPR spectrum to monitor the oxidation state of the cluster. The average of three titrations gave $E_m = +305 \pm 10$ mV, a value similar to that reported for the Rieske protein in other cytochrome bc_1 complexes [1–4,12,39,41]. Unlike the case for the cytochrome components of the *R. rubrum* complex (see below), the E_m value of the Rieske protein was not affected by the conditions used for storage of the complex.

Although studies with inhibitor-resistant mutants, cited above, suggested that the binding site for stimatellin is likely to involve, at least in part, the cytochrome *b* subunit of these complexes, in species other than *R. rubrum* stigmatellin has been shown to also cause changes in the midpoint potential and EPR spectrum of the Rieske iron-sulfur protein [12,42]. Fig. 3A demonstrates that stigmatellin produces a large positive shift in the E_m value of the *R. rubrum* Rieske protein, similar to that previously observed with other cytochrome bc_1 complexes [12,42]. Fig. 3B shows that stigmatellin also shifts the *g* values observed in the EPR spectrum of the reduced *R. rubrum* Rieske protein in a manner similar to that observed with cytochrome bc_1 complexes from other species [12,42]. It has been demonstrated that shifts in the EPR spectrum of the reduced Rieske protein can also be observed, in the absence of inhibitors of the complex, when the quinol oxidizing site of the complex (also referred to as the Q_z site) is occupied by quinol rather than by quinone [12,43–45]. No change in the EPR spectrum of the reduced Rieske protein in the solubilized *R. rubrum* complex was observed when the

