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Amino Acid Sequences and Distribution of High-Potential Iron–Sulfur Proteins That Donate Electrons to the Photosynthetic Reaction Center in Phototropic Proteobacteria*

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Abstract. High-potential iron-sulfur protein (HiPIP) has recently been shown to function as a soluble mediator in photosynthetic electron transfer between the cytochrome bc_1 complex and the reaction-center bacteriochlorophyll in some species of phototrophic proteobacteria, a role traditionally assigned to cytochrome c_2. For those species that produce more than one high-potential electron carrier, it is unclear which protein functions in cyclic electron transfer and what characteristics determine reactivity. To establish how widespread the phenomenon of multiple electron donors might be, we have studied the electron transfer protein composition of a number of phototrophic proteobacterial species. Based upon the distribution of electron transfer proteins alone, we found that HiPIP is likely to be the electron carrier of choice in the purple sulfur bacteria in the families Chromatiaceae and Ectothiorhodospiraceae, but the majority of purple nonsulfur bacteria are likely to utilize cytochrome c_2. We have identified several new species of phototrophic proteobacteria that may use HiPIP as electron donor and a few that may use cytochromes c other than c_2. We have determined the amino acid sequences of 14 new HiPIPs and have compared their structures. There is a minimum of three sequence categories of HiPIP based upon major insertions and deletions which approximate the three families of phototrophic proteobacteria and each of them can be further subdivided prior to construction of a phylogenetic tree. The comparison of relationships based upon HiPIP and RNA revealed several discrepancies.

Key words: High-potential iron-sulfur proteins — Phototrophic proteobacteria — Cytochromes c — Reaction center — Amino acid sequences

Introduction

High-potential iron–sulfur protein (HiPIP) is generally believed to participate in electron transfer reactions similar to those of the c-type cytochromes. HiPIP is a small soluble protein, located in the bacterial periplasmic space, which has a relatively high redox potential comparable to those of the c-type

*The new taxonomic classification for phototrophic bacteria is according to Imhoff (2001) and abbreviations are according to the International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of Phototrophic Bacteria (*Int. J. Syst. Bacteriol.* **49**, 925–926 [1999]).

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cytochromes. In purple photosynthetic bacteria such as *Rhodoferax fermentans*, *Rubrivivax gelatinosus*, *Rhodocyclus tenuis*, *Rhodopila globiformis*, *Allochromatium vinosum*, *Marichromatium purpuratum*, *Ectothiorhodospira vacuolata* and *Halorhodospira halophila*, HiPIP has been shown to function as mediator between the cytochrome bc_1 complex and the membrane-bound tetraheme cytochrome c of the photosynthetic reaction center, although it may not be the only mediator (Hochkoeppler et al. 1995, 1996; Schoepp et al. 1995; Menin et al. 1997, 1998; Osyczka et al. 1999a, b). However, soluble cytochrome c_2 was shown to donate electrons to the photoactivated special pair bacteriochlorophyll in the majority of purple nonsulfur bacterial species, either with or without participation of the membrane-bound tetraheme cytochrome c (Pettigrew and Moore 1987). The interactions of cytochromes c_2 with reaction centers appear to be dominated by electrostatic contacts, but the HiPIP–reaction center interaction is primarily hydrophobic and appears to require the participation of the tetraheme reaction center cytochrome c (Osyczka et al. 1999a, b).

Another possible role of HiPIP was found during extensive studies of the thermophilic and chemoorganotrophic bacterium *Rhodothermus marinus*. HiPIP is associated with the membrane of this bacterium, where it acts as an electron carrier between the novel cytochrome bc_1 complex and the cytochrome oxidase complex (Pereira et al. 1994, 1999). Such a role of HiPIP in the respiratory chain was also proposed for the facultative photosynthetic bacteria *Rhodoferax fermentans* and *Rhodovibrio salinarum* (formerly known as *Rhodospirillum salinarum*) (Bonora et al. 1999). A ferrous iron-oxidizing enzyme was isolated from the chemolithotrophic bacterium *Thiobacillus ferrooxidans*, strain Fe1, by Kusano et al. (1992), who identified the decameric protein as a HiPIP homologue.

HiPIPs are found primarily in purple phototrophic bacteria. With the exception of a halophilic *Paracoccus* sp., the chemolithotrophic (*Acidi*)*thiobacillus ferrooxidans*, *Ralstonia metallidurans* (formerly known as *Alcaligenes eutrophus*), *Ralstonia solanacearum*, and the thermophilic *Rhodothermus marinus*, all the other known HiPIP-containing bacteria are photosynthetic (Bartsch 1991; Kusano et al. 1992; Perreira et al. 1994). HiPIP contains a single cubane [4Fe–4S] center with 3+ and 2+ as the physiologically accessible oxidation states (Carter et al. 1972) . The redox potential ranges from 50 to 500 mV and averages 300 mV (Luchinat et al. 1994; Banci et al. 1995b; Heering et al. 1995). Bacterial ferredoxins have the same iron–sulfur cluster as HiPIPs but they differ in redox potential (average, about −400 mV) and they use the +1 and +2 oxidation states (Carter et al. 1972). In general, HiPIP cannot be reduced to the +1 level and bacterial ferredoxins cannot be oxidized to the +3 level without alteration of the iron–sulfur cluster. It is still uncertain what structural features determine which redox states will be accessible to the iron–sulfur cluster, although the degree of hydrogen bonding and relative exposure to solvent appear to be major contributors (Bentrop et al. 1996; Cowan et al. 1998; Babini et al. 1999).

Usually, HiPIP is an abundant protein in soluble cell extracts of phototrophic proteobacteria in the families Chromatiaceae and Ectothiorhodospiraceae; it is less abundant in the purple nonsulfur bacteria, family Rhodospirillaceae, and isozymes occur frequently. The amino acid sequences of a number of HiPIPs, and of genes coding for HiPIPs, have been published (Dus et al. 1973; Fischer 1980; Tedro et al. 1974, 1976, 1977, 1979, 1981, 1985a, b; Kusano et al. 1992; Moulis et al. 1993; Ambler et al. 1993b, 1994, 1999; Brüser et al. 1997a; Van Driessche et al. 1997). Besides these published data, there are HiPIP genes in the genomes of *Rhodopseudomonas palustris*, *Ralstonia metallidurans*, and *Ralstonia solanacearum* (http://www.jgi.doe.gov/; Salanoubat et al. 2002). The HiPIP sequences are not highly conserved and it is difficult to align them. Five three-dimensional structures of HiPIPs have been determined by X-ray crystallography and/or NMR, which aid in sequence alignment, but they do not cover the full range of variation (Carter et al. 1974; Freer et al. 1975; Holden et al. 1986; Rayment et al. 1992; Bertini et al. 1993, 1995, 1996, 1997; Banci et al. 1994, 1995a; Benning et al. 1994; Kerfeld et al. 1998; Nogi et al. 2000). Because HiPIPs are small and the insertions and deletions can be relatively large, it is expected that these insertions and deletions will affect either the redox properties or functional interactions, or both, to a certain degree.

Cytochromes c other than c_2 have also been implicated in photosynthetic electron transfer. There is at least one instance, in *Rhodocyclus tenuis*, where a cytochrome c_8 may assume the role of electron donor to the reaction center under certain conditions (Menin et al. 1998). In *Allochromatium vinosum*, cytochrome c_8 was found to be a good electron donor in vitro (Van Grondelle et al. 1977), although its cellular concentration is only a fraction of that of HiPIP, which would appear to preclude significant participation. Yet another high-potential cytochrome was reported in *Ach.*[1] *vinosum* (Cusanovich and Bartsch

[1]*Genus abbreviations used: Ach., Allochromatium*; *Atb., Acidithiobacillus; Blc., Blastochloris; Ect., Ectothiorhodospira*; *Hch., Halochromatium*; *Hlr., Halorhodospira*; *Ich., Isochromatium*; *Mch., Marichromatium*; *Psp., Phaeospirillum*; *Ral., Ralstonia*; *Rba., Rhodobacter*; *Rbi., Rhodobium*; *Rcs., Rhodocista*; *Rcy., Rhodocyclus*; *Rfx., Rhodoferax*; *Rmi., Rhodomicrobium*; *Rpi., Rhodopila*; *Rps., Rhodopseudomonas*; *Rsp., Rhodospirillum*; *Rhv., Rhodovibrio*; *Rdv., Rhodovulum*; *Rvi., Rubrivivax*; *Tch., Thermochromatium*; *Tba., Thiobacillus*; *Tca., thiocapsa*; *Tco., Thiococcus*; *Tcs., Thiocystis*.

1969), but the amino acid sequence has not been reported, and so far, its role in photosynthetic electron transfer is unknown. In the Ectothiorhodospiraceae, HiPIP is generally dominant, but some species do not appear to have any at all, such as *Hlr. halochloris* and *Hlr. abdelmalekii*. In these cases, it is likely that a cytochrome c_5 homologue rather than c_2 or c_8 may be the electron donor.

In this paper, we report the high-potential electron transfer protein compositions and the complete amino acid sequences of 14 HiPIPs from purple phototrophic bacteria and compare them with previously reported sequences. The present study pinpoints the species toward which further research should be directed to understand the structure/function relationship, especially as it relates to the roles of HiPIPs and cytochromes as electron donors to reaction centers.

Materials and Methods

Materials

HiPIPs were generally purified by the methods described by Bartsch (1978a) and Meyer (1994). HiPIPs iso-1 and iso-2 from *Ectothiorhodospira shaposhnikovii* were isolated, purified, and described by Kusche and Trüper (1984), and *Allochromatium warmingii* by Wermter and Fischer (1983). *Thiocystis violacea* HiPIP was isolated and purified by Fischer and Meyer (unpublished). The isolation of *Rhodobium marinum* (formerly known as *Rhodopseudomonas marina*) HiPIP was described by Meyer et al. (1990). *Halochromatium salexigens*, *Ectothiorhodospira vacuolata* iso-III, *Ectothiorhodospira mobilis* iso-I and iso-II, *Rhodomicrobium vannielii*, and *Rhodopseudomonas cryptolactis* HiPIPs were purified in Tucson. *Isochromatium buderi* HiPIP was isolated by Bartsch (unpublished).

