^{1}H NOE studies of oxidized high potential iron sulfur protein II from *Ectothiorhodospira halophila*

Lucia Banci, Ivano Bertini*, Fabrizio Briganti, Andrea Scozzafava, Margarita Vicens Oliver
Department of Chemistry, University of Florence, Via Gino Capponi 7, 50121 Florence (Italy)

and **Claudio Luchinat**
Institute of Agricultural Chemistry, University of Bologna, Bologna (Italy)

(Received September 4, 1990)

Abstract

The ^{1}H NMR spectra of oxidized HiPIP II from *Ectothiorhodospira halophila* have been recorded at 600 MHz. Nuclear Overhauser effect measurements have allowed the assignment of the cysteine β-CH_2 resonances. Four β-CH_2 signals are downfield shifted and four upfield shifted. Through a theoretical model and on the ground of Mössbauer data on analogous systems we propose that the upfield signals are those of the cysteines bound to the iron(III) ions and those downfield of the cysteines bound to the mixed valence pair Fe(III)–Fe(II).

Introduction

The ^{1}H NMR spectra of the oxidized high potential iron–sulfur proteins (HiPIPs hereafter) have been known for many years [1–7]. Among the many reported ^{1}H NMR spectra of iron–sulfur proteins, HiPIPs show the unusual feature of having, besides several signals shifted far downfield by isotropic hyperfine interaction, also two or four signals shifted *upfield* and quite far from the diamagnetic region. Since the isotropic hyperfine shifts of β-CH_2 cysteinyl protons in iron–sulfur proteins are mainly contact in origin, and the contact coupling constants for such systems are usually positive (downfield shifts) [8–10], these signals have up to now constituted an unsolved puzzle for the researchers in the area. To complicate the picture, two signals in the downfield region show an anti-Curie temperature dependence when the upfield shifted signals are two [4–6, 11], whereas no signals with anti-Curie behavior have been reported for those HiPIPs having four upfield shifted signals [4]. The Mössbauer data on oxidized *C. vinosum* HiPIP and on a synthetic analogue indicate that two of the four iron ions are in the oxidation state +3, whereas the other two ions constitute a mixed valence pair with average oxidation state +2.5 [12–15].

We have recently proposed a theoretical model that is in agreement with the Mössbauer data, and predicts four β-CH_2 protons to be shifted upfield and the other four to be shifted downfield [9, 16]. The four upfield shifted signals would arise from the cysteines coordinated to the two iron(III) ions, whereas the four downfield shifted signals would arise from the cysteines coordinated to the irons constituting the mixed valence pair. Such a model is being tentatively used by us to account for the ^{1}H NMR spectrum of HiPIP from *C. vinosum* [17]. Since *C. vinosum* shows only two upfield shifted signals, a strong inequivalence between the two iron(III) ions must be postulated.

In order to test our theoretical model, we have decided to study the HiPIP II from *Ectothiorhodospira halophila*, which shows four upfield and four downfield shifted signals. Unfortunately Mössbauer data are not yet available for this system. We show in this paper, by using ^{1}H NMR NOE experiments, that the four upfield shifted signals indeed belong to two β-CH_2 pairs, and the four downfield shifted signals to the other two pairs. This strongly supports the theory which describes the electronic structure of the cluster and suggest a general solution of the problem of the assignment of ^{1}H NMR spectra of oxidized HiPIPs.

Experimental

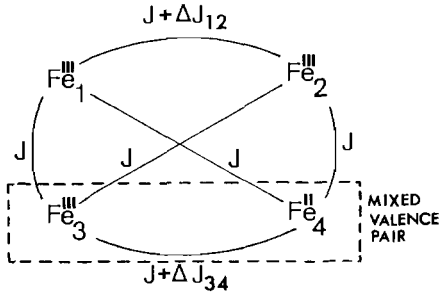
All chemicals used throughout were of the best quality available. *E. halophila* HiPIP II was purified

*Author to whom correspondence should be addressed.

