Extremophiles (2006) 10:191–198
DOI 10.1007/s00792-005-0486-8

ORIGINAL PAPER

**Matthieu Nouailler · Patrice Bruscella
Elisabeth Lojou · Régine Lebrun · Violaine Bonnefoy
Françoise Guerlesquin**

Structural analysis of the HiPIP from the acidophilic bacteria: *Acidithiobacillus ferrooxidans*

Received: 27 September 2005 / Accepted: 2 October 2005 / Published online: 8 April 2006

Abstract Hip is a high-potential iron–sulfur protein (HiPIP) isolated from the acidophilic bacterium, *Acidithiobacillus ferrooxidans*. In the present work, a structural model of Hip suggests that the role of proline residues is essential to stabilize the protein folding at very low pH. The presence of an unusual disulfide bridge in Hip is demonstrated using mass spectrometry and nuclear magnetic resonance. This disulfide bridge is necessary to anchor the N-terminal extremity of the protein, but is not involved in the acid stability of Hip. The structural parameters correlated with the pH dependence of Hip redox potential are also analysed on the basis of this model. Given that the same structural features can enhance acidic stability and lead to elevated redox potentials, modulation of the redox potentials of electron carriers may be necessary to achieve electron transfer at very low pH.

Keywords HiPIP · *Acidithiobacillus ferrooxidans* · Acid stable · Mass spectrometry · NMR spectroscopy · Electrochemistry · Proline residues · Disulfide bridge

Communicated by K. Horikoshi

M. Nouailler · E. Lojou · F. Guerlesquin (✉)
Unité de Bioénergétique et Ingénierie des Protéines, CNRS,
31 chemin Joseph Aiguier, 13402 Marseille, Cedex 20, France
e-mail: guerlesq@ibsm.cnrs-mrs.fr
Tel.: +33-491-164379
Fax: +33-491-164578

P. Bruscella · V. Bonnefoy
Laboratoire de Chimie Bactérienne, CNRS,
31 chemin Joseph Aiguier, 13402 Marseille, Cedex 20, France

R. Lebrun
Institut de Biologie Structurale et de Microbiologie, CNRS,
31 chemin Joseph Aiguier, 13402 Marseille, Cedex 20, France

Introduction

High-potential iron–sulfur proteins (HiPIPs) are commonly found in the purple phototrophic bacteria as periplasmic electron carriers between the bc_1 complex and the reaction centre (Schoepp et al 1995; Hochkoeppler et al. 1995; Hochkoeppler et al. 1996; Menin et al. 1997; Nagashima et al. 2002;Verméglio et al. 2002). Nonetheless, HiPIPs have also been shown in bacteria to transfer electrons from the bc_1 complex to a terminal oxidase (Bonora et al. 1999; Pereira et al. 1999). *Acidithiobacillus ferrooxidans* is a gram-negative acidophilic chemolithoautotroph which obtains energy for its growth in oxic conditions mainly from the oxidation of ferrous iron or reduced sulfur compounds (Ingledew 1982). The bioenergetic metabolism of this acidophilic bacterium involves several periplasmic acid-stable proteins with very high redox potentials. In this non-photosynthetic bacterium a HiPIP protein has been purified from two different strains: Fe-1 (Fukumori et al. 1988; Kusano et al. 1992) and BRGM (Cavazza et al. 1995). This HiPIP was proposed to be the first electron acceptor in the electron transfer chain between Fe^{2+} and oxygen and was referred to as Iro (Kusano et al. 1992;Yamanaka and Fukumori 1995). However, in more recent studies with *A. ferrooxidans* ATCC 23270 (the type strain), ATCC19859 and ATCC33020, the only gene encoding a HiPIP was not located downstream from *purA* as in strains Fe-1 and BRGM (Kusano et al. 1992; Bruscella et al. 2005), but was located downstream from *petC2* encoding the cytochrome c_1 of the second *A. ferrooxidans* cytochrome bc_1 complex (Bruscella et al. 2005; Brasseur et al. 2002). Therefore, it was suggested that this HiPIP is involved in an electron transfer chain between a cytochrome bc_1 complex and a terminal oxidase and it was proposed to refer to it as Hip, until its physiological role is determined (Bruscella et al. 2005). A His-tagged form of the *A. ferrooxidans* protein, Hip, was overproduced in *Escherichia coli* (Bruscella et al. 2005). This protein is acid stable and holds the highest redox

potential among the HiPIP family (510 mV). To further evaluate the acid stability of *A. ferrooxidans* periplasmic proteins, we have generated a model of Hip that we compare to the structures of mesophilic HiPIPs. Since NMR is well adapted to study proteins at extreme conditions (low or high pH or temperature), we have investigated the structural properties of Hip at different pH using 2D-heteronuclear NMR.