Removal of Iron and Modification of the Apoprotein

The procedure followed for the removal of the iron–sulfur clusters was described by Hong and Rabinowitz (1967). The dried protein was dissolved in 15 m*M* Tris–HCl, pH 7.2, containing 20–25% trichloroacetic acid. After incubation in an ice bath for 1 h, the precipitated apoprotein was redissolved in water and desalted by ultrafiltration through a Centricon-3 membrane (Amicon, Beverly, MA) with 3 vol water. Cysteines were modified to carboxymethyl cysteine (Crestfield et al. 1963), pyridylethyl cysteine (Andrews et al. 1987), or aminopropyl cysteine (Jue et al. 1993). After incubation at 37°C for 2 to 4 h, the excess salts and reagents were removed by ultrafiltration as described above or by gel filtration through Sephadex SG25 (Pharmacia, Uppsala, Sweden), equilibrated, and eluted with 0.1 *M* ammonium bicarbonate, pH 7.4.

Enzymatic Digestions

Digestions or subdigestions with trypsin (Boehringer, Mannheim, FRG), *Staphylococcus aureus* V8 protease (Boehringer), Lys-C endoproteinase (Boehringer or Wako, Osaka, Japan), and/or Asp-N endoproteinase (Boehringer) were carried out in 50–100 m*M* Tris–HCl buffer, pH 7.5–8, for 2–4 h at 37°C at an enzyme-to-substrate ratio (mass/mass) of 1/40 to 1/100.

Peptide and Protein Purifications

At an earlier stage of this work, peptides obtained after enzymatic digestions were separated by high-performance liquid chromatography on a 214TP54 C4 column (Vydac, Hesperia, CA). The chromatographic equipment consisted of an 870 three-headed piston pump, an 8800 gradient controller, a UV spectrophotometer (DuPont, Wilmington, DE) set at 220 nm, and a Rheodyne injector equipped with a 100-µl loop. The eluted fractions were collected manually at a flow rate of 1 ml/min. At a later stage of the work, peptides were separated on a C18 column (2.1 × 100 mm) using the SMART chromatographic equipment (Pharmacia).

N-Terminal Sequence and Amino Acid Composition Analyses

Automated N-terminal sequence analyses of peptides were performed on a pulsed liquid-phase 477A, 476A, and/or 475A sequenator, equipped with an on-line 120A PTH amino acid analyzer (Applied Biosystems, Foster City, CA). Amino acid composition analyses were performed on a 420A Derivatizer with an on-line 130A PTC amino acid separation system (Applied Biosystems). Gas-phase hydrolyses were carried out in borosilicate glass tubes of 5 × 55 mm placed in a Pierce hydrolysis vial, using 6 *N* HCl for 20 h at 106°C.

C-Terminal Analyses

Several techniques were performed to confirm the C-terminal sequence of different HiPIPs. At an earlier stage of this work, different commercially available carboxypeptidases (Boehringer) were used to cleave the C-terminal residues of native or apoproteins and to analyze the released amino acids at different time intervals on a 420A Derivatizer, equipped with an on-line 130A separation system. After the introduction of biological mass spectrometry in our laboratories, we also analyzed the carboxypeptidase-digested native or apoprotein with these techniques to measure the mass of the enzymatically shortened protein. At a later stage of the work, the native proteins were subjected to automated C-terminal sequence analysis on the sequenator as described by Samyn et al. (2000).

Mass Analyses

In the earlier stages of our work, electrospray mass spectrometry was performed on a Bio-Q quadrupole mass spectrometer equipped with an electrospray ionisation source (Micromass, Altrincham, UK). Ten microliters of sample solution in 50% acetonitrile/1% formic acid was injected manually into the 10-µl loop of a Rheodyne injector and pumped to the source at a flow rate of 6 µl/min. The solvent of 50% acetonitrile/1% formic acid was delivered by the 140A Solvent Delivery System (Applied Biosystems). At a later stage, all samples were analyzed on a hybrid nanoelectrospray ionization quadrupole and time-of-flight (Q-TOF) mass spectrometer (Micromass, Wythenshawe, UK), equipped with an improved Z-nanospray ionization system. Tandem MS fragmentation of blocked and some selected peptides by collision-induced dissociation (CID) were performed using argon as the collision gas at a collision energy of 20–40 V. The MS/MS spectra were transformed using the MassLynx Sequence Software supplied with the mass spectrometer.

Overall Sequencing Strategy

As a starting point, we subjected the native HiPIPs to automated N-terminal sequence analysis, for which the sequencer was pro-

grammed to identify the maximum number of residues unambiguously in a single run. Mass spectrometric analysis was used to determine the mass of the proteins with and without the iron–sulfur cluster. After these analyses, the iron sulfur cluster of the proteins was removed and the cysteines were modified to a stable derivative for unambiguous identification during Edman degradation. We digested the modified apoproteins with two different enzymes, selected according to the number of cleavable basic and acidic residues such as arginines, lysines, and aspartic and glutamic acids, obtaining overlaps between different peptides and new sequence information to complete the final primary structure determination. In the case of blocked native proteins, the Edman-resistant peptide, obtained after chromatographic purification by reversed-phase HPLC, was selected and used for tandem MS fragmentation and amino acid composition analysis. Evidence for the C-terminal sequence of some HiPIPs, at an earlier stage of this work, was obtained after digestion of the native or modified apoproteins with carboxypeptidases, of which the released amino acids, following a time course of incubation, were analyzed by amino acid analysis. The combination of electrospray ionization mass spectrometric analysis and enzymatic digestion with carboxypeptidases on native or modified apoprotein proved to be a powerful tool for confirmation of the C terminus. At a later stage, we used automated C-terminal degradation to prove the correctness of the C-terminal sequence following the procedure described by Samyn et al. (2000).

Computer Graphics and Sequence Analysis

The structures of HiPIPs from *Thermochromatium tepidum* (1YET), *Marichromatium purpuratum* (3HIP), *Halorhodospira halophila* iso 1 (2HIP), *Ectothiorhodospira vacuolata* iso 2 (1HPI), and *Rhodocyclus tenuis* (1ISU) were superimposed on the structure of *Allochromatium vinosum* HiPIP (1BOY) using the SwissPdbViewer (http://us.expasy.org/spdbv/), and insertions and deletions as well as the overlapping segments were identified as discussed by Benning et al. (1994) and Van Driessche et al. (1997) .

Multiple sequence alignments were created using the ClustalW option of the MacVector software package (Oxford Molecular Group, Oxford, UK). From this alignment, similarity coefficients were calculated without taking the gaps into account. A phylogenetic tree, using the UPGMA method, was subsequently constructed with respect to the different insertion/deletion patterns; see Discussion.

Results

Distribution of Redox Proteins

Over the years, many species of phototrophic proteobacteria have been studied with respect to their content of electron transfer proteins (Bartsch, 1978a, b; 1991). It has been shown that cytochrome c_2 is the principal electron donor to reaction centers in the purple nonsulfur bacteria (Donohue et al. 1988; Knaff et al. 1991). However, it has recently been found that, in some instances, HiPIP and cytochrome c_8 can also function in photosynthetic electron transfer instead of cytochrome c_2.

From continuating literature data, we have updated the survey reported by Bartsch (1991) concerning available information on high-potential electron transfer proteins present in purple phototrophic bacteria that might conceivably function in cyclic electron transfer. In addition, we have surveyed a number of new species with an emphasis on HiPIP.

As shown in Table 1, there are twice as many phototrophic proteobacterial species producing HiPIP as those having cytochrome c_2. It is difficult, if not impossible, to prove the absence of a particular redox protein short of having the complete genome sequences available. Thus, it is not certain that the purple sulfur bacteria generally do not have cytochrome c_2 since no published genome sequences exist for these species. It is only in the case of *Thermochromatium tepidum*, for which there is a preliminary genome sequence (Integrated Genomics) , that we can postulate the absence of cytochrome c_2. For the four purple nonsulfur bacteria for which partial genome sequences exist, it is likely that HiPIP is absent in three of the four species (Joint Genome Institute and Integrated Genomics). Three of the four sequenced genomes (see Table 1) contain cytochrome c_2 isozymes, as well as the well- characterized soluble c_2 produced in abundance under phototrophic growth conditions. However, the tetraheme reaction-center cytochrome (THRC) and cytochrome c_8 are apparently absent in these four species. It is assumed that cytochrome c_2 is present in most nonsulfur phototrophic proteobacteria but we have no definitive evidence for its ubiquitous occurrence. Furthermore, it is not certain that all purple sulfur bacteria contain HiPIP and the tetraheme reaction-center cytochrome, but they have been found in nearly all species that have been examined. Therefore, it is likely that HiPIP functions as the principal electron donor to the reaction center in most, if not all, Chromatiaceae and in some Rhodospirillaceae.