0020-1693/91/$3.50

as previously reported [18]. Experiments in D_2O 99.95%, 30 mM NaP_i were performed by solvent exchange utilizing an ultrafiltration Amicon cell equipped with a YM5 membrane; at least 5 changes of deuterated buffer were performed to assure satisfactory solvent exchange. The protein samples (2–4 mM) were oxidized by addition of small amounts of 0.1 M potassium hexacyanoferrate(III), in 30 mM NaP_i, D_2O 99.9%. The pH values are reported as uncorrected pH-meter readings (pH*).

High resolution Fourier transform ^1H NMR measurements were carried out on a Bruker AMX 600 spectrometer running at 600.13 MHz. Typically



Scheme 1.

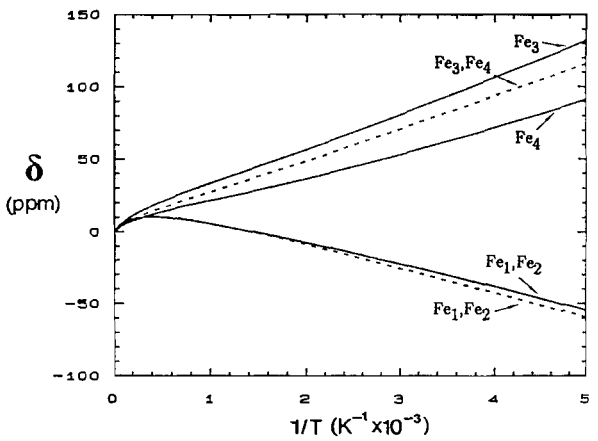


Fig. 1. Temperature dependence of the ^1H NMR isotropic shifts of oxidized HiPIPs calculated using eqn. (2) according to ref. 17, with $J = 300$ cm^{-1}, $\Delta J_{12}/J = -\Delta J_{34}/J = 0.333$, and $B_{34}/J = 0$ (——), or $J = 300$ cm^{-1}, $\Delta J_{12}/J = 0.3$, $\Delta J_{34}/J = 0$, and $B_{34}/J = 1.3$ (· · · · ·). ($Fe_1 = Fe_2 = Fe_3 = Fe(III)$ and $Fe_4 = Fe(II)$.)

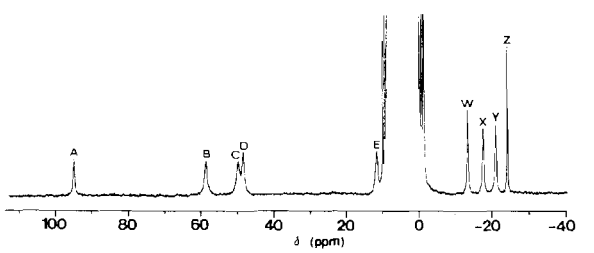


Fig. 2. 600 MHz ^1H NMR spectrum of oxidized *E. halophila* HiPIP II in D_2O pH* 5.1 and 298 K.

1000–5000 transients were acquired utilizing the Super-WEFT (180-τ-90-AQ) pulse sequence [19].

^1H NOE measurements were performed by collecting 8–16 K data points over a 125 KHz bandwidth. The water signal was suppressed using the Super-WEFT pulse sequence with recycle times of 60–120 ms and delay times τ of 50–100 ms. The resonances under investigation were saturated utilizing a selective decoupling pulse of 0.01–0.02 W kept on for 9/10 of the delay time τ. Difference spectra were collected directly by applying the decoupler frequency alternatively according to the scheme

$$\omega_2 - (\omega_2 + \delta) - \omega_2 - (\omega_2 - \delta)$$

where ω_2 is the frequency of the irradiated signal and δ a small off-resonance offset, typically of the order of twice the irradiated signal linewidth; the receiver phase was alternated in such a way that the scans with the decoupler frequency on-resonance were added and those with the decoupler frequency off-resonance subtracted. This sequence scheme allows the obtainement of good difference spectra minimizing hardware instabilities. Each experiment, run in block-averaging mode, consisted usually of 5–15 blocks of 32 K scans each. Exponential multiplication of the free induction decay improved the signal to noise ratio introducing 10–40 Hz additional line broadening.

T_1 measurements were performed utilizing the inversion recovery pulse sequence [20].