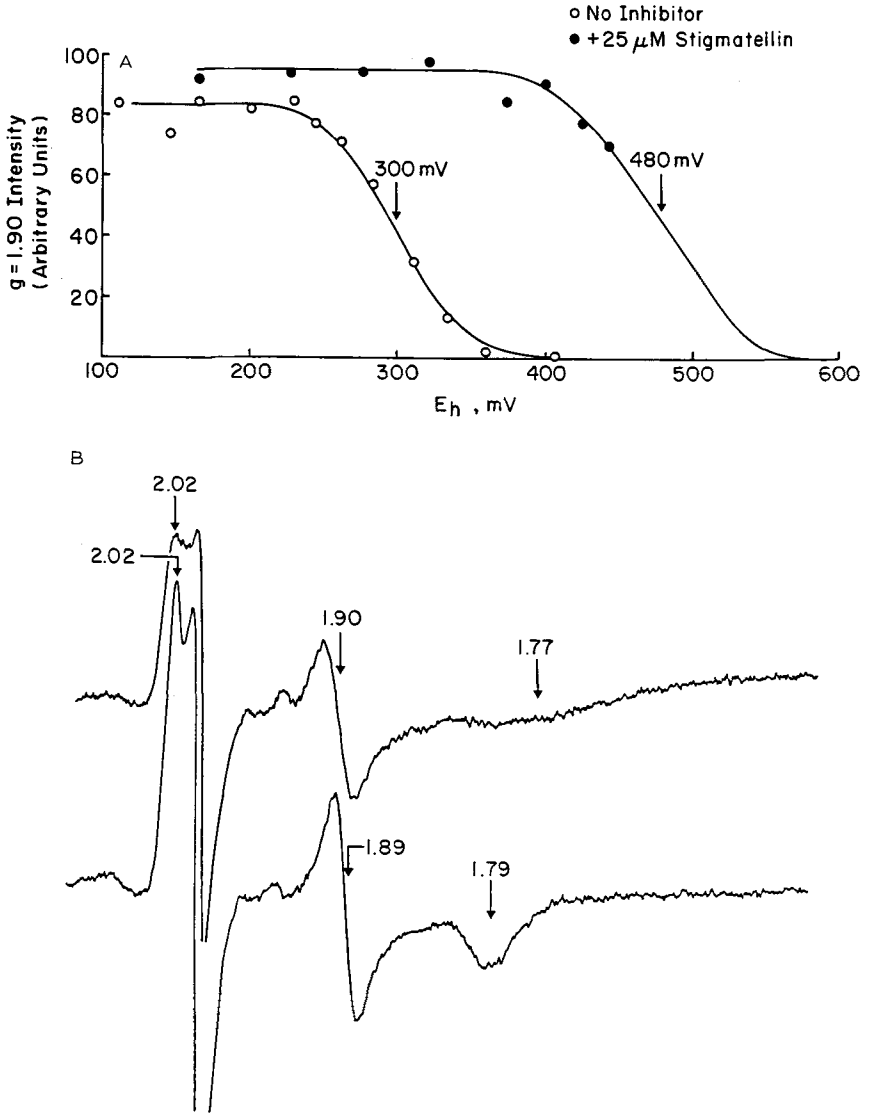

Fig. 3. The effect of stigmatellin on the E_m value and EPR spectrum on the *R. rubrum* Rieske iron-sulfur protein. (A) Oxidation-reduction titrations. The reaction mixture contained the *R. rubrum* cytochrome bc_1 complex at a concentration equivalent to 24 μM cytochrome c_1 in 35 mM Mops buffer (pH 7.4) containing 1 mM $MgSO_4$ and 0.1 mg/ml dodecyl maltoside and the following oxidation-reduction mediators: 40 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine; 40 μM quinhydrone; 20 μM phenazine methosulfate and 20 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. Samples equilibrated at the indicated E_h values were transferred anaerobically to EPR tubes and frozen. Stigmatellin was present at a concentration of 25 μM, where indicated. EPR conditions: Frequency, 9.33 GHz; power, 1 mW; modulation amplitude, 10 G; temperature, 20 K. (B) EPR spectra. The upper trace shows the EPR spectrum of the Rieske iron-sulfur protein in the absence of stigmatellin and the lower trace shows the EPR spectrum in the presence of 25 μM stigmatellin. The spectra were obtained with samples that had been poised at E_h values of +168 mV and +174 mV, respectively, prior to freezing. Other conditions were as in (A).

E_h value of the sample was lowered from +200 mV to less than −300 mV (data not shown). The absence of any observable shift in Rieske protein g values over a range of E_h values where the ubiquinone known to be present in the solubilized *R. rubrum* complex [14] should go from fully oxidized to completely reduced suggests either that this ubiquinone is not at the Q_z site or the the g value shift does not occur in the solubilized *R. rubrum* complex.

Previous oxidation-reduction titrations of the solubilized *R. rubrum* complex resolved the two different E_m values of the two inequivalent protohemes of cytochrome *b* [15]. The values obtained in this earlier study, −30 and −90 mV for the high potential (cytochrome b_H) and low potential (cytochrome b_L) components, respectively, differed somewhat from values obtained with solubilized cytochrome bc_1 complexes isolated from other photosynthetic bacteria [8,12,17] and from values obtained with intact *R. rubrum* membranes [46]. Some, but not all, of these differences could perhaps be attributed to the different pH values at which the measurements were conducted, as cytochrome *b* has been reported to have pH-dependent E_m values [1–4,17,47]. As our earlier titrations were performed electrochemically, using an optically transparent gold mesh electrode, while the other studies used chemical oxidants and reductants to adjust E_h values, it seemed appropriate to repeat the redox titrations using conventional rather than electrochemical methods. In the course of these measurements, it became clear that considerable variability could be observed in the E_m values measured for cytochromes b_H and b_L. This variability correlated with the manner in which the complex was stored rather than the manner in which the titrations were performed. Fig. 4A shows the results of a titration on a sample that had been stored unfrozen in 50% glycerol. Average E_m values, from redox titrations of three separate samples of the *R. rubrum* complex that had been stored unfrozen in 50% glycerol at −20 °C, of +20 and −85 ± 35 mV were obtained for cytochromes b_H and b_L, respectively. The significant variation observed from one titration to another (indicated by the ±35 mV average deviation) appears to arise, in part, from slow equilibration of the sample because of presence of residual glycerol in some of the samples. Considerably more negative E_m values (e.g., as low as −50 mV for cytochrome b_H and −175 mV for cytochrome b_L) were obtained with samples that had been stored frozen at 77 K in 20% glycerol (data not shown). As samples stored unfrozen in 50% glycerol exhibited considerably higher ubiquinol : cytochrome *c* oxidoreductase activity than those that had been stored frozen at 77 K, it seems reasonable to take the more positive values as most representative of the native E_m values. These E_m values of +20 and −85 mV for cytochromes b_H and b_L, respectively, are also in closer agreement with the E_m values of +35 and −40 mV determined for the two hemes of cytochrome *b* in intact *R. rubrum* membranes [46], than the more negative values obtained with samples that had been stored frozen.

