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The ubiquinol:cytochrome c_2/c oxidoreductase of *Chromatium vinosum*

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Chromatophore membranes isolated from the photosynthetic purple sulfur bacterium *Chromatium vinosum* exhibit a quinol:cytochrome $c_2(c)$ oxidoreductase activity that is sensitive to two specific inhibitors of cytochrome bc_1 complexes, antimycin A and myxothiazol. Digests of *C. vinosum* DNA hybridize to probes constructed from portions of the *pet*(*fbc*) operons that code for the cytochrome bc_1 complexes of the photosynthetic purple non-sulfur bacteria *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*. Despite the fact that it has not yet proven possible to isolate a detergent-solubilized, purified cytochrome bc_1 complex from *C. vinosum*, these new results, when combined with spectroscopic and kinetic data available from the literature, indicate that this purple sulfur bacterium contains a cytochrome bc_1 complex similar in structure to the well-characterized complexes found in purple non-sulfur bacteria. Equine cytochrome *c* and cytochromes c_2 isolated from the purple non-sulfur bacteria *Rhodospirillum rubrum* and *Rhodopseudomonas viridis* all function as effective electron acceptors from the *C. vinosum* cytochrome bc_1 complex. In contrast, HiPIP (high-potential iron protein) isolated from two purple sulfur bacteria, *C. vinosum* and *Chromatium tepidum*, are reduced only at low rates by quinol in the presence of *C. vinosum* membranes and their reduction is not inhibited by either antimycin A or myxothiazol. These observations support the hypothesis that a protein structurally related to cytochrome c_2, rather than HiPIP, is the physiological electron acceptor for the *C. vinosum* cytochrome bc_1 complex. Although it has not yet proven possible to purify the putative *C. vinosum* cytochrome c_2 to homogeneity, we report here that digests of *C. vinosum* DNA hybridize with probes constructed from portions of the *cycA* genes coding for cytochromes c_2 from *Rb. capsulatus*, *Rps. viridis* and *Rb. sphaeroides*. These DNA hybridization results provide further support for the hypothesis that a cytochrome c_2-like protein is present in *C. vinosum*.

Introduction

Cytochrome bc_1 complexes have been isolated and purified to homogeneity from four photosynthetic purple non-sulfur bacteria: *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum* and *Rhodopseudomonas viridis* (See Ref. 1 for a recent review). The role of these complexes in vivo, under conditions of photosynthetic growth, is to catalyze electron transfer from ubiquinol to cytochrome c_2 during light-driven cyclic electron flow and couple this electron transfer to the creation of an electrochemical proton gradient across the bacterial membrane [1–6]. The genes encoding the three electron-carrying subunits of the cytochrome bc_1 complexes in these four bacteria are located in similarly arranged operons (referred to as either *pet* or *fbc*) and the operons have all been cloned and sequenced [1,7]. In contrast, no cytochrome bc_1 complex has yet been purified from any photosynthetic purple sulfur bacterium nor is anything known about the arrangement of *pet* genes in this family of bacteria. Electron paramagnetic resonance (EPR) spectra of chromatophores (membrane vesicles) isolated from the purple sulfur bacterium *Chromatium vinosum* contain features characteristic of the Rieske iron-sulfur protein, one of the constituents of all cytochrome bc_1 complexes [8,9] and evidence exists that the membranes of this bacterium also contain cytochrome c_1 [10,11]. *C. vinosum* membranes also contain a *b*-type cytochrome with properties similar to those expected for a component of a cytochrome bc_1 complex [11–13]. Furthermore, kinetic studies [14,15] and the effects of specific inhibitors of cytochrome bc_1 complexes on the absorbance spectrum [16] and photoreduction [12–16] of *C. vinosum* cytochrome *b* suggest

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Abbreviations: BChl, bacteriochlorophyll; DBH, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzohydroquinone; SSC, 0.015 M sodium citrate buffer (pH 7.0) containing 0.15 M NaCl.

that this bacterium has a cytochrome bc_1 complex that functions in a manner similar to the cytochrome bc_1 complexes present in purple non-sulfur bacteria.

