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Isolation and characterization of soluble cytochromes, ferredoxins and other chromophoric proteins from the halophilic phototrophic bacterium *Ectothiorhodospira halophila*

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A cytochrome *c*-551 and a pair of 'high redox-potential' ferredoxins (iso-high-potential iron-sulfur proteins) were found to be the major soluble electron-transport proteins in *Ectothiorhodospira halophila*. Smaller amounts of 'bacterial' ferredoxin and cytochrome *c'* were also observed. With the exception of cytochrome *c*-551, these proteins are commonly encountered in the purple sulfur bacteria, family Chromatiaceae and less frequently in the purple bacteria, family Rhodospirillaceae. In addition to the cytochromes and ferredoxins, *E. halophila* synthesizes substantial amounts of a small yellow-colored protein, which has a chromophore spectrally similar to flavins having oxygen, nitrogen or sulfur substituents in place of the 8-methyl group such as roseoflavin and the methanogen cofactor F-420. A purple-colored protein was only partially purified, but it is spectrally similar to iron proteins having a tyrosine ligand, such as transferrin, catechuate dioxygenase, and especially the purple acid phosphatases. Neither the yellow protein nor the purple one has previously been observed in phototrophic bacteria, but may in some way be required for survival in extremely halophilic habitats. The only feature common to halophiles including *E. halophila* is the very acidic nature of their proteins.

Introduction

A wide variety of soluble electron-transport proteins have been characterized in phototrophic bacteria, especially in the purple bacterial families Chromatiaceae and Rhodospirillaceae [1,2]. The Chromatiaceae are primarily autotrophic bacteria, which fix carbon dioxide at the expense of reduced sulfur compounds such as sulfide and thiosulfate. Elemental sulfur is an intermediate which accumulates within the cells. These purple sulfur bacteria have a very limited ability to take up organic compounds and are generally strictly anaerobic. The soluble electron-transport proteins found in the Chromatiaceae generally are dominated by HiPIP, cytochrome *c'*, flavocytochrome *c*, and bacterial ferredoxin. (HiPIP is an acronym for high-potential iron-sulfur protein, now known as a distinct class of high redox-potential ferredoxins which use the 2^+, 3^+ redox states of the four-iron, four-sulfur cluster. Bacterial ferredoxin is a distinct class of low redox potential ferredoxin using the 1^+, 2^+ redox states). The Rhodospirillaceae generally are intolerent of hydrogen sulfide, but assimilate a relatively wide range of organic compounds, and many have alternate capability for aerobic growth. Those few species able to use hydrogen sulfide do not accumulate elemental sulfur. Soluble electron transport proteins in the Rhodospirillaceae generally are

Abbreviation: HiPIP, high-potential iron-sulfur protein.

dominated by cytochrome c_2, cytochrome c', and bacterial ferredoxin. As is true of most generalizations, there are prominant exceptions.

The species grouped in the genus *Ectothiorhodospira* are considered to be anomalous members of the family Chromatiaceae. Although they utilize reduced sulfur compounds, elemental sulfur is deposited outside the cells. In this genus, there are three known species of extremely halophilic bacteria: *Ectothiorhodospira halophila, Ectothiorhodospira halochloris,* and *Ectothiorhodospira abdelmalekii* [3–6] in addition to several marine forms and a fresh water species. Based on the above generalizations, it is possible that the soluble electron-transport protein content of these halophilic bacteria might give some indication as to which family they might be more closely related, and may also increase our understanding of the process of adaptation to halophilic environments. In this preliminary report, the purification and properties of electron-transport proteins and other colored substances are described. These studies suggest that *E. halophila* was correctly typed as a distant relative of the purple sulfur bacteria in the family Chromatiaceae.

Results

E. halophila strain BN 9626 was kindly provided by Dr. Johannes Imhoff and grown under tungsten illumination at 40°C on the following medium: 0.25 g $Na_2S \cdot 9H_2O$/1 g $Na_2S_2O_3$/0.5 g KH_2PO_4/0.8 g NH_4Cl/0.1 g $MgCl_2 \cdot 6H_2O$/0.05 g $CaCl_2 \cdot 2H_2O$/150 g NaCl/1 g KCl/7 g Na_2CO_3/2 g malic acid/0.5 g yeast extract (Difco), and 1 ml trace elements solution per l of medium. Sulfide, thiosulfate and carbonate were autoclaved separately and the pH adjusted to 8.5 after mixing. The cell yield after 6 days anaerobic growth in 20 l carboys was 3 g/l wet weight.

