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Unusual high redox potential ferredoxins and soluble cytochromes from the moderately halophilic purple phototrophic bacterium *Rhodospirillum salinarum*

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Two soluble cytochromes and two high redox potential ferredoxins (HiPIP) were purified from extracts of the halophilic non-sulfur purple phototrophic bacterium *Rhodospirillum salinarum*. All four proteins are highly acidic, as are those from other halophilic phototrophs. Cytochrome *c′* is otherwise like those of other species. Cytochrome *c*-551 has a low redox potential (−143 mV), and appears to be monomeric (12 kDa). We did not find a protein similar to the cytochrome c_2 in most species of non-sulfur purple bacteria. The two HiPIP isozymes in *R. salinarum* differ in native molecular weight (iso-1, 10000 and iso-2, 45000), although iso-2 HiPIP may be a tetramer (subunit size 11 kDa). The redox potential of Iso-1 HiPIP is 265 mV, but iso-2 HiPIP is labile to ferricyanide and other oxidants tested, thus the redox potential was not measured. This is to our knowledge the first report of either an aggregated or a labile HiPIP. Abundant membrane bound *c*-type cytochromes were observed in *R. salinarum* by difference spectroscopy. About one-half of the heme (alpha peak maximum 553 nm) could be reduced by ascorbate, whereas the remainder of the heme was reduced by dithionite (552 nm maximum). These observations suggest the presence in *R. salinarum* of a tetraheme reaction center cytochrome which overshadows the cytochrome bc_1 complex.

Introduction

Electron transfer proteins have received less attention in halophiles than in fresh-water or marine species of phototrophic bacteria [1]. HiPIP and cytochrome *c′* from the extreme halophile, *Ectothiorhodospira halophila* (salt optimum, 23%) were reported to be similar to their counterparts from non-halophiles, but were more acidic and had lower redox potentials [2]. Cytochromes c_2 and *c′* from the moderate halophile, *Rhodospirillum salexigens* (salt optimum, 7%) were recently found to have redox potentials similar to those of non-halophiles but they were more acidic than proteins from fresh water bacteria [3]. Both species contain an unusual photoactive yellow protein, which has not been isolated from any other source [2–5]. We have also reexamined the marine phototrophic bacterium, *Rhodopseudomonas marina* (salt optimum 1–5%), which has a complex assemblage of redox proteins [6,7]. To extend our observations on halophiles, we have now examined *Rhodospirillum salinarum*, a non-sulfur purple phototroph which has a salt optimum for anaerobic growth of 12–18% [8]. This bacterium differs from *R. salexigens* in its higher salt optimum, in having vesicular intracytoplasmic membranes, and in complex growth factor requirements. We now find that it is somewhat different from the other halophiles in its complement of electron transfer proteins.

Materials and Methods

Rhodospirillum salinarum type strain (ATCC 35394) was a gift of Dr. J. Imhoff. It was grown on a 12% NaCl medium according to Nissen and Dundas [8]. 1 kg cells was suspended in 4 liters 0.1 M Tris-HCl buffer (pH 7.3) and broken in the Ribi Cell Fractionator (an Automated French Press) at 20000 psi, then centrifuged 2 h in the Beckman Type 45 Ti rotor to remove membranes. The supernatant was desalted, buffered with 1 mM Tris-HCl (pH 7.3) and adsorbed to an 8 × 12 cm DEAE-cellulose column (Whatman DE52). The column was developed as described in Results.

Abbreviations: HiPIP, high-potential iron-sulfur protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; FMN, flavin mononucleotide.

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The approximate redox potential of HiPIP was measured by titration of oxidized protein with ferrocyanide in an open cuvet. The amount of ferricyanide produced was calculated from the extent of reduction of HiPIP. The redox potential of the ferri-/ferrocyanide couple was assumed to be 413 mV in 50 mM phosphate (pH 7) [9]. The reverse reaction was not studied. The redox potential of cytochrome *c*-551 was measured by anaerobic photititration of a solution containing 40 μM FMN, 10 mM EDTA, and 20 mM phosphate (pH 7.0). The potential of FMN was assumed to be -205 mV [10].

The native molecular weight was measured using a Pharmacia FPLC system with a Bio-Rad TSK125 column. The elution buffer contained 150 mM phosphate and 150 mM NaCl (pH 7.0). Elution peaks were monitored with a Hewlett-Packard Diode Array Spectrophotometer at two wavelengths, 280 nm and the visible absorption maximum (400 nm for HiPIP and cytochrome *c'* and 408 nm for cytochrome *c*-551). Subunit sizes were measured by SDS-PAGE using the Pharmacia Phast System, with 20% crosslinked gels, stained with Coomassie Blue R-250. The Bio-Rad low-molecular-weight standard kit was used for calibration.