Only a few species of nonsulfur phototrophic proteobacteria such as *Rpi. globiformis*, *Rmi. vannielii*, *Rbi. marinum*, *Rps. cryptolactis*, and *Rps. palustris* have both c_2 and HiPIP. Therefore, it will be of interest to determine which is the more efficient electron donor. Cytochrome c_8 is not very common in phototrophic proteobacteria, but most of those species containing this hemoprotein possess HiPIP as well. *Rcy. purpureus* is the only species that apparently has cytochrome c_8 but not HiPIP. There are both high- and low-potential versions of cytochrome c_8 in *Rvi. gelatinosus* that are differentially expressed under photosynthetic and aerobic growth conditions, indicative of different functional roles (Menin et al. 1999). No HiPIP has been detected in *Hlr. halochloris* or *Hlr. abdelmalekii* according to Then and Trüper (1983) or by our own survey, but all other *Ectothiorhodospiraceae* species contain HiPIP isozymes. All three extremely halophilic *Halorhodospira* species contain a relative of cytochrome c_5 which has been called *Ectothiorhodospira* cytochrome c-551. The cytochrome c_5 family of proteins also includes green

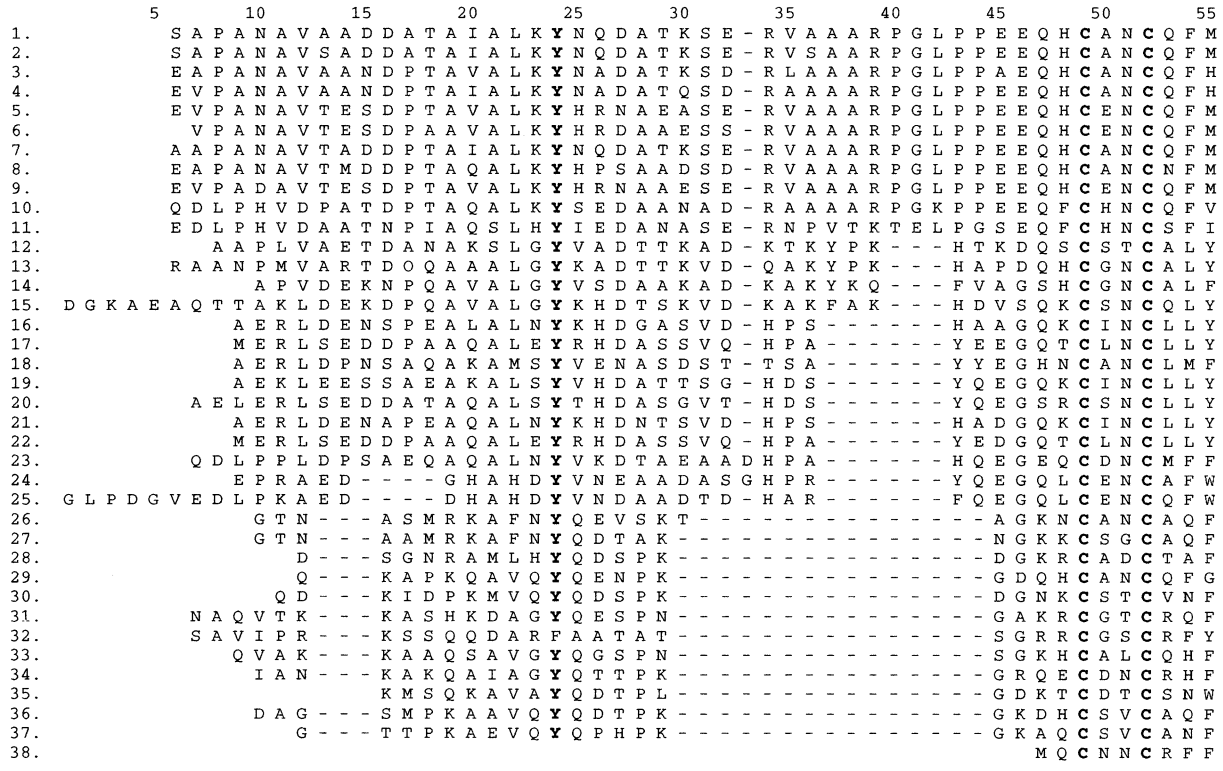
Table 1. Occurrence of high-potential electron carriers in purple and green bacteria that could function in photosynthesis as primary or secondary electron donors to reaction centers (nonphototrophic species containing HiPIP are also listed)

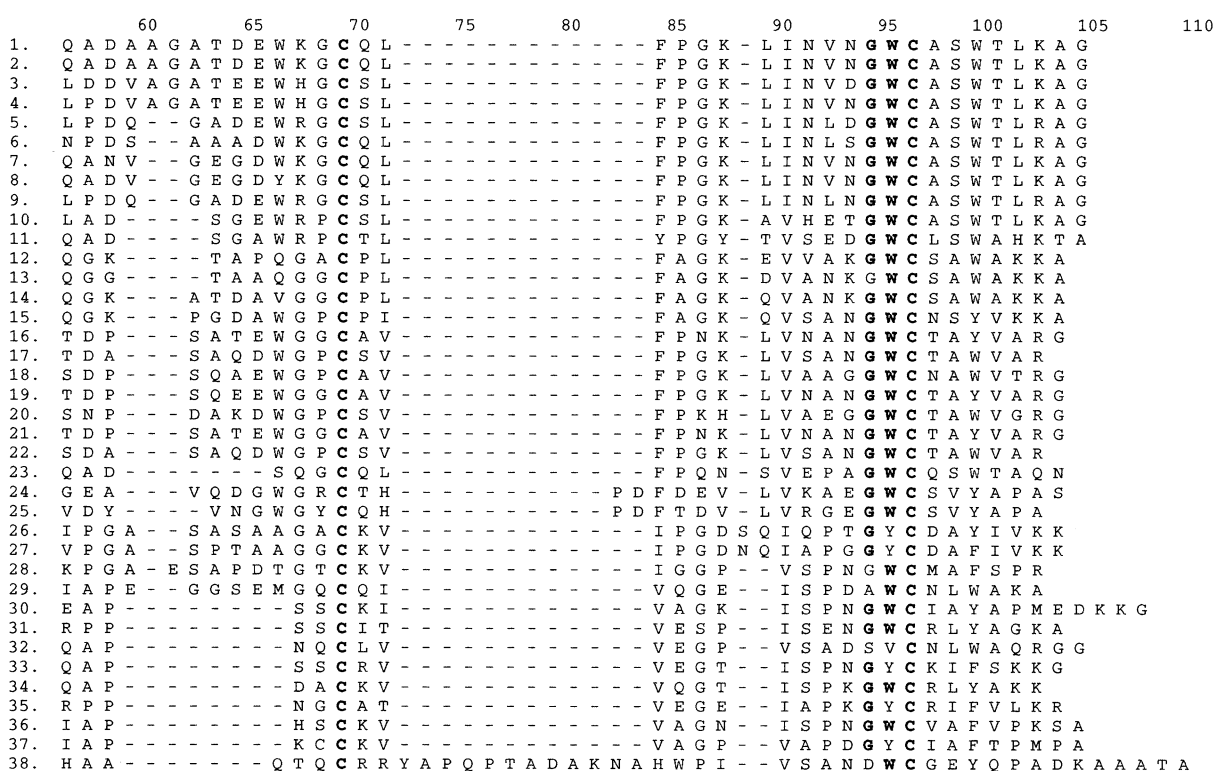
	HiPIP	THRC	C_2	C_8	C_5	C_4	Copper
Chromatiaceae							
Allochromatium vinosum	+	+		+		+	
Allochromatium warmingii	+						
Thiocapsa roseopersicina	+	+				+	
Thiocapsa sp. strain 5811	+	+					
Marichromatium gracile	+	+				+	
Marichromatium purpuratum	+	+		+		+	
*Thermochromatium tepidum**	+	+	–	–	–	+	–
Thiocystis violaceae	+						
Isochromatium buderi	+						
Halochromatium salexigens	+	+				+	
Thiococcus pfennigii	+	+				+	
Ectothiorhodospiraceae							
Ectothiorhodospira vacuolata	+ + +	+				+	
Ectothiorhodospira shaposhnikovii	+ +	+				+	
Ectothiorhodospira mobilis	+ +	+				+	
Halorhodospira halophila	+ +	+			+		
Halorhodospira halochloris		+			+		
Halorhodospira abdelmalekii		+			+		
Rhodospirillaceae							
Rhodoferax fermentans	+	+		+ +			
Rubrivivax gelatinosus	+	+		+ +		+	
Rhodocyclus tenuis	+	+		+		+	
Rhodocyclus purpureus		+		+			
Rhodovibrio salinarum	+ +	+					
Rhodovibrio sodomensis	+						
Rhodopila globiformis	+	+	+				
Blastochloris viridis		+	+				
Rhodomicrobium vannielii	+		+				
Rhodopseudomonas acidophila			+				
Rhodobium marinum	+	+	+				
Rhodocista centenaria		+	+ +				
Rhodopseudomonas cryptolactis	+ +		+ +				
*Rhodopseudomonas palustris**	+ +	–	+ +	–	–	+	–
*Rhodobacter capsulatus**	–	–	+ + +	–	–	–	+
*Rhodobacter sphaeroides**	–	–	+ + +	–	–	+ +	+
Rhodovulum adriaticum			+				
Rhodovulum sulfidophilum		+	+				
*Rhodospirilium rubrum**	–	–	+	–	–	+	+
Rhodospirilium photometricum			+				
Phaeospirilium molischianum		+	+ +				
Phaeospirilium fulvum			+ +				
Chloroflexaceae							
*Chloroflexus aurantiacus**	–	+	–	–	–	–	+ + +
Nonphototrophs							
Paracoccus sp.	+					+ +	
*Ralstonia metallidurans**	+	–	–	+ +	–	+ + + +	–
*Ralstonia solanacearum**	+ +	–	–	+ + +	+ +	+ + + +	–
*Acidithiobacillus ferrooxidans**	+	–	–	+ + +	–	+ + + +	+
Rhodothermus marinus	+						

Note. (+) Indicates strong evidence for the presence of one, or more (+ +, + + +, + + + +) isozymes. A blank space indicates that the gene is not expressed, that there is too little protein to identify, that the protein is located in the membrane making it difficult to prove, or that no attempt has been made to identify it. (–) Not present, as indicated from nearly complete genome sequences. *Partial genome sequence; when completed, additional components may be discovered. THRC, tetraheme reaction-center cytochrome c.

bacterial cytochrome c-555 and cyanobacterial cytochrome c_6. It is conceivable that c_5 too could function as an electron donor to the reaction center.