Materials and methods

Protein expression and purification

Hip expression in *E. coli* and purification of the recombinant protein have been previously reported (Bruscella et al. 2005). The ^{15}N-labelled Hip was produced by growing *E. coli* BL21(DE3) strain carrying the pET21-*hip* plasmid at 37°C in 1 l of M9 medium supplemented with $^{15}NH_4Cl$ (1 g l^{-1}) and ampicillin (50 μg ml^{-1}). After purification, the recombinant ^{15}N-labelled Hip was dialysed against 50 mM (pH 7.6) phosphate buffer.

Mass spectrometry

Mass analyses were performed with a MALDI-TOF mass spectrometer (PerSeptive Voyager DE-RP, Applied Biosystems, USA). External calibration was made on the mono-charged ion $[M+H]^+$ at 16,951.56 Da and the doubly charged ion $[M+2H]^{2+}/2$ at 8,476.28 Da of apomyoglobin.

Redox potential measurements

Square-wave voltammetries were carried out using a PAR 263A potentiostat controlled by PAR PowerSuit software. A conventional three-electrode system was used consisting of a Metrohm Ag/AgCl/saturated NaCl reference electrode, a platinum auxiliary electrode and a membrane-pyrolytic graphite-working electrode. Potentials versus the normal hydrogen electrode have been obtained by adding 210 mV to the measured potentials. The membrane electrode technology was described in a previous paper (Haladjian et al. 1994). Prior to each experiment, the solutions were deoxygenated by bubbling high-purity nitrogen. All experiments were carried out at room temperature (about 23°C) under nitrogen atmosphere.

NMR experiments

NMR samples were prepared in 10 mM, pH 7.0 phosphate buffer in 10% D_2O. Homonuclear NMR experiments (NOESY and TOCSY) necessary for tentative assignment were performed on a sample of Hip at 1 mM concentration. Heteronuclear NMR spectra (^{1}H–^{15}N HSCQ) were recorded on a sample of ^{15}N-labelled Hip at 0.1 mM concentration. NMR experiments were carried out on a Bruker Avance DRX500 spectrometer at 300 K, equipped with a HCN probe and self-shielded triple axis gradients. Spectrum processing was done with XwinNMR from Bruker. ^{1}H chemical shifts were referenced indirectly with H_2O resonance calibrated at 4.792 ppm and ^{15}N chemical shifts referenced indirectly using $^1H/^{15}N$ frequency ratio 0.1013291118 (Wishart et al. 1995).

Model building

Modelling was performed with the model-default option of the programme MODELLER 7v7 (Sali and Blundell 1993) using the structure of the HiPIP from *R. tenuis* (pdb: 1ISU) as template (33% identities). The calculation includes the [4Fe–4S] cluster and a disulfide bridge between C4 and C36.

Results and discussion

Sequence analysis

When Hip sequence is compared to sequences of HiPIPs from several sources (Fig. 1), the four cysteines required to bind the FeS cluster are absolutely conserved. There are only a few positions that are highly conserved such as Y15, G48, W49 and W/F53 (Hip numbering) (van Driessche et al. 2003). Y15 has three important functions: in shielding the FeS from solvent, in forming H-bonds to backbone and side chains and in making electrostatic interactions with the cluster. For G48, the lack of a side chain appears to be due to structural constraints imposed by its interaction with Y15. The highly conserved aromatic residues at position 15, 49 and 53 generally appear to protect the FeS cluster from solvent. The Hip sequence reveals two particular features: the presence of two additional cysteine residues (C4 and C36) and a very high content of proline residues (15.8%). Figure 1 shows that Hip sequence contains nine proline residues while only four are present in the sequence of the HiPIP from *Rhodocyclus tenuis* (Isu). Proline residues are mainly located at the N- and C-terminal extremities of Hip sequence. Five of the nine proline residues of Hip are conserved in the similar protein Iro from *A. ferrooxidans* and therefore they are probably playing an important role in the folding of these two acid-stable proteins. In order to clarify the role of proline residues in Hip structure, we have generated a structural model of this protein.

Fig. 1 Sequence alignment of HiPIPs from *Acidithiobacillus ferrooxidans* (Iro and Hip) (Bruscella et al. 2005), *Rhodocyclus tenuis* (Isu) (Rayment et al. 1992), *Ectothiorhodospira vacuolata* (HPI) (Benning et al. 1994) and *Thermochromatium tepidum* (IUA) (Liu et al. 2002). Cysteine residues are in *green*, proline residues are in *red*, conserved residues are in *pink*. In Hip, the cysteine residues involved in disulfide bridge are *underlined* and the acidic residues are in *blue*