Isoelectric points were also measured with the Pharmacia Phast System using Pharmacia pH 4–6.5 gels. The Pharmacia pH 3–10 broad calibration kit was used as standard.

Protein was determined by the Pierce BCA protein assay method using bovine serum albumin as standard [11].

Results

DEAE-cellulose chromatography

An extract of *R. salinarum* was prepared as described in Materials and Methods and adsorbed to DEAE-cellulose. The column was developed with a stepwise salt gradient and electron transfer proteins were eluted in the following order. Approx. 120 μmol of HiPIP was eluted with 40–80 mM NaCl in 20 mM Tris-HCl buffer. About 10 μmol of a low-redox-potential cytochrome *c*-551 was eluted with 100–150 mM NaCl in the same buffer. About 25 μmol of cytochrome *c'* was eluted with 250–300 mM NaCl plus buffer. The column was finally washed with 0.4 M and with 0.5 M NaCl, which eluted a very large amount of orange-brown and green materials, respectively, which at first appeared to be flavins, but which had absorption maxima at about 490 nm. Both substances could be bleached by added dithionite and if they are flavin derivatives, there

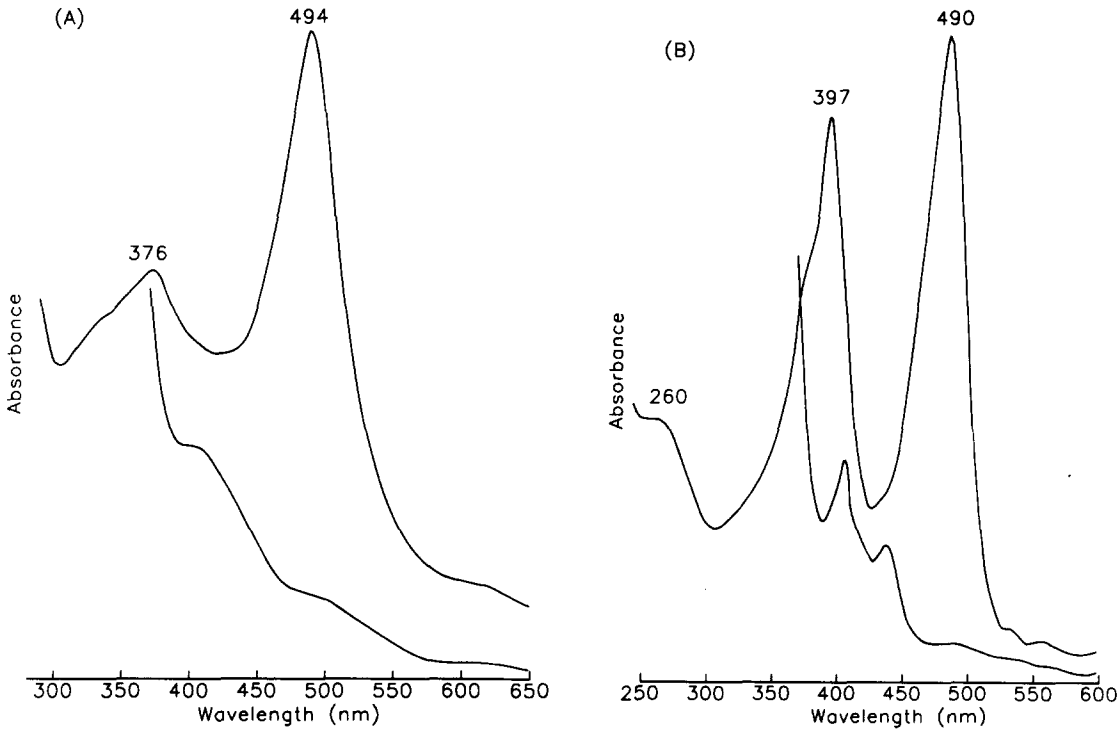

Fig. 1. Ultraviolet-visible absorption spectra of *R. salinarum* small-molecular-weight fractions from Sephadex G-75 chromatography of the 0.4 M eluate of DEAE-cellulose. (A) Oxidized and dithionite reduced 'brown' fraction following cytochrome *c'* and ferredoxin off the column. (B) Oxidized and dithionite reduced 'green' fraction following the 'brown'. It took approx. 0.5 h to reduce the 'brown' and 5 min to reduce the 'green'. Note the small 260 nm peak in the 'green' fraction indicating the absence of protein contamination.

was about 200 μmol or more (using an assumed extinction coefficient of 12 $mM^{-1} \cdot cm^{-1}$).

