The D13C variant of *Bacillus schlegelii* 7Fe ferredoxin is an 8Fe ferredoxin as revealed by ^{1}H-NMR spectroscopy

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Abstract The N-terminal cluster binding motif Cys^8-XXXXXXXCys^{16}....Cys^{49} of *Bacillus schlegelii* 7Fe ferredoxin, which provides the ligands to the $[Fe_3S_4]^+$ cluster, was modified by the mutation Asp^{13} → Cys. The mutant D13C is expressed in *Escherichia coli* as an 8Fe ferredoxin, with NMR properties similar to those of clostridial-type ferredoxins. The full assignment of the hyperfine shifted resonances indicates that Cys^{13} serves as ligand to the new fourth iron atom in the N-terminal cluster despite the atypical binding sequence CysXXXXCysXX-Cys....Cys. The Cα–Cβ–S–Fe dihedral angles of all cysteine ligands to the two $[Fe_4S_4]^{2+}$ clusters of the D13C variant are similar to those observed in other 8Fe and 4Fe ferredoxins.

Key words: *Bacillus schlegelii*; Ferredoxin; ^{1}H-NMR; Iron–sulfur cluster

1. Introduction

The *Azotobacter*-type Fe_7S_8 ferredoxin from the thermophilic bacterium *Bacillus schlegelii* has recently been investigated by ^{1}H-NMR and the sequence specific assignment of the cysteinyl cluster ligands has been obtained by taking advantage of a structural model of this protein [1]. The ligands to the $[Fe_3S_4]^+$ cluster in wild-type (WT) are Cys^8, Cys^{16}, and Cys^{49}. There is an aspartate in position 13 which is a candidate fourth ligand to the cluster when a bivalent metal ion (Fe^{2+}) is added to the protein under reducing conditions (unpublished).

In the present paper, we report on the D13C mutant of *B. schlegelii* 7Fe ferredoxin. The substitution of Asp^{13} with a cysteine can provide an eighth ligand for the formation of an engineered Fe_8S_8 protein with an unusual sequence motif (CysXXXXCysXXCys....Cys) for the binding of the N-terminal $[Fe_4S_4]^{2+}$ cluster.

2. Materials and methods

The expression vector of the D13C mutant of *B. schlegelii* 7Fe ferredoxin was prepared by the unique-site-elimination mutagenesis procedure [2] with a Chameleon Double-Stranded, Site-Directed Mutagenesis kit (Stratagene). Two oligonucleotides (a: 5′-CTCGACGC-AACTCGCACATTTGGTCCCGATACACG-3′ and b: 5′-AAGCT-TGGCTGCAAGTCGACGGATCCCCGG-3′) were used to introduce the mutation at position 13 from Asp to Cys and to eliminate the *Pst*I site in the expression vector of the wild-type *B. schlegelii* ferredoxin (pKKFd54) [3] as the template for the mutation, respectively. Mutagenesis was performed according to the manufacturer's protocol. The construct was sequenced to ensure that the desired mutation and no replication errors were introduced in the *B. schlegelii* ferredoxin structural gene. The resulting plasmid, pKKFd D13C, was transformed into *E. coli* strain JM109 for protein expression.

The D13C variant of *B. schlegeliis* 7Fe ferredoxin was expressed in *E. coli* and purified to homogeneity according to the procedure reported for the WT protein [3]. NMR samples contained approximately 3.5 mM of protein in 20 mM potassium phosphate buffer (pH 6.5) either in 99.8% D_2O or 90% H_2O/10% D_2O (v/v). The latter solutions contained 0.05% NaN_3 to prevent microbial growth.

All ^{1}H-NMR spectra were recorded on a Bruker DRX 500 spectrometer. 1D NOE difference spectra were performed according to previously described acquisition schemes [4,5] with irradiation times of 90 ms and a repetition rate of 4.6 s^{-1} at 282 K. Fast repetition 2D spectra for the detection of scalar and dipolar connectivities of the hyperfine shifted signals were recorded at 282 and 290 K over a spectral window of 26 ppm with the following parameters: TOCSY [6] with 8 ms spin-lock time, 2K data points in F2 and 425 or 490 increments in F1; WEFT-NOESY [7] with 6 ms mixing time, 2K data points in F2 and 490 or 400 increments in F1. Between 688 and 800 transients per increment were accumulated. In all cases, phase sensitivity was achieved by application of the TPPI scheme [8]. The data matrices of the 2D spectra were processed to a final size of 1K×1K data points using shifted squared sine-bell window functions prior to Fourier transformation.