Structural analysis

Disulfide bridge

The first step of the structural analysis of Hip was to determine the presence of a disulfide bridge involving the two additional cysteine residues (C4 and C36). Mass spectra were recorded on carbamidomethylated apoHip in the absence and the presence of dithiothreitol (DTT). In the absence of DTT, two major masses were observed at 7,145 and 7,260 Da, which correspond to Hip forms including two and four alkylated cysteine residues, respectively. A major form was observed at 7,380 Da in the presence of DTT corresponding to six alkylated cysteine residues. These results suggested the presence of a disulfide bridge in Hip. 1D-NMR spectra of Hip in the presence and absence of DTT revealed an important effect on the chemical shift of the βCH_2 of one of the cysteine ligands of the FeS cluster. This effect was explained by the reduction of the disulfide bridge involving C4 and C36 which would affect the FeS ligand, C37. The presence of DTT also induced significant modifications of the $^{1}H–^{15}N$ HSQC spectrum (Fig. 2). Partial assignment of NH resonances in HSQC spectrum indicated that the N-terminal extremity (G6, T7, K10, A11) was particularly affected. However, the optical spectra of Hip in the presence or absence of DTT were the same and the redox potential of the protein was poorly modified by the presence of DTT (Fig. 3). These results indicated that DTT did not alter the FeS binding.

Acid stability

The general folding motif of HiPIPs is rather simple, consisting of a series of reverse turns. For this reason and due to the high content of proline residues (15.8%), we obtained only few dipolar restraints in the NMR spectra of Hip. A structural model was generated on the basis of *R. tenuis* HiPIP (pdb: 1ISU) presenting 33% sequence identities with Hip (Fig. 4). The only major secondary structural element found in *R. tenuis* HiPIP (Isu) was a short helix composed of residues 4–9 (Rayment et al. 1992). This part of the sequence, rich in proline residues in Hip, was not favourable for a helical structure. The presence of the disulfide bridge was necessary to stabilize the N-terminal extremity of Hip.

An important point is that the unique salt bridge present in Isu (R7 / D46) is missing in Hip. The proline residue P43 in Hip, substituting D46 in Isu, may counterbalance the structural effect of this non-bonded interaction. In contrast, most of the hydrogen bonds in Isu may exist in Hip. However, it is to be noticed that the additional proline residues in Hip are located in the

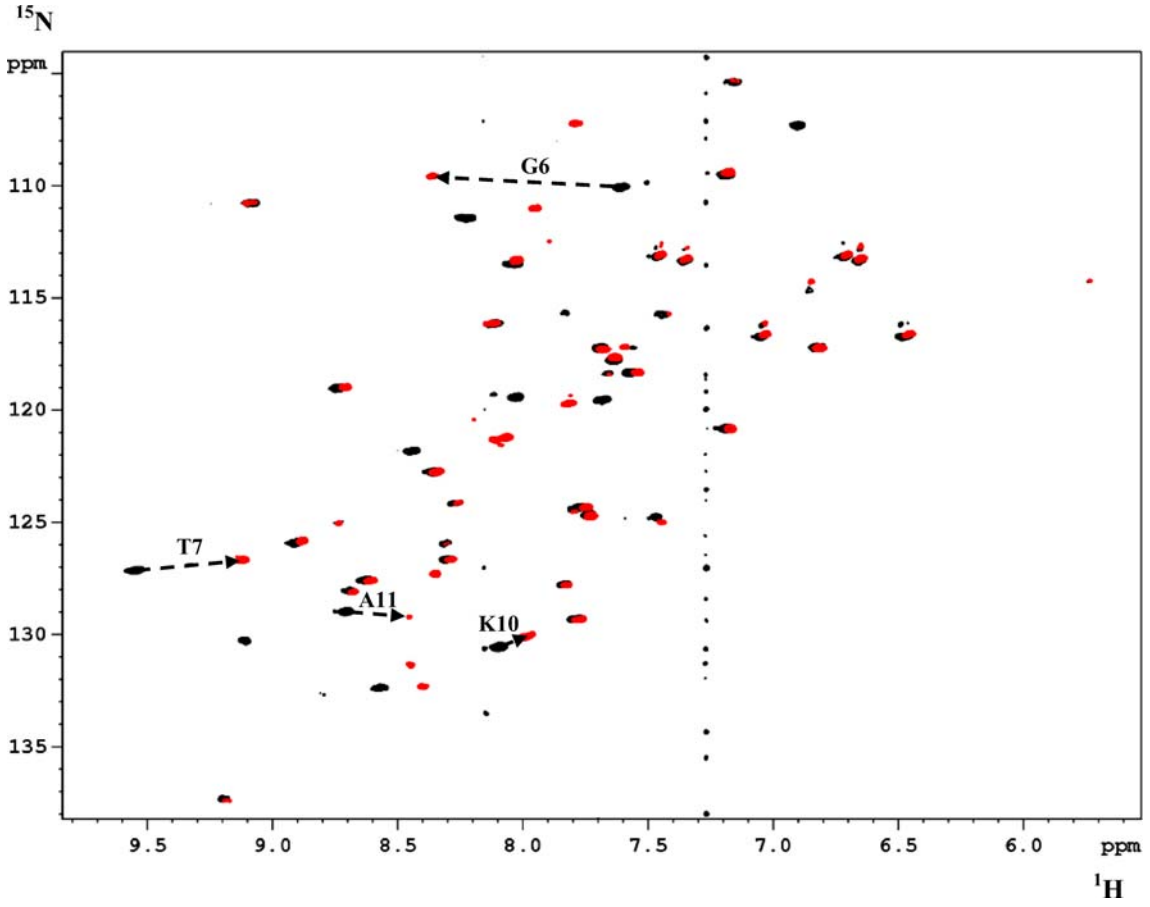

Fig. 2 ^{1}H–^{15}N HSQC of Hip in the presence (*red*) and in the absence (*black*) of 10 mM DTT. NMR spectra were recorded at 300 K pH 2.7