1500 g cells were suspended to 5 l in 0.1 M Tris-HCl buffer at pH 7.5 containing a few mg DNAase and broken in a Ribi cell fractionator (an automated French press) operated at 152 MPa. The broken cells were centrifuged in the Spinco 45 Ti rotor (235 000 × *g* maximal) for 3 h at 4°C. The supernatant solution was desalted using Sephadex G-25-C and adjusted to pH 7.5 with 1 mM Tris-HCl buffer, then adsorbed to an 8 × 15 cm column of DEAE-cellulose (Whatman DE-52). A considerable amount of unsedimented membrane material passed through without being adsorbed,

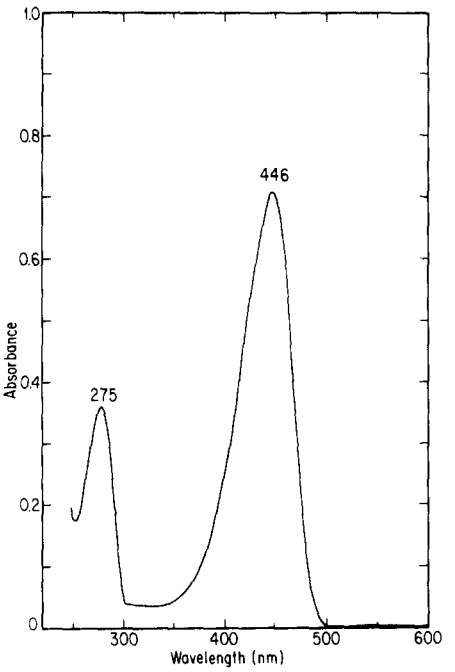

Fig. 1. Absorption spectrum of 'yellow protein' in 0.1 M potassium phosphate (pH 7.0).

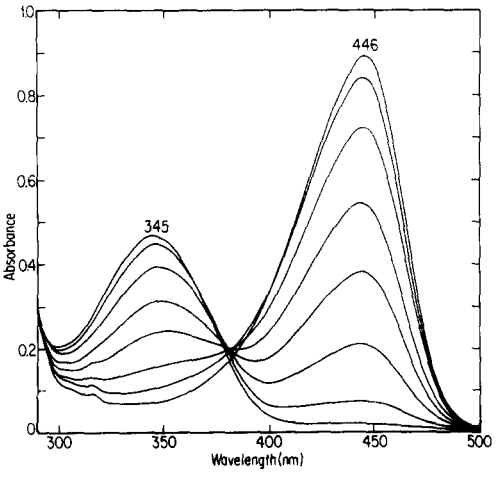

Fig. 2. Effect of pH on the absorption spectrum of yellow protein. Starting pH was 7.51 in 5 mM sodium acetate, potassium phosphate. Successive additions of 1 M HCl resulted in a shift of the absorption spectrum from 446 to 345 nm over the pH range: 3.60, 3.18, 2.82, 2.61, 2.33, 1.93 and 1.34. Note that the titration is isosbestic only between pH 3.60 and 2.33.

TABLE I

AMINO ACID COMPOSITIONS OF *E. HALOPHILA* PROTEINS

The numbers in parentheses are from the sequence of cytochrome *c*-551 and the two high-potential iron-sulfur proteins, and are otherwise the average or extrapolated best integers for the other proteins. The total number of amino acid residues was adjusted to approximate the subunit molecular weights for the latter.