The effect of sample storage on the E_m value of cytochrome c_1 was also investigated. Fig. 4B shows a representative oxidation-reduction titration of cytochrome c_1 in a sample of the *R. rubrum* cytochrome bc_1 complex that was stored unfrozen at −20 °C in 50% glycerol. An average value for three titrations of +280 ± 15 mV was obtained for the E_m of the cytochrome in these samples, compared to an average value of +265 ± 20 mV (three titrations) for the E_m of cytochrome c_1 in samples of the *R. rubrum* cytochrome bc_1 complex

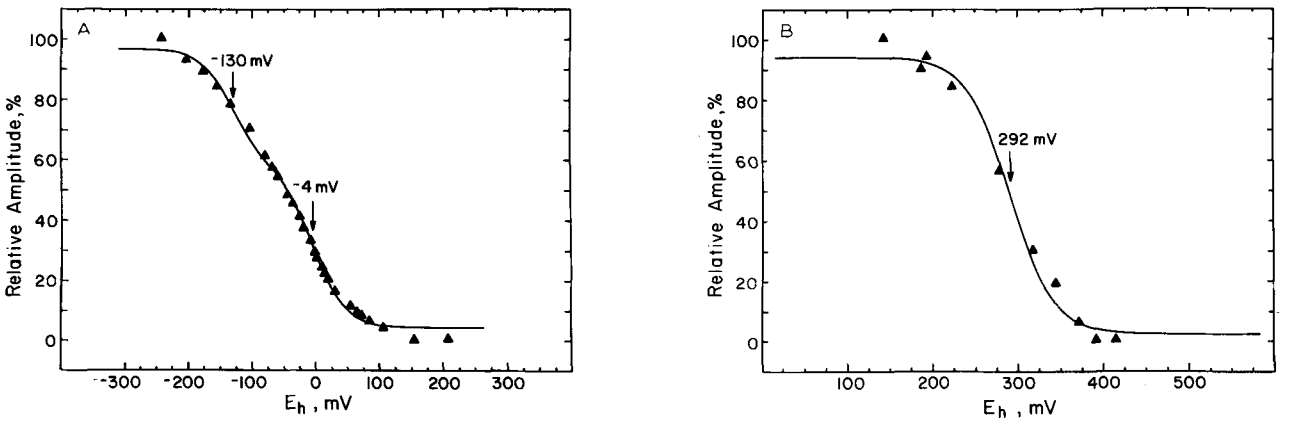

Fig. 4. Oxidation-reduction titrations of *R. rubrum* cytochrome bc_1 complex hemes. (A) Cytochromes b_H and b_L. The reaction mixture contained the *R. rubrum* cytochrome bc_1 complex at a concentration equivalent to 2 μM cytochrome c_1 in 35 mM MOPS buffer (pH 7.4) containing 100 mM NaCl and 1 mM $MgSO_4$ and the following redox mediators: 20 μM phenazine methosulfate; 20 μM phenazine ethosulfate, 20 μM phenazine; 20 μM pyocyanine; 25 μM benzoquinone; 25 μM 1,2-naphthoquinone; 25 μM 1,4-naphthoquinone; 50 μM duroquinone; 70 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine and 15 μM 2-hydroxy-1,4-naphthoquinone. Absorbance changes were monitored at 562 minus 577 nm and the data plotted to give the best fit to the Nernst equation for two $n = 1$ components. (B) Cytochrome c_1. Conditions were as in 4A except that observation and reference wavelengths of 552 and 540 nm, respectively, were used and the data fitted to a single $n = 1$ component.