A portion of the orange-brown 0.4 M NaCl elute was chromatographed on Sephadex G-75. Three bands were apparent. The first band contained a small amount of poorly resolved cytochrome *c'* and 'bacterial' ferredoxin, then there were well-resolved brown and green bands (the absorption spectra are shown in Fig. 1). They were not further purified and decomposed on storage at −20 °C after several months.

HiPIP

HiPIP fractions were concentrated on a small DEAE-cellulose column, eluted with 0.5 M NaCl and then precipitated with ammonium sulfate. The HiPIP precipitated over two ranges of ammonium sulfate concentrations, without completely resolving the two fractions. Approximately two-thirds of the HiPIP precipitated at 40–60% saturation and one-third at 70–90% saturation. The two HiPIP fractions were next chromatographed on Sephadex G-75 in 100 mM NaCl plus 20 mM Tris-HCl (pH 7.3). That fraction of HiPIP which precipitated at the lower ammonium sulfate concentration separated into two bands, the first of which was the more abundant and of larger molecular weight. The 70–90% saturation ammonium sulfate fraction of HiPIP also separated into two bands on gel filtration. The first band was a minor component and the second was the principal, lower-molecular-weight HiPIP component. The smaller and larger-molecular-weight HiPIP fractions from the two columns were combined and labeled iso-1 and iso-2 HiPIP, respectively. Final purification was achieved by DEAE-cellulose chromatography using a linear gradient of 0–150 mM NaCl in 20 mM Tris-HCl (pH 7.3). Each HiPIP eluted at about 60 mM NaCl and was in the reduced form. 20 μmol iso-1 and 40 μmol iso-2 HiPIP were recovered in an overall yield of about 50%. The best ratio of 280 nm to 385 nm absorbance was 2.2 for iso-1 and 1.9 for iso-2 HiPIP. The absorption spectrum of iso-1 HiPIP is shown in Fig. 2 and of iso-2 HiPIP in Fig. 3. The native size of iso-1 HiPIP is 10 kDa and the subunit 9 kDa, whereas the native size of iso-2 HiPIP is 45 000 and the subunit appears to be 11 000, and thus may be a tetramer. The far-UV circular dichroism spectra of the two HiPIPs are shown in Fig. 4. These spectra indicate the absence of alpha helical secondary structure in both proteins and about 20% beta structure in iso-1 and 23% beta structure in iso-2 HiPIP. The N-terminus of iso-1 HiPIP is blocked and was therefore not amenable to sequence analysis without modification. The first 29 residues of iso-2 HiPIP were determined by automated Edman degradation on the Beckman 890 sequencer as follows:

MQSNNSRFFJAAQTQSRRYAPWPTADA .....

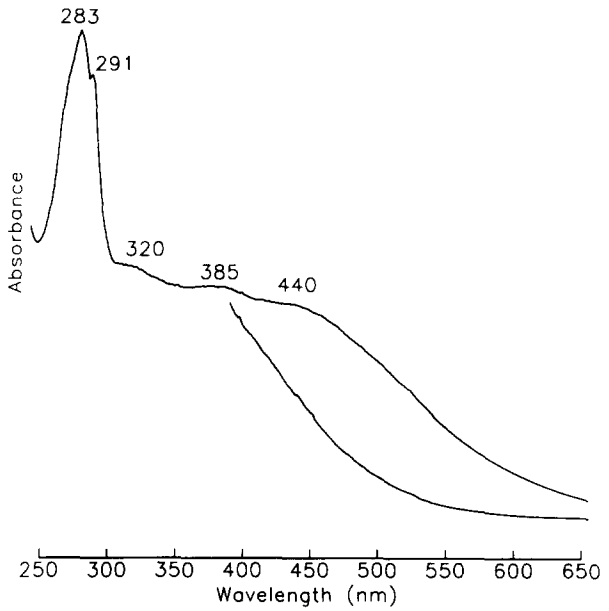

Fig. 2. Ultraviolet-visible absorption spectra of oxidized and (dithionite) reduced *R. salinarum* iso-1 HiPIP in 0.1 M phosphate (pH 7.0).