	c-551	Iso-I HiPIP	Iso-II HiPIP	*c'*	Yellow	Bacterial ferredoxin
Asp	9.8 (10)	8.4 (8)	14.0 (14)	19.6 (20)	17.8 (18)	12.4 (13)
Thr	3.4 (4)	1.7 (1)	1.9 (2)	6.3 (6)	10.7 (11)	4.9 (5)
Ser	5.6 (7)	2.7 (3)	1.1 (1)	9.9 (10)	9.5 (10)	2.6 (3)
Glu	8.9 (7)	10.3 (11)	9.8 (9)	22.2 (22)	13.2 (13)	16.6 (17)
Pro	5.7 (5)	5.4 (4)	4.4 (4)	2.9 (3)	4.7 (5)	14.3 (14)
Gly	6.6 (7)	6.6 (7)	7.4 (7)	9.7 (10)	12.0 (12)	4.0 (4)
Ala	10.3 (10)	9.9 (9)	7.4 (7)	15.4 (15)	10.2 (10)	5.8 (6)
Cys	(2)	(4)	(4)	1.8 (2)	1.4 (2)	7.1 (8)
Val	3.7 (4)	3.8 (5)	6.4 (7)	8.2 (8)	8.6 (9)	4.5 (5)
Met	2.2 (2)	0 (0)	0 (0)	3.5 (4)	4.7 (5)	1.9 (2)
Ile	2.6 (3)	0 (0)	0 (0)	4.4 (5)	4.9 (5)	3.7 (4)
Leu	6.0 (6)	2.4 (2)	3.9 (4)	5.1 (5)	7.4 (8)	7.4 (8)
Tyr	2.5 (3)	2.6 (3)	3.5 (4)	2.3 (3)	4.8 (5)	2.1 (2)
Phe	0.4 (0)	2.1 (2)	3.1 (3)	5.6 (6)	7.8 (8)	2.3 (2)
His	1.0 (1)	3.3 (4)	3.6 (4)	4.3 (4)	2.1 (2)	4.7 (5)
Lys	2.0 (2)	1.0 (1)	1.3 (1)	3.3 (3)	9.1 (9)	6.1 (6)
Arg	4.6 (4)	3.0 (3)	1.8 (2)	6.3 (6)	2.3 (2)	0 (0)
Trp	(1)	(3)	1.9 (3)	1.7 (2)	1.1 (2)	4.0 (4)
Total	(78)	(70)	(76)	(134)	(137)	(108)

but a cytochrome-containing band was visible near the middle of the column. The column was developed with a stepwise gradient from 0 to 0.6 M NaCl starting with 0.02 M increments in 0.02 M Tris-HCl buffer (pH 7.5). A yellow protein was eluted at 0.12 M NaCl, a cytochrome *c*-551 at 0.14–0.16 M NaCl, a flavoprotein at 0.16–0.18 M NaCl, a ferredoxin (iso-I-HiPIP) at 0.2–0.24 M NaCl, another ferredoxin (iso-II-HiPIP) and some cytochrome *c'* at 0.28 M NaCl, and a mixture of cytochrome *c'*, a third ferredoxin (bacterial ferredoxin) and a purple protein at 0.6 M NaCl.

Fractions containing the yellow protein were diluted and concentrated on a small DEAE-cellulose column, eluted with 0.5 M NaCl in buffer, further concentrated by ammonium sulfate precipitation, and chromatographed on Sephadex G-75. The yellow protein and traces of cytochrome *c*-551 eluted in a single band, although the yellow protein was at the leading edge, indicating a larger size. The yellow protein was adsorbed to a small DEAE-cellulose column and chromatographed with a linear gradient from 0.1–0.2 M NaCl in buffer. It was eluted near 0.15 M NaCl and was closely followed by a colorless protein, necessitating several repetitions of the chromatography to separate them completely. Approx. 6.5 μmol was recovered. The best fraction had a purity index (280–446 nm absorbance ratio) near 0.5.

Pure yellow protein had the ultraviolet-visible absorption spectrum shown in Fig. 1. The spectrum was unaltered by dithionite or ferricyanide. The absorption spectrum was pH dependent as shown in the titration of Fig. 2. At least three spectral changes took place between pH 7.5 and 1.3. The changes were reversible if the pH change was rapid, but upon neutralization at the end of a 2 h titration, the wavelength maximum was shifted to 407 nm. In the titration, there was approx. a 5% reduction in absorbance at 446 nm between pH 7.5 and 3.6. There was then an isosbestic shift of the 446 nm peak to about 350 nm between pH 3.6 and 2.0 (pK 2.7). There was finally a small shift with about 10% increase in intensity at 345 nm

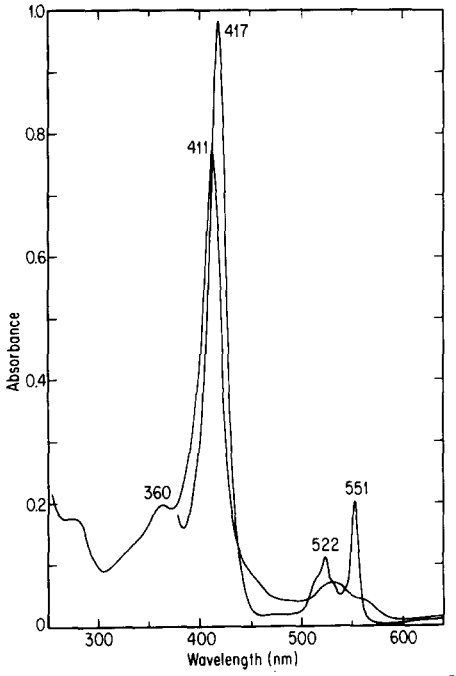

Fig. 3. Absorption spectra of cytochrome *c*-551 in 0.1 M potassium phosphate (pH 7.0). The reduced form was obtained by adding a few crystals solid sodium dithionite.