In purple non-sulfur bacteria, the acceptor of electrons from the cytochrome bc_1 complex is known to be the periplasmically-located soluble cytochrome c_2 [1–7]. Evidence obtained with intact cells [17] and spheroplasts [18] of *C. vinosum* suggests that a soluble *c*-type cytochrome, structurally similar to cytochrome c_2, functions as an electron carrier during light-driven cyclic electron flow in this photosynthetic purple sulfur bacterium. A soluble *c*-type cytochrome has been detected in *C. vinosum* homogenates and purified sufficiently to establish that it has an absorbance spectrum, molecular mass and oxidation-reduction midpoint potential (E_m) similar to those of cytochromes c_2 isolated from a number of purple non-sulfur bacteria [19,20]. However, the *C. vinosum* cytochrome could not be purified to homogeneity and thus it was not possible to obtain amino acid sequence data establishing that the cytochrome was in fact a member of the cytochrome c_2 family [19,20].

The absence of a completely purified cytochrome c_2, isolated from *C. vinosum*, has raised some doubts as to whether this bacterium actually contains such a cytochrome [21]. The many similarities between photosynthesis in purple sulfur and purple non-sulfur bacteria [5] made it appear unlikely to us that *C. vinosum* would not utilize a protein related to cytochrome c_2 as the mobile electron carrier linking the cytochrome bc_1 complex and the reaction center. However, if *C. vinosum* does in fact lack a cytochrome c_2, it would be important to identify the electron carrier in this purple sulfur bacterium that plays the role filled by cytochrome c_2 in purple non-sulfur bacteria. *C. vinosum* does contain large amounts of a soluble iron-sulfur protein known as HiPIP, which has an oxidation-reduction midpoint potential (E_m) value similar to those of cytochromes c_2 [21,22]. It has recently been suggested that in bacteria, such as *C. vinosum*, which contain a tetraheme subunit in the reaction center, HiPIP, rather than cytochrome c_2, may function as the mobile, high potential electron carrier in the cyclic electron transport chain [23]. Below, we present evidence that *C. vinosum* contains both a cytochrome bc_1 complex and a cytochrome c_2 similar to those found in purple non-sulfur bacteria and that, as is the case in purple non-sulfur bacteria, the *C. vinosum* cytochrome bc_1 complex uses cytochrome c_2 and not HiPIP as the electron acceptor.

Materials and Methods

C. vinosum cells were grown and chromatophores isolated as described previously [12]. In order to render the closed chromatophore membranes permeable to protein electron acceptors, the chromatophores (at a final bacteriochlorophyll *a* (BChl *a*) concentration of 500 μM) were suspended in 20 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 0.1% (w/v) dodecyl β-D-maltoside and incubated for 1 h at 4°C. After centrifugation for 30 min at 17000 rpm in a Beckman JA-20 rotor, the supernatant was collected and used for the cytochrome bc_1 activity assays.

Rps. viridis cytochrome c_2 was prepared as described previously [24]. *R. rubrum* cytochrome c_2 was prepared by a modification of the procedure of Bartsch [25]. The bacteriochlorophyll-free soluble portion of a *R. rubrum* cell homogenate in 2 mM Tris-HCl buffer (pH 8.0) was chromatographed on a DEAE-cellulose (Whatman DE-52) anion-exchange column (2.5 × 30 cm) which had been equilibrated with this buffer. After washing the column with 700 ml of this buffer and 700 ml of 10 mM Tris-HCl buffer (pH 8.0), the cytochrome was eluted with 30 mM Tris-HCl buffer (pH 8.0) and concentrated using an Amicon ultrafiltration apparatus with a YM-5 membrane. The concentrated sample was then chromatographed on a Sephadex G-75 gel filtration column (2.5 × 120 cm) in 50 mM Tris-HCl buffer (pH 8.0) and concentrated by ultrafiltration. Both cytochromes c_2 were at least 95% pure as judged by absorbance spectra and by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS) using a Pharmacia Phast electrophoresis system and Pharmacia 10–15% Phast gradient gels (gels were stained for protein with Coomassie brilliant blue). The cytochromes c_2 and equine cytochrome (Sigma Type VI) *c* were stored at −20°C in 25 mM potassium phosphate buffer (pH 7.0). Immediately before cytochrome bc_1 activity assays were performed, the cytochromes were oxidized with a slight excess of potassium ferricyanide and the excess ferricyanide was removed by chromatography on a Dowex 1 anion-exchange column (1.5 × 5 cm) equilibrated with 25 mM potassium phosphate buffer (pH 7.0). *C. vinosum* HiPIP was prepared according to the method of Bartsch [22]. *Chromatium tepidum* cells (a gift from Prof. R.E. Blankenship) were disrupted by sonication and *C. tepidum* HiPIP purified from the soluble fraction of the *C. tepidum* homogenate using the same procedure used for the *C. vinosum* HiPIP. The absorbance spectra of both oxidized and reduced *C. tepidum* HiPIP in the visible and near ultraviolet regions are very similar to those of *C. vinosum* HiPIP. Both HiPIP's were at least 95% pure, as judged by absorbance spectra and polyacrylamide gel electrophoresis in the presence of SDS. The HiPIP's were stored and oxidized as described above for the cytochromes *c* and c_2.