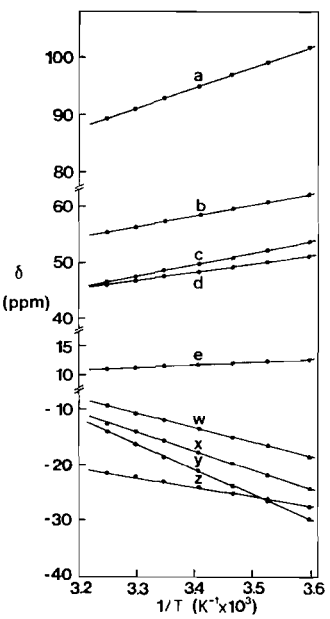


Fig. 3. Experimental temperature dependence of the ^1H NMR isotropically shifted signals of oxidized HiPIP II from *E. halophila* at pH* 5.1.

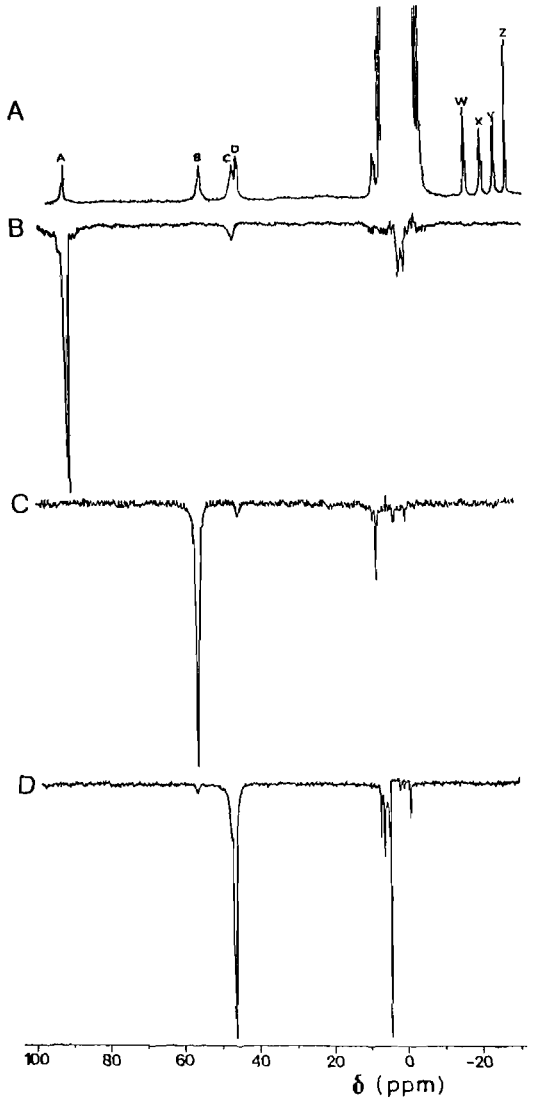


Fig. 4. 600 MHz ^{1}H steady state NOE difference spectra of oxidized *E. halophila* HiPIP II in D_2O pH* 5.1 and 298 K. Trace A, reference spectrum; traces B–D, steady state NOE difference spectra obtained by saturating peaks A, B and D, respectively.