The redox potential of iso-1 HiPIP was determined to be 265 mV in 50 mM phosphate buffer (pH 7). The redox potential of iso-2 HiPIP could not be accurately measured because the iron-sulfur cluster was slowly and irreversibly bleached following a brief initial increase in

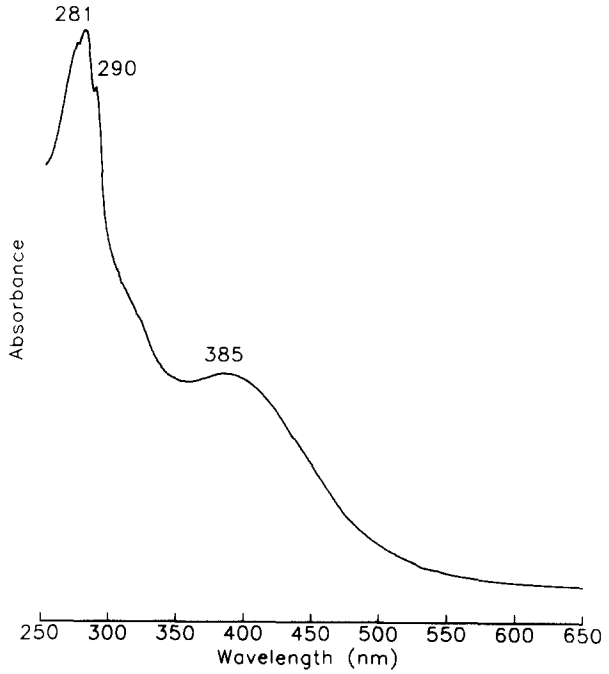

Fig. 3. Ultraviolet-visible absorption spectrum of *R. salinarum* iso-2 HiPIP in 0.1 M phosphate (pH 7.0). There was an initial increase in absorbance upon addition of ferricyanide followed by an irreversible bleach of the spectrum. There was no change in the absorbance of native protein upon addition of dithionite which suggests that it was already completely reduced as isolated.

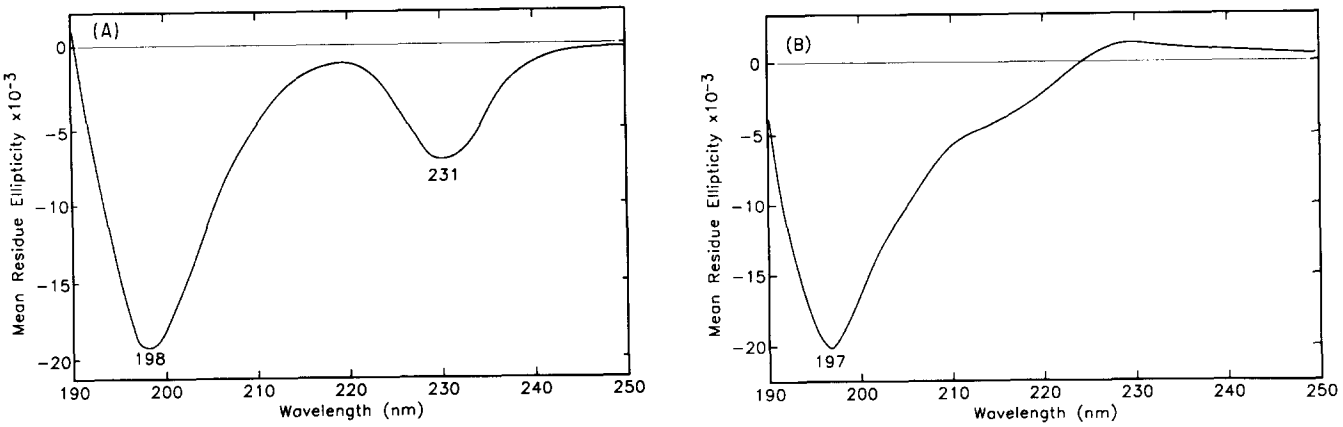

Fig. 4. Far-ultraviolet circular dichroism spectra of *R. salinarum* HiPIP's. (A) Iso-1 HiPIP. (B) Iso-2 HiPIP.

absorbance, presumably due to destructive oxidation by ferricyanide. Protein oxidants which were tested gave similar results. Horse cytochrome *c* ($E_{m,7} = 260$ mV) and *Euglena viridis* cytochrome *c*-552 ($E_{m,7} = 370$ mV) were slowly reduced in the presence of iso-2 HiPIP, but without a corresponding increase in absorbance of HiPIP in the visible region, also suggesting destruction of the iron-sulfur cluster. A crude upper estimate of the redox potential based on the initial absorbance change with a single addition of ferricyanide is 480 mV, although this is considered to be high because there was some iron-sulfur destruction which occurred during the time of the experiment (approx. 1 min). Assuming there was redox equilibration with *Euglena* cytochrome *c*-552, the potential may be higher than 370 mV, although horse cytochrome *c* should not have oxidized the HiPIP at all if the potential was in fact that high. A conservative estimate is that the potential is at least as high as or higher than iso-1 HiPIP.

