Isolation and Characterization of Soluble Electron Transfer Proteins from *Chromatium purpuratum*†

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ABSTRACT: Several soluble electron transfer proteins were isolated and characterized from the marine purple-sulfur bacterium *Chromatium purpuratum*. The *C. purpuratum* flavocytochrome *c* is similar in molecular mass (68 kDa) and isoelectric point (6.5) to flavocytochromes isolated from other phototrophs. Redox titrations of the flavocytochrome *c* hemes show two components with midpoint potential values of +15 and −120 mV, behavior similar to that observed with the flavocytochrome isolated from the thermophilic *Chromatium tepidum.* Moreover, N-terminal amino acid sequence analysis of both the flavin and the cytochrome subunit indicates substantial homology to the primary structure of the flavocytochrome *c* of *Chromatium vinosum*. In contrast, the *C. purpuratum* high-potential iron−sulfur protein (HiPIP) differs from those isolated from other photosynthetic bacteria in its relatively high midpoint potential (+390 mV) and the possibility that it exists as a dimer in solution. Two low molecular mass *c*-type cytochromes were also characterized. One appears to be a high-potential (+310 mV) c_8-type cytochrome. Amino acid sequencing suggests that the second cytochrome may be a homologue of the low-potential cytochrome *c*-551, previously described in two species of Ectothiorhodospirillaceae.

Purple phototrophic bacteria transform light energy into a transmembrane electrochemical gradient using light-driven cyclic electron flow involving both mobile electron carriers and two multisubunit, integral-membrane protein complexes: the photosynthetic reaction center (PRC)[1] and the cytochrome bc_1 complex. This potential is used for reduction of NAD^+, production of ATP, active transport, and motility (Drews & Imhoff, 1991). The phototrophic eubacteria are taxonomically subdivided based on the source of electrons utilized in photosynthesis. Purple bacteria that oxidize reduced sulfur compounds comprise the Chromatiaceae and Ectothiorhodospirillaceae, in contrast to the purple non-sulfur bacteria, the Rhodospirillaceae. Comparison of the membrane-bound components of the photosynthetic apparatus of both purple-non-sulfur and purple-sulfur bacteria indicates that they are quite similar in the two groups of organisms. However, the soluble electron transfer proteins functioning in photosynthesis in purple-sulfur bacteria are less well characterized. In most purple-non-sulfur bacteria, cyclic electron transfer between the membrane-bound complexes in photosynthesis is mediated by a soluble, high-potential c_2-type cytochrome located in the periplasmic space [summarized in Bartsch (1991)]. In contrast, although some evidence from DNA hybridization with heterologous probes does exist for the possible presence of a c_2-type cytochrome in *C. vinosum* (Tan et al., 1993), c_2-type cytochromes have not been definitively identified at the protein level in the purple-sulfur bacteria. Instead, a high-potential cytochrome c_8 (formerly designated *c*-551; Ambler, 1991) has been suggested to be the donor to the photooxidized PRC (Bartsch, 1991; Meyer & Donohue, 1995). A second soluble electron transfer protein found in all species of *Chromatium* characterized thus far, the high-potential iron−sulfur protein (HiPIP), has recently been shown to reduce the PRC in two species of purple-non-sulfur bacteria (Hochkoeppler et al., 1995a,b; Schoepp et al., 1995).

Chromatium purpuratum is an anaerobic, marine species of photosynthetic purple-sulfur bacteria. It is the only marine species of the Chromatiaceae from which the photosynthetic apparatus has been extensively characterized (Kerfeld et al., 1994a,b). The PRC of this organism has a subunit composition similar to those of other *Chromatium* species, including a tetraheme cytochrome containing two high-potential and two low-potential hemes *c* (Kerfeld, Robertson, and Knaff, unpublished results). As part of the characterization of photosynthetic electron transfer in *C. purpuratum*, we have purified and characterized several soluble redox proteins. This investigation of the electron transfer components from a marine species of *Chromatium* also provides preliminary data for understanding specific characteristics of proteins adapted for a moderately halophilic environment through

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[1] Abbreviations: E_m, oxidation−reduction midpoint potential; HiPIP, high-potential iron−sulfur protein; HPLC-SEC, size-exclusion high-performance liquid chromatography; pI, isoelectric point; PRC, photoreaction center.

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comparison to the homologous proteins characterized from the mesophile *Chromatium vinosum* and the thermophile *Chromatium tepidum*. Furthermore, soluble redox proteins are one of several molecular markers that have proven useful for the classification of photosynthetic bacteria and for gaining insight into the evolution of photosynthetic function (Dickerson, 1980).