One of the most commonly encountered cytochromes in genome sequencing projects is cytochrome c_4, a protein that to date appears to be twice as common as cytochrome c_2. It occurs in all three families of the phototrophic proteobacteria, in both monoheme and diheme versions, although its usual membrane location makes it difficult to assess its





presence. Tiny amounts are often found in the soluble fraction, but in *Ectothiorhodospira* species, it appears to be a fully soluble protein. Thus, cytochrome c_4 is a potential candidate for photosynthetic electron donor in some species. *Chloroflexus aurantiacus* is interesting in that the partial genome sequence (Joint Genome Institute) indicates the presence of the tetraheme reaction-center cytochrome but the ab-

Fig. 1. HiPIP sequence comparisons: (1) *Ach. vinosum*, (2) *Ach. warmingii*, (3) *Tca. roseopersicina*, (4) *Thiocapsa* sp., strain 5811, (5) *Mch. gracile*, (6) *Mch. purpuratum*, (7) *Tch. tepidum*, (8) *Tcs. violaceae*, (9) *Ich. buderi*, (10) *Hch. salexigens*, (11) *Tco. pfennigii*, (12) *Rfx. fermentans*, (13) *Ral. solanacearum* iso-1, (14) *Rvi. gelatinosus*, (15) *Ral. metallidurans*, (16) *Ect. vacuolata* iso-1, (17) *Ect. vacuolata* iso-2, (18) *Ect. vacuolata* iso-3, (19) *Ect. mobilis* iso-1, (20) *Ect. mobilis* iso-2, (21) *Ect. shaposhnikovii* iso-1, (22) *Ect. shaposhnikovii* iso-2, (23) *Paracoccus* sp., (24) *Hlr. halophila* iso-1, (25) *Hlr. halophila* iso-2, (26) *Rcy. tenuis* 3761, (27) *Rcy. tenuis* 2761, (28) *Ral. solanacearum* iso-2, (29) *Rhv. salinarum* iso-1, (30) *Rpi. globiformis*, (31) *Rps. palustris* iso-1, (32) *Rps. palustris* iso-2, (33) *Rmi. vannielii*, (34) *Rbi. marinum*, (35) *Rps. cryptolactis*, (36) *Tba. ferrooxidans* strain Fe1, (37) *Atb. ferrooxidans* strain 23270 (genome), and (38) *Rhv. salinarum* iso-2. The N terminus of some HiPIPs, whose genes have been cloned and characterized, are hypothetical where the proteins have not yet been isolated. Minimal insertions and deletions are placed according to comparison of the available three-dimensional structures shown in Fig. 2. Highly conserved residues are in *boldface*.

sence of HiPIP or any of the soluble c-type cytochromes (Pierson 1985) typical of purple or green bacteria. Instead, *Chloroflexus* contains three small membrane-bound copper proteins, called auracyanin, that are likely candidates to participate in photosynthesis (Van Driessche et al. 1999; Bond et al. 2001). Genome sequences also indicate that *Rba. capsulatus*, *Rba. sphaeroides*, and *Rsp. rubrum*, but not *Rps. palustris*, contain genes for the blue copper protein pseudoazurin (Joint Genome Institute and Integrated Genomics). It has not been reported as a soluble protein in any of these species but should also be considered as a possible photosynthetic electron donor.

Sequence Determination

Following sequencing and mass analysis of peptides obtained after digestion of modified apoprotein by one or two different enzymes, the theoretical calculated mass of the complete amino acid sequence was compared with the measured masses of native or denaturated HiPIPs. For some HiPIPs, electrospray ionization mass spectrometry showed that the protein sample was composed of two different species, of which the B component differed from the A component by +14 Da (*Rps. cryptolactis*), +43 Da (*Ect. mobilis* iso 1), +111 Da (*Rmi. vannielii*), and −184 Da (*Rbi. marinum*). The mass difference of −184 Da corresponds very well to the sum of the theoretical masses of the first two N-terminal residues of the major component (Ile–Ala, 184 Da). The cleaved, but minor, component was also observed during the N-terminal sequence analysis of otherwise native protein. Concerning the other three observed mass differences, they could be explained after tandem mass spectrometric fragmentation of selected peptides that also exhibit the above-mentioned mass differences between the theoretical and the calculated masses of peptides after mass screening. Component B from *Rmi. vannielii* HiPIP corresponds with the mass of an extra pyroglutamic residue (111 Da) at the N terminus of the protein. Carbamylation at the N terminus of *Ect. mobilis* iso-1 and mutation of valine (99 Da) to isoleucine (113 Da) at position 38 of *Rps. cryptolactis* resulted in a mass increment of 43 and 14 Da, respectively, as observed during the electrospray ionization mass spectrometric analyses of native protein samples. It is thus likely that *Rps. cryptolactis* has more than one HiPIP gene. Carbamylation is a rare post-translational modification that was reported by Lapko and co-workers (2001), but this is the first report of such a posttranslational modification at the N terminus of a prokaryotic electron transport protein (Van Driessche et al. 2002).

HiPIP Sequences

In view of the fact that HiPIP may be as important as cytochrome c_2 in phototrophic proteobacterial photosynthesis, although relatively neglected until recently, we have isolated and characterized additional examples. The amino acid sequences of 14 new HiPIPs are shown in Fig. 1, in comparison with those of species previously published. They generally fall into three size categories, according to the family of phototrophic proteobacteria from which they were isolated. Thus, we have found that all species of Chromatiaceae that have been examined contain HiPIP, that they are of the largest type, and that the sequences are very similar to one another. We have characterized five new species in this family which are fairly typical (from *Ach. warmingii*, *Ich. buderi*, *Hch. salexigens*, *Tcs. violaceae*, and *Thiocapsa* sp.). About half the species of Chromatiaceae HiPIP contain a two-residue deletion at positions 60–61 (Fig. 1 numbering) as determined by comparison of the three-dimensional structures of *Ach. vinosum*, *Tch. tepidum*, and *Mch. purpuratum* (Carter et al. 1974; Kerfeld et al. 1998; Nogi et al. 2000) (Fig. 2A). This deletion must have occurred more than once, based upon the percentage identities shown in Fig. 3, yet it is located in exactly the same place. Two species, *Ha1ochromatium salexigens* and *Thiococcus pfennigii*, contain a single four-residue deletion in the same region as the two-residue deletion of the other species, although it cannot be precisely located at the present time, nor can we establish whether it is the result of a single event or two sequential events. These two species of HiPIP are also the most divergent of their family on a percentage basis.

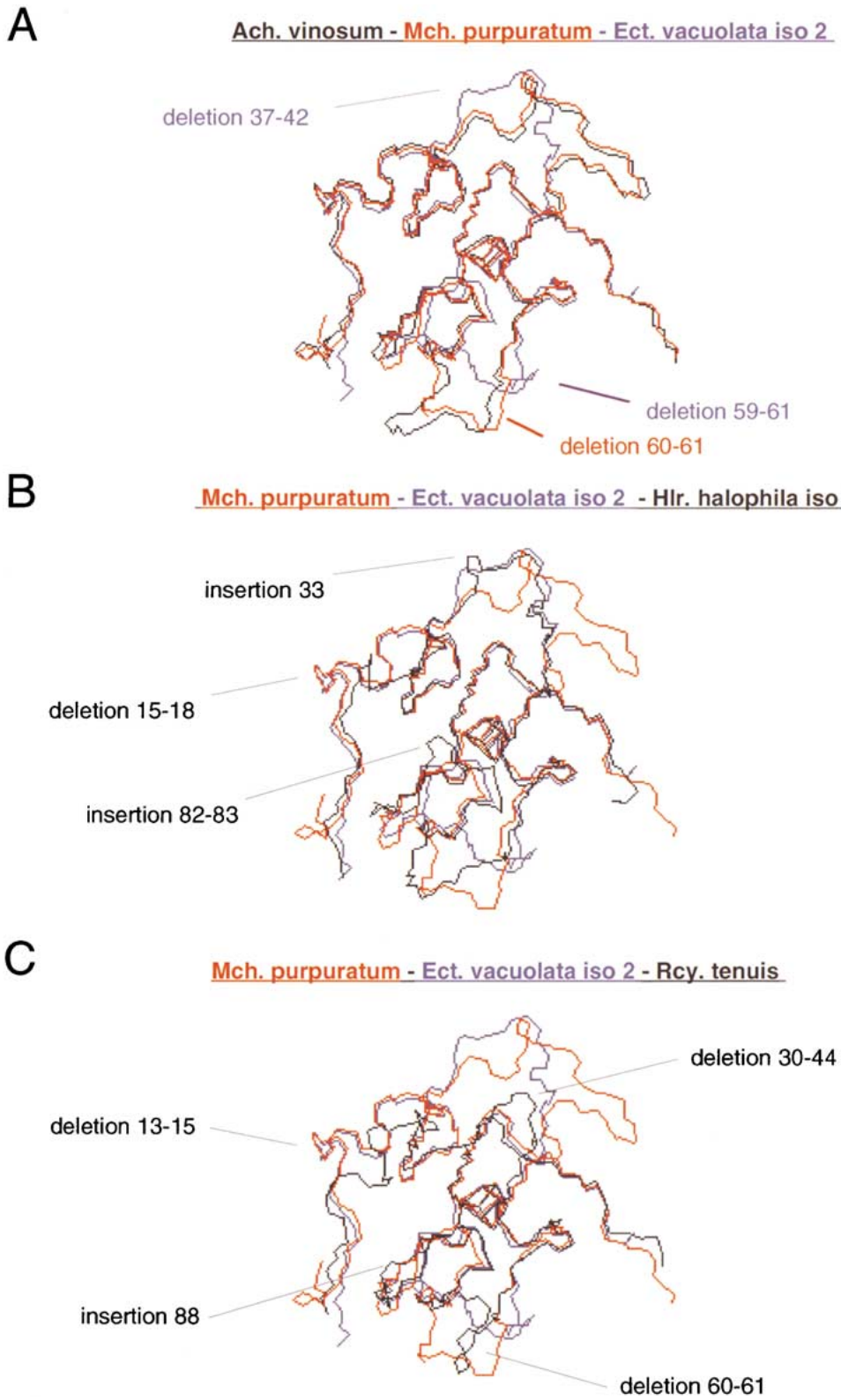


Fig. 2. **A** The structure of *Ach. vinosum* (black), superimposed upon the structures of *Mch. purpuratum* (red) and *Ect. vacuolata* iso-2 (blue). **B** The structures of *Mch. purpuratum* (red) and *Ect. vacuolata* iso-2 (blue) superimposed upon the structure of *Hlr. halophila* iso-1 (black). **C** The *Rcy. tenuis* structure (black) superimposed upon the structures of *Mch. purpuratum* (red) and *Ect. vacuolata* iso-2 (blue). Significant insertions and deletions discussed in the text and shown in Fig. 1 are indicated.