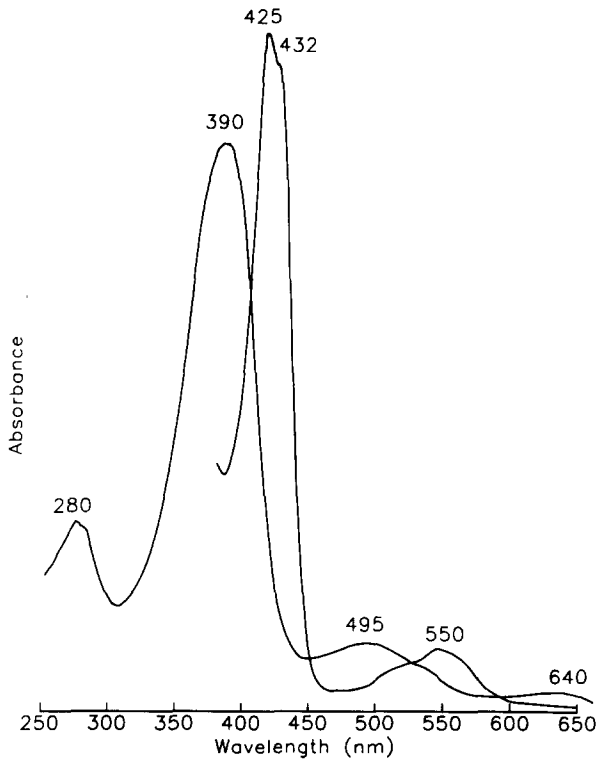

Fig. 5. Ultraviolet-visible absorption spectra of oxidized and (dithionite) reduced *R. salinarum* cytochrome *c'* in 0.1 M phosphate (pH 7.0).

Cytochrome c'

Cytochrome c' which was eluted from the initial DEAE-cellulose column, was concentrated by ultrafiltration on a Diaflo YM5 membrane and then precipitated at 60–90% saturation ammonium sulfate. It was redissolved and chromatographed on Sephadex G-75. It was next chromatographed on DEAE-cellulose using a linear gradient of 160 to 260 mM NaCl plus 20 mM Tris-HCl buffer, the main protein fraction eluted at about 240 mM NaCl. Final purification involved chromatography on hydroxyapatite using a linear gradient of 0–200 mM phosphate (pH 7.0) plus a constant 100 mM NaCl. The protein eluted at about 150 mM phosphate. 6 μmol cytochrome *c'* was recovered in an overall yield of about 25%. The best ratio of 280 nm to 390 nm absorbance was 0.33. The absorption spectra are shown in Fig. 5.

Cytochrome c-551

Pooled fractions containing cytochrome *c*-551 were precipitated by 40–70% saturation ammonium sulfate and chromatographed on Sephadex G-75. Early fractions off the column (large molecular weight) contained some high potential cytochrome *c*-550 (about 0.5 μmol) along with iso-2 HiPIP, whereas a small amount of iso-1 HiPIP followed the low-potential cytochrome *c*-551. The quantity of high-potential cytochrome *c*-550 was too little to continue purification, but the size was determined by FPLC; four heme-containing bands were apparent at 350, 175, 90, and 47 kDa, which suggests aggregation of a single cytochrome *c*-550. The minimum