Absorbance spectra were measured using Shimadzu Model UV-2100 and UV-265 spectrophotometers. BChl *a* concentrations were determined after extraction into 7:2 (v/v) acetone/methanol according to the proce-

dure of Clayton [26]. Protein was measured according to the procedure of Lowry et al. [27] using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of SDS was performed on a Pharmacia Phast system according to protocols supplied by the manufacturer.

The cytochrome bc_1 activity assays were conducted using a 1.0 cm optical pathlength with 1.0 ml reaction mixtures that contained detergent-treated *C. vinosum* membranes (equivalent to 0.84 μM BChl for cytochrome c_2/c reduction and 2.5 μM BChl for HiPIP reduction) and 100 μM DBH in 1 mM sodium phosphate buffer (pH 7.0). A concentration of 100 μM DBH, as the electron donor, was shown to be saturating for the rate measurements. The ionic strengths of the reaction mixtures were adjusted by additions of NaCl. The reduction of equine cytochrome *c* and *R. rubrum* cytochrome c_2 were monitored by following the increase in absorbance at 550 nm, using reduced *minus* oxidized $\Delta\epsilon$ values of 21.1 $mM^{-1}cm^{-1}$ [28] and 22.0 $mM^{-1}cm^{-1}$ [29], respectively. Reduction of *Rps. viridis* cytochrome c_2 was monitored by following the increase in absorbance at 550.5 nm, using a reduced *minus* oxidized $\Delta\epsilon$ of 19.6 $mM^{-1}cm^{-1}$ [24]. The reduction of both *C. vinosum* and *C. tepidum* HiPIP's were followed by monitoring the decrease in absorbance at 480 nm, using an oxidized *minus* reduced $\Delta\epsilon$ of 10 $mM^{-1}cm^{-1}$ [22]. The electron acceptor and the detergent-treated membranes were added initially and allowed to incubate for 2 min and the reaction was then started by the addition of DBH. Control experiments, in which no detergent-treated membranes were added, were run to determine whether any direct reduction of the electron acceptor by DBH occurred and this rate, if not zero, was subtracted from the rate measured in the presence of the membranes to arrive at the catalyzed rates reported below. In reactions in which inhibitors were used, the inhibitors were added in small volumes from concentrated stock solutions in ethanol prior to the initiation of the reaction by addition of DBH. Control experiments, in which ethanol alone was added, demonstrated that, at the concentrations used, ethanol itself had no inhibitory effect.

C. vinosum chromosomal DNA was isolated from 1 g (wet weight) of cells which had been washed in doubly distilled water, collected by centrifugation at 4000 rpm in a Beckman JA-20 rotor and resuspended in 10 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl and 1 mM EDTA. The DNA was isolated according to the procedures of Ausubel et al. [30]. Contaminating RNA was removed by treatment with RNAase after the precipitated DNA was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA [31]. The *C. vinosum* DNA was digested with a series of restriction enzymes following protocols supplied by the vendors.