3. Results

The diamagnetic part of the ^{1}H-NMR spectrum of the D13C mutant of *B. schlegelii* ferredoxin in the 0–10 ppm chemical shift range is very similar to the WT spectrum. As far as the hyperfine shifted resonances are concerned (Fig. 1), it is similar to the spectra of the 8Fe ferredoxins from *Clostridium pasteurianum* and *C. acidi-urici* [9], but different from the spectrum of *Chromatium vinosum* 8Fe ferredoxin [10]. The downfield region between 10 and 17 ppm contains numerous resonances (Fig. 1, A-I) that are not solvent exchangeable. The temperature dependence of the chemical shifts of all these resonances is of the anti-Curie type, the only exception being signal H whose shift is not sensitive to temperature (Fig. 2). Due to their chemical shifts, temperature dependence, and large line widths, the resonances A-G and I are attributed to βCH2 protons of the cysteinyl cluster ligands.

Fast repetition TOCSY and NOESY spectra at two temperatures and 1D NOE difference experiments (performed by saturation of signals A-H) allowed the identification of the geminal and vicinal partners of the hyperfine shifted signals. With the exception of a weak dipolar coupling between signal B and D there are no dipolar or scalar correlations among the hyperfine shifted resonances indicating that each of them represents a βCH_2 proton of a distinct cysteine. Indeed, signals B and D belong to different cysteines as evidenced by their scalar and dipolar couplings: D shows a TOCSY and NOESY cross-peak with a broad resonance at 4.92 ppm (assigned as

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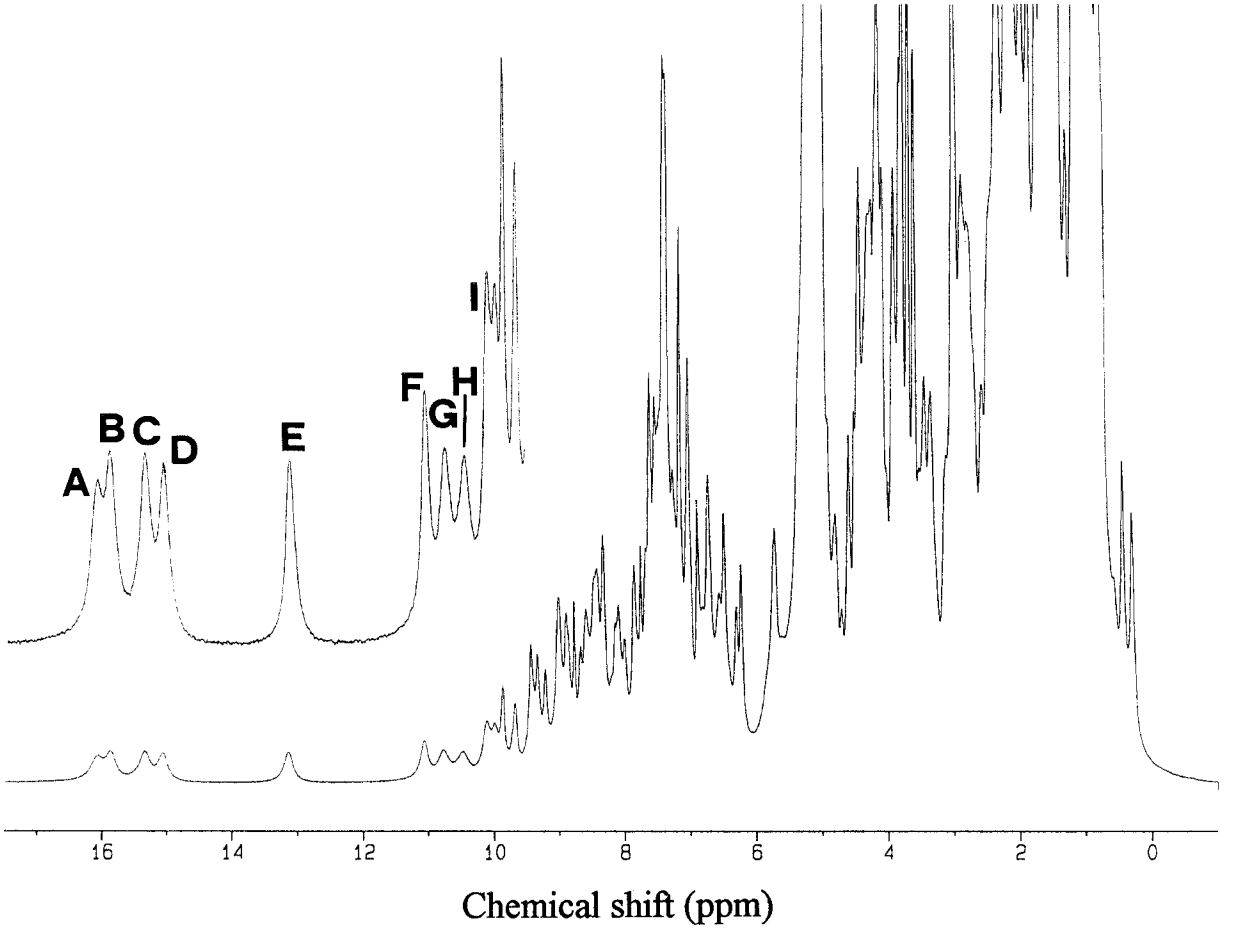