between pH 2 and 1. The changes in the 260–280 nm region were only about 10% throughout the titration. Attempts were made to remove the chromophore from the protein for additional characterization, but were unsuccessful. The color was reversibly bleached on boiling for 1 min followed by cooling and this effect could be repeated several times. However, continuous boiling for 10–15 min resulted in irreversible loss in color although the protein never coagulated. The color was also reversibly bleached in 8 M urea when followed by dilution.

The millimolar extinction coefficient of yellow protein at 446 nm (48 $mM^{-1} \cdot cm^{-1}$) was determined using amino acid analyses for estimation of protein. The size of the yellow protein was approximated by sodium dodecyl sulfate polyacrylamide gel electrophoresis to be 15.3 kDa and was used to estimate the total number of amino acid residues in the analyses shown in Table I. Therefore any error in molecular weight determination will result in an error in the extinction coefficient.

Cytochrome *c*-551 was eluted from DEAE-cellulose following the yellow protein and preceding the flavoprotein. It was purified by chromatography on Sephadex G-75 followed by chromatography on DEAE-cellulose using a linear gradient of 0.12–0.2 M NaCl in buffer. The best purity index (280–417 nm ratio) was 0.15. The yield of pure protein was 53 μmol. The redox potential was measured using the Fe-EDTA couple in 20 mM potassium phosphate (pH 7), containing 10 mM EDTA/1 mM $FeCl_3$/1 mM K_4 $FeCN_6$/1 mM methyl viologen. The potential of the solution was varied using a potentiostat. The redox potential was found to be 58 mV with $N = 1$ at 23°C and ionic strength about 0.1 M. The redox reaction was completely reversible. The ultraviolet–visible absorption spectra are shown in Fig. 3. The amino acid composition is shown in Table I along with that based on the sequence to indicate the accuracy to be expected in those compositions for which there is no corresponding sequence. The error in this case was about 10%.

The flavoprotein was not further purified. Its molecular weight was estimated by chromatography on Sephadex to be about 47000. It had ultraviolet-visible absorption peaks at 370 and 450 nm. Approx. 5 μmol was recovered.

The first ferredoxin band to be eluted from the initial DEAE-cellulose column was recognized as a member of the class of high redox potential ferredoxins based on ultraviolet–visible absorption spectra [7]. The second ferredoxin band was also identified as a HiPIP although both proteins were found to be completely oxidized as isolated.

Iso-I-HiPIP was purified by Sephadex G-75 followed by DEAE-cellulose chromatography utilizing a linear gradient of 0.16–0.32 M NaCl in 0.02 M Tris-HCl (pH 7.5). The protein was then adsorbed to hydroxylapatite and chromatographed using a linear gradient of 0.02–0.1 M potassium phosphate, pH 7.0 in 0.1 M NaCl. The final purification step was ammonium sulfate precipitation. Very little protein precipitated until the solution was saturated with ammonium sulfate and as much as one third had to be adsorbed on DEAE-cellulose from the saturated supernatant solution and eluted with NaCl. Approx. 28 μmol pure protein was recovered. The best purity index for oxidized protein (280–375 nm ratio) was 2.1. The ultra-