Epicurian coli cells (XL-1 Blue, Stratagene) were either purchased competent or were made competent [32] and then transformed with plasmids containing the *cycA* genes encoding either *Rb. capsulatus* cytochrome c_2 [33], *Rb. sphaeroides* cytochrome c_2 [34] or *Rps. viridis* cytochrome c_2 [35] according to the protocol supplied by Stratagene and were grown in Luria-Bertani (LB) medium in the presence of ampicillin (50 μg/ml) at 37°C. Similar transformations were carried out with plasmids p17-4 and p14-3 [36] containing the genes for *Rb. capsulatus* cytochromes *b* and c_1 (*petB* and *petC*) and the gene for the *Rb. capsulatus* Rieske iron-sulfur protein (*petA*), respectively, and the transformed cells were grown under conditions similar to those described above for *E. coli* cells transformed with *cycA*-containing plasmids. Plasmid DNA was isolated using a QIAGEN isolation kit following the protocol supplied by the manufacturer. The *Rps. viridis cycA* probe used for Southern blots was constructed by labeling a 477 bp fragment, prepared by excising the gene from the plasmid pUC-NA477 [35] by digestion with *Eco*RI/*Hin*dIII, with digoxigenin using the Boehringer Mannheim Genius non-radioactive labeling kit according to the protocol supplied by the vendor. The *Rb. sphaeroides* and *Rb. capsulatus cycA* probes were prepared by similar labeling of a 411 bp *Bam*HI fragment excised from the plasmid pC2PS19 [34,37] and a 211 bp fragment excised from the plasmid pBc_2 [33] by digestion with *Eco*RI/*Sma*I, respectively. The *Rb. capsulatus petA* and *petBC* probes were prepared by similar non-radioactive labeling of a 521 bp fragment of the plasmid p14-3 [36] excised by digestion with *Eco*RI/*Bst*EII and a 1041 bp fragment of the plasmid p17-4 [36] excised by digestion with *Nae*I, respectively. The *Rb. sphaeroides petBC* probe, which contained all of the *petC* gene and most of the *petB* gene, was prepared by labeling a 1.71 kb *Sma*I fragment of the plasmid pBC9 [38]. The *R. rubrum petC* probe was prepared by labeling a 875 bp *Eco*RV/*Pst*I fragment of the plasmid pBS5 [39]. All DNA used for labeling was size-selected by electrophoresis in Tris/Borate/EDTA (TBE) or Tris/acetate/EDTA (TAE) buffers [40] on agarose gels (Promega) in a Fotodyne electrophoresis apparatus and then isolated using either Geneclean II or Mermaid kits (Bio101). Ethidium bromide (0.5 μg/ml) was used for DNA visualization and *Bst*EII-digested or *Hin*dIII-digested λ DNA were used as molecular mass standards.

Southern blotting of genomic *C. vinosum* DNA digests was carried out according to the procedure of Sambrook et al. [41] using Nytran membranes (Schleicher & Schuell). Hybridization was carried out using digoxigenin-labeled, denatured probes in 5 × SSC solution after prehybridization for 2 h in 5 × SCC solution containing 1% blocking reagent (Boehringer-Mannheim), 0.1% *N*-laurylsarcosine and 0.02% SDS.

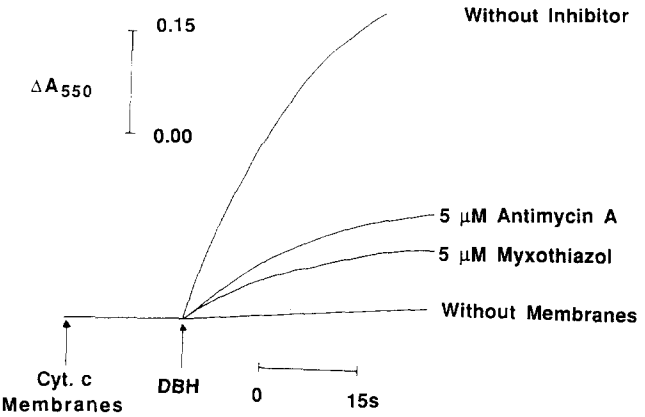

Fig. 1. The reduction of equine cytochrome *c* catalyzed by detergent-treated *C. vinosum* membranes. The reaction mixture was as described in Materials and Methods, with cytochrome *c* present at a concentration of 20 μM.

Details of the washing and hybridization conditions are given in the figure legends below. Immunological detection of the probes was carried out using the Boehringer-Mannheim Genius kit following the protocol supplied by the vendor.

Results

Fig. 1 shows typical time-courses for the reduction of equine cytochrome *c* by DBH, catalyzed by detergent-treated *C. vinosum* membranes, in the absence and presence of inhibitors of cytochrome bc_1 complexes. For comparison, an experiment is shown in which no detergent-treated *C. vinosum* membranes were included, demonstrating that little uncatalyzed direct reduction of the cytochrome by DBH occurs. Both antimycin A and myxothiazol result in significant inhibition of the catalyzed reaction. Similar results were obtained using *R. rubrum* and *Rps. viridis* cytochromes c_2 as electron acceptors (Table I). Fig. 2 shows that, in contrast to the results obtained with these three *c*-type cytochromes as electron acceptors, *C. vinosum* HiPIP is a relatively poor acceptor of electrons from DBH in the reaction catalyzed by detergent-solubilized *C. vinosum* membranes (note that the concentration of detergent-treated *C. vinosum* membranes used for the experiment of Fig. 2 was 3-fold higher than that used for the experiment of Fig. 1 and the concentration of HiPIP was 2.5-fold higher than that of cytochrome *c*), although there is a substantial uncatalyzed, direct reduction of HiPIP by DBH. Furthermore, neither antimycin A nor myxothiazol, two specific inhibitors of cytochrome bc_1 complexes [1–6], produced any inhibition of HiPIP reduction (Table I), suggesting that the component present in *C. vinosum* membranes responsible for catalyzing the slow reduction of HiPIP illustrated in Fig. 2 is not the cytochrome bc_1 complex. Results very similar to those illustrated in Fig. 2 were obtained using HiPIP isolated from *C. tepidum* (Table I).