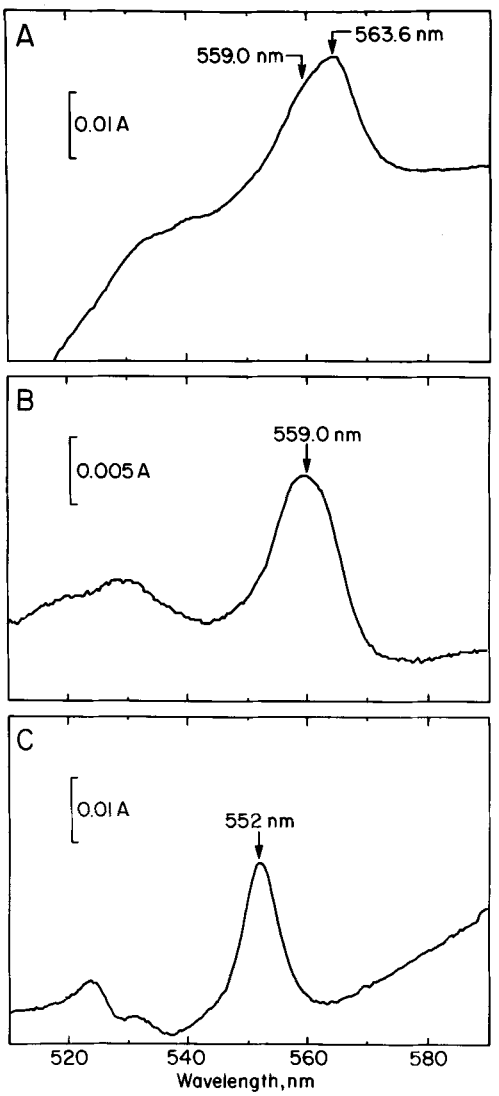

Fig. 5. Reduced minus oxidized difference spectra for the cytochrome components of the *R. rubrum* cytochrome bc_1 complex. Reaction conditions were as in Fig. 4. (A) Cytochrome b_L; (B) cytochrome b_H; (C) cytochrome c_1.

that had been stored frozen in 20% glycerol at 77 K. Thus, storage conditions for the complex had little or no effect on the E_m value of cytochrome c_1. The E_m value obtained for cytochrome c_1 in samples of the *R. rubrum* complex that had been stored unfrozen is similar to values obtained for cytochrome c_1 in detergent-solubilized complexes isolated from other photosynthetic bacteria [8,12,17] and the value measured for cytochrome c_1 in intact *R. rubrum* membranes [46]. Both the E_m values obtained in this study with samples that had been stored unfrozen and those that had been stored frozen were slightly less positive than the +320 mV value previously obtained in our laboratory using electrochemical methods. The reasons for this discrepancy are not known.