Ach. vinosum
97 *Ach. warmingii*
89 **88** *Tch. tepidum*
78 **77** **85** *Tcs. violacea*
81 **78** **77** **72** *Tca. roseopersicina*
82 **80** **78** **73** **91** *Tca.* sp., strain 5811
72 **71** **72** **73** **71** **75** *Ich. buderi*
72 **71** **72** **72** **73** **75** **95** *Mch. gracile*
73 **72** **74** **74** **70** **72** **85** **84** *Mch. purpuratum*
59 **58** **59** **59** **62** **64** **60** **59** **56** *Hch. salexigens*
40 38 40 38 40 38 36 38 33 **55** *Tco. pfennigii*
36 35 32 29 36 35 27 27 29 31 29 *Rfx. fermentans*
35 38 38 35 42 40 31 31 34 34 31 **74** *Ral. solanacearum* 1
38 38 35 35 41 38 35 34 36 35 30 **57** **67** *Rvi. gelatinosus*
36 36 36 32 36 36 36 35 35 38 32 **51** **55** **57** *Ral. metallidurans*
32 33 32 32 38 36 38 35 36 31 22 32 39 37 **48** *Ect. vacuolata* 1
33 35 33 32 38 38 36 35 35 31 24 33 41 34 **48** **93** *Ect. shaposhnikovii* 1
40 42 38 33 39 40 40 39 39 31 25 36 34 34 **42** **73** **73** *Ect. mobilis* 1
37 37 39 38 37 38 41 39 42 39 31 32 37 35 **48** **65** **66** **62** *Ect. vacuolata* 2
35 35 38 37 37 37 39 38 41 38 31 33 37 34 **48** **64** **66** **59** **97** *Ect. shaposhnikovii* 2
32 35 32 31 30 30 32 32 32 34 26 29 33 32 39 **53** **54** **58** **66** **66** *Ect. mobilis* 2
35 33 32 32 35 35 35 35 33 32 25 29 28 35 35 **51** **50** **61** **52** **52** **54** *Ect. vacuolata* 3
37 35 35 38 31 33 35 32 38 38 30 29 31 39 34 35 38 35 36 33 32 33 *Paracoccus* sp.
24 24 28 26 21 23 28 26 28 26 22 27 25 33 25 30 31 38 35 33 31 34 32 *Hlr. halophila* 1
27 27 27 30 27 28 27 25 31 30 22 25 28 24 31 33 30 38 31 30 21 30 35 **75** *Hlr. halophila* 2
22 22 21 21 22 22 21 21 21 22 20 22 25 29 20 29 30 30 25 25 23 33 25 20 19 *Rcy. tenuis* 3761
24 24 21 21 21 23 21 21 21 19 19 24 27 32 22 28 30 32 24 24 28 33 25 19 19 **74** *Rcy. tenuis* 2761
21 21 21 23 17 25 23 21 23 20 24 24 25 25 23 32 32 32 36 37 30 32 23 21 21 **44** **44** *Ral. solanacearum* 2
19 19 19 21 17 15 15 17 17 19 21 23 28 25 21 19 19 21 19 20 23 25 23 18 17 32 36 39 *Atb. ferrooxidans,* strain 23270
26 26 26 26 25 26 23 21 21 21 23 25 25 28 28 26 25 28 27 26 25 28 23 22 19 38 **43** **45** **66** *Tba. ferrooxidans, strain* Fe1
22 22 22 24 19 20 20 19 19 20 22 28 23 26 31 26 28 30 25 27 21 26 21 22 22 32 36 **47** **47** **53** *Rpi. globiformis*
33 33 31 31 25 27 24 22 24 20 24 22 24 27 27 22 22 26 24 24 22 27 22 16 18 33 35 39 34 **45** **43** *Rmi. vannielii*
30 30 28 28 25 25 23 23 23 23 28 32 32 30 27 23 23 26 23 23 21 25 25 22 22 36 34 33 36 **47** **42** **51** *Rbi. marinum*
35 35 33 33 32 30 32 33 28 31 31 25 29 25 32 21 23 25 25 25 23 32 25 25 29 30 23 25 39 **47** **42** **43** **51** *Rhv. salinarum* 1
22 22 20 22 18 22 20 18 18 20 24 25 23 24 21 18 19 22 16 16 22 22 15 16 18 31 29 33 25 36 **43** **50** **47** 35 *Rps. palustris* 1
24 24 24 24 24 25 24 24 24 20 18 24 26 24 17 22 22 25 20 20 20 25 20 17 16 32 41 28 35 **43** 37 **45** 41 35 **45** *Rps. cryptolactis*
20 20 20 20 20 18 16 18 16 20 29 24 25 24 22 22 25 27 27 27 27 27 22 19 18 15 16 24 23 21 23 31 36 35 33 25 *Rps. palustris* 2
24 24 24 24 19 22 22 19 19 24 27 22 24 22 32 28 28 28 36 31 19 25 31 34 31 19 14 24 25 28 31 31 31 31 28 11 28 *Rhv. salinarum* 2

Fig. 3. Percentage identities of HiPIP according to the alignment in Fig. 1. Significant values higher than 41% are in *boldface.*

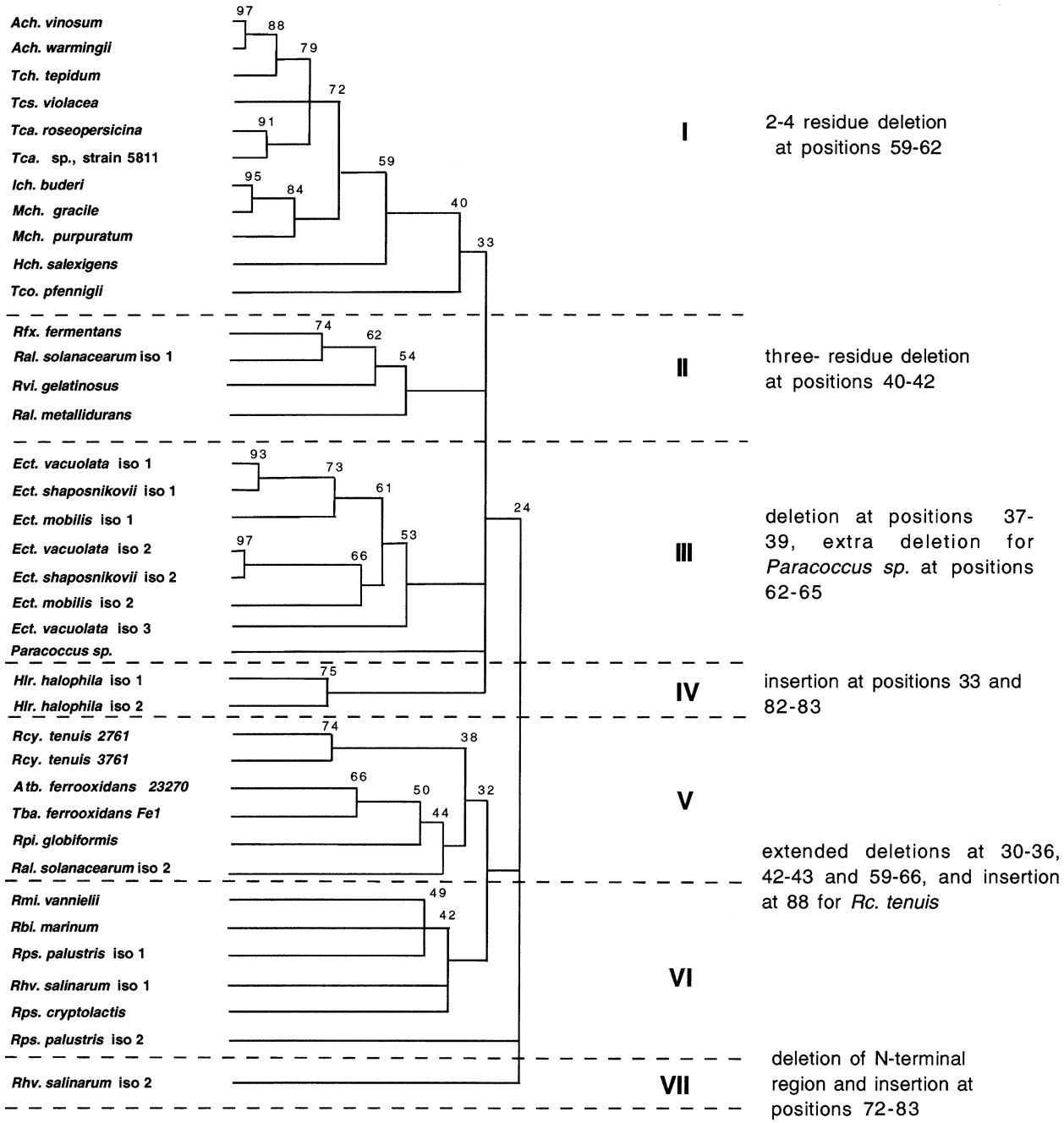


Fig. 4. UPGMA evolutionary tree for HiPIP from the data in Fig. 3 and on insertions and deletions. The separation of species according to the occurrence of significant shared insertions and deletions is indicated by *dashed lines*. When the rms deviation (3%) was larger than the difference between groups, they were combined and averaged. As described in the discussion, groups I and II reside in major group 1, groups III and IV in major group 2, and groups V, VI, and VIII in major group 3.