TABLE I

The effect of two electron transfer inhibitors on the reduction of different acceptors catalyzed by detergent-treated C. vinosum membranes

The assays were carried out as described under Materials and Methods. 100% corresponds to initial rates of 3.04 for the reduction of equine cytochrome *c*, 1.66 for the reduction of *R. rubrum* cytochrome c_2, 1.25 for the reduction of *Rps. viridis* cytochrome c_2, 0.167 for the reduction of *C. tepidum* HiPIP and 0.50 for the reduction of *C. vinosum* HiPIP. The units for all of the rates are mmol acceptor reduced(g protein)$^{-1}$s^{-1}. All assays were conducted in 10 mM sodium phosphate buffer (pH 7.0), except for the equine cytochrome *c* assay, which was conducted in 1 mM sodium phosphate buffer (pH 7.0). Both inhibitors were present at concentrations of 5 μM.

Electron acceptor	Initial reduction rate (%)	
	+ antimycin A	+ myxothiazol
Equine cytochrome *c*	36	27
R. rubrum cyt. c_2	42	19
Rps. viridis cyt.c_2	22	29
C. tepidum HiPIP	100	100
C. vinosum HiPIP	100	100

Considerable evidence exists for the hypothesis that the cytochrome bc_1 complex-catalyzed reduction of cytochrome c_2 in photosynthetic purple non-sulfur bacteria involves electrostatic interactions between positive charges on lysine residues surrounding the heme of cytochrome c_2 and negatively charged groups on cytochrome c_1 [42–46]. In order to determine whether similar electrostatic interactions may occur between the cytochrome bc_1 complex of *C. vinosum* and its

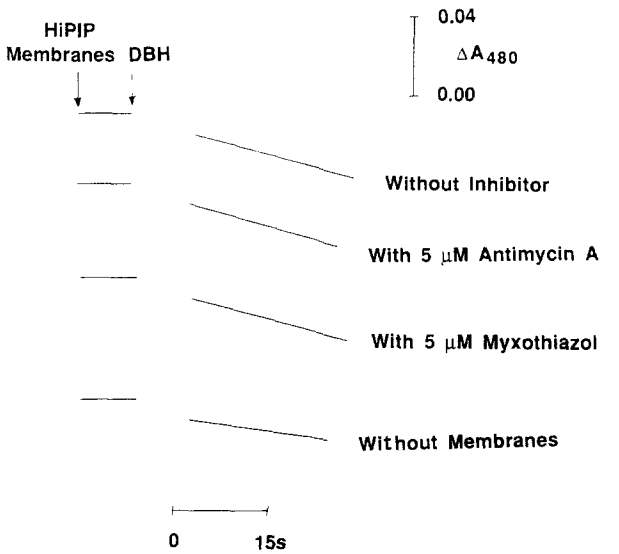

Fig. 2. The reduction of *C. vinosum* HiPIP catalyzed by detergent-treated *C. vinosum* membranes. The reaction mixture was as described in Materials and Methods, with HiPIP present at a concentration of 50 μM.

TABLE II

Michaelis constants (K_m) and maximal velocities (V_{max}) for the reduction of equine cytochrome c, R. rubrum cytochrome c_2 and Rps. viridis cytochrome c_2 catalyzed by detergent-treated C. vinosum membranes

The V_{max} values are in μmol cytochrome c (c_2) reduced(g protein)$^{-1}$ s^{-1}, the K_m values are in μM and the ionic strengths are in mM. The assays were conducted as described under Materials and Methods using 10 mM sodium phosphate buffer pH (7.0) and the ionic strength was adjusted by addition of NaCl.