Fig. 1. The 500 MHz 1D ^{1}H-NMR spectrum of the D13C mutant of *B. schlegelii* 7Fe ferredoxin (recorded at 282 K in 20 mM potassium phosphate buffer (pH 6.5), 99.8% D_2O) shows numerous downfield shifted resonances that are typical for 8Fe ferredoxins.

its geminal partner) and a NOESY cross-peak at 6.85 ppm (assigned as Hα of the corresponding cysteine); both of these resonances have anti-Curie temperature dependence. Signal B shows two intense NOESY cross-peaks, one with a broad signal at 4.23 ppm (assigned as its geminal partner) and one with a sharp resonance at 6.7 ppm (assigned as Hα); again, both of these resonances are anti-Curie. In a similar manner, assignments for the spin systems of all other coordinated cysteines could be established (Table 1). Support for these assignments comes also from analogy with published NMR data on 4Fe and 8Fe ferredoxins [9–15] and from comparison of the present mutant with WT [1].

Table 1 provides also a sequence-specific assignment of the eight identified cysteinyl cluster ligands. The assignment of resonances B and D is straightforward since they show an interresidual dipolar connectivity that can only be expected for βCH_2 protons of Cys^{16} and Cys^{45}, as described for the analogous NOE of WT [1]. Comparison of the 1D NOE difference spectra of B and D with those of the corresponding WT signals (data not shown) and analogy with the NMR properties of the 8Fe ferredoxins from *C. pasteurianum* and *C. acidi-urici* [9] permit the assignment of resonance D to Cys^{45} Hβ_1 and of resonance B to Cys^{16} Hβ_1. Following similar arguments, all hyperfine shifted resonances attributed to the 4Fe cluster in WT 7Fe ferredoxin can be recognized in the D13C mutant resulting in the assignment of signal C to Cys^{42} Hβ_2 and of signal G to Cys^{39} Hβ_1. Both signal G and its geminal partner resonating at 8.55 ppm show dipolar connectivities to a resonance at 7.50 ppm. Due to its chemical shift, the latter is tentatively assigned to the NH proton of the corresponding cysteine. Signal F is tentatively assigned to Cys^{20} Hβ_2. The latter resonance is characterized by its NOE to a resonance at 4.24 ppm, presumably Asp^{23} Hα. This assignment is supported by the fact that the structurally characterized cubane ferredoxins share a turn involving the fourth cysteine of the cluster binding sequence (C(IV)PXX) as a highly conserved structural element [16–23]. Owing to this turn, a strong NOE connectivity is expected between CysIV Hβ_2 and the Hα of amino acid *i*+3 with respect to CysIV (Asp^{23} in the present case).