MATERIALS AND METHODS

(A) Cell Growth and Protein Preparation. Cells of *C. purpuratum* obtained from 152 L of culture (yielding 300 g wet weight) were fractionated into a membrane and soluble fraction as previously described (Kerfeld et al., 1994a). The soluble fraction (with an OD_{280} of 0.888) was filtered to remove particulates and dialyzed against 20 mM Tris-HCl, pH 8.0; 350 mL was adsorbed to a Pharmacia XK26/20 column packed with 80 mL of Whatman DE-52 DEAE-cellulose anion exchange resin (Fisher Scientific, Tustin, CA) and equilibrated in 20 mM Tris-HCl, pH 8.0, containing 0.01% NaN_3 (buffer A) at a flow rate of 5.5 mL/min. After washing with 1 column volume of buffer A, a linear gradient to 50% buffer B (buffer A containing 1 M NaCl) was used to elute the proteins. The ionic strength of the eluting buffer was held constant during the elution of very abundant proteins (e.g., HiPIP). The eluent was monitored at 280 nm, and 1.2 mL fractions were collected. After separately pooling fractions corresponding to a high-potential cytochrome, HiPIP, and flavocytochrome *c*, each pool was concentrated and dialyzed in a ProDiCon apparatus (Spectrum Medical Industries, Houston, TX) against 50 mM MES, pH 6.5, using 10 kDa MW cutoff membranes. Small volumes (<3 mL) were concentrated by centrifugation in Centricon cells (Amicon, Beverly, MA). A TSK 3000 HPLC size-exclusion column (Novex Co., San Diego, CA) equilibrated in 25 mM sodium phosphate, pH 7.0, 100 mM sodium sulfate, with 0.03% sodium azide was used for HPLC size-exclusion chromatography (HPLC-SEC). Elution was monitored at 280 nm. Chromatography fractions were assayed for the presence of heme-containing polypeptides by a "dot blot": 1 μL from of each fraction was spotted onto nitrocellulose that had been pretreated in Tris-HCl, pH 8.0, and allowed to dry; the nitrocellulose was then stained for heme using the method of Thomas et al. (1976). Pools were analyzed for protein composition by SDS−PAGE with the PHAST System (Pharmacia, Uppsala, Sweden) and stained for protein with Coomassie Brilliant Blue or for heme (Thomas et al., 1976). SDS−PAGE low molecular weight markers were obtained from Bio-Rad (Hercules, CA). Isoelectric focusing was also performed using the PHAST System.

(B) Spectroscopy and Analytical Procedures. Absorbance spectra were recorded on Shimadzu UV 160 and UV2100 spectrophotometers (Cole Scientific, Moorpark, CA) at a spectral resolution of ±1 nm. The nature of the heme prosthetic group was determined by the method of Drabkin (1942). Protein concentration was determined by the Bradford method (Bio-Rad), using bovine serum albumin as a standard.

(C) Ion Spray Mass Spectroscopy. HiPIP was prepared for mass spectroscopy by dissolving crystals in deionized water and dialyzing/concentrating the protein against 5 volumes of deionized water in Microcon 3 concentration cells (Amicon Co.). Cytochrome c_8 purified by HPLC was prepared for mass spectroscopy in a similar fashion. Ion spray mass spectroscopy data were recorded at the UCLA Center for Molecular and Medical Sciences Spectroscopy.

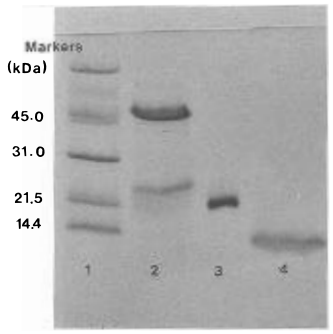

FIGURE 1: Coomassie-stained SDS−PAGE (20%) of *C. purpuratum* flavocytochrome *c* (lane 2) and HiPIP (lanes 3 and 4); HiPIP was boiled in SDS sample buffer for 3 min prior to loading lane 4.

(D) Amino Acid Sequencing. Cytochrome c_8 and the flavocytochrome subunits were isolated by Tricine−SDS−PAGE (Schägger & Von Jagow, 1987). The proteins were transferred onto Immobilon (Millipore, Bedford, MA), and sequencing was carried out either at Texas Tech University (using automated Edman degradation on a Porton Model 2020 protein sequencer) or at the Microsequencing Facility at UCLA. Purified cytochrome c_8 was also sequenced from solution after exhaustive dialysis against 25 mM sodium carbonate buffer. HiPIP was sequenced from solution after a similar preparative procedure.

(E) Oxidation−Reduction Titrations. Oxidation−reduction midpoint potentials (E_m) were determined at 4 °C in a 0.3 mm optical path length cell containing an optically-transparent gold electrode by the spectrochemical technique described previously (Hirasawa et al., 1994), using the criteria for the selection of oxidation−reduction mediators described by Dutton (1971). The data for the HiPIP and cytochrome c_8 titrations were fitted to the Nernst Equation for a single one-electron carrier using Cricket Graph. The data for the flavocytochrome *c* titrations were fitted to multiple $n = 1$ Nernst equations as described previously (Garcia-Castillo et al., 1994a).

(F) Electron Transfer Assays. Assays for the flavocytochrome *c*-catalyzed electron transport from sulfide to either equine cytochrome *c*, or *C. purpuratum* HiPIP, or cytochrome c_8 were carried out essentially as described for the *C. vinosum* flavocytochrome *c*-catalyzed electron transfer from sulfide to equine cytochrome *c* (Gray & Knaff, 1982).