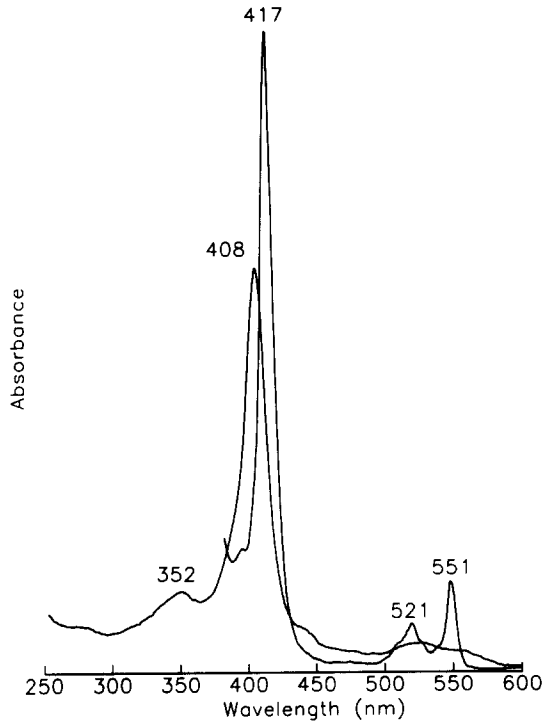

Fig. 6. Ultraviolet-visible absorption of oxidized and (dithionite) reduced *R. salinarum* cytochrome *c*-551 in 0.1 M phosphate (pH 7.0). Note the near absence of absorption in the protein region at 280 nm.

size could not be measured because the cytochrome smeared on SDS-PAGE.

The cytochrome *c*-551 was next chromatographed on DEAE-cellulose, using a 40–160 mM NaCl linear gradient plus 20 mM Tris-HCl buffer (pH 7.5). The cytochrome *c*-551 eluted at about 80 mM NaCl. Final purification involved chromatography on hydroxyapatite using a 0–100 mM phosphate linear gradient plus a constant 100 mM NaCl. Cytochrome *c*-551 eluted at about 45 mM phosphate. 3 μmol protein was recovered in an overall yield of about 30%. The best ratio of 280 nm to 408 nm absorbance was 0.11. The absorption spectra are shown in Fig. 6. Both native and subunit sizes appear to be 12 kDa. There was 7.0 mg protein per μmol heme, which suggests that there could be two hemes per protein. The redox potential is −143 ± 10 mV. The far-ultraviolet circular dichroism spectrum, shown in Fig. 7, indicates about 26% helical secondary structure.

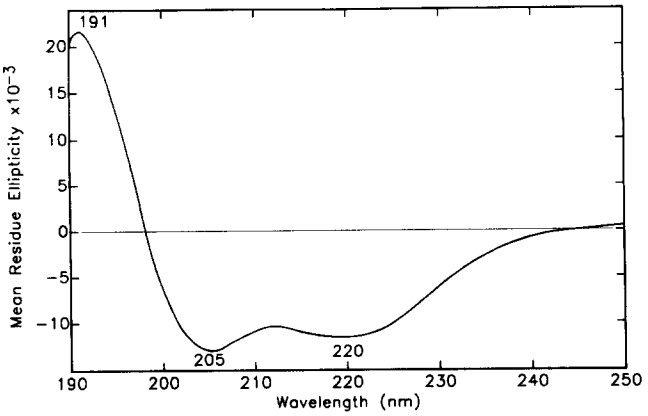

Fig. 7. Far-ultraviolet circular dichroism spectrum of *R. salinarum* cytochrome *c*-551.

Membrane-bound cytochromes

Photosynthetic membranes from the high-speed centrifugation were suspended in 0.1 M phosphate buffer (pH 7.0), homogenized, and centrifuged at low speed for a few minutes. Difference spectra of the suspension were recorded on an Aminco DW2 spectrophotometer as shown in Fig. 8. One-quarter of the total heme was found to be reduced relative to the reference sample oxidized by addition of a crystal of potassium ferricyanide. Addition of about 1 mM ascorbate resulted in reduction of another one-quarter of the heme. The remaining heme required dithionite for reduction. The wavelength maximum shifted slightly from 553 nm for half reduction to 552 nm for full reduction. There was no long-wavelength shoulder on the 552 nm maximum of the dithionite minus ascorbate difference spectrum (not shown) which might indicate some *b*-type cytochrome with wavelength maximum in the vicinity of 560 nm. The Soret maximum of this spectrum was at 423 nm. The membrane bound cytochrome was abundant and taking all characteristics into account may be tenta-