Fig. 5A and B shows reduced minus oxidized difference spectra for cytochromes b_L and b_H, respectively, obtained during an oxidation-reduction titration of a sample of the *R. rubrum* complex that had been stored unfrozen. A spectrum recorded at an ambient potential of −79 mV was subtracted from one recorded at −244 mV to produce the difference spectrum of cytochrome b_L in Fig. 5A, which shows an asymmetric α-band with a maximum at 563 to 564 nm and a shoulder at 559 nm. The reduced minus oxidized difference spectrum obtained for the cytochrome b_L component of the solubilized *R. rubrum* cytochrome bc_1 complex differs from those reported for cytochrome b_L in the complexes isolated from *Rb. capsulatus* and *Rb. sphaeroides,* which exhibit a split α-band with two maxima located at 565 to 566 nm and 558 nm, respectively (Refs. 8, 12, 47 and Robertson, D.E., unpublished observations). In intact *R. rubrum* membranes the component with $E_m = -40$ mV that is likely to be cytochrome b_L has been demonstrated to have a reduced-minus-oxidized difference spectrum similar to that of Fig. 5A, with an α-band maximum at 564 nm and a shoulder at 558 nm [46]. It is thus possible that *R. rubrum* cytochrome b_L has an α-band reduced minus oxidized difference spectrum that differs somewhat from those of this component in other photosynthetic bacteria and does not exhibit two well-separated maxima in the α-band region. The β-band region in the difference spectrum of *R. rubrum* cytochrome b_L exhibits a broad maximum centered at 522 nm. Reduced-minus-oxidized difference spectra for cytochrome b_L obtained using *R. rubrum* cytochrome bc_1 complex samples that had been stored frozen were similar to that of Fig. 5A except that the α-band was slightly broader.

The reduced-minus-oxidized difference spectrum of cytochrome b_H (Fig. 5B), obtained by subtracting a spectrum recorded at an ambient potential of +134 mV from one recorded at $E_h = -4$ mV, shows a single, symmetric α-band centered at 559 ± 1.0 nm, a value similar to that observed for the α-band absorbance maxima of cytochrome b_H in other cytochrome bc_1 complexes [8,12,47] and to that observed for the component detected in intact *R. rubrum* membranes that is likely to be cytochrome b_H [46]. The β-band maximum in the cytochrome b_H difference spectrum is centered at 520 nm. Reduced-minus-oxidized difference spectra for cytochrome b_H, obtained using samples that had been stored frozen, exhibited an α-band centered at 560 nm and the band was somewhat broader than that shown in Fig. 5B.

Fig. 5C shows the reduced-minus-oxidized difference spectrum in the α- and β-band regions for cytochrome c_1 in the solubilized *R. rubrum* complex. This difference spectrum was obtained by subtracting a spectrum, measured during the course of one of the oxidation-reduction titrations described above, recorded at an ambient potential of +392 mV for one recorded at $E_h = +187$ mV. The α-band maximum, located at 552 ± 1.0 nm, is

identical to that previously observed in ascorbate-reduced-minus-ferricyanide-oxidized difference spectra of the complex [14,15]. The *c*-type cytochrome with $E_m = +260$ mV, likely to be cytochrome c_1, that has been detected in intact *R. rubrum* membranes exhibits an α-band maximum at 553 nm [46], a value that is identical (within the experimental uncertainties) to that observed for cytochrome c_1 in the solubilized complex. The reduced-minus-oxidized difference spectrum contains maxima at 514 and 521 in the β-band region. Storage conditions for the complex had no detectable effect on the reduced-minus-oxidized difference spectrum of cytochrome c_1. The shape of the difference spectrum shown in Fig. 5C is also essentially identical to that of the previously reported ascorbate-reduced-minus-ferricyanide-oxidized complex [14,15], indicating that cytochrome c_1 is the only ascorbate-reducible cytochrome in the solubilized *R. rubrum* complex.

One important property of cytochrome bc_1 complexes in situ is the ability to couple electron flow from quinol to cytochrome *c* to proton translocation across the membrane in which the complex is located [1–4]. It has proven possible to demonstrate this property with several detergent-solubilized, purified cytochrome bc_1 complexes, including the beef-heart mitochondrial complex [48] and the three subunit complex isolated from the non-photosynthetic bacterium *Paracoccus denitrificans* [49], after incorporation of the complexes into liposomes. As mentioned above, it is at least possible in principle that the *R. rubrum* cytochrome bc_1 complex contains four subunits in situ and the fourth subunit is lost during purification. If the putative fourth subunit were to play some role in 'vectorial' proton translocation by the complex, then the purified complex would not be expected to couple electron flow to proton translocation. It was thus of interest to determine whether the *R. rubrum* complex, after incorporation into liposomes, was capable of proton translocation. Before attempting such measurements with the *R. rubrum* complex, a series of control experiments were conducted using liposomes containing the beef-heart cytochrome bc_1 complex. This system had previously been shown to translocate protons, with the ratio of protons translocated to electrons transferred (H^+/e^-) approaching 2.0 [48].