RESULTS

(A) Flavocytochrome c. Flavocytochrome *c* eluted from the DE-52 column at a concentration of 210 mM sodium chloride. A native molecular mass of 85 kDa was estimated from the HPLC-SEC purification step. SDS−PAGE (Figure 1) resolved the protein into 2 subunits of 45 and 23 kDa. The smaller subunit stains for heme. Isoelectric focusing indicates that the oxidized protein has a p*I* of 6.5 (data not shown). The absorption spectrum of the reduced protein exhibits maxima at 552 nm (α), 523 nm (β), and 421 nm (γ) as shown in Figure 2. Alkaline pyridine hemochrome analysis indicates the presence of only heme *c* and allowed estimation of a value of 22.7 mM^{-1} cm^{-1} per heme for the extinction coefficient at 552 nm of the dithionite-reduced flavocytochrome *c*. Assuming a molecular mass of 68 kDa (i.e., that the protein contains one heme-containing subunit

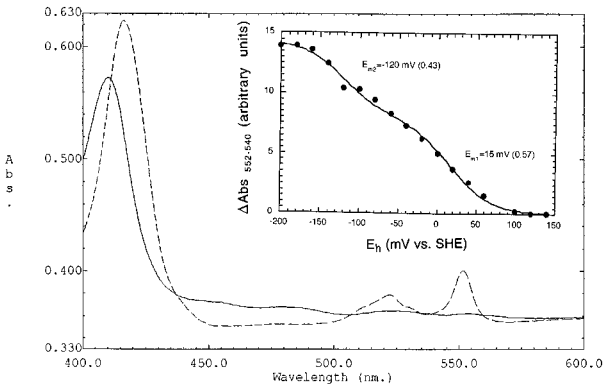

FIGURE 2: Ultraviolet−visible absorbance spectrum of *C. purpuratum* flavocytochrome *c* in 25 mM MES, pH 6.4. The solid line shows the spectrum of a sample oxidized with ferricyanide, and the dashed line shows the spectrum of a sample reduced with dithionite. Inset: Oxidation−reduction titration of *C. purpuratum* flavocytochrome *c* obtained by monitoring $\Delta A_{552-540nm}$. The solid line represents the best fit of the data to the sum of the Nernst equations for two ($n = 1$) components. Mediators: 10 μM duroquinone ($E_m = +50$ mV) and 10 μM 2-hydroxy-1,4-naphthoquinone ($E_m = -135$ mV). Buffer: 10 mM potassium phosphate, pH 8.0. Protein concentration: 7.25 μM. Temperature: 4 °C.

and one flavin-containing subunit and that the molecular masses of the subunits are accurately determined by SDS−PAGE), the molar ratio of heme/protein is 1.7 ± 0.2. This calculation, and the similarity of the size of the heme-containing subunit of *C. purpuratum* to the cytochrome subunit of other *Chromatium* flavocytochromes *c*, suggests there are two hemes per cytochrome subunit. The oxidized protein displays a charge-transfer band with a maximum at 696 nm (data not shown), indicative of a methionine axial ligation to at least one of the two proposed heme prosthetic groups. Similar charge transfer bands were observed with the flavocytochromes isolated from *C. vinosum* (Meyer et al., 1991; Garcia-Castillo et al., 1994a) and *C. tepidum* (Garcia-Castillo et al., 1994b).

Attempts to fit the data obtained from oxidation−reduction titrations of the *C. purpuratum* flavocytochrome *c*, using the absorbance in the α-band region to monitor the oxidation state of the heme, to a single $n = 1$ component were unsuccessful. However, as shown in the inset of Figure 2, the data give an excellent fit to two $n = 1$ components with E_m values of +15 mV and −120 mV, respectively, at pH 8.0 (Figure 2). The two components contribute 57% and 43% of the total absorbance change at 552 nm *minus* 540 nm, respectively. The presence of two components with different E_m values was observed previously in oxidation−reduction titrations of the hemes of the *C. tepidum* flavocytochrome *c* (Garcia-Castillo et al., 1994a).

The flavin subunit of the *C. purpuratum* flavocytochrome *c* is visible as a yellow band during SDS−PAGE and on Immobilon after electroblotting, suggesting that flavin cofactor, like the hemes, is covalently bound as it is in the *C. vinosum* flavocytochrome *c* (Chen et al., 1994). N-Terminal amino acid sequences for the flavin and the cytochrome subunits are shown in Table 1.

In contrast to the flavocytochromes *c* isolated from *C. vinosum* (Fukumori & Yamanaka, 1979; Gray & Knaff, 1982) and *C. tepidum* (Garcia-Castillo et al., 1994), which can catalyze electron transfer from sulfide to a number of soluble high-potential electron acceptor proteins, *C. purpuratum* flavocytochrome-mediated catalysis of electron transfer from sulfide to either *C. purpuratum* HiPIP or *C. purpuratum* cytochrome c_8 or equine cytochrome *c* could not be detected (data not shown).