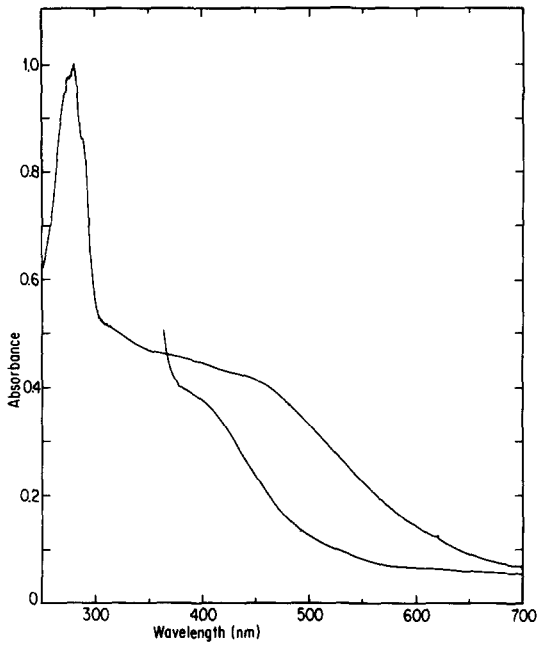

Fig. 4. Absorption spectra of iso-I-HiPIP in 0.1 M potassium phosphate (pH 7.0). Note that the fine structure is a little less distinct in the oxidized form as compared with iso-II-HiPIP.

violet–visible absorption spectra are shown in Fig. 4. The amino acid composition is shown in Table I and agrees within 12% with that based on the sequence.

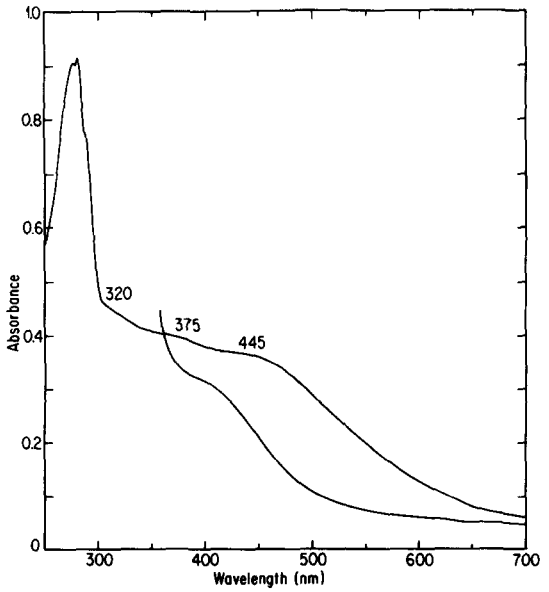

Fig. 5. Absorption spectra of iso-II-HiPIP in 0.1 M potassium phosphate (pH 7.0).

Iso-II-HiPIP was purified by Sephadex G-75 chromatography and DEAE-cellulose chromatography using a linear gradient from 0.2 to 0.4 M NaCl in 0.02 M Tris-HCl, pH 7.5. The DEAE-cellulose chromatography was repeated with a shallower gradient from 0.2 to 0.32 M NaCl. The results of ammonium sulfate precipitation were very similar to those for the iso-I-HiPIP. The best purity index (280–375 nm ratio) was 2.2. Approx. 45 μmol were isolated. The ultraviolet–visible absorption spectra are shown in Fig. 5. The amino acid composition is shown in Table I and is within 10% agreement with that derived from the sequence.

The cytochrome *c'* which was mixed with iso-II-HiPIP, was completely resolved on Sephadex G-75, but the cytochrome *c'*, bacterial ferredoxin and purple protein mixture was more difficult to separate. Although all three proteins were eluted in a single band on Sephadex, the purple protein was at the leading edge, cytochrome *c'* was in the middle and bacterial ferredoxin was at the trailing edge. Therefore, judicious division of fractions resulted in a cytochrome *c'*/ferredoxin mixture and a cytochrome *c'*/purple protein mixture. It was found that ammonium sulfate was an effective means of resolving these two mixtures, because cytochrome *c'* behaved like the high-potential iron sulfur proteins in that it precipitated at very high concentrations of ammonium sulfate (80–100% saturation), whereas bacterial ferredoxin precipitated primarily in the 60–80% saturation range and the purple protein in the region 70–90% saturation. The best cytochrome *c'* ammonium sulfate fraction was chromatographed on DEAE-cellulose using a linear gradient from 0.24 to 0.4 M NaCl in buffer. Although there were about 10 μmol cytochrome *c'* in the mixtures from the first DEAE-cellulose column, the final yield of purified protein was only approx. 1 μmol. The best purity index attained (ratio of 280–400 nm absorbance) was 0.35. The molecular weight estimated on Sephadex was about 30000 and by SDS-polyacrylamide gel electrophoresis was 13000 which indicates that the native protein is dimeric like most cytochromes *c'*. The absorption spectra were also typical as shown in Fig. 6. The amino acid composition is presented in Table I. The redox potential was found to be 30 mV at pH 7, 23°C,