Fig. 6A shows the results of a typical proton ejection that occurs when liposomes containing beef-heart cytochrome bc_1 complex catalyze electron flow from Q_2H_2 to cytochrome *c* (cytochrome *c*, originally present in the reduced form, is oxidized by the addition of potassium ferricyanide to initiate the electron flow from Q_2H_2 through the complex). After pH equilibrium was reached, the uncoupler CCCP was added to render the liposome membranes freely permeable to protons and a second aliquot of ferricyanide added. The protons released under these conditions are the 'scalar' protons released by the oxidation of Q_2H_2, as no contribution from the accumulation of vectorially translocated protons is possible in the presence of CCCP. The ratio of the pH changes produced by the addition of equal amounts of ferricyanide, in the absence and presence of CCCP, was taken as a measure of the H^+/e^- ratio for electron flow through the beef-heart complex. Ratios between 1.6 and 2.0 were routinely observed with liposomes containing the beef-heart complex. The average value of 1.8 is equal, within the experimental uncertainties of the measurements, to the value of 2.0 predicted by the Q-cycle model for electron flow [2–4].

Fig. 6B shows the results of a similar proton translocation experiment, conducted with liposomes containing the *R. rubrum* cytochrome bc_1 instead of the beef heart complex. The results are qualitatively similar to those shown in Fig. 6A, indicating that the solubilized, purified *R. rubrum* complex is capable of proton transloca-

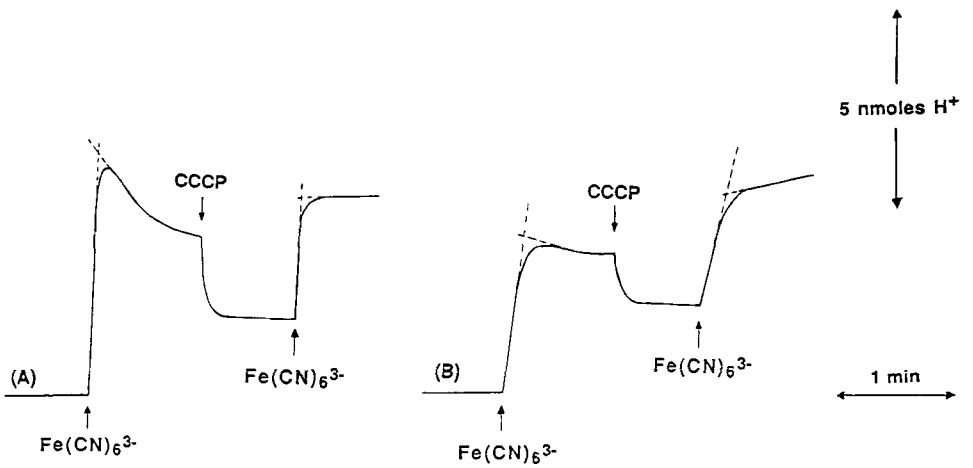

Fig. 6. Proton translocation by liposomes containing cytochrome bc_1 complexes. Reaction conditions were as described in Materials and Methods. The final liposome concentrations were equivalent to 0.4 mg protein/ml. (A) Liposomes containing the beef-heart complex; (B) liposomes containing the *R. rubrum* complex.