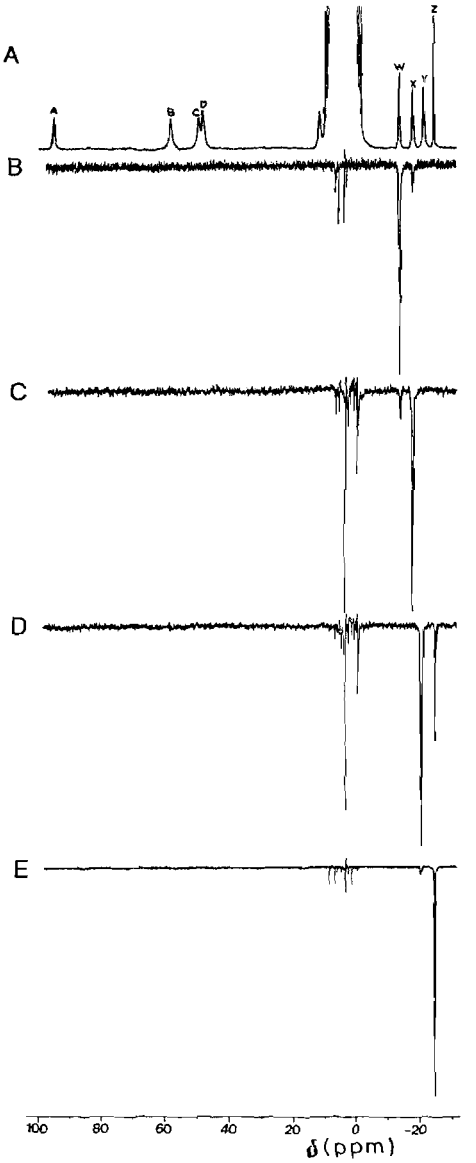


Fig. 5. 600 MHz ^{1}H steady state NOE difference spectra of oxidized *E. halophila* HiPIP II in D_2O pH* 5.1 and 298 K. Trace A, reference spectrum; traces B–E, steady state NOE difference spectra obtained by saturating peaks W, X, Y and Z, respectively.

Theoretical background

The simplest coupling scheme for a Fe_4S_4 cluster is summarized in Scheme 1. It has been shown that the relative energies of the levels in the coupled systems are given by

$$E_i(S'_{12_i}S'_{34_i}S'_i) = (J/2)[S'_i(S'_i+1)] + (\Delta J_{12}/2)[S'_{12_i}(S'_{12_i}+1)] + (\Delta J_{34}/2)[S'_{34_i}(S'_{34_i}+1)] \quad (1)$$

where S'_i ranges between $(S_1+S_2)+(S_3+S_4)$ and $|(S_1+S_2)-(S_3+S_4)|$, S'_{12_i} ranges between S_1+S_2 and $|S_1-S_2|$, and S'_{34_i} ranges between S_3+S_4 and $|S_3-S_4|$. Since it is known from Mössbauer data on the analogous protein from *C. vinosum* and on a synthetic analogue that at least one Fe(II)–Fe(III) pair in oxidized HiPIPs is delocalized, i.e. the two iron ions have an averaged +2.5 oxidation state [12–15], one additional term of the type $\pm B_{34}(S'_{34_i}+1/2)$ can be parametrically introduced in eqn. (1) [21].

TABLE 1. Steady state nuclear Overhauser effects measured[a] between the isotropically shifted resonances of the oxidized HiPIP II from *E. halophila*

Observed signals							Saturated signals[b]
B	C	D	W	X	Y	Z	
	3.2(1.5)						A
		2.3(1.5)					B
2.2(1.7)							D
				14.2(1.8)			W
			11.8(1.7)				X
						21.2(1.6)	Y
					7.7(1.6)		Z

[a]The data were recorded at 600 MHz, 298 K and pH* 5.1 and are reported as percent decrease in signal intensity. Estimated distances (Å) are given in parentheses. The estimated errors are ±0.1 Å. [b]The NOE from C to A has not been checked.

$$E_i(S'_{12_i}S'_{34_i}S'_i) = (J/2)[S'_i(S'_i+1)] \pm B_{34}(S'_{34_i}+1/2) + (\Delta J_{12}/2)[S'_{12_i}(S_{34_i}+1)] + (\Delta J_{34}/2)[S'_{34_i}(S_{34_i}+1)] \quad (2)$$