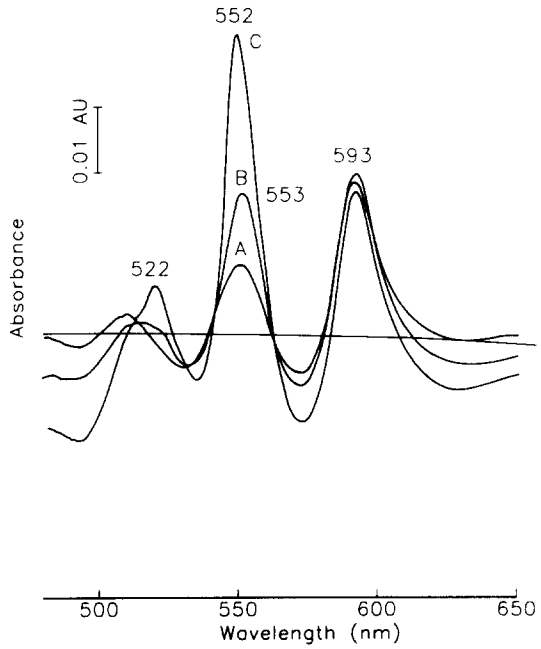

Fig. 8. Membrane difference absorption spectra for *R. salinarum* (A) as is minus ferricyanide. (B) Ascorbate minus ferricyanide. (C) Dithionite minus ferricyanide. All spectra were recorded in 0.1 M phosphate (pH 7.0). The spectra were arbitrarily adjusted to zero absorbance at 542 and 564 nm. The peak at 593 nm is due to bacteriochlorophyll, whereas the peaks at 552 and 522 are the alpha and beta peaks of *c*-type cytochrome.

tively identified as a tetraheme reaction center cytochrome similar to that characterized in *Rps. viridis* [12].

Discussion

Four soluble electron transfer proteins were purified from extracts of *R. salinarum*, including two HiPIP's, cytochrome c' and a low-potential cytochrome *c*-551. Small amounts of a 'bacterial' ferredoxin and a high-potential cytochrome *c*-550 were observed, but there was not enough of either for purification. The photosynthetic membranes also appear to contain a tetraheme reaction center cytochrome *c*. Phototrophic bacteria nearly always contain an abundant soluble high-potential cytochrome *c*, which in the non-sulfur purple bacteria is usually a cytochrome c_2. *Rc. gelatinosus, Rc. tenuis* and *Rc. purpureus* [13] are the only non-sulfur purple bacteria which have a high-potential cytochrome which is not a cytochrome c_2, although it is homologous to cytochrome c_2. *R. salinarum* is the only species of non-sulfur purple bacteria which does not appear to have an abundant soluble high-potential cytochrome of any kind. The small amount of soluble high-potential cytochrome *c*-550 we did observe was highly aggregated, which suggests that it may be hydrophobic and predominantly membrane-bound in vivo. Cytochromes c_2 are synthesized with a hydrophobic signal peptide, which is normally cleaved once the cytochrome is transported across the membrane to the periplasmic space [14,15]. *R. salinarum* cytochrome *c*-550 could possibly be a cytochrome c_2, which retains the signal peptide. We are now attempting to obtain a larger amount of cytochrome *c*-550 for purification and to determine cellular location in order to test this idea.

Cytochrome c' is the most frequently observed cytochrome in purple photosynthetic bacteria. It is apparently absent in only four of the species thus far examined: *Rps. viridis, Rm. vannielii, Rps. acidophila* and *Rp. globiformis* [1,16]. It is almost always dimeric, and the cytochrome c' from *R. salinarum* is typical in this regard.

HiPIP is not observed in the non-sulfur purple bacteria as often as are cytochromes c_2 and c', but is present in all the purple sulfur bacteria examined [17]. The non-sulfur purple bacteria which have HiPIP are *Rc. gelatinosus, Rc. tenuis, Rm. vannielii* [17], *Rp. globiformis* [16] and *Rps. marina* [7]. All HiPIPs characterized to date are monomeric and have molecular weights ranging from 7000 to 10000 [17]. Thus, *R. salinarum* iso-2 HiPIP is the first HiPIP which has a large apparent molecular weight and which is composed of subunits.

It was previously noted that the iso-HiPIPs from the extreme halophile, *E. halophila*, have much lower redox potentials (50 mV and 150 mV) than HiPIPs from fresh-water bacteria [2]. The redox potentials of the 14 known HiPIPs average 289 ± 83 mV, with extremes of 50 and 445 mV. *R. salinarum* iso-1 HiPIP is thus close to the overall average (265 mV). On the other hand, the five previously characterized HiPIP's from halophiles have potentials which range from 50 to 290 mV (average 174 ± 81 mV). The non-halophiles average 353 ± 26 mV, with the extremes 300 and 445 mV. In this respect, *R. salinarum* iso-1 HiPIP appears to be more like the examples from other halophiles and less like the fresh-water species.