Electron acceptor	Ionic strength	K_m	V_{max}
Equine cytochrome *c*	40	8.2	4.3
Equine cytochrome *c*	70	14.2	3.4
R. rubrum cyt. c_2	40	50.6	5.9
Rps. viridis cyt. c_2	40	31.1	3.1
Rps. viridis cyt. c_2	60	53.7	3.1

electron acceptor, the effects of ionic strength on the kinetic parameters for the reductions of two possible model acceptors, equine cytochrome *c* and *Rps. viridis* cytochrome c_2, catalyzed by detergent-treated *C. vinosum* membranes were investigated. The reduction of these two cytochromes, and of *R. rubrum* cytochrome c_2, catalyzed by the *C. vinosum* membranes all obeyed Michaelis-Menten kinetics, with K_m values (at 40 mM ionic strength) ranging from 8.3 μM for equine cytochrome *c* to 50.6 μM for *R. rubrum* cytochrome c_2 (Table II). As can be seen from the results summarized in Table II, the K_m values for both *c*-type cytochromes tested increase significantly when the ionic strength was increased to 70 mM, but there was little or no effect of ionic strength on V_{max}. These results, which are similar to those previously obtained with the cytochrome bc_1 complexes of the photosynthetic purple non-sulfur bacteria *R. rubrum*, *Rb. sphaeroides* and *Rb. capsulatus* [43–46], are consistent with electrostatic interactions playing a role in the reduction of cytochromes *c* and c_2 by the *C. vinosum* cytochrome bc_1 complex. The rates for the reduction of *C. vinosum* and *C. tepidum* HiPIP's catalyzed by the detergent-treated *C. vinosum* membranes were not affected by ionic strength [47].

The observations, reported above, that the cytochrome bc_1 complex of a purple sulfur bacterium displays inhibitor sensitivity and a mode of interaction with its electron acceptor similar to those observed in photosynthetic purple non-sulfur bacteria, when added to previous spectroscopic and kinetic studies on the prosthetic groups present in the membranes of *C. vinosum* (see above), suggested the possibility that structural similarities could exist at the molecular level between the cytochrome bc_1 complexes in these two families of purple photosynthetic bacteria. To test this hypothesis, we have carried out Southern hybridization experiments between digests of *C. vinosum* DNA and probes containing portions of the *pet* operon coding for the cytochrome subunits of the cytochrome bc_1 complex of the photosynthetic purple non-sulfur bacterium *Rb. capsulatus* [36]. Fig. 3 shows that a probe constructed from the *Rb. capsulatus petBC* genes, coding for cytochromes *b* and c_1 in this bacterium, hybridizes with a 3.1 kb DNA fragment prepared by digesting *C. vinosum* chromosomal DNA with *Eco*RI and with 2.6 and 2.1 kb fragments prepared by digesting *C. vinosum* chromosomal DNA with *Eco*RI *plus Bam*HI. Positive hybridization results were also obtained using digests of *C. vinosum* DNA and the *Rb. capsulatus petA* probe and with probes constructed from portions of the *pet* operons of two other photosynthetic purple bacteria, *Rb. sphaeroides* and *R. rubrum*. The 521 bp probe constructed from the *Rb. capsulatus petA* gene, coding for the Rieske iron-sulfur protein in this bacterium, hybridizes with a 2.0 kb fragment of DNA prepared by digesting *C. vinosum* chromosomal DNA with *Bam*HI and with 1.8 and 0.97 kb DNA fragments prepared by digesting *C. vinosum* chromosomal DNA with *Eco*RI *plus Bam*HI. The 1.71 kb *Rb. sphaeroides petBC* probe described above hybridized with 7.8 and 3.0 kb DNA fragments obtained