The remaining resonances A, E, and I must originate from the new 4Fe cluster in the D13C mutant that is ligated by Cys^8, Cys^{13}, Cys^{16}, and Cys^{49}. Signal A exhibits only one interresidual NOE with a resonance at 1.45 ppm. By chemical shift analogy to 4Fe and other 8Fe ferredoxins it is assigned as Cys^{13} Hβ_2. Thus, signals E and I correspond to the up to now unassigned Cys^8 and Cys^{49}. Both E and I show NOEs with resonances in the Hα chemical shift range (E with resonances at 4.58 and 4.30 ppm, I with a resonance at 4.40 ppm). Nevertheless, based on (i) chemical shift analogy with 8Fe ferredoxins and (ii) dipolar connectivities that are similar to those observed for the Cys^{49} βCH_2 protons in WT, signal E is

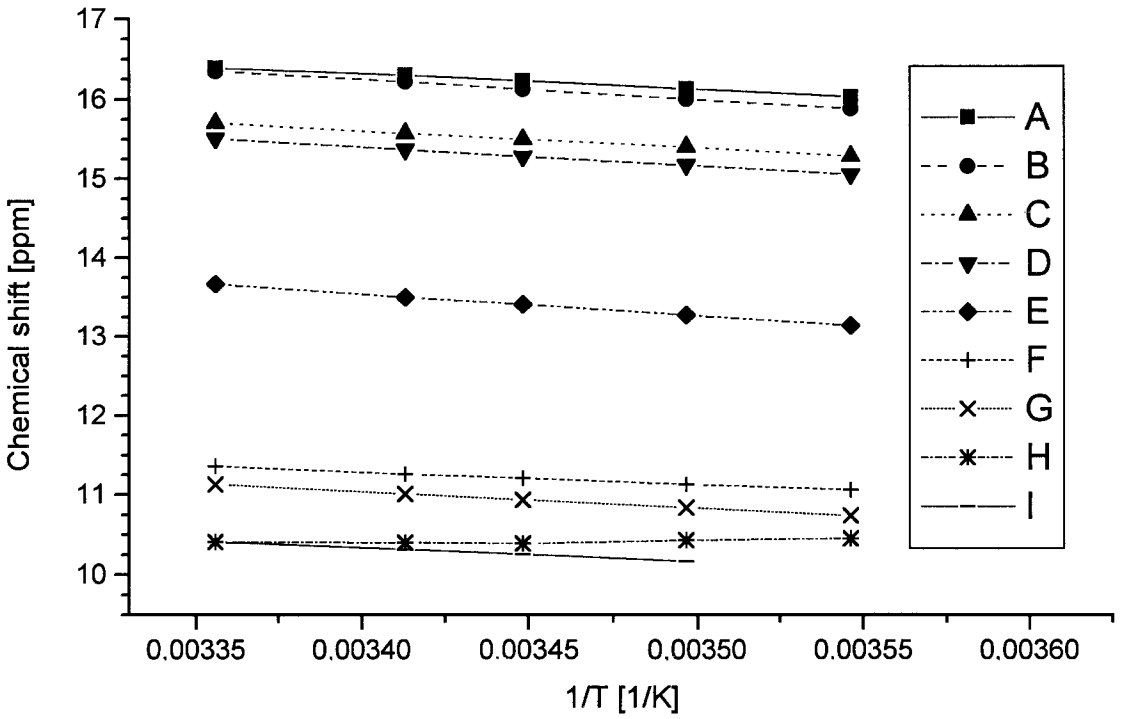

Fig. 2. Temperature dependence of the hyperfine-shifted resonances of the D13C mutant of *B. schlegelii* 7Fe ferredoxin in the temperature range 282–298 K in a chemical shift vs. 1/T plot.