Table 1: N-Terminal Amino Acid Sequences of *C. purpuratum* Redox Proteins and Homologs

A.) Flavocytochrome Cytochrome Subunit

*C. purpuratum**	GDATP**EML**ANAX**AGCHGT**E?N**S**M?PAAP?I
C. vinosum[a]	EPTA**EML**TNNC**AGCHGT**HGN**S**VGPASPSI
Chlorobium[b]	APEQSKSIPRG**EIL**SLSC**AGCHGT**DGK**S**ESIIPTIY

B.) Flavocytochrome Flavin Subunit

C. purpuratum	ASHR**VVVVGGGT**?**GA**TA**AKY**LKRA**D**
C. vinosum[a]	AGRK**VVVVGGGT**G**GA**TA**AKY**IKLA**D**
Chlorobium[b]	GTKR**VVVVGGG**FG**GA**ST**AKY**LRKL**D**

C.) High Potential Iron Sulfur Protein (HiPIP)

C. purpuratum	EV**PANAV**TED**DPTA**V
C. gracile[c]	EV**PANAV**TES**DPTA**V
C. vinosum[d]	SA**PANAV**AAD**DATA**I

D.) Cytochrome c8[+]

*C. purpuratum**	SPE**L**AQQS**GC**LN**CH**QXRDKIF**GP**AYRDVA
C. vinosum[e]	DLV**L**AQKS**GC**TV**CH**SVEAAIY**GP**AYADVAK
P. aeruginosa[f]	EDPEV**L**FKNK**GC**VA**CH**AIDTKMV**GP**AYKDVAAKF

E.) Low Potential Cytochrome c-551

*C. purpuratum**	MR**IY**VGN-L**P**YX**S**VX**H----**TD?**EL**R?FAGYF
E. halophila[g]	DGES**IY**INGTA**P**TC**S**SC**H**DRGVAGAP**EL**NAPEDW

[a] The Cys which covalently binds the heme prosthetic group appeared as a blank cycle (X) in the corresponding chromatogram. (?) denotes an ambiguous amino acid identification. Alignments by FASTA and BESTFIT of the GCG Sequence Analysis Software Package (Sneath & Sokal, 1973). Amino acids shown in boldface type are identical in all of the sequences shown. [b] Van Beeumen et al. (1991). [c] Van Beeumen et al. (1990). [d] Tedro et al. (1981). [e] Dus et al. (1973). [f] The designation of cytochrome c_8 is that of Ambler (1991); formerly known as cytochrome *c*-551. [g] Samyn et al. (1996). [h] Dickerson (1980). [i] Ambler et al. (1993).

(B) High-Potential Iron−Sulfur Protein (HiPIP). HiPIP was the most abundant protein present in the *C. purpuratum* soluble fraction. It elutes relatively early from the anion exchange column (130 mM NaCl), despite its acidic isoelectric point: 4.3 (reduced), 4.0 (oxidized; data not shown). Ion spray mass spectroscopy and silver-stained SDS−PAGE both indicated that the protein preparation was pure and homogeneous. The purity index (A_{283}/A_{388}) for the reduced protein is 2.71. The sequence of the first 15 amino acids of the *C. purpuratum* HiPIP, obtained by automated Edman degradation, is shown in Table 1.

The monomeric molecular mass of *C. purpuratum* HiPIP was determined by ion spray mass spectroscopy to be 9372 Da. Evidence has been obtained suggesting the possibility that the *C. purpuratum* HiPIP may be unusual in that it appears to behave as a dimer in solution. Its apparent molecular mass, as determined by gel filtration using either HPLC (TSK3000 column) or FPLC Superose 12 and 6 columns linked in series, is 17 kDa, approximately twice the value determined for a monomer by mass spectroscopy. In both chromatographic systems, care was taken to minimize the possibility of protein binding to the chromatographic matrix (i.e., buffers of ionic composition were used that

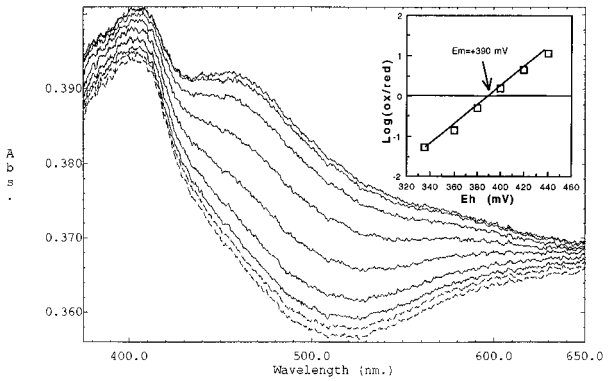

FIGURE 3: Spectra of *C. purpuratum* HiPIP obtained during an oxidation−reduction titration. The spectra, in descending order, were obtained at ambient potentials of 460, 440, 420, 400, 380, 360, 340, 320, and 280 mV, respectively. The inset shows the best fit of the data to the Nernst equation for a single ($n = 1$) component, with the extent of HiPIP oxidation measured by monitoring $\Delta A_{405-520nm}$. Mediators: 10 μM 1,1′-dimethylferrocene ($E_m = +341$ mV), 10 μM DAD ($E_m = +300$ mV), and 10 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine ($E_m = +260$ mV). Buffer: 10 mM Tris-HCl, pH 8.0. Protein concentration: 10 μM. Temperature: 4 °C.