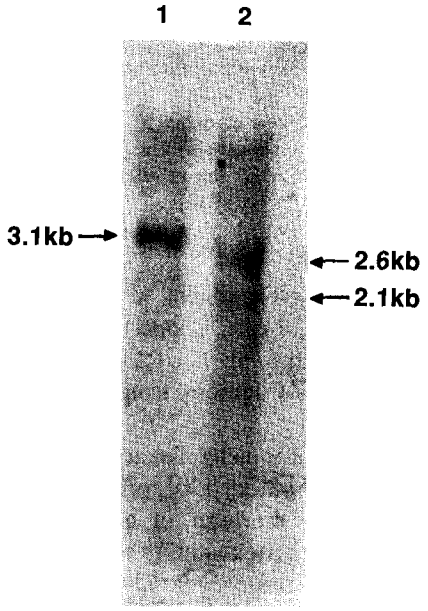

Fig. 3. Southern blot analysis of *C. vinosum* genomic DNA digests with a probe constructed from the *Rb. capsulatus petBC* genes. The probe used was the *petBC* probe coding for *Rb. capsulatus* cytochromes *b* and c_1, described in Materials and Methods. Electrophoresis of the *C. vinosum* digests was conducted using a 1% agarose gel. Hybridization was performed at 62°C at a probe concentration of 35 ng/ml. After the hybridization, the membrane was washed twice for 5 min with 100 ml of 2×SSC solution containing 0.1% SDS at room temperature and then twice for 15 min with 100 ml of 0.1×SSC solution containing 0.1% SDS at62°C. Approx. 20 μg of *C. vinosum* DNA was present in each well. Lanes 1 and 2 represent *C. vinosum* DNA digested with *Eco*RI and *Eco*RI plus *Bam*HI, respectively. The sizes of the hybridizing bands, given in kb, were determined by comparison to the migration distance of λ DNA standards.

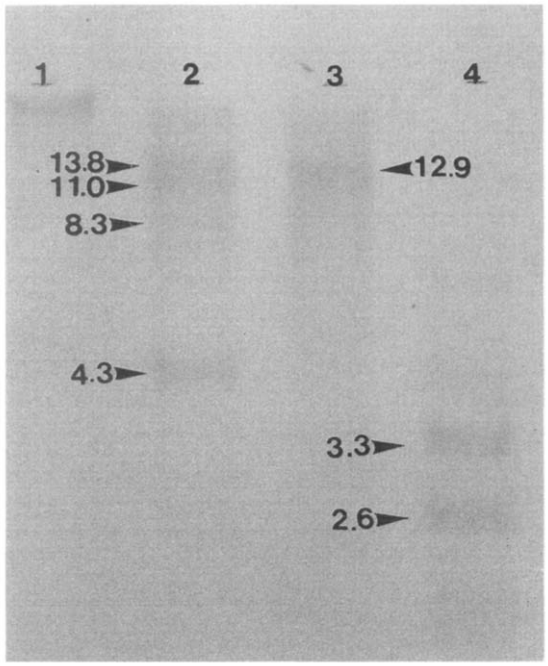

Fig. 4. Southern blot analysis of *C. vinosum* genomic DNA digests with a probe constructed from the *cycA* gene of *Rps. viridis*. The hybridization was carried out at 57°C in 2×SSPE buffer containing 0.1% *N*-laurylsulfate, 0.01% SDS, at a probe concentration of 50 ng/ml. Two 15 min posthybridization washes were carried out at 57°C with 2×SSPE buffer, followed by a 30 min wash at the same temperature with 2×SSPE buffer containing 0.1% SDS. The undigested *C. vinosum* DNA (lane 1) and *C. vinosum* DNA digested with *Eco*RI (lane 2), *Bam*HI (lane 3) or *Sal*I (lane 4) were subjected to electrophoresis. The sizes of the hybridizing bands, given in kb, were determined by comparison to the migration distance of l DNA standards.

from a *Eco*RI/*Bam*HI digest of *C. vinosum* DNA. The 875 bp *R. rubrum petC* probe hybridized with a 1.7 kb DNA fragment from an *Eco*RI digest of *C. vinosum* DNA and a 1.6 kb fragment from a *Eco*RI/*Bam*HI digest.

Previous investigations by Takamiya and co-workers [19] and in our laboratory [20], provided evidence for the presence in *C. vinosum* of a soluble *c*-type cytochrome with properties similar to those of the well-characterized cytochrome c_2 family. The new evidence, presented above, that two cytochromes c_2 and the related equine cytochrome *c* can serve as effective electron acceptors from the *C. vinosum* cytochrome bc_1 complex, made it seem useful to search for additional evidence for the presence of a cytochrome c_2-like protein in *C. vinosum*. Fig. 4 shows the results of Southern blots of *C. vinosum* DNA digested with restriction enzymes and hybridized with a probe constructed from the *Rps. viridis cycA* gene. *Eco*RI digestion produced *C. vinosum* DNA fragments of 13.8, 11.0, 8.3 and 4.3 kb that hybridized with the *Rps. viridis cycA* gene probe; *Bam*HI digestion produced a single 12.9 kb fragment of *C. vinosum* DNA that hybridized with the probe and *Sal*I produced fragments of 3.3 and 2.6 kb that hybridized with the probe. Positive hybridization results were also obtained from similar experiments using probes constructed from portions of the *Rb. sphaeroides* and *Rb. capsulatus cycA* genes (data not shown). In hybridizations conducted at 60°C, a large number of *C. vinosum* DNA fragments produced by digestion with *Eco*RI, *Hin*dIII, *Bam*HI, *Nae*I, *Sal*I or *Sma*I and ranging in size from 0.55 to 8.1 kb, were found to hybridize with the *Rb. capsulatus cycA* probe. In hybridizations conducted at 68°C, hybridization to the *Rb. capsulatus cycA* probe was observed only with a 3.0 kb fragment of *Eco*RI-digested *C. vinosum* DNA and a 2.0 kb fragment of *Sma*I-digested *C. vinosum* DNA. In hybridizations conducted at 60°C, a large number of *C. vinosum* DNA fragments produced by digestion with *Eco*RI, *Sal*I, *Eco*RI plus *Bam*HI and *Eco*RI plus *Sal*I, ranging in size from 0.93 to 6.0 kb, were found to hybridize with the *Rb. sphaeroides cycA* probe.