assigned to Cys^{49} $H\beta_2$. This assignment is also consistent with the expected dipolar connectivities of Cys^{47} $H\beta_2$ in the X-ray structure of *C. acidi-urici* 8Fe ferredoxin [19] since this proton (corresponding to Cys^{49} $H\beta_2$ in the D13C mutant of *B. schlegeliis* ferredoxin) is close to two Hα protons (those of residues $i-3$ and $i+3$, respectively). Signal I can be tentatively assigned to Cys^8. In the case of WT, Cys^8 is the only unassigned cluster ligand, as it does not display any detectable hyperfine shifted resonance outside of the diamagnetic shift range [1]. The assignments of Cys^{20} and Cys^8 are tentative and could, in principle, be interchanged. However, the analogy in chemical shift patterns of the Hα and βCH_2 protons with clostridial ferredoxins strongly suggests the present assignment (Table 1).

As far as signal H is concerned, no assignment can be provided. It shows a TOCSY and a NOESY cross-peak with a resonance at 5.29 ppm. Additional 1D NOEs with resonances at 3.46, 3.12, 2.74, and 1.15 ppm were detected. On the basis of these connectivities, it can be speculated that H represents a very slowly exchanging NH proton with a Hα at 5.29 ppm. It is worth noting that the dipolar connectivities of H match exactly those of the unassigned signal G of WT [1]. More information about the identity of H can only be provided by a full assignment of the present mutant.

Table 1
Summary of the assignments for the hyperfine shifted resonances of the D13C mutant of *B. schlegelii* ferredoxin

Signal	Chem. shift (ppm)	Assignment	Connectivities with geminal and vicinal partners[a]		Cysteine	Cluster
A	16.1	$H\beta_2$	10.0 ppm (T,N)	$H\beta_1$ (aC) broad	13	I
			9.66 ppm (N)	Hα (aC) sharp		
B	15.9	$H\beta_1$	6.72 ppm (N)	Hα (aC) sharp	16	I
			4.23 ppm (N)	$H\beta_2$ (aC) broad		
C	15.3	$H\beta_2$	9.40 ppm (T, N)	$H\beta_1$ (aC) broad	42	II
			9.25 ppm (N)	Hα (aC) sharp		
D	15.0	$H\beta_1$	6.85 ppm (N)	Hα (aC) sharp	45	II
			4.92 ppm (T,N)	$H\beta_2$ (aC) broad		
E	13.15	$H\beta_2$	9.00 ppm (T, N)	$H\beta_1$ (aC) broad	49	I
			3.90 ppm (T)	Hα (ind)		
F	11.06	$H\beta_2$	5.48 ppm (T, N)	$H\beta_1$ (aC) broad	20[b]	II[b]
			4.98 ppm (T)	Hα[b] (ind)		
G	10.77	$H\beta_1$	8.55 ppm (T, N)	$H\beta_2$ (aC) broad	39	II
I	10.12	Hβ	8.48 ppm (T, N)	Hβ′ (aC) broad	8[b]	I[b]
			5.48 ppm (T)	Hα[b] (weak Curie)		

All shifts are given at 282 K and are referenced to a shift of 5.00 ppm for the residual HDO signal relative to DSS. The stereospecific assignments, except those for Cys^{16} and Cys^{45}, are tentative and based on the analysis of experimental linewidths.
[a]T, TOCSY cross-peak observed; N, NOESY cross-peak or 1D NOE observed; aC, anti-Curie temperature dependence; ind, chemical shift independent of temperature; broad or sharp, qualitative line width in 1D NOE difference spectra.
[b]Tentative assignment.