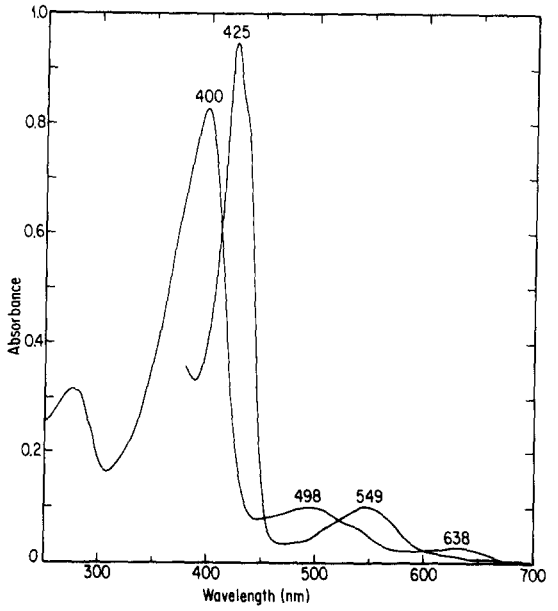

Fig. 6. Absorption spectra of cytochrome *c'* in 0.1 M potassium phosphate (pH 7.0).

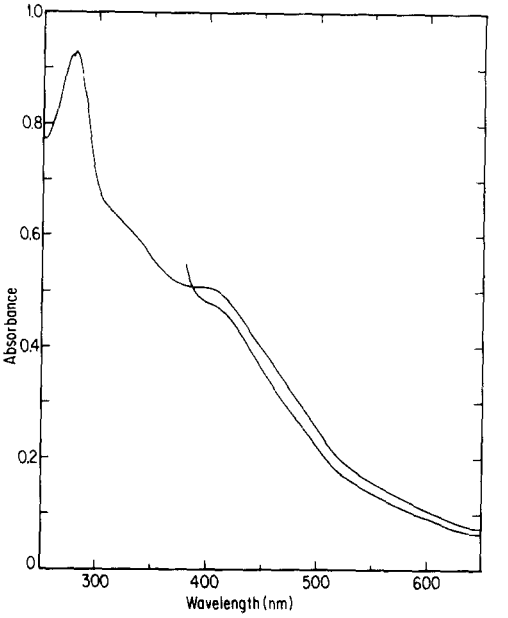

Fig. 7. Absorption spectra of the 'bacterial' ferredoxin in 0.1 M potassium phosphate (pH 7.0).

and ionic strength 0.1 M.

The best bacterial ferredoxin fraction from ammonium sulfate precipitation was chromatographed on DEAE-cellulose using a linear gradient from 0.2 to 0.4 M NaCl. The final yield of purified protein was only about 3 μmol, but there was approx. 15 μmol in the initial mixture (assuming an extinction coefficient at 390 nm of 32 $mM^{-1} \cdot cm^{-1}$ corresponding to two [4Fe-4S] clusters per protein). The molecular weight was estimated on Sephadex to be approx. 20000 and by SDS-polyacrylamide gel electrophoresis to be 22000. The amino acid composition, Table I, shows that there are about 8 cysteines and approx. 1.9 iron sulfur clusters per 108 amino acid residues (formula weight, approx. 11000). This indicates that both native and denatured protein are likely to be dimers. (Although mercaptoethanol is added in the SDS-polyacrylamide gel electrophoresis experiments to split disulfide bonds, with ferredoxins one often observes a molecular weight ladder composed of multiples of the subunit because of the high cysteine content). The absorption spectra are shown in Fig. 7. It is to be noted that the best purity index obtained (ratio of 280–390 nm absorbance) was 1.85, which is rather high compared with typical bacterial ferredoxins with two clusters. Most bacterial ferredoxins have few aromatics and usually no tryptophans, whereas *E. halophila* ferredoxin has about four tryptophans, which easily can account for the high 280 nm absorbance.