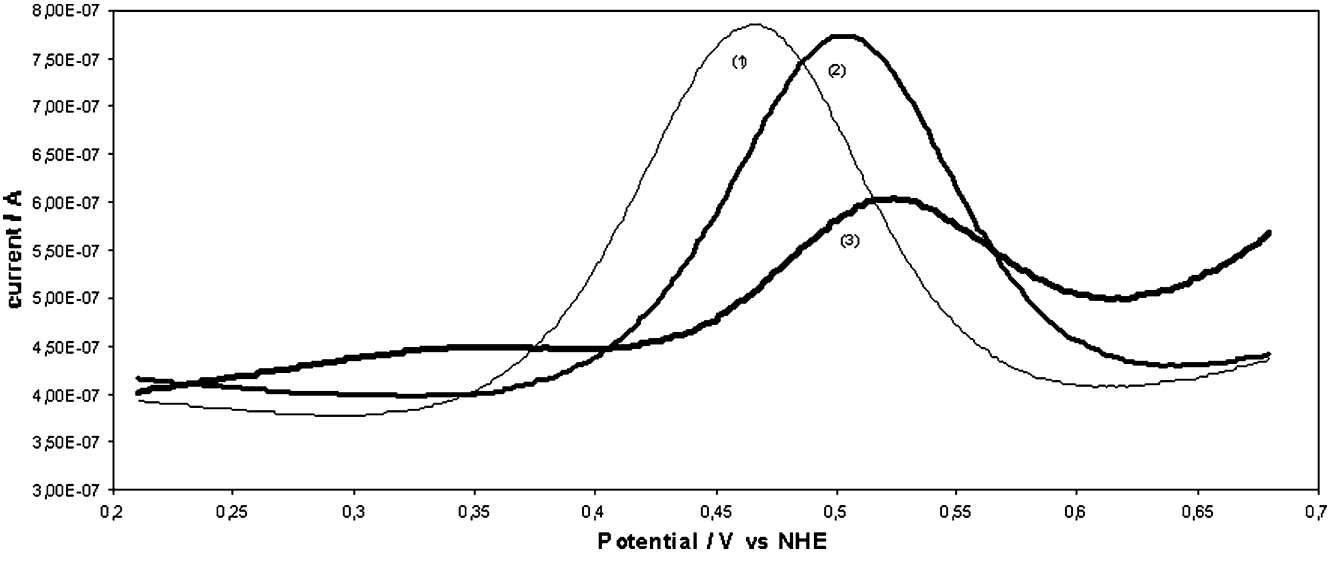

Fig. 3 Square-wave voltammograms at the membrane-pyrolytic graphic electrode of 2 µl Hip (100 µM) in 40 mM phosphate buffer pH 7 (*curve 1*), after addition of 300 mM NaCl (*curve 2*), after addition of 10 mM DTT (*curve 3*). SW peak potentials are respectively, 470 , 500 and 530 ± 5 mV versus NHE