The presence of such a term has been already put on firm theoretical ground for Fe_3S_4 systems [22–24]. For the oxidized HiPIP from *C. vinosum* and a synthetic analogue, Mössbauer data have shown that the $S'=1/2$ ground state of the coupled system is made up by an $S'_{34}=9/2$ subsystem, arising from the mixed valence pair, antiferromagnetically coupled to an $S'_{12}=4$ subsystem arising from the two Fe(III) ions [12–15]. We have shown that such ground state can be easily obtained from eqns. (1) or (2) with $\Delta J_{34}<0$ and $\Delta J_{12}>0$, independently of the magnitude of B_{34} because of the large covariance between B_{34} and ΔJ_{34} [9]. Similarly large covariance is encountered in Fe_3S_4 systems [25]. No matter how the correct ground state is obtained, the contact hyperfine shifts for the protons belonging to the cysteines bound to the individual ions follow the temperature dependences of the type shown in Fig. 1. It appears that, for $kT \leqslant J$, the two iron(III) ions induce upfield shifts for the nuclei belonging to their coordinated cysteines. Therefore the sign and temperature dependence of the hyperfine shifts observed in *E. halophila* HiPIP would be correctly predicted if the four downfield signals belonged to the two β-CH_2 proton pairs of the two cysteines coordinated to the irons constituting the mixed valence pair, and the four upfield signals belonged to the two β-CH_2 proton pairs of the two cysteines coordinated to the two iron(III) ions.

Results and discussion

The 600 MHz ^1H NMR spectra of the oxidized form of HiPIP II from *E. halophila* at 300 K is shown in Fig. 2, and the temperature dependence of the shifts of the isotropically shifted signals is shown in Fig. 3. Besides the better resolution of the present spectra, the data are in complete agreement with those already reported [4].

In order to test the theoretical predictions, we must demonstrate that the four downfield signals belong to two β-CH_2 pairs and the four upfield signals to the other two. This can be accomplished by looking for the nuclear Overhauser effects within each proton pair; these effects are relatively strong even in paramagnetic systems due to the short interproton distance in methylene groups (1.5–1.7 Å).

The nuclear Overhauser effect is defined as the fractional intensity change of a NMR resonance i upon saturation of another resonance j. The steady state NOE η_{ij} is given by [26, 27]

$$\eta_{ij} = \sigma_{ij}/\rho_i \quad (3)$$

where ρ_i is the intrinsic longitudinal relaxation rate for the nucleus i and σ_{ij} is the cross relaxation rate between i and j given by

$$\sigma_{ij} = \frac{\mu_0}{4\pi^2}\frac{\hbar^2\gamma^4}{10r_{ij}^6} f(\tau_c) \quad (4)$$

where, if local motions are not present, τ_c is the protein tumbling time, and r_{ij} is the internuclear distance. In the present case $f(\tau_c) = -\tau_c$.

NOEs in paramagnetic systems are relatively small [26–30]. However, the paramagnetism quenches spin diffusion allowing the selective detection of primary NOEs in large proteins [28–30].

By using the T_1 values which are in the range 2–7 ms for the downfield signals and in the range 7–25 ms for the upfield signals, and an estimate of the rotational correlation time of the protein of $3–4\times10^{-9}$ s, we predict steady state NOE effects around 1–3% for the downfield signals and 10–20% for the upfield signals.

The NOE difference spectra are reported in Figs. 4 and 5, and the η values and the estimated distances are collected in Table 1. The eight signals can be straightforwardly grouped into four pairs, namely, A–C, B–D, W–X, Y–Z. The estimated distances are in good agreement with the expected value for a geminal β-CH_2 proton pair.

We have thus demonstrated that the four upfield shifted signals belong to two β-CH_2 cysteinyl proton pairs and solved the puzzle of the interpretation of the ^1H NMR spectra of oxidized HiPIPs. According to the theoretical model described earlier, if the ground state of *E. halophila* is the same as that found in *C. vinosum* HiPIP and a synthetic analogue through Mössbauer spectroscopy, the upfield signals belong to the cysteines coordinated to the ions forming the mixed valence pair. This finding opens the way to future work aimed at performing the individual assignment of the iron ions in the protein frame, through extension of the NOE or NOESY experiments to other protons in the neighborhoods of the β-CH_2 protons.