The purple protein was not completely purified, but the best fraction from ammonium sulfate precipitation was chromatographed on DEAE-cellulose using a linear gradient from 0.24 to 0.4 M NaCl. The absorption spectrum shown in Fig. 8 indicates a single charge-transfer band centered near 550 nm. The ultraviolet maximum was near 258 nm and the best purity index (ratio of 258–550 nm absorbance) was about 75, indicative of nucleic acid contamination. There was also a very small amount of cytochrome *c'* remaining as evidenced by the shoulder at 400 nm. There was about 9 μmol of crude purple protein assuming an extinction coefficient at 550 nm of 3 $mM^{-1} \cdot cm^{-1}$ similar to that of the purple acid phosphatases [8,9]. The protein color was completely bleached in phosphate buffer. The protein was slowly reduced by 2-mercaptoethanol without shifting the wavelength maximum and was reversibly reduced by dithionite. The molecular weight was very roughly estimated on Sephadex to be about 40000. On SDS-polyacrylamide gel electrophoresis, there were only two bands, corresponding to molecular

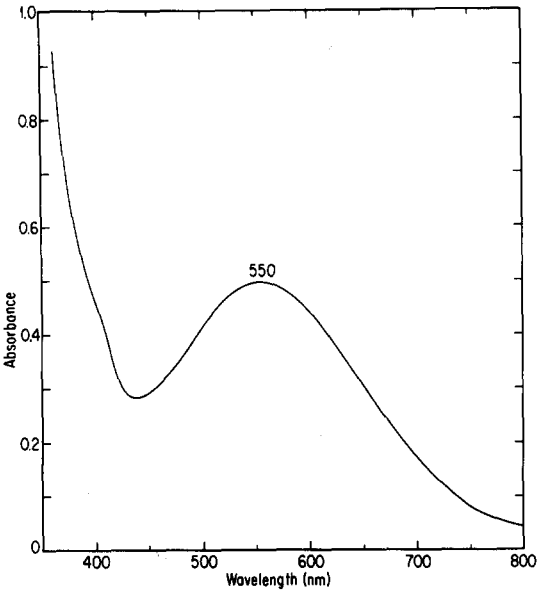

Fig. 8. Absorption spectrum of partially purified 'purple protein' in 0.1 M Tris-HCl buffer (pH 7.5). The color is reversibly bleached on addition of dithionite followed by potassium ferricyanide.

weights of 40 000 and 12 000. The smaller molecular weight band stained about twice as intensely as the larger. The amount of *c'* in the sample was less than 1% and could not account for the amount of small molecular-weight material. One of these two protein bands on the gel is probably from contamination, but at the present level of purification, it cannot be determined which.

Discussion

Eight chromophoric proteins were observed in soluble extracts of *E. halophila,* six of which were completely purified. The cytochrome *c*-551 is not particularly distinctive in any of its physico-chemical properties and could not a priori be assigned to any one group of low-spin cytochromes occurring in phototrophic bacteria. Based on ultraviolet-visible absorption spectra, redox potential and size, it could not be decided whether it was a small cytochrome c_2 such as found in about half the species of Rhodospirillaceae or whether it was related to the *Pseudomonas* cytochromes *c*-551, which currently have been found in only three species of purple phototrophs, viz., *Rhodopseudomonas gelatinosa, Rhodospirillum tenue,* and *Rhodocyclus purpureus.* None of the cytochromes c_2 have such a low redox potential as the *E. halophila* cytochrome *c*-551 ($E_{m,7} = 58$ mV), but *R. gelatinosa* cytochrome *c*-551 is comparable in this regard ($E_{m,7} =$ 28 mV). *E. halophila* cytochrome *c*-551 is actually homologous to, but not closely related to these two groups of proteins. It should be regarded as belonging to a new subclass of small cytochromes based on its amino acid sequence (Ambler, R.P., Meyer, T.E. and Kamen, M.D., unpublished data). A cytochrome *c*-551 from another halophilic purple bacterium, *Ectothiorhodospira halochloris* (Ambler, R.P., Meyer, T.E. and Kamen, M.D., unpublished data) also belongs to this new sequence class.

The presence of both HiPIP and cytochrome *c'* in *E. halophila* is an indication that this species may be more closely related to the chromatiaceae in general and to the atypical Rhodospirillaceae, *R. gelatinosa* and *R. tenue* than it is to the majority of the Rhodospirillaceae. HiPIP isozymes were found in *Ectothiorhodospira shaposhnikovii* [10] and now in *E. halophila* and *Ectothiorhodospira vacuolata* (Meyer, T.E. and Fischer, U., unpublished data). HiPIP isozymes seem to be confined to the genus *Ectothiorhodospira,* but at least one species, *E. halochloris,* appears to have none at all. The *E. halophila* HiPIP isozymes appear to have diverged relatively recently judging by amino acid sequences which are closer to one another than to other species (Tedro, S.M., Meyer, T.E. and Kamen, M.D., unpublished data). The redox potentials of the *E. halophila* HiPIP isozymes (50 and 120 mV) are the lowest yet reported for HiPIP's [12]. The *E. shaposhnikovii* and *E. vacuolata* HiPIP's have about the same redox potentials as one another, but intermediate to the others (150–155 and 260–270 mV) [5,7]. The only other HiPIP with unusually low redox potential is that from a halophilic *Paracoccus* sp. (280 mV) [13,14]. Most have potentials about 330–350 mV. Two species of Rhodospirillaceae have iso-cytochromes c_2 which differ both in amino acid sequence [15] and redox potential [16]. Cytochrome *c'* isozymes are also unusual in purple bacteria. In *Rhodopseudomonas palustris,* the cytochrome *c'* isozyme has different heme ligands as well as a distinct