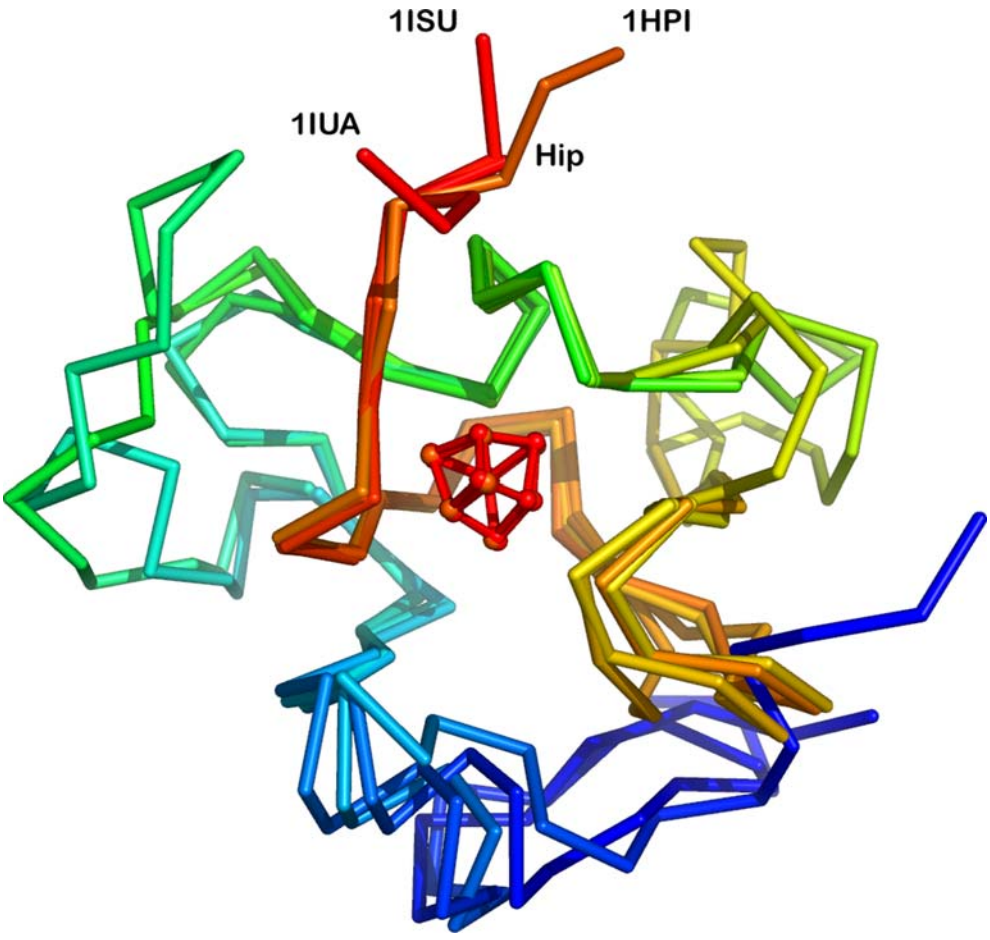

Fig. 4 Superimposition of the 3D structures of HiPIPs from *R. tenuis* (1ISU), *E. vacuolata* (1HPI) and *T. tepidum* (1IUA) and the structural model of Hip. The FeS cluster is in *red* and the structural elements in the backbones are coloured from N-terminal in *blue* to C-terminal in *red*

vicinity of the residues whose amide group is involved in one of the conserved hydrogen bonds. P17 and P19 are located in Hip around H18: the amide group of the equivalent T15 in Isu is involved in a hydrogen bond with the conserved Q13 adjacent to the essential Y12 (Isu numbering). P55 and P57 induce the orientation of M56 in Hip: the amide group of the equivalent K61 in Isu is involved in a hydrogen bond with G24 which is in close contact to the FeS cluster. In conclusion, these additional proline residues would stabilize the folding of the protein at low pH by introducing new geometric constraints to the backbone at the position of the requisite hydrogen bonds (Fig. 5).

The high content of proline residues is typical of *A. ferrooxidans* periplasmic electron transfer proteins. Rusticyanin contains 14 prolines over 155 residues (Botuyan et al. 1996;Walter et al. 1996). Similarly, cytochromes c_4 from *A. ferrooxidans* have a very high number of proline residues. As the comparison of rusicyanin structure with cupper proteins from mesophilic organisms being more difficult to interpret, we have analysed the three-dimensional structure of *A. ferrooxidans* Cyc41 (pdb: 1H10). Proline residues play also an important role in the folding of this protein. Two proline residues (P88 and P89) are found in the linker peptide of the two hemic domains; these two residues are probably essential for the conformation of the protein at low pH. Moreover, a proline residue (P132) replaces a salt bridge involving D131 and R38 in *Pseudomonas stutzeri* cytochrome c_4. It is to be noticed that the high number of proline residues was previously emphasized in the X-ray structure of Maltose-Maltodextrine-binding protein from the thermoacidophile *Alicyclobacillus acidocaldarius* (Schäfer et al. 2004). But in this case, the authors concluded that the proline residues were involved in the thermostability and not the acid stability of the protein.

Modulation of the very high redox potential

In HiPIP, the iron–sulfur cluster is completely inaccessible to water and surrounded by hydrophobic residues. The large differences in redox potentials are caused by differences in the polarity of the cluster environment and ability to delocalize electrons by the amino acid residues around the cluster (Heering et al. 1995). The role of the cysteine ligand in the very high redox potential modulation was analysed on the basis of the structural model of Hip. When considering HiPIP structures (Fig. 3), C37 (Hip numbering) is found exposed to the solvent in most of the HiPIPs; C28 is exposed in the low redox potential proteins and buried in the medium range redox potential

proteins. Surprisingly, in Hip, C28 is exposed to the solvent but C37 is particularly buried. A model of Hip, generated without the (C4/C36) disulfide bridge restores the C37 exposure. However, cyclic voltammetry analysis of Hip in the presence of DTT revealed a slight increase in the redox potential (30 mV) indicating that the disulfide bridge, and thus, C37 exposure is not responsible for the high redox potential of Hip (Fig. 3). Reduction of the disulfide bridge has only a slight effect on the stability of the protein at either high or low pH. Figure 2 shows the 2D-NMR spectra of Hip in the presence and absence of DTT at pH 2.7; one can observe that except for the residues of the N-terminal extremity, the global NMR spectrum and thus the folding of the protein are conserved in the absence of the disulfide bridge at extreme pH. In conclusion, the exposure of the cysteine ligands is not a determinant parameter for redox potential modulation in Hip, and it is difficult to distinguish the parameters involved in the acid stability and in the modulation of the very high redox potential.