equaled or exceeded manufacturers' recommendations). The molecular mass was estimated by comparison of the elution volume for HiPIP to those for *Pseudomonas aeruginosa* cytochrome *c*-551, horse heart cytochrome *c*, and BioRad gel filtration standards (Kerfeld et al., in press and manuscript in preparation). *C. purpuratum* HiPIP was completely retained by a 10 kDa cutoff Centricon ultrafiltration membrane. In contrast, *Pseudomonas aeruginosa* cytochrome *c*-551 (a protein with essentially the same monomeric molecular mass as *C. purpuratum* HiPIP) was not retained to a significant extent by the same ultrafiltration membrane. Moreover, *C. purpuratum* HiPIP migrates with an apparent molecular mass of 20 kDa (essentially the mass expected for a dimer) during SDS−PAGE in the presence of β-mercaptoethanol, unless the sample is boiled prior to loading. Similar migration during SDS−PAGE has been observed for the *Thiocapsa pfennigii* HiPIP, but in this case, the anomalously high apparent mass was attributed to aggregation through disulfide exchange (Meyer et al., 1973).

Figure 3 shows spectra of the *C. purpuratum* HiPIP obtained during the course of an oxidation−reduction titration of the protein. The spectra of the oxidized and reduced forms of the protein and the oxidized *minus* reduced spectrum are similar to those of HiPIP's isolated from other photosynthetic bacteria. Data obtained from oxidation−reduction titrations (see the inset to Figure 3) gave a good fit to the Nernst equation for a single $n = 1$ component with a midpoint potential of +390 mV, one of the highest among HiPIP's characterized to date.

(C) High-Potential Cytochrome c_8 and Putative Low-Potential Cytochrome c-551. The only *c*-type cytochrome to elute in the reduced state from the anion exchange column was contained in a broad peak that precedes HiPIP in DE-52 chromatography (eluting at 13−15 mM NaCl). Fractions corresponding to this peak were pooled and dialyzed against 25 mM MES, pH 6.4. SEC-HPLC further resolved this fraction into three peaks with apparent molecular masses of 29.8 and 15.8 kDa (HPLC-A) and 7.5 kDa (HPLC-B; Figure 4). All three peaks stained positively for heme-dependent peroxidase activity, with HPLC-B staining most intensely. The 29.8 kDa pool stained for heme only very faintly and was not investigated further.

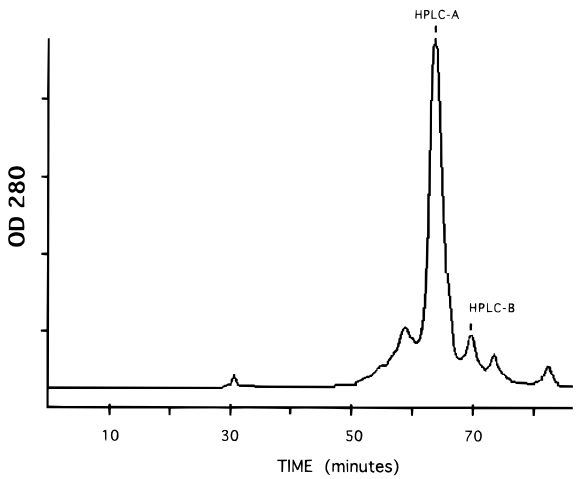

FIGURE 4: Separation of the DE-52 fraction containing the high-potential cytochrome into HPLC-A and HPLC-B fractions.

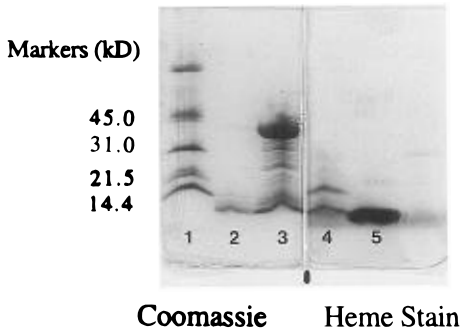

FIGURE 5: 10−15% SDS−PAGE of HPLC-A (lanes 2 and 5), HPLC-B (lanes 3 and 4), and molecular mass markers (lane 1). Lanes 1−3 are stained with Coomassie Blue; lanes 4 and 5 are stained for heme.

When examined by SDS−PAGE (Figure 5), HPLC-A shows two heme-staining proteins with apparent molecular masses of 12 and 18 kDa and an additional component with an apparent molecular mass of 43 kDa that does not stain for heme. The amino acid sequence of the 12 kDa protein shows some homology to small low-potential *c*-type cytochromes *c*-551 from Ectothiorhodospirillaceae species (Table 1). Amino acid sequencing of the 18 kDa band indicated a mixture of this putative low potential *c*-type cytochrome sequence and trace amounts of a second protein that no longer sequenced after the eighth cycle of Edman degradation. Amino acid sequencing of the 43 kDa protein indicated the presence of three equally abundant forms of a single protein that differed only in the amount of proteolysis at the N-terminus. Its anomalous migration on SDS−PAGE (i.e., as a larger protein than expected from its mass estimated by HPLC-SEC) is perhaps due to aggregation. The longest sequence obtained for this component, 28 residues, is not significantly homologous to any in the sequence data base (data not shown). IEF-PAGE indicates that the p*I*s of the three proteins found in the HPLC-A fraction are between 5 and 6 (data not shown).