Discussion

Southern blot hybridizations using heterologous *cycA* gene probes from three different photosynthetic purple non-sulfur bacteria, *Rb. capsulatus*, *Rb. sphaeroides* and *Rps. viridis*, have established that *C. vinosum* genomic DNA contains sequences homologous to those present in the three *cycA* genes. The fact that these hybridizations could be observed at high stringencies strongly suggests that *C. vinosum* does contain a gene coding for a protein homologous to the well-characterized cytochromes c_2 of purple non-sulfur bacteria. These results strengthen the earlier conclusion, based on partial purification and characterization of a soluble *C. vinosum c*-type cytochrome, that this photosynthetic purple sulfur bacterium contains a cytochrome c_2 [18–20]. Southern blots of *C. vinosum* DNA digests using probes constructed from portions of the *pet* operons, coding for components of the cytochrome bc_1 complex, of three different photosynthetic purple non-sulfur bacteria (*Rb. capsulatus*, *Rb. sphaeroides* and *R. rubrum*) reenforce the earlier conclusion, based on spectroscopic and kinetic measurements (see above) that *C. vinosum*, a photosynthetic purple sulfur bacterium, contains a cytochrome bc_1 complex similar in structure to that found in photosynthetic purple non-sulfur bacteria. Efforts to clone and sequence *C. vinosum cycA* and *pet* genes and to explore the operon organization, if any, of the *C. vinosum pet* genes are currently underway in our laboratory.

The data presented in Fig. 1 and in Tables I and II represent the first demonstration that *C. vinosum* membranes can catalyze electron transfer from a ubiquinol analog to cytochrome c_2/c. At first glance, it

may appear that the rates of this reaction are low, compared to those reported for photosynthetic purple non-sulfur bacteria. However, *C. vinosum* membranes contain much less cytochrome bc_1 complex and greater amounts of antenna BChl than purple non-sulfur bacteria [15]. These two factors limit the rates that can be obtained at membrane concentrations low enough so that BChl absorbance does not seriously diminish instrument signal/noise ratios. It should also be pointed out that the rates given in this communication for the *C. vinosum* complex are reported on a per cytochrome c_1 basis, as is usual in describing the activities of the bacterial complexes. As it is difficult to accurately estimate the amount of cytochrome c_1 or cytochrome *b* present in *C. vinosum* chromatophores [10,15], the true specific rates could be significantly higher than those reported if the amount of cytochrome c_1 has been overestimated. It may also be possible that the treatment of *C. vinosum* chromatophores (which are sealed membrane vesicles) with the low concentration of detergent we have used to render the membranes permeable to small proteins does not provide full access for the cytochromes to the site on the inside face of the chromatophore membrane where they are reduced. Finally, it should be pointed out that equine cytochrome *c* and the cytochromes c_2 used as electron acceptors in this study are likely homologs of the putative *C. vinosum* cytochrome c_2, rather than the true physiological acceptor and that this may further lower the observed rates compared to those obtainable if *C. vinosum* cytochrome c_2 itself were available as an electron acceptor for the assays. Nevertheless, the observation that membranes isolated from *C. vinosum* are able to catalyze cytochrome c/c_2 reduction that is sensitive to two highly specific inhibitors of cytochrome bc_1 complexes provides further support for the hypothesis that a cytochrome of the cytochrome c_2 family serves as the physiological acceptor of electrons from the *C. vinosum* cytochrome bc_1 complex in vivo. In contrast, our inability to observe any inhibitor-sensitive reduction of HiPIP's isolated from two different *Chromatium* species by the *C. vinosum* cytochrome bc_1 complex argues strongly against a role for HiPIP in light-driven cyclic electron flow in this photosynthetic purple sulfur bacterium.

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