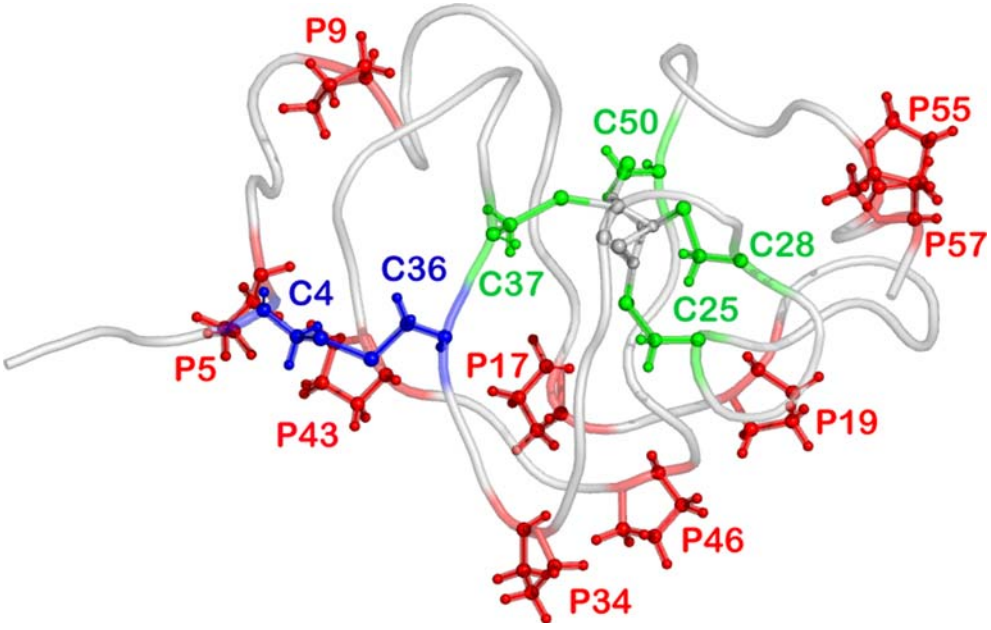

Fig. 5 Structural model of Hip represented by PyMOL software (DeLano 2002). Those in *green* are the cystein ligands of the FeS cluster; those in *blue* are the two cysteines involved in the disulfide bridge; those in *red* are proline residues

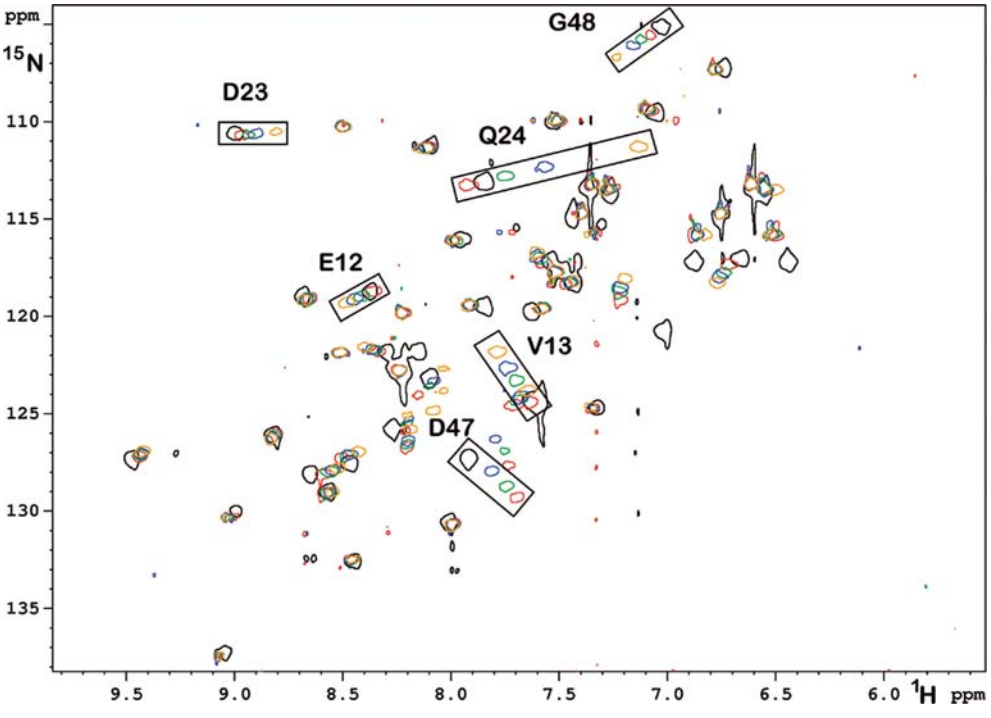

Fig. 6 Overlay of Hip HSQC spectra at different pH (*black* pH 7.0; *red* pH 5.3; *green* pH 4.1; *blue* pH 3.8; *orange* pH 2.7)