SDS−PAGE analysis of HPLC-B indicates a single band of molecular mass of approximately 12 kDa that stains for heme (Figure 5). However, mass spectroscopy analysis indicates the presence of two equally abundant proteins of 9088 and 9107 Da and trace amounts of a contaminating protein of 9297 Da. Amino acid sequencing of this fraction from solution also indicates the presence of two equally abundant proteins. One of the sequences is identical to that of the putative low-potential cytochrome *c*-551 (sequenced

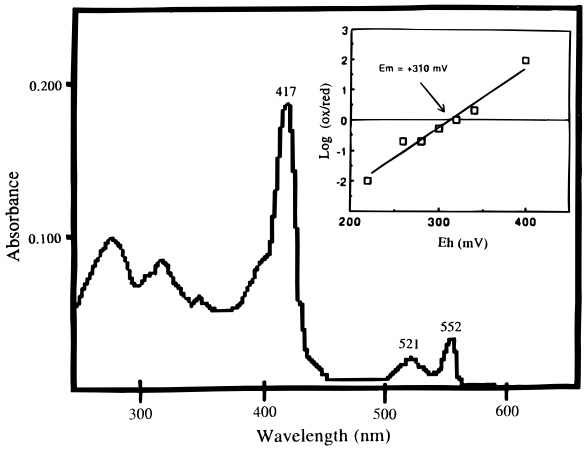

FIGURE 6: UV−Visible absorption spectrum of HPLC-B. Inset: Oxidation−reduction titration of *C. purpuratum* cytochrome c_8 obtained by monitoring $\Delta A_{552-537nm}$. Mediators: 10 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD, $E_m = +300$ mV) and 10 μM *N*-methylphenazinemethosulfate ($E_m = +80$ mV). Buffer: 10 mM potassium phosphate, pH 7.5. Protein concentration: 5.0 μM. Temperature: 4 °C.

from HPLC-A). After subtraction of this sequence, the remaining amino acid sequence indicates the second cytochrome is related to c_8-type cytochromes (Table 1). The amino acid sequencing of HPLC-B was repeated from solution and from an Immobilon blot in order to verify the amino acid identities. Isoelectric focusing of the HPLC-B fraction also reveals the presence of two proteins, one of which is more intensely stained by Coomassie Blue than the other. The reduced form of this component has a p*I* of 4.3, and the oxidized form has a p*I* of 3.5 (data not shown).

The UV−visible absorbance spectrum for HPLC-B is shown in Figure 6. It displays absorbance maxima at 552 nm (α), 521 nm (β), and 417 nm (γ), and it does not autoxidize. The presence of an absolutely conserved methionine in cytochrome c_8 sequences (Ambler, 1991; Bartsch, 1991) would suggest that if the high-potential *C. purpuratum* cytochrome *c* is indeed a member of the cytochrome c_8 family, it would also have histidine/methionine axial heme ligation. However, the absorption spectrum of the oxidized protein in the region around 695 nm shows no evidence for the sulfur-to-metal charge-transfer band that might be expected if the heme had a methionine axial ligand. As the extinction coefficient for this feature in the 695 nm region is small ($\sim$1 mM^{-1} cm^{-1}), it may be below the level of detection. Furthermore, there are *c*-type cytochromes with a methionine axial ligand that do not show this absorbance feature (Finnegan et al., 1996). The alkaline pyridine hemochrome spectrum of this fraction shows a single, narrow, symmetric band centered at 550 nm, indicating that the only heme present in this fraction is heme *c*. The midpoint potential for the cytochrome is estimated to be +310 mV at pH 6.5 (Figure 6). This high potential is consistent with the fact that the reduced form of the protein is stable against oxidation by ambient oxygen.

DISCUSSION

(A) Flavocytochrome c. Flavocytochromes *c*, like the PRC-associated tetraheme cytochrome, appear to be present in all purple-sulfur organisms and have also been isolated from green-sulfur bacteria. The subunit composition of flavocytochromes is similar among the phototrophs, consisting of an $\sim$45 kDa flavin-binding subunit and a second subunit that covalently binds 1 heme *c* per ca. 10 kDa protein. The molecular mass, subunit composition, spectral characteristics, and N-terminal amino acid sequences of both the flavin and cytochrome subunits of the *C. purpuratum* flavocytochrome *c* indicate a high degree of homology to flavocytochromes *c* of *C. vinosum* and *C. tepidum*. However, the isoelectric point of the *C. purpuratum* flavocytochrome *c* is significantly higher than those isolated from other *Chromatium* species. This is somewhat surprising since proteins derived from halophilic organisms tend to be more acidic than those from their mesophilic counterparts (Lanyi, 1974). The p*I* of *C. purpuratum* flavocytochrome *c* is more similar to that of the green-sulfur phototroph *Chlorobium limicola* [p*I* = 6.7; summarized in Pettigrew and Moore (1987)].