The Ectothiorhodospiraceae are interesting in that all but *Hlr. halochloris* and *Hlr. abdelmalekii* contain HiPIP. All species with HiPIP contain from one to three isozymes, and the sequences contain a three-residue deletion in the same area as the two- and four-residue deletions of the Chromatiaceae. In addition, they contain a six-residue deletion at positions 37 to 42 (Fig. 1 numbering) as determined from comparison of the three-dimensional structures of *Ach. vinosum*, *Ect. vacuolata* iso-2, and *Hlr. halophila* iso-1 HiPIPs (Breiter et al. 1991; Benning et al. 1994) (Figs. 2A and B). Based upon its size, the six-residue deletion is far more significant (Pascarella and Argos 1992) than the smaller ones described above and is not likely to have occurred more than once. It clearly distinguishes the Ectothiorhodospiraceae HiPIPs from those of the Chromatiaceae. We have added five new HiPIPs to this family (*Ect. shaposhnikovii* iso-1 and iso-2, *Ect. mobilis* iso-1 and iso-2, *and Ect. vacuolata* iso-3) and now believe that all *Ectothiorhodo-*

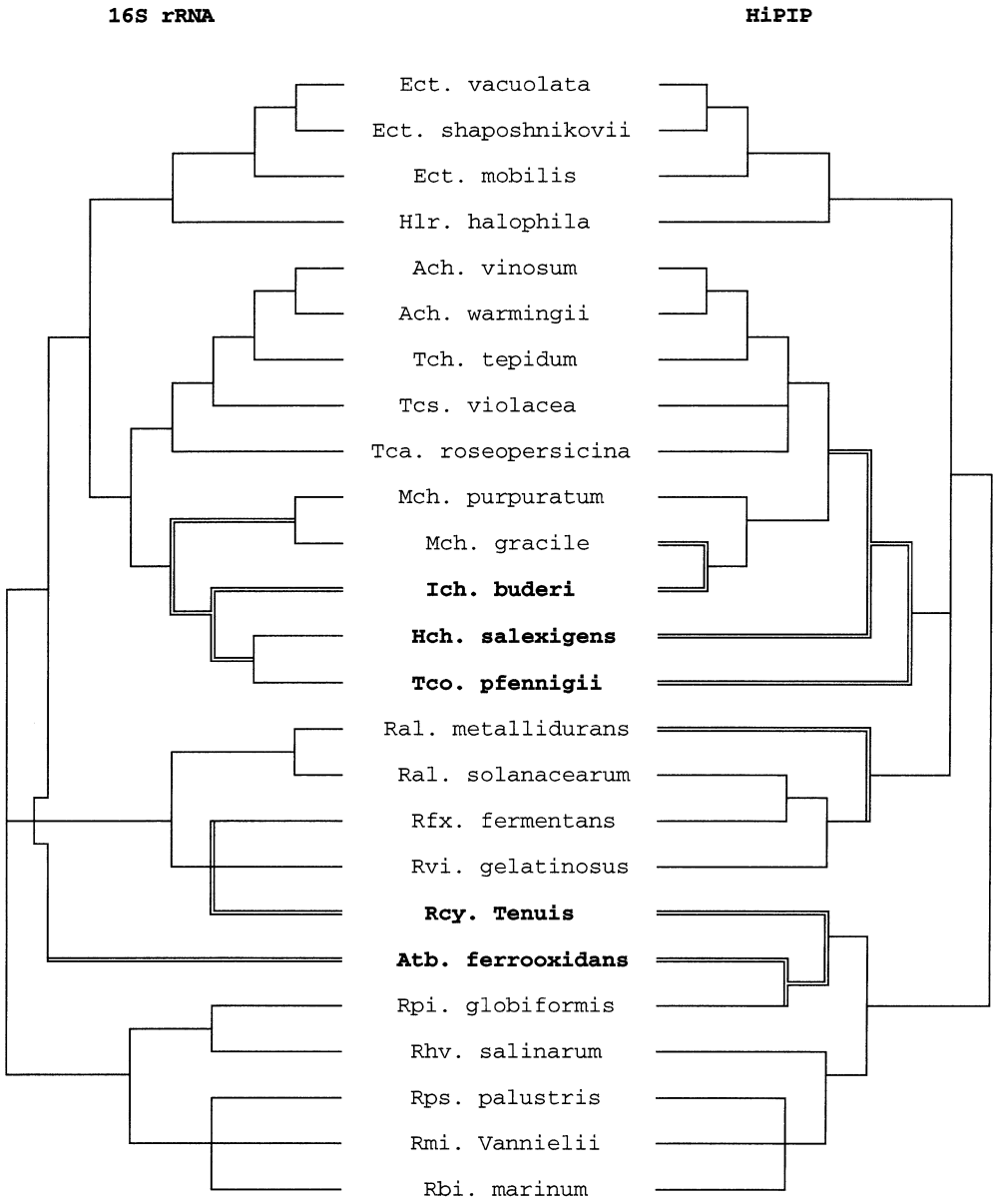


Fig. 5. A classification of species based upon our own analysis of rRNA data compiled at http://rrna.uia.ac.be and by Imhoff et al. (1998) and Kelly and Wood (2000) compared and contrasted with divisions based upon the HiPIP tree in Fig. 4. Species which are connected differently based on analysis of the two molecules are in *boldface*. These regions of disagreement are indicated by *double lines*.

spira species may contain three or more isozymes. *Hlr. halophila* is unique in that both isozymes contain additional insertions and deletions beyond those of the family as a whole that define a distinctive subgroup of HiPIPs. Both isozymes have a two-residue insertion between position 82 and position 83, and they have a four-residue deletion at positions 15 to 18 (Fig. 1 numbering). Iso-1 HiPIP has a single-residue insertion between position 27 and position 28 (*Ach. vinosum* numbering, shared with the halophilic *Paracoccus* HiPIP). Iso-2 HiPIP has an extra-residue deletion in the region of the three-residue deletion shared by the other species. The halophilic *Paracoccus* HiPIP is most similar to the *Ectothiorhodospira* HiPIPs but has a seven-residue deletion, whereas the others have a three-residue deletion and may have the four-residue deletion near the N terminus like those of *Hlr. halophila* based upon sequence evidence alone.

HiPIPs from the Rhodospirillaceae group into two major sequence categories based upon insertions and deletions. Those from *Rubrivivax gelatinosus* and *Rhodoferax fermentans*, plus the nonphototrophic *Ralstonia metallidurans* and *Ralstonia solanacearum* iso-1, contain a three- to four-residue deletion in the same region as the two- to four-residue deletions of the Chromatiaceae, but in addition, they contain a three-residue deletion in the vicinity of positions 34 to 36 of *Ach. vinosum* HiPIP (40 to 42 in Fig. 1). These

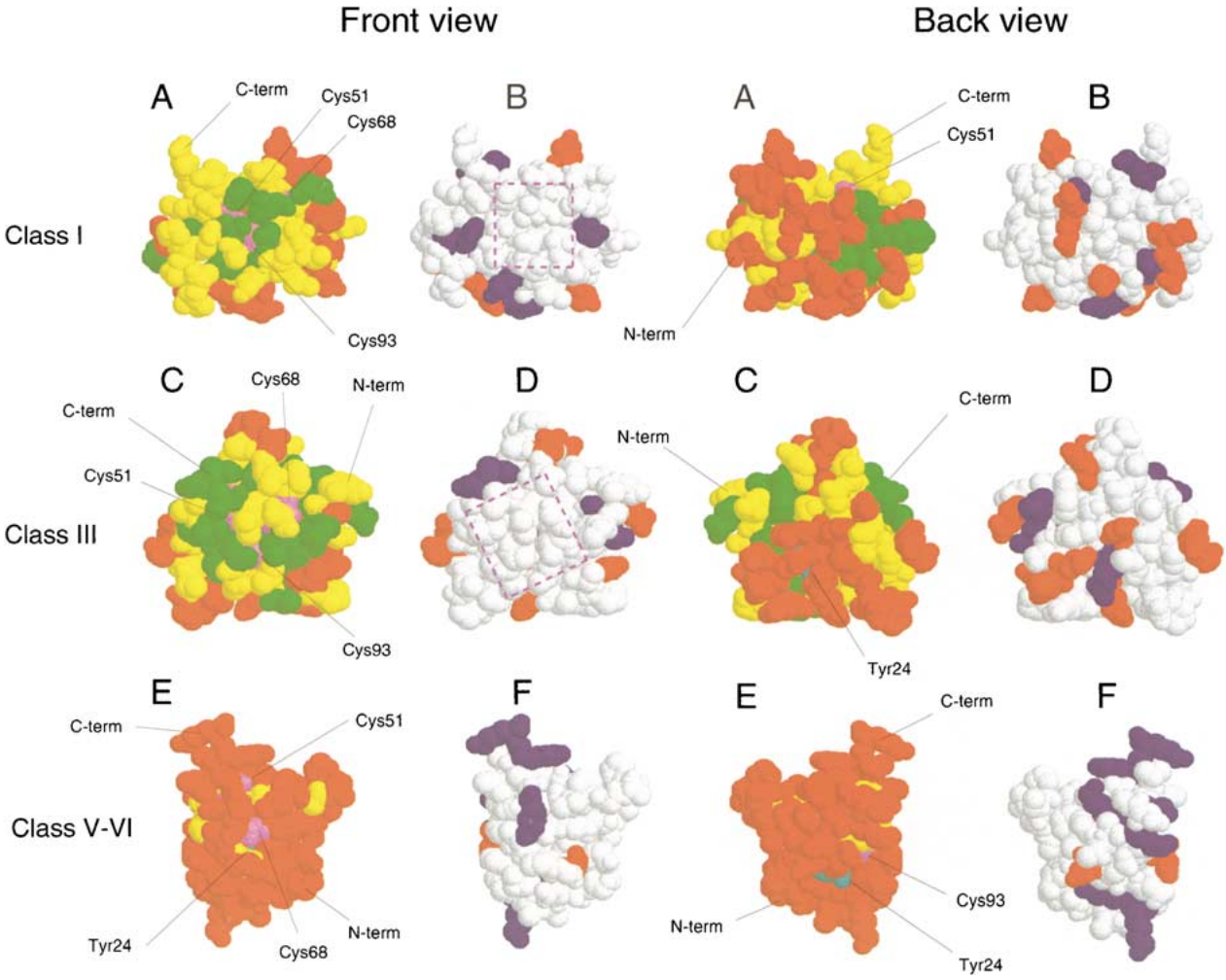


Fig. 6. Protein surface showing identical (green), conservatively substituted (yellow), and radically changed (red) amino acids mapped onto the three-dimensional structure of *Ach. vinosum* (**A**), *Ect. vacuolata* iso-2 (**C**). and *Rcy. tennis* (**E**) HiPIP. Distributions, of charged amino acids (basic, blue; acidic, red) are shown on the three-dimensional structures of *Ach. vinosum* (**B**), *Ect. vacuolata* iso-2 (**D**), and *Rcy. tenuis* (**F**) HiPIP. The conserved tyrosine and the cysteines are colored cyan and violet, respectively.

gaps cannot be placed more precisely because of the lack of a three-dimensional structure for this group, but the three-residue deletion appears to be in the same location as the six-residue deletion in the proteins from the Ectothiorhodospiraceae. Thus, these four HiPIPs define a subgroup that appears to be intermediate between those of the Chromatiaceae and those of the Ectothiorhodospiraceae, although slightly closer to those of the Chromatiaceae.