tion, but differ quantitatively from those obtained with liposomes containing the beef-heart cytochrome bc_1 complex. In particular, the H^+/e^- ratios obtained with liposomes containing the *R. rubrum* complex ranged from 1.3 to 1.6, indicating a lower efficiency of coupling between proton translocation and electron flow than was observed with liposomes containing the beef heart complex. Removal of the glycerol in which the *R. rubrum* complex was stored by precipitating the complex with ammonium sulfate prior to incorporation of the complex into the liposomes did not increase the H^+/e^- ratio observed for the *R. rubrum* complex. Likewise, initiating the reaction by adding Q_2H_2 to a reaction mixture containing ferricyanide and cytochrome *c* rather than using the reverse sequence did not increase the H^+/e^- ratio observed for the *R. rubrum* complex. In fact, the H^+/e^- ratios observed were consistently lower for both liposomes containing the *R. rubrum* complex and those containing the beef heart complex when electron flow was initiated by the addition of Q_2H_2 compared to those observed when electron flow was initiated by the addition of ferricyanide. The liposomes containing the *R. rubrum* cytochrome bc_1 complex also exhibited 'respiratory control'. The rate of electron flow from quinol to cytochrome *c* observed in continuous electron flow assays [14] was approximately 3-fold higher in the presence of the uncoupler CCCP than that in the absence of an uncoupler (data not shown).

One other difference in the properties of the two types of liposomes, in addition to the difference in H^+/e^- ratios, should be mentioned. As can be seen in Fig. 6A, the initial proton ejection observed with liposomes containing the beef-heart complex is followed by a slow proton uptake, presumably due to some leakage of protons back across the liposome membrane. This proton uptake was typically slower and less extensive with liposomes containing the *R. rubrum* complex (Fig. 6B) and in some cases no proton uptake was observed (data not shown). The reasons for this difference in apparent proton leakage between the two types of liposome are not known.

Discussion

Evidence presented above indicates that the prosthetic groups of the *R. rubrum* cytochrome bc_1 complex have oxidation-reduction characteristics very similar to those observed with the cytochrome bc_1 complexes isolated from other photosynthetic bacteria. Storage conditions for the complex have effects on both the E_m values and spectral properties of the cytochromes in the complex and on its activity. The cytochrome *b* and c_1 subunits of the *R. rubrum* complex are recognized by antibodies raised against the corresponding subunits of the *Rb. capsulatus* and *Rb. sphaeroides* complexes. These results, combined with the earlier observation of antigenic similarities between the Rieske iron-sulfur proteins of *R. rubrum* and *Rb. capsulatus* [15], no doubt reflect the structural similarities between the complexes of these three photosynthetic bacteria that could be expected from the amino acid sequence homologies that have been found in their three prosthetic group-containing subunits (Ref. 16 and Shanker, S., Daldal, F., Moomaw, C., Güner, S., Knaff, D.B. and Harman, J.G., unpublished results).

Results presented above clearly indicate that the solubilized, purified three subunit *R. rubrum* cytochrome bc_1 complex is sensitive to four specific inhibitors of these complexes at inhibitor concentrations similar to those necessary for inhibition of complexes in other prokaryotic species and in eukaryotes. Thus, if the *R. rubrum* complex were to contain a fourth, 12–14 kDa subunit in situ, this subunit would appear to play no role in binding the inhibitors antimycin A, myxothiazol, stigmatellin and UHDBT. The fact that one can observe the antimycin A-induced red shift in the α-band of cytochrome b_H and stigmatellin-induced changes in the E_m value and EPR *g*-values of the Rieske iron-sulfur cluster with the isolated *R. rubrum* complex further suggest that a fourth subunit is not required for normal binding of these quinone analogs.