The oxidation−reduction properties of the hemes of the *C. purpuratum* flavocytochrome *c* (Figure 2) resemble those reported for the flavocytochrome *c* isolated from *C. tepidum* (Garcia-Castillo et al., 1994b), in that two components separated by approximately 130 mV in E_m were observed, and differ from those observed for the *C. vinosum* flavocytochrome *c*, for which only a single component with an E_m value near +15 mV was observed (Meyer et al., 1991; Garcia-Castillo et al., 1994b).

Although it has been proposed that the physiological role of flavocytochrome *c* in phototrophic bacteria is to catalyze the oxidation of sulfur to S^0 [see Brune (1989) for a review], this role has recently been questioned. The genes for both the flavin and cytochrome subunits of the *C. vinosum* flavocytochrome *c* contain leader sequences, suggesting that the enzyme is located in the periplasm (Dolata et al., 1993). It is difficult to reconcile a periplasmic location with the proposed function of flavocytochrome *c* in oxidizing sulfide to elemental sulfur when, *in vivo*, elemental sulfur granules accumulate in the cytoplasm. Moreover, not all organisms that use sulfide as an electron source contain flavocytochrome *c* (Brune, 1989; Meyer & Cusanovich, 1989). The observation (see above) that the *C. purpuratum* flavocytochrome *c* does not catalyze electron transfer from sulfide to equine cytochrome *c* (in contrast to the flavocytochromes of *C. vinosum* and *C. tepidum*) or to either *C. purpuratum* cytochrome c_8 or HiPIP is also inconsistent with a functional role for flavocytochrome *c* as a sulfide dehydrogenase (Fukumori & Yamananka, 1980; Gray & Knaff, 1982; Garcia-Castillo et al., 1994).

(B) Low-Potential Cytochrome c-551. A low-potential, soluble cytochrome *c* has also been detected in several species of phototrophic bacteria (Tedro et al., 1979; Gray et al., 1983; Meyer et al., 1983). The amino acid sequence of one of the soluble *C. purpuratum* cytochromes detected in this study, cytochrome *c*-551, exhibits homology to that reported for a low-potential cytochrome *c* isolated from *Ectothiorhodospira* species (Table 1). On the basis of this sequence homology, we have tentatively proposed that *C. purpuratum* cytochrome *c*-551 is also a low-potential cytochrome. Oxidation−reduction titrations are planned to determine the exact E_m value of this cytochrome. One high-potential and one low-potential soluble, low molecular mass cytochrome *c* have also been detected in *C. vinosum* (Gray et al., 1983; Tomiyama et al., 1983). However, no amino acid sequence data for these cytochromes were obtained, precluding an evaluation of their relationship to the low molecular mass cytochromes *c* in *C. purpuratum*.

(C) C. purpuratum HiPIP and Cytochrome c_8. In our investigation of the soluble fraction of *C. purpuratum*, we found no evidence for the presence of a c_2-type cytochrome similar to those which function as a soluble electron carrier in the photosynthetic electron transfer pathway of purple-non-sulfur bacteria. In an attempt to locate a soluble, high-potential electron carrier that might function in *C. purpuratum* in a manner analogous to that of cytochrome c_2, we have isolated large quantities of the high-potential [4Fe-4S]-containing HiPIP and a small amount of a low molecular mass, high-potential *c*-type cytochrome. We have assigned the latter protein to the cytochrome c_8 class, using the designation of Ambler [1991; see Moore and Pettigrew (1990) for an alternative cytochrome nomenclature], typified by the high-potential, class I *Pseudomonas* cytochrome *c*-551. HiPIP is abundant in the phototrophic purple-sulfur bacteria, but is less common in the phototrophic non-sulfur bacteria.

Mass spectroscopy of the purified *C. purpuratum* HiPIP used in the present study, and for crystallization, indicates a homogeneous population of HiPIP molecules with a molecular mass of 9372 Da. However, the biochemical and crystallographic data on *C. purpuratum* HiPIP suggest it may form dimers in solution. The protein, consistently eluted with the apparent molecular mass expected for a dimer in FPLC or HPLC gel filtration chromatography, was retained by a 10 kDa molecular mass cutoff Centricon filter and migrated as a dimer on SDS−PAGE unless the sample was boiled prior to loading of the gel (Figure 1). The structure of *C. purpuratum* HiPIP has been determined to 2.7 Å resolution by molecular replacement. The asymmetric unit contains 3 molecules which comprise 1.5 dimers (Kerfeld et al., manuscript in preparation). Previous crystallographic studies of HiPIPs derived from other organisms have suggested that HiPIP may form dimers. Molecules in crystals of the *Ectothiorhodospira vacuolata* HiPIP pack as dimers (Benning et al., 1994), as do those in *Rubrivivax gelatinosus* HiPIP crystals (Rayment et al., 1992). Although *C. vinosum* HiPIP does not dimerize in the crystal lattice (Carter et al., 1974), EPR studies of the protein indicate that two slightly different dimeric forms are present after freezing in 0.1−0.2 M NaCl (Dunham et al., 1991).