pH dependence of Hip redox potential

Electrochemical measurements have revealed a pH dependence of Hip redox potential in the range of pH 3.5–5.0 (Bruscella et al. 2005). HSQC spectra of Hip were recorded in this range of pH (Fig. 6). Among the few affected resonances, six resonances were respectively assigned to E12 and V13, D23 and Q24, and D47 and G48. The pK values for these resonances were around 4, indicating that the pH dependence of Hip redox potential is modulated by one of these three acidic residues (E12, D23 and D47). Sequence alignment (Fig. 1) shows that D23 located at 6 Å of the cluster in the hydrophobic core of Hip is conserved in Iro. The similar pH dependence of Iro redox potential (Bonora et al. 1999) suggests that D23 may be responsible for this phenomenon in the two proteins.

pH dependence of redox potentials is a common property of periplasmic electron transfer proteins in *A. ferrooxidans*. A pK value above 7 was observed for the redox potential of rusticyanin (Guidici-Orticoni et al. 1999). A structural model of the cytochrome c_4-rusticyanin complex has shown that E121 from the cytochrome forms a hydrogen bond with H143 of rusticyanin (Abergel et al. 2003) leading to a change in the redox potential of rusticyanin of 100 mV upon complex formation. Considering the redox potentials of the free states of rusticyanin (680 mV) and of cytochrome c_4 (480 mV), the authors proposed that the electron transfer between the copper and the heme, thermodynamically unfavourable, necessitates an induced redox switch to allow electron transfer between the two redox partners. By analogy, D23 may be involved in a similar redox switch in Hip, including a basic residue of the physiological partner to form a hydrogen bond during complex formation.

It has been previously demonstrated that the structural features, which enhance acid stability of rusticyanin, lead also to an elevated redox potential (+680 mV), making these two properties interrelated (Walter et al. 1996). It is to be noticed that the redox proteins isolated from this acidophilic bacteria have a particularly high redox potential. The modulation of the redox potentials of these acid-stable proteins may be a general process necessary to achieve electron transfer reactions at extreme pH.

Acknowledgements The authors gratefully acknowledge J.A. DeMoss (Laboratoire de Chimie Bactérienne, Marseille, France) for a critical reading of the manuscript.

References

Abergel C, Nitschke W, Malarte G, Bruschi M, Claverie JM, Giudici-Orticoni MT (2003) The structure of Acidithiobacillus ferrooxidans c(4)-cytochrome: a model for complex-induced electron transfer tuning. Structure 11:547–555

Benning M, Meyer TE, Rayment I, Holden HM (1994) Molecular structure of the oxidized high-potential iron-sulfur protein isolated from *Ectothiorhodospira vacuolata*. Biochemistry 33:2476–2483

Bonora P, Principi II, Monti B, Ciurli S, Zannoni D, Hochkoeppler A (1999) On the role of high-potential iron-sulfur proteins and cytochromes in the respiratory chain of two facultative phototrophs. Biochim Biophys Acta 1410:51–60

Botuyan MV, Toy-Palmer A, Chung J, Blake RC II, Beroza P, Case DA, Dyson HJ (1996) NMR solution structure of Cu(I) rusticyanin from Thiobacillus ferrooxidans: structural basis for the extreme acid stability and redox potential. J Mol Biol 263:752–767

Brasseur G, Bruscella P, Bonnefoy V, Lemesle-Meunier D (2002) The *bc*(1) complex of the iron-grown acidophilic chemolithotrophic bacterium *Acidithiobacillus ferrooxidans* functions in the reverse but not in the forward direction. Is there a second *bc*(1) complex? Biochim Biophys Acta 1555:37–43

Bruscella P, Cassagnaud L, Ratouchniak J, Brasseur G, Lojou E, Amils R, Bonnefoy V (2005) The HiPIP from the acidophilic *Acidithiobacillus ferrooxidans* is correctly processed and translocated in *Escherichia coli*, in spite of the periplasm pH difference between these two micro-organisms. Microbiology 151:1421–31

Cavazza C, Guigliarelli B, Bertrand P, Bruschi M (1995) Biochemical and EPR characterization of high potential iron-sulfur protein in *Thiobacillus ferrooxidans*. FEMS Microbiol Lett 130:193–200

DeLano WL (2002) The PyMOL Molecular Graphics System. DeLano Scientific, San Carlos, CA, USA

Fukumori Y, Yano T, Sato A, Yamanaka T (1988) FeII oxidizing enzyme purified from *Thiobacillus ferrooxidans*. FEMS Microbiol Lett 20:169–172

Giudici-Orticoni MT, Guerlesquin F, Bruschi M, Nitschke W (1999) Interaction-induced redox switch in the electron transfer complex rusticyanin-cytochrome c(4). J Biol Chem 274:30365–30369