The majority of HiPIPs from the Rhodospirillaceae, of which we have characterized four new examples (*Rmi. vannielii*, *Rbi. marinum*, *and Rps. cryptolactis* iso-1 and iso-2), generally share two rather large and significant deletions, one of 13 residues between position 30 and position 44 (Fig. 1 numbering) and another of 8 based upon comparison of the three-dimensional structures of *Mch. purpuratum* and *Rcy. tennis* HiPIPs (Rayment et al. 1992) (Fig. 2C). The latter deletion of eight residues between position 59 and position 66 cannot be precisely placed because of the lack of a three-dimensional structure for the smallest proteins. The *Rcy. tenuis* and *Rhodovibrio salinarum* iso-1 proteins have a two-residue deletion and the *Ral. solanacearum* iso-2 has a single-residue deletion rather than the eight-residue deletion present in most species, which defines an intermediate but distinct subgroup. The 13- and 8-residue deletions are quite unusual and clearly set the majority of Rhodospirillaceae HiPIPs apart from the other two families. *Rcy. tenuis* appears to have a three-residue deletion near the N terminus (at positions 13 to 15) which may be shared by some of the other HiPIPs, but because of its position near the N terminus, it is hard to be certain whether it is real. *Rcy. tenuis* also has a single-residue insertion between position 69 and position 70 of the *Ach. vinosum* sequence. The new HiPIPs from *Rps. cryptolactis*, *Rbi. marinum*, and *Rmi. vannielii* are typical of the group as a whole. The two *Rps. palustris* HiPIP genes, discovered as a result of genome sequencing

(Joint Genome Institute), both conform to the pattern of small HiPIPs. However, the *Ral. solanacearum* HiPIPs (Salanoubat et al. 2002) fall into two categories, based upon insertions and deletions that suggest that one was acquired by gene transfer or that the smaller HiPIPs in our group VI were directly derived from those in group II through duplication and divergence.

The most unusual HiPIP is the *Rhodovibrio salinarum* iso-2, in which the N-terminal 40 residues have been deleted and 12 residues inserted in the region of positions 72–83 (Ambler et al. 1999). It has a seven-residue deletion in the same region as the eight-residue deletion of most of the other small HiPIPs. *Rhodovibrio salinarum* iso-2 HiPIP has a very high potential of 500 mV and the iron–sulfur cluster decomposes when oxidized, probably as a result of the large reduction in protein size that is likely to increase exposure of the cluster to solvent.

The percentage identities among the HiPIPs were calculated based upon the alignment in Fig. 1 as shown in Fig. 3. The most significant numbers are highlighted and these data were used in turn to construct an evolutionary tree as shown in Fig. 4. Both percentage identities and insertions and deletions were taken into account separately in tree construction. *Halochromatium salexigens* and *Thiococcus pfennigii*, both from group I, share a gap of four residues and are 55% identical to each other. However, *Hch. salexigens* is 60% identical to the other species from group I and *Tco. pfennigii* is only 40% identical to the others. Since these values are based on an average of 9 and 10 species, respectively, they are more significant than the 55% identity. The same conclusion was arrived at for *Rhodocyclus tenuis*, *Ralstonia solanacearum* iso-2, and *Rhodovibrio salinarum* iso-1. The observation that *Rcy. tenuis* HiPIPs have significantly less percentage identity to the group VI HiPIPs than do *Rhv. salinarum* iso-1 and *Ral. solanacearum* iso-2, but that the latter have insertions and deletions, similar to those of *Rcy. tenuis*, suggests that the eight-residue deletion at positions 59–66 is less significant.

For group I, the nearest neighbors of *Thiocystis violaceae* are *Thermochromatium tepidum*, *Allochromatium vinosum*, and *Allochromatium warmingii* at 80% (rms = ±3%), whereas *Thiocapsa roseopersicinia* and *Thiocapsa* sp., strain 5811, connect to the above group at 78% (rms = ±4%). Because the errors of 3 and 4% are larger than the difference in the branch lengths, the values of 78 and 80% were averaged to 79%, and *Tcs. violaceae* and *Tca. roseopersicinia–Thiocapsa* sp. should be considered equidistant from the *Ach. vinosum–Ach. warmingii–Teh. tepidum* group. The same conclusion was also reached for the species in groups V and VI: *Rhodomicrobium vannielii*, *Rhodobium marinum*, and *Rhodopseudomonas palustris* iso-1 should be considered equidistant because the differences in branch lengths are smaller than the errors.

Discussion

Electron Transfer Protein Distribution

With the growing interest in alternative electron donors to photosynthetic reaction centers, it is appropriate to establish which high-potential electron carriers in phototrophic proteobacteria might conceivably interact with reaction centers. As shown in Table 1, all of the species of the Chromatiaceae that have been examined contain HiPIP. Our own experience indicates that it is one of the most abundant of the soluble electron carriers. Most, if not all, species have the membrane-bound tetraheme reaction-center cytochrome c as well. On the other hand, soluble high-potential cytochromes have not been characterized in many species of Chromatiaceae, perhaps because they are much less abundant than is HiPIP. These findings are consistent with the observation that the HiPIP gene in *Allochromatium vinosum* is essential for photoorganotrophic growth (Brüser et al. 1997b; Kerfeld et al. 1998; C. Dahl, personal communication). HiPIP is probably the preferred electron donor to reaction centers under most conditions, as shown for *Ach. vinosum* and *Mch. purpuratum* (Menin et al. 1998).

It has been demonstrated that a small soluble cytochrome c-551 can donate electrons to reaction centers in *Ach. vinosum* (Van Grondelle et al. 1977), and we have identified this protein as a cytochrome c_8 through previous sequence determination (Samyn et al. 1996). It is relatively closely related to the prototypical cytochrome c_8 from *Pseudomonas aeruginosa*. *Marichromatium purpuratum* is the only other species of purple sulfur bacteria that has been reported to have a cytochrome c_8 (Kerfeld et al. 1996). A high-potential cytochrome c extracted from *Ach. vinosum* membranes with acetone (Cusanovich and Bartsch 1969) is a diheme cytochrome c_4 (unpublished results) related to the prototypic cytochrome c_4 from *Azotobacter vinelandii* (Ambler et al. 1984). We have observed small quantities in the soluble fraction of several other species of purple sulfur bacteria based upon spectral analysis as shown in Table 1. It is thus possible that both cytochrome c_4 and cytochrome c_8 could function in photosynthesis under some conditions.

Unlike the Chromatiaceae, we found HiPIP in only four of six Ectothiorhodospiraceae species examined as shown in Table 1. However, HiPIP is abundant in these four species. There are two major components and one or more minor forms of HiPIP

in each. We have determined the sequences of the major HiPIPs and of a minor component as well. The differences among the *Ectothiorhodospira* HiPIP sequences and redox potentials are large enough to suggest that they may have different functional roles, although it is unclear which of the HiPIP isozymes may function in photosynthesis. The work of Menin et al. (1998) suggests that it is the higher potential iso-1 HiPIP in *Ect. vacuolata* that mediates electron transfer to the reaction center.

There are soluble cytochromes in the Ectothiorhodospiraceae, but we have found that the protein from *Ect. vacuolata* is like the *Ach. vinosum* cytochrome c_4 and that the *Hlr. abdelmalekii* protein is like the *Hlr. halophila* and *Hlr. halochloris* c-551 (unpublished results). The *Halorhodospira* c-551 proteins are part of the greater cytochrome c_5 family (prototype from *Azotobacter vinelandii*) related to *Chlorobium* cytochromes c-555 and to cyanobacterial cytochromes c_6 (Ambler et al. 1993a). Considering the apparent absence of HiPIP in *Hlr. halochloris* and *Hlr. abdelmalekii*, it is likely that the cytochrome c_5 homologue may function as electron donor to reaction centers, although the role of this cytochrome in *Hlr. halophila* is unclear. A cytochrome other than c_5 appears to be an electron donor *in Hlr. halophila*, although the c_5 homologue has the potential to be an effective mediator (Menin et al. 1998). Judging by the strong similarity among *Ect. shaposhnikovii*, *Ect. vacuolata*, and *Ect. mobilis* HiPIPs, it is likely that the high-potential cytochromes from *Ect. shaposhnikovii* and *Ect. mobilis* will be like the cytochromes c_4 from *Ect. vacuolata* and *Ach. vinosum*. A difference is that the *Ectothiorhodospira* cytochromes c_4 are more abundant in the soluble fraction than is the *Ach. vinosum* protein.