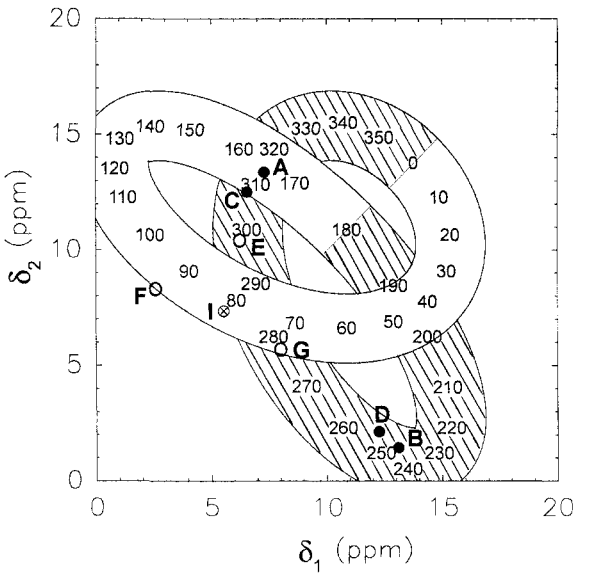

Fig. 3. Determination of the Cα–Cβ–S–Fe dihedral angles for the cysteine ligands of the two $[Fe_4S_4]^{2+}$ clusters in the D13C mutant of *B. schlegelii* ferredoxin. The hyperfine shifts of the βCH_2 protons, δ_1 and δ_2, were calculated from the chemical shift values in Table 1 minus 2.8 ppm [9]. Their relative relaxation rates R_1/R_2 were estimated from the linewidths in 1D and 1D NOE difference spectra. The figure depicts the relationship between δ_1 and δ_2 for different dihedral angles, as represented by numbers in the ribbon [12]. The hatched part of the ribbon represents values of $R_1/R_2 < 1$, and the open ribbon values of $R_1/R_2 > 1$. Cysteines with $R_1/R_2 < 1$ (●) and cysteines with $R_1/R_2 > 1$ (○) are labeled according to the most shifted resonance (Table 1). The indices 1 and 2 refer to the stereospecific assignments in Table 1. When a stereospecific assignment was not available or R_1/R_2 could not be estimated, δ_1 and δ_2 were defined arbitrarily (symbol for Cys^8 (signal I)).

4. Discussion

The data and assignments presented above provide clear evidence that the D13C mutant of *B. schlegeliis* 7Fe ferredoxin, as isolated in the oxidized state, is a Fe_8S_8 protein with two intact $[Fe_4S_4]^{2+}$ clusters that give rise to eight hyperfine shifted signals with anti-Curie temperature dependence in the ^{1}H-NMR spectrum (Fig. 1, resonances A-G and I). It therefore has to be assumed that the mutated residue (Cys^{13}) became a ligand to the fourth Fe ion, converting the 3Fe cluster of WT into a 4Fe cluster with the unusual cluster binding sequence CysXXXXCysXXCys....CysPro. The second 4Fe cluster, ligated by Cys^{39}, Cys^{42}, Cys^{45}, and Cys^{20}, is very little affected by the mutation and the introduction of another 4Fe cluster nearby. The 1D NOE difference spectra obtained by irradiation of its hyperfine shifted resonances are generally very similar to those of the corresponding WT signals (data not shown). The least similar 1D NOE difference spectrum and the biggest chemical shift difference with respect to WT are observed for Cys^{20}.

The question of how the protein frame of *B. schlegeliis* 7Fe ferredoxin structurally accommodates a second 4Fe cluster, especially the conformation of the unusually long linking sequence between Cys^8 and Cys^{13}, can only be partially addressed by the presently available data. The overall tertiary structure of *B. schlegeliis* ferredoxin maintains the pseudo 2-fold symmetry of all dicluster ferredoxins [19,21,22,24,25]. This is evident from the similarity of the 1D spectra of the two proteins and the observation of the expected NOE between $H\beta_1$ of CysIII of cluster I and $H\beta_1$ of CysIII of cluster II in both cases.

On a more local structural level, we have applied to the D13C mutant a recently proposed approach to determine the Cα–Cβ–S–Fe dihedral angles of cysteines that coordinate $[Fe_4S_4]^{2+}$ clusters from the chemical shifts and relaxation rates of their βCH_2 protons [9,12]. The dihedral angles of all cysteine residues have values that are compatible with the previously derived relationship between the hyperfine shifts (δ_1, δ_2) of the βCH_2 protons and their relative relaxation rates R_1/R_2 (Fig. 3). Only one minor deviation from the allowed region of the δ_1/δ_2 plane is observed for Cys^{49} (signal E), the presumed last ligand of cluster I. The mutant protein rearranges the conformation of amino acid 13 and the neighboring residues in such a way that this new ligand can bind a fourth Fe ion in the first cluster with a geometry that is very similar to the one in 4Fe and 8Fe ferredoxins with the CysXXCysXXCys binding sequence. Apparently, the spatial requirements of the amino acid linker Ile^9–Gly^{10}–Thr^{11}–Lys^{12} do not lead to distortions of the cysteine ligands to cluster I.