Haladjian J, Bianco P, Nunzi F, Bruschi M (1994) A permselective-membrane electrode for the electrochemical study of redox proteins: application to cytochrome c_{552} from *Thiobacillus ferrooxidans*. Anal Chim Acta 289:15–20

Heerring HA, Bulsink BM, Hagen WR, Meyer TE (1995) Influence of charge and polarity on the redox potentials of high potential iron sulfur proteins: evidence for the existence of two groups. Biochemistry 34(45):14675–14686

Hochkoeppler AS, Ciurli G, Venturoli G, Zannoni D (1995) The high potential iron-sulfur protein (HiPIP) from *Rhodoferax fermentans* is competent in photosynthetic electron transfer. FEBS Lett 357:70–74

Hochkoeppler AS, Zannoni D, Ciurli S, Meyer TE, Cusanovich MA, Tollin G (1996) Kinetics of photo-induced electron transfer from high-potential iron-sulfur protein to the photosynthetic reaction center of the purple phototroph *Rhodoferax fermentans*. Proc Natl Acad Sci 93:6998–7002

Ingledew WJ (1982) *Thiobacillus ferrooxidans*. The bioenergetics of an acidophilic chemolithotroph. Biochim Biophys Acta 683:89–117

Kusano T, Sugawara K, Inoue C, Takeshima T, Numata M, Shiratori T (1992) Electrotransformation of *Thiobacillus ferrooxidans* with plasmids containing a *mer* determinant. J Bacteriol 174:6617–6623

Liu L, Nogi T, Kobayashi M, Nozawa T, Miki K (2002) Ultrahigh-resolution structure of high-potential iron-sulfur protein from *Thermochromatium tepidum*. Acta Crystallogr 58:1085–1091

Menin L, Schoepp B, Parot P, Verméglio A (1997) Photoinduced cyclic electron transfer in *Rhodocyclus tenuis* cells: participation of HiPIP or cyt c_8 depending on the ambient redox potential. Biochemistry 36:12183–12188

Nagashima KV, Matsuura K, Shimada K, Verméglio A (2002) High-potential iron-sulfur protein (HiPIP) is the major electron donor to the reaction center complex in photosynthetically growing cells of the purple bacterium *Rubrivivax gelatinosus*. Biochemistry 41:14028–14032

Pereira MM, Carita JN, Teixeira M (1999) Membrane-bound electron transfer chain of the thermohalophilic bacterium *Rhodothermus marinus*: characterization of the iron-sulfur centers from the dehydrogenases and investigation of the high-potential iron-sulfur protein function by in vitro reconstitution of the respiratory chain. Biochemistry 38:1276–1283

Rayment L, Wesenberg G, Meyer TE, Cusanovich MA, Holden HM (1992) Three-dimensional structure of the high-potential iron-sulfur protein isolated from the purple phototrophic bacterium *Rhodocyclus tenuis* determined and refined at 1.5 A resolution. J Mol Biol 228:672–686

Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 234:779–815

Schäfer K, Magnusson U, Scheffel F, Schiefner A, Sandgren MO, Diederichs K, Welte W, Hülsmann A, Schneider E, Mowbray SL (2004) X-ray structures of the maltose-maltodextrin-binding protein of the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* provide insight into acid stability of proteins. J Mol Biol 335:261–274

Schoepp B, Parot P, Menin L, Gaillard J, Richaud P, Vermeglio A (1995) In vivo participation of a high potential iron-sulfur protein as electron donor to the photochemical reaction center of *Rubrivivax gelatinosus*. Biochemistry 34:11736–11742

van Driessche G, Vandenberghe I, Devreese B, Samyn B, Meyer TE, Leigh R, Cusanovich MA, Bartsch RG, Fisher U, van Beeumen JJ (2003) Amino acid sequences and distribution of high-potential iron-sulfur proteins that donate electrons to the photosynthetic reaction center in phototropic proteobacteria. J Mol Evol 57:181–199

Verméglio AJ, Schoepp-Cothenet B, Pratt N, Knaff DB (2002) The role of high-potential iron protein and cytochrome c(8) as alternative electron donors to the reaction center of *Chromatium vinosum*. Biochemistry 41:8868–8875

Walter RL, Ealick SE, Friedman AM, Blake RC II, Proctor P, Shoham M (1996) Multiple wavelength anomalous diffraction (MAD) crystal structure of rusticyanin: a highly oxidizing cupredoxin with extreme acid stability. J Mol Biol 263:730–751

Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, Olfield E, Markley JL, Sykes BD (1995) 1H, 13C and 15 N chemical shift referencing in biomolecular NMR. J Biomol NMR 6:135–140

Yamanaka T, Fukumori Y (1995) Molecular aspects of the electron transfer system which participates in the oxidation of ferrous ion by *Thiobacillus ferrooxidans*. FEMS Microbiol Rev 17:401–413