The Rhodospirillaceae are quite different from the purple sulfur bacteria in that HiPIP was found in only 10 of 22 species examined and cytochrome c_2 are present in 16 species. Five species have both HiPIP and c_2 (*Rmi. vannielli*, *Rbi. marinum*, *Rpi. globiformis*, *Rps. cryptolactis*, and *Rps. palustris*). The *Rps. palustris* HiPIP isozymes are not normally expressed as soluble proteins during photosynthetic growth according to our analysis, and the *Rmi. vannielii* and *Rps. cryptolactis* HiPIPs are minor components. This leaves *Rbi. marinum* and *Rpi. globiformis* HiPIPs as major soluble constituents comparable in concentration to cytochrome c_2 and, therefore, candidates for electron donor to the reaction center. HiPIP has in fact been found to function as photosynthetic mediator in *Rpi. globiformis* but not in *Rhodobium marinum* (Menin et al. 1998). HiPIP has not yet been found in the *Rba. capsulatus*, *Rba. sphaeroides*, or *Rsp. rubrum* preliminary genome sequences and is likely to be absent. We have not yet found a cytochrome c_2 in *Rhodovibrio salinarum*, although there are small quantities of a high-potential cytochrome that aggregates and has not yet been completely purified. It cannot be positively identified without sequence data. Unless the minor soluble cytochrome is a major membrane protein, it is most likely that *Rhodovibrio salinarum* iso-1 HiPIP is the electron donor to the reaction center. Cytochrome c_2 isozymes have been found in the Rhodospirillaceae, including membrane-bound forms which were found to function in photosynthesis under some conditions (Jenney et al. 1994).

Four species of Rhodospirillaceae that are particularly interesting, because a cytochrome c_8 is present instead of the more common cytochrome c_2, are *Rvi. gelatinosus*, *Rfx. fermentans*, *Rcy. tenuis*, and *Rcy. purpureus*. It has been shown that *Rvi. gelatinosus* and *Rfx. fermentans* HiPIPs can function in photosynthesis (Hochkoeppler et al. 1995, 1996; Schoepp et al. 1995; Osyczka et al. 1999a, b). A low-potential c_8 was previously characterized in *Rvi. gelatinosus* (Ambler et al. 1979a, b) and a high-potential c_8 in *Rfx. fermentans* (Hochkoeppler et al. 1997). More recently, it has been shown that *Rvi. gelatinosus* can make both high- and low-potential c_8 isozymes, the former under aerobic and the latter under anaerobic photosynthetic conditions (Menin et al. 1999). It thus appears that HiPIP is likely to be the major electron donor to reaction centers in *Rvi. gelatinosus* and *Rfx. fermentans*. *Rcy. tenuis* c_8 and HiPIP have both been shown to be electron donors to the photosynthetic reaction center (Menin et al. 1997). The *Rcy. tenuis* HiPIP is as abundant as the c_8 and the ambient redox potential appears to determine which of the two is functional at any time. *Rcy. purpureus* does not appear to have either HiPIP or cytochrome c_2, thus the c_8 is most likely to function in photosynthesis.

HiPIP Sequence Comparisons

We have shown that HiPIP sequences can be divided into three major groups on the basis of shared insertions and deletions which generally correspond to the three families of phototrophic proteobacteria. Each of these three groups can be further subdivided by the same criteria, resulting in seven distinct subgroups of HiPIP that are consistent with other electron transfer characteristics (see Table 1). Ribosomal RNA has been used in the past to determine phylogenetic relationships among the purple photosynthetic bacteria, including those in the current study (Imhoff et al. 1998). One should observe the same results whether using HiPIP or RNA; thus the two are in agreement concerning the relationships among the Ectothiorhodospiraceae, as shown in Fig. 5. There is also fairly good agreement among the Chromatiaceae, except for the position of *Tco.*

pfennigii and *Hch. salexigens* with respect to the other species. Our HiPIP data clearly show that they are the most divergent of the Chromatiaceae in terms of both insertions and deletions and percentage identity, but in the RNA tree they are connected to the *Mch. gracile*/*Mch. purpuratum*/*Ich. buderi* branch. The relationship of *Ich. buderi* to the other two species also differs in that the HiPIP is most similar to that of *Mch. gracile*, but the RNA is more like that of *Tco. pfennigii* and *Hch. salexigens*. This deserves further study.

Another difference between HiPIP and RNA is in the position of *Rvi. gelatinosus* and *Rfx. fermentans* with respect to *Rcy. tenuis*. In RNA trees, all three species are on the same branch, but *Rcy. tenuis* HiPIP groups with the majority of the Rhodospirillaceae and the *Rvi. gelatinosus*/*Rfx. fermentans* pair branches nearer to the Chromatiaceae. In this case, it is plausible that a small HiPIP gene may have been transferred to *Rcy. tenuis* from a species containing a small HiPIP gene. A similar situation exists with *Acidithiobacillus ferrooxidans*, whose RNA appears to be similar to those of Chromatiaceae according to Kelly et al. (2000), but the HiPIPs are like those of the majority of purple nonsulfur bacteria. The HiPIPs from different strains of *Tba.* and *Atb. ferrooxidans* are only about 66% identical (Kusano et al. 1992; Institute for Genome Research) but the small copper protein rusticyanin is 92–98% identical in the same strains (Bengrine et al. 1998). The comparison of additional proteins or genes will be required to resolve these discrepancies.

Conserved Residues

The HiPIPs have no absolutely conserved residues besides the four cysteines required to bind the four-iron/four-sulfur cluster. There are only a few positions that are highly conserved, such as tyrosine 24 (Fig. 1 numbering), glycine 94, and tryptophans 95 and 99. *Rps. palustris* iso-2 is the only HiPIP in which Tyr 24 has been substituted (by a Phe). *Rhodovibrio salinarum* iso-2 is missing the whole N terminus, including Tyr 24. The function of Tyr 24 has been studied by mutagenesis, which shows that it has three important roles: in shielding the iron–sulfur cluster from solvent, in forming H-bonds to backbone and side chains, and through electrostatic interactions with the cluster (Iwagami et al. 1995; Agarwal et al. 1995). The Phe and Trp mutants were found to be relatively stable, whereas other substitutions resulted in marked instability, particularly for the oxidized form. The function of the second most highly conserved residue, glycine at position 94, is not as well defined, although the lack of a side chain appears to be due to structural constraints imposed by its interaction with Tyr 24 (Benning et al. 1994). It is substituted in *Rps. palustris* iso-2 by Ser, in *Rhodovibrio salinarum* iso-1 by Ala, and in iso-2 of the same species by Asp. The highly conserved aromatic and hydrophobic residues in addition to Tyr 24, such as at positions 22, 54, 55, 66, 71, 84, 90, 95, and 99 (Fig. 1 numbering) generally appear to protect the iron–sulfur cluster from solvent.

The necessity for such protection may contribute to a hydrophobic site of interaction with reaction partner proteins, as determined by studies of the kinetics of oxidation by photosynthetic reaction centers (Osyczka et al. 1999a, b). The site of electron transfer is located where the iron–sulfur cluster is nearest to the protein surface and centered over positions 21, 22, 54, 71, 84, 99, and 101. Thus, the hydrophilic and charged residues in HiPIP are peripheral to the site of interaction and may contribute to binding and electron transfer in specific instances. On the contrary, the site of interaction of Class I cytochromes c with reaction centers is normally dominated by positively charged residues, surrounding the heme, that form salt bridges with negatively charged residues on the reaction centers (Osyczka et al. 2001). The two types of reaction centers, those that interact with HiPIP and those that use soluble cytochromes, differ in that the latter contain a negatively charged residue at the center of the interaction site on the membrane-bound tetraheme reaction-center cytochrome (Glu 67 in *Blastochloris viridis* vs. Val 65 in *Rvi. gelatinosus*), which promotes binding and electron transfer with the cytochromes but inhibits the reaction with HiPIP (Osyczka et al. 2001). The tetraheme reaction-center cytochrome has a hydrophobic residue at the equivalent position in *Ach. vinosum*, *Tch. tepidum*, and *Ect. shaposhnikovii*, consistent with HiPIP being the electron donor. On the other hand, *Rhodobium marinum* has a Glu at that position which would favor interaction with cytochrome c_2, as found in functional analyses (Menin et al. 1998).

To determine which region on the protein surface may interact with reaction partner proteins, we mapped identical, conservatively substituted, and radically changed amino acids of 11 sequences from the family Chromatiaceae onto the three-dimensional HiPIP structure of *Ach. vinosum* (Fig. 6A), which also shows the distribution of charged amino acids (Fig. 6B). From this map, it is clear that the surface, composed of identical and conservatively substituted amino acids, is located where the iron–sulfur cluster is nearest to the surface, at the front side of the protein. The center of this surface contains no charged residues, which are instead located at the edges around this surface. Thus, the hypothetical site of interaction with reaction partner proteins is most likely to be at this conserved surface. This site also

forms a dimerization interface for HiPIPs during crystallization, as discussed by Kerfeld et al. (1998), and the conserved residues involved in dimerization are Leu 22, Arg 39, Phe 54, and Pro 85. Whether the monomer or the dimer is more reactive with reaction centers remains to be established. On the other hand, most of the radically changed amino acids are located at the opposite side of the protein, where most of the charged residues also occur. As expected from the sequence alignment, the same observation was made when the seven *Ectothiorhodospira* sequences were mapped onto the structure of *Ect. vacuolata* iso-2 HiPIP (Fig. 6C). The area of identical and conservatively substituted amino acids around the cluster-binding cysteines is greater than those for the Chromatiaceae and covers the front view of the protein almost completely (Fig. 6D). We believe that the center of the distribution of conserved residues is more important than the size of the conserved region, which is dependent on the overall degree of sequence divergence of the species chosen for analysis. In contrast to the proteins from the Chromatiaceae and Ectothiorhodospiraceae, 11 small HiPIP sequences from the Rhodospirillaceae, with little similarity to each other, show a very low degree of conservation, resulting in a virtually red-colored map (Fig. 6E). This is undoubtedly an artifact resulting from the fact that these sequences are too divergent for this method to work. Conserved amino acids are located around tyrosine 24 and the cluster-binding cysteines, and most of them are buried in the protein. With the exception of a surface formed by amino acids at positions 27 to 39 (*Rcy. tenuis* numbering), most of the charged residues are evenly distributed at the surface of the protein (Fig. 6F). Some of them, Asp 14, Lys 14, and Asp 56, are located in the direct vicinity of the conserved tyrosine and cysteines. Neverthelesss, we feel that these proteins use the same site for electron transfer as in the other HiPIPs but that it will require more closely related sequences to demonstrate it. This does not exclude the possibility that some of these HiPIPs may have an interaction surface with substitutions suited to a particular functional role.

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