Among HiPIP's characterized, that of *C. purpuratum* has one of the highest midpoint potential values reported. At +390 mV, it is exceeded only by that of the HiPIP isolated from *Rhodopseudomonas globiformis* (E_m = +450 mV; Bertini et al., 1993). A relatively high E_m has been suggested to correlate with a higher percentage of acidic amino acids in the protein. Like the HiPIP of *C. vinosum*, that of *C. purpuratum* has an acidic p*I*, with the oxidized form of the protein being slightly more acidic than the reduced form.

The N-terminal 15 amino acids of *C. purpuratum* HiPIP are identical to the primary structure of *Chromatium gracile* HiPIP and highly homologous to *C. vinosum* HiPIP (Table 1). The complete amino acid sequence of *C. purpuratum* HiPIP is being determined from averaged electron density maps (Kerfeld et al., manuscript in preparation).

The HPLC-B fraction separated in gel filtration chromatography of the *C. purpuratum* soluble proteins (Figure 4) contains a reduced *c*-type cytochrome and appears as a single, Coomassie-staining band on SDS−PAGE that stains intensely for heme (Figure 5). The absorbance spectrum, shown in Figure 6, obtained without the addition of any exogenous reductant, indicates that the protein remains reduced during purification. This behavior is consistent with the +310 mV E_m value estimated in this study (Figure 6). The N-terminal sequence of the protein (Table 1) indicates homology to c_8 cytochromes [as designated by Ambler (1991)], and the high E_m value is consistent with this assignment. Cytochromes c_8 have been found in other phototrophic organisms that contain a PRC with an associated tetraheme cytochrome, and have been suggested to function in a manner analogous to c_2 cytochromes of the Rhodospirillaceae in reducing the photooxidized PRC (Bartsch, 1991). It is tempting to speculate that *C. purpuratum* cytochrome c_8 is related to the high-potential cytochrome *c*-551 found in *C. vinosum* (Tomiyama et al., 1983; Gray & Knaff, 1982) that was shown to mediate cyclic electron flow (Knaff et al., 1980). Moreover, in the same study, *P. aeruginosa* cytochrome c_8 was shown to be as effective as the *C. vinosum* cytochrome *c*-551 in mediating cyclic electron flow in *C. vinosum* spheroplasts (Knaff et al., 1980). However, although the *C. purpuratum* and *C. vinosum* high-potential cytochromes have similar absorbance spectra and E_m values, the molecular mass of the *C. vinosum* cytochrome was estimated to be ~15 kDa (Tomiyama et al., 1983; Gray & Knaff, 1982).

A high-potential, soluble cytochrome *c*, cytochrome *c*-551, has been detected in *C. vinosum* and shown, in flash photolysis studies using intact *C. vinosum* cells, to be competent in reducing the photooxidized PRC (van Grondelle et al., 1977). However, it has not been established whether the soluble cytochrome identified in these flash photolysis experiments is the same cytochrome isolated by Tomiyama et al. (1983) and by Gray and Knaff (1982). The low yields obtained during purification of this cytochrome and its relatively poor stability (Gray & Knaff, 1982; Tomiyama et al., 1983) have made it difficult to characterize the cytochrome in detail, and no amino acid sequence data are available for it.

The cytochrome we have tentatively identified as a c_8-type is the only high-potential, soluble cytochrome detectable in *C. purpuratum*. Cytochromes of the c_8 family have also been identified in three species of purple-non-sulfur bacteria that contain a PRC-associated tetraheme cytochrome (*Rhodocyclus purpureus*, *Rhodocyclus tenuis*, *Ru. gelatinosus*) and have been suggested to serve as donors to the PRC in these organisms (Bartsch, 1991) and also in *Rhodoferax fermentans* (A. Hochkoeppler, personal communication). In *Ru. gelatinosus* and *Rx. fermentans*, HiPIP has been shown to mediate photosynthetic electron flow (Hochkoeppler et al., 1995a,b; Schoepp et al., 1995). It is thus possible that these organisms utilize HiPIP and/or cytochrome c_8 as a soluble high-potential electron carrier to the tetraheme PRC-associated subunit, rather than cytochrome c_2. HiPIP and cytochrome c_8 from *C. purpuratum* resemble each other in several ways (they are similar in size and isoelectric point, and both have a high oxidation−reduction potential), suggesting that both proteins could perform the same function, i.e., that they serve as interchangeable electron donors to the PRC in this purple-sulfur bacterium. In the Pseudomonads, cytochrome c_8 is functionally interchangeable with the copper protein azurin [summarized in Meyer and Cusanovich (1989)]. Azurin is homologous to the cyanobacterial and plant copper-containing protein plastocyanin, which also has a functionally interchangeable cytochrome analog, cytochrome c_6 (Ho & Krogmann, 1984). The two members of each of these pairs of functionally interchangeable electron transfer proteins

(*Pseudomonas* azurin and cytochrome c_8, and plastocyanin and cytochrome c_6) are similar in size, isoelectric point, and midpoint potential. An investigation of the electron transfer from *C. purpuratum* HiPIP and cytochrome c_8 to the PRC of *C. purpuratum* will determine if these two proteins represent another instance of an interchangeable pair of electron transfer proteins that function in photosynthesis.

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