It has also been clearly established in this study that the solubilized, three subunit *R. rubrum* cytochrome bc_1 complex, after incorporation into liposomes, is capable of coupling electron flow to the translocation of protons across a membrane and exhibits respiratory control. Thus the presence of a fourth, 12–14 kDa subunit is not an absolute requirement for proton translocation by the complex. However, the maximum H^+/e^- ratio observed was 1.6, significantly lower than the 2.0 value predicted by the Q-cycle [2–4] and the average H^+/e^- value of 1.5 was lower than the average value of 1.8 observed for liposomes containing the beef-heart complex. It should be mentioned that kinetic studies conducted with *R. rubrum* chromatophores support the likely operation of a Q-cycle pathway during light-driven cyclic electron flow [50]. The reason for suboptimal proton translocation by liposomes reconstituted with the *R. rubrum* complex is not clear, but it cannot be attributed to the presence of different liposome populations with opposite orientations of the cytochrome bc_1 complex with respect to the lipid membrane. As the electron acceptor used in the assay, cytochrome *c*, cannot cross the lipid membrane of the liposome, only those liposomes containing *R. rubrum* complex with cytochrome c/c_2-binding site facing out towards the external medium will be active in electron transfer and proton translocation. The ferricyanide anion, due to its substantial negative charge, would also be expected to be unable to cross the liposome membrane. Liposomes, if any are present, containing cytochrome bc_1 complexes

with orientations other than that resulting in proton ejection, cannot produce proton translocation in the opposite direction that would diminish the H^+/e^- ratio, as such liposomes will not catalyze electron flow due to the absence of an accessible electron acceptor.

In summary, it seems clear that the three peptide subunit *R. rubrum* cytochrome bc_1 complex is capable of catalyzing electron flow from quinol to cytochrome c/c_2 at high rates and that the complex exhibits high affinity, 'normal' binding of four specific, quinone analog inhibitors of cytochrome bc_1 complexes. Previous observations of the interaction of a ubiquinone photoaffinity analog with the *R. rubrum* complex also indicated that a fourth subunit is not required for binding of the analog to cytochrome *b* [15]. Thus neither quinol : cytochrome c/c_2 activity of the cytochrome bc_1 nor the interaction of quinone with the complex appears to require any components other than the three prosthetic group-containing subunits of the complex in *R. rubrum*. A similar situation appears to be true for the *Rb. capsulatus* cytochrome bc_1 complex (Robertson, D.E. and Daldal, F., unpublished observations).

The three subunit *R. rubrum* complex is also capable of coupling electron transfer to proton translocation, albeit with a H^+/e^- ratio less than the value of 2.0 predicted by Q-cycle models [2–4] and observed for the beef heart (see Ref. 48 and Fig. 6A) and *Rb. sphaeroides* complexes (Yu, L. and Yu, C.-A., unpublished observations). Thus it is possible that, if the *R. rubrum* complex does contain a fourth subunit in situ, then this subunit could perhaps play some role in optimal proton translocation. It is also possible that the fourth subunit, if one exists, is involved in some still unrecognized function. However, such speculations must await the appearance of evidence for the existence of such a subunit in *R. rubrum*. At the present, there is no evidence for the presence of a fourth subunit in the *R. rubrum* cytochrome bc_1 complex, nor does evidence exist for the presence of more than three subunits in the corresponding complex isolated from *Rps. viridis* [5,14,17]. The situation for the complex isolated from *Rb. capsulatus* is less clear. Earlier reports [1,9], based on the appearance of four protein-staining bands after electrophoresis under denaturing conditions, concluded that four discrete subunits were present in the *Rb. capsulatus* complex. Recent evidence, based on recognition by monoclonal antibodies and on peptide sequencing, has revealed that the isolated *Rb. capsulatus* complex, in fact, contains only three subunits (Robertson, D.E. and Daldal, F., unpublished observations; also see Ref. 5 for a discussion). Should the *R. rubrum, Rps. viridis* and *Rb. capsulatus* cytochrome bc_1 complexes turn out to contain only three peptide subunits in situ and the fourth subunit found associated with preparations of the detergent-solubilized *Rb. sphaeroides* complex [9,10,12,13] prove to be a bona fide constituent of the complex and not a fortuitous contaminant, an interesting case of species differences in this energy-transducing electron transfer complex will exist in closely related bacteria.

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