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References

[1] Aono, S., Bertini, I., Cowan, J.A., Luchinat, C., Rosato, A. and Viezzoli, M.S. (1996) JBIC 1, 523–528.
[2] Deng, W.P. and Nickoloff, J.A. (1992) Anal. Biochem. 200, 81–88.
[3] Aono, S., Nakamura, S., Aono, R. and Okura, I. (1994) Biochem. Biophys. Res. Commun. 201, 938–942.
[4] Johnson, R.D., Ramaprasad, S. and La Mar, G.N. (1983) J. Am. Chem. Soc. 105, 7205–7206.
[5] Banci, L., Bertini, I., Luchinat, C., Piccioli, M., Scozzafava, A. and Turano, P. (1989) Inorg. Chem. 28, 4650–4656.
[6] Davis, D.G. and Bax, A. (1985) J. Am. Chem. Soc. 107, 2820–2821.
[7] Chen, Z.G., de Ropp, J.S., Hernandez, G. and La Mar, G.N. (1994) J. Am. Chem. Soc. 116, 8772–8783.
[8] Marion, D. and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 113, 967–974.
[9] Bertini, I., Capozzi, F., Luchinat, C., Piccioli, M. and Vila, A.J. (1994) J. Am. Chem. Soc. 116, 651–660.
[10] Huber, J.G., Gaillard, J. and Moulis, J.-M. (1995) Biochemistry 34, 194–205.
[11] Macedo, A.L., Palma, P.N., Moura, I., LeGall, J., Wray, V. and Moura, J.J.G. (1993) Magn. Reson. Chem. 31, S59–S67.
[12] Davy, S.L., Osborne, J.M., Breton, J., Moore, G.R., Thomson, A.J., Bertini, I. and Luchinat, C. (1995) FEBS Lett. 363, 199–204.
[13] Donaire, A., Gorst, C.M., Zhou, Z.H., Adams, M.W.W. and La Mar, G.N. (1994) J. Am. Chem. Soc. 116, 6841–6849.
[14] Wildegger, G., Bentrop, D., Ejchart, A., Alber, M., Hage, A., Sterner, R. and Roesch, P. (1995) Eur. J. Biochem. 229, 658–668.
[15] Busse, S.C., La Mar, G.N. and Howard, J.B. (1991) J. Biol. Chem. 266, 23714–23723.
[16] Adman, E.T., Sieker, L.C. and Jensen, L.H. (1976) J. Biol. Chem. 251, 3801–3806.
[17] Fukuyama, K., Nagahara, Y., Tsukihara, T. and Katsube, Y. (1988) J. Mol. Biol. 199, 183–193.
[18] Kissinger, C.R., Sieker, L.C., Adman, E.T. and Jensen, L.H. (1991) J. Mol. Biol. 219, 693–715.
[19] Duée, E.D., Fanchon, E., Vicat, J., Sieker, L.C., Meyer, J. and Moulis, J.-M. (1994) J. Mol. Biol. 243, 683–695.

[20] Sery, A., Housset, D., Serre, L., Bonicel, J., Hatchikian, C., Frey, M. and Roth, M. (1994) Biochemistry 33, 15408–15417.
[21] Bertini, I., Donaire, A., Feinberg, B.A., Luchinat, C., Piccioli, M. and Yuan, H. (1995) Eur. J. Biochem. 232, 192–205.
[22] Moulis, J.-M., Sieker, L.C., Wilson, K.S. and Dauter, Z. (1996) Prot. Sci. 5, 1765–1775.
[23] Macedo-Ribeiro, S., Darimont, B., Sterner, R. and Huber, R. (1996) Structure 4, 1291–1301.
[24] Stout, C.D. (1989) J. Mol. Biol. 205, 545–555.
[25] Fujii, T., Hata, Y., Oozeki, M., Moriyama, H., Wakagi, T., Tanaka, N. and Oshima, T. (1997) Biochemistry 36, 1505–1513.