R. salinarum iso-2 HiPIP is unusual in several respects. It is the only aggregated HiPIP presently known (it appears to be a tetramer), the N-terminal sequence is unlike that of any other known HiPIP, and the 4-Fe-S cluster is labile when oxidized either by ferricyanide or by the protein oxidants tested: horse cytochrome *c* and *Euglena* cytochrome *c*-552. It is known that 'bacterial' ferredoxins are labile to ferricyanide [18], but this is the first HiPIP which is decomposed upon oxidation. The cytochromes would not be expected to cause destruction of the iron-sulfur clusters by the same mechanism as ferricyanide (formation of Prussian blue) and the loss of the iron-sulfur cluster indicates that the oxidized HiPIP is inherently unstable. The reduced iso-2 HiPIP partially decomposed on long-term storage in the freezer. These observations suggest that the *salinarum* iso-2 HiPIP is structurally very different from the others and may be representative of a new class of HiPIP, perhaps unrelated in sequence. In fact, there is no obvious similarity to any of the known HiPIP's in the first 29 N-terminal residues.

The far-ultraviolet circular dichroism spectra of the two HiPIP's are dominated by random structure (negative peaks below 200 nm) with only a small admixture of about 20% beta structure. This is consistent with the crystal structure of *Ch. vinosum* HiPIP, which shows 9% alpha helix and 12% beta secondary structure [19]. An interesting feature of the *R. salinarum* iso-1 HiPIP far-ultraviolet spectrum is a negative peak at 231 nm, which was attributed to trptophan 80 in *Ch. vinosum* HiPIP [20]. *R. salinarum* Iso-2 HiPIP lacks this feature as does *Rc. tenuis* HiPIP, which has no tryptophan in its amino-acid sequence [21].

Low-redox-potential cytochromes, such as *R. salinarum* cytochrome *c*-551, are even less commonly observed than HiPIP in purple bacteria [1]. They have been reported in *Rps. palustris* and *Rb. sphaeroides* [22], *R. rubrum* [23], *Ch. vinosum* [24], *Rc. tenuis* [25], *R. salexigens* [3] and *Rps. marina* [7]. The most completely characterized is from *Rb. sphaeroides* [26], which is a diheme 16 kDa and which has a redox potential of -254 mV. *R. salinarum* cytochrome *c*-551 appears to be a 12 kDa monomer, but there is one heme per 7 kDa protein, which suggests either that it is a diheme protein which is slightly impure or that it is a monoheme protein which runs anomalously on SDS-PAGE. The

amino-acid sequence is needed to determine the actual size and heme content and to establish whether this protein represents an additional class of low potential cytochrome in purple bacteria. None of the low-potential cytochromes from purple bacteria has been sequenced to date.

Membrane-bound cytochromes characterized in purple bacteria include the cytochrome bc_1 complex and the tetraheme reaction center cytochrome. The difference spectra we measured for *R. salinarum* show the presence of the tetraheme reaction center cytochrome by the following criteria. The cytochrome has both high- and low-potential *c*-type heme and the high-potential heme absorbs light at longer wavelengths than the low-potential heme. The cytochrome is so abundant, it masks any absorbance changes expected from the bc_1 complex, in particular that of the low-potential *b*-type heme, which should have an alpha peak near 560 nm. The tetraheme reaction center cytochrome has been thoroughly characterized in *Rps. viridis* [12] and has been observed in *Rc. gelatinosus* [27], *Ch. vinosum* [28], *Th. pfennigii* [29], *Ec. halophila* [30], *Ec. halochloris* [31], *Ch. tepidum* [32], *R. salexigens* [3] and *Rps. marina* [7]. Thus, there are now five non-sulfur purple bacteria known to have the tetraheme reaction center cytochrome and four which do not (*R. rubrum, Rps. palustris, Rb. sphaeroides* and *Rb. capsulatus*). All of the purple sulfur bacteria examined appear to have it.

R. salinarum does not appear to be very closely related to any other purple bacterium in terms of electron transfer proteins. The apparent absence of an abundant high potential cytochrome *c* sets it apart from the majority of non-sulfur purple bacteria, whereas the presence of HiPIP suggests some similarity to *Rc. gelatinosus* and *Rc. tenuis*, which have HiPIP, but not cytochrome c_2. Considering the three halophiles which have been examined with respect to electron transfer proteins, *Ec. halophila* [2], *R. salexigens* [3] and now *R. salinarum*, it appears that each is more closely related to non-halophiles than to one another, suggesting that adaptation to high salt has occurred repeatedly.

Acknowledgement

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