amino acid sequence and is known as cytochrome *c*-556 [17].

Bacterial ferredoxin isozymes are more common. The amino acid sequences of the iso-ferredoxins from green sulfur bacteria show that both are likely to contain a pair of [4Fe-4S] clusters [18] but there appear to be both single cluster and double cluster ferredoxins in the purple bacterium, *Rhodospirillum rubrum* [19]. Only one bacterial ferredoxin was found in *E. halophila* and it appears to have two [4Fe-4S] clusters like the typical proteins from the green bacteria and the purple sulfur bacteria such as *Chromatium vinosum* [20], but may have a larger peptide chain.

The yellow protein of *E. halophila* is not identical to anything previously reported, but the chromophore appears to be similar to flavins which have an oxygen, nitrogen or sulfur in place of the 8-methyl group. At pH values slightly above neutrality, these flavins exist in a paraquinoid form which is redox inactive [21]. The absorption spectrum of the yellow protein is like the paraquinoid form of 8-hydroxyflavins and it is redox inactive. On the other hand, the hydroxyl group ionizes with a pK near neutrality in the model compounds. The spectrum of the yellow protein shifts with pH, but not in exactly the same way, 8-demethyl-8-hydroxyl-FAD and -FMN derivatives (maximal wavelength, 472 nm; extinction coefficient, 41 $mM^{-1} \cdot cm^{-1}$) are thought to be naturally occurring inactive aerobic degradation products of normal flavin and flavoproteins [22]. *E. halophila* is an anearobic bacterium, and 8-hydroxyflavin would have had to be formed during purification, if it were an abnormal product of air oxidation. However, there was no observed change in spectral properties during purification. Roseoflavin [23] is a naturally occurring 8-dimethylaminoflavin which appears to have a wavelength maximum shifted too far to the red (505 nm) to be the chromophore in the *E. halophila* yellow protein. Cofactor F-420 from the anaerobic methanogenic bacteria, is a naturally occurring derivative of 8-hydroxy-7,8-demethyl-5-deazaflavin (maximal wavelength, 420 nm; extinction coefficient, 45 $mM^{-1} \cdot cm^{-1}$) [24,25]. 7,8-Demethyl-8-hydroxy-5-deazaflavins also function in the DNA photoreactivating enzymes from *Streptomyces griseus* and various blue-green phototrophic bacteria [27–29] which split thymine dimers when irradiated by visible or near-ultraviolet light. The molecular weight of the *Anacystis nidulans* photoreactivating enzyme is 93000 [30] as contrasted with the *E. halophila* yellow protein which is 15000. A photolyase from *Escherichia coli* [31] and yeast [32] contains normal FAD but it is present as the neutral radical which imparts a blue color to the enzyme [11]. Acid precipitation, high-temperature coagulation or urea denaturation of proteins are standard procedures for removal of noncovalently bound flavins, but the yellow protein reversibly loses its color instead. This suggests that the chromophore is not a flavin, but on the other hand, the loss of color may be due to formation of a cyteinyl adduct of the supposed flavin which may also be covalently bound.

The purple protein also has no precedent among the phototrophic bacteria. It is most similar in properties to the purple acid phosphatases from rat spleen [8] and pig uterus [9]. The wavelength maxima coincide and the molecular weights are similar. However, mercaptoethanol causes a shift of the visible maximum of the phosphatases to shorter wavelengths, but merely reduces the *E. halophila* purple protein. The peak at 550 nm in phosphatase is apparently due to a tyrosine to iron charge transfer, which is also responsible for the color in catechuate dioxygenases and transferrins [26]. However, the latter proteins have wavelength maxima closer to 430–460 nm.

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