References

1 W. D. Phillips, in G. N. La Mar, W. DeW. Horrocks, Jr. and R. H. Holm (eds.), *NMR of Paramagnetic Molecules, Principles and Applications,* Academic Press, New York, 1973, Ch. 11.
2 W. D. Phillips and M. Poe, in W. Lovenberg (ed.), *Iron–Sulfur Proteins,* Vol. 3, Academic Press, New York, 1977, Ch. 7.
3 D. G. Nettesheim, J. L. Meyer, B. A. Feinberg and J. D. Otvos, *J. Biol. Chem., 258* (1983) 8235.
4 R. Krishnamoorthi, J. L. Markley, M. A. Cusanovich, C. T. Prysiecki and T. E. Meyer, *Biochemistry, 25* (1986) 60.
5 R. Krishnamoorthi, M. A. Cusanovich, T. E. Meyer and C. T. Prysiecki, *Eur. J. Biochem., 181* (1989) 81.
6 M. Sola, J. A. Cowan and H. B. Gray, *Biochemistry, 28* (1989) 5261.
7 W. D. Phillips, M. Poe, C. C. Mc Donald and R. G. Bartsch, *Proc. Natl. Acad. Sci. U.S.A., 67* (1970) 682.
8 I. Bertini and C. Luchinat, *NMR of Paramagnetic Molecules in Biological Systems,* Benjamin/Cummings, Menlo Park, CA, 1986.
9 L. Banci, I. Bertini and C. Luchinat, *Struct. Bonding (Berlin), 72* (1990) 113.
10 M. T. Werth, D. M. Kurtz, I. Moura and J. LeGall, *J. Am. Chem. Soc., 109* (1987) 273.
11 J. A. Cowan and M. Sola *Biochemistry, 29* (1990) 5633.
12 P. Middleton, D. P. E. Dickson, C. E. Johnson and J. D. Rush, *Eur. J. Biochem., 104* (1980) 289.
13 T. H. Moss, A. J. Bearden, R. G. Bartsch and M. A. Cusanovich, *Biochemistry, 7* (1968) 1591.
14 D. P. E. Dickson, C. E. Johnson, R. Cammack, M. C. W. Evans, D. O. Hall and K. K. Kao, *Biochem. J., 139* (1974) 105.
15 V. Papaefthymiou, M. M. Millar and E. Münck, *Inorg. Chem., 25* (1986) 3010.
16 L. Banci, I. Bertini, F. Briganti and C. Luchinat, in *Physics and Chemistry of Metallic Clusters in Proteins,* special issue of *New J. Chem.,* in press.
17 I. Bertini, F. Briganti, C. Luchinat, A. Scozzafava and M. Sola, *J. Am. Chem. Soc., 113* (1991) 1237.
18 T. E. Meyer, *Biochim. Biophys. Acta, 806* (1985) 175.
19 T. Inubushi and E. D. Becker, *J. Magn. Reson., 51* (1983) 128.
20 R. L. Vold, J. S. Waugh, M. P. Klein and D. E. Phelps, *J. Chem. Phys., 48* (1968) 3831.
21 L. Noodleman, *Inorg. Chem., 27* (1988) 3677.
22 E. Münck, V. Papaefthymiou, K. K. Surerus and J.-J. Girerd, in L. Que, Jr., (ed), *Metal Clusters in Proteins,* ACS Symposium Series 372, 1987, p. 302.
23 V. Papaefthymiou, J.-J. Girerd, I. Moura, J. J. G. Moura and E. Münck, *J. Am. Chem. Soc., 109* (1987) 4703.
24 G. Blondin and J.-J. Girerd, *Chem. Rev., 90* (1990) 1359.
25 I. Bertini, F. Briganti and C. Luchinat, *Inorg. Chem. Acta, 175* (1990) 9.
26 J. H. Noggle and R. E. Shirmer, *The Nuclear Overhauser Effect,* Academic Press, New York, 1971.
27 D. Neuhaus and M. Williamson, *The Nuclear Overhauser Effect in Structural and Conformational Analysis,* VCH, New York, 1989.
28 L. B. Dugad, G. N. La Mar and S. W. Unger, *J. Am. Chem. Soc., 112* (1990) 1386.
29 V. Thanabal, J. S. de Ropp and G. N. La Mar, *J. Am. Chem. Soc., 109* (1987) 265.
30 S. W. Unger, Ju. T. Lecomte and G. N. La Mar, *J. Magn. Reson., 64* (1985) 521.