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2D ^{1}H NMR studies of oxidized 2(Fe_4S_4) ferredoxin from *Clostridium pasteurianum*

Ivano Bertini[1], Fabrizio Briganti[1], Claudio Luchinat[2], Luigi Messori[1], Roberto Monnanni[1], Andrea Scozzafava[1] and Giovanni Vallini[3]

[1]*Department of Chemistry, University of Florence, Florence, Italy,* [2]*Institute of Agricultural Chemistry, University of Bologna, Bologna, Italy* and [3]*Centro di Studio per la Microbiologia del Suolo, CNR, Pisa, Italy*

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Oxidized ferredoxin from *Clostridium pasteurianum*, containing two Fe_4S_4 clusters, has been investigated using 2D ^{1}H NMR spectroscopy at 600 MHz. 2D NMR experiments allowed complete assignment of the sixteen isotropically shifted signals corresponding to the β-CH_2 protons of the eight metal coordinated cysteines. Geminal connectivities of Cys β-CH_2 protons were identified through magnitude COSY experiments and confirmed through 2D NOESY experiments. A few additional signals could be assigned to the corresponding α-CH protons. The importance of 2D experiments to achieve firm assignments of isotropically shifted signals in paramagnetic metalloproteins is stressed.

Ferredoxin; Iron–sulfur cluster; 2D NMR; Metalloprotein

1. INTRODUCTION

The 2(Fe_4S_4) ferredoxin from *Clostridium pasteurianum* is a low molecular weight iron sulfur protein [1–3]; in the oxidized state each cluster formally contains two iron (III) and two iron (II) ions [4]. Owing to antiferromagnetic exchange coupling the ground state is S=0 [4,5]. At room temperature some paramagnetism arises from occupancy of the excited states; the overall magnetic moment at 298 K is ≈4.1 BM per cluster [6].

2D ^{1}H NMR experiments have been developed and extensively utilized for the determination of the solution structure of diamagnetic compounds [7,8]. In these systems the 2D pulse sequences take place in a time negligible with respect to proton relaxation times; magnetization transfer occurs in a time shorter or of the same order of magnitude. On the other hand, paramagnetic systems are characterized by drastic enhancements of nuclear relaxation rates [9,10]; so it often happens that the spin system reaches equilibrium without transferring detectable amounts of magnetization from one spin set to another. This fact has heavily hindered the application of 2D NMR techniques to paramagnetic systems [11,12].

Only in recent years 2D NMR experiments of paramagnetic compounds have been reported, in particular for systems containing low spin iron (III), for which the nuclear relaxation rates are only slightly increased with respect to diamagnetic systems [13–17].

In this frame, we here report a 2D NOESY and COSY study of the oxidized 2(Fe_4S_4) ferredoxin from *Clostridium pasteurianum*. We demonstrate that, by an accurate choice of the experimental parameters, 2D NMR experiments can be fruitfully applied to the investigation of paramagnetic iron–sulfur clusters and can provide valuable information for firm assignments of the isotropically shifted lines.

Abbreviations: CpFd, *Clostridium pasteurianum* 2(Fe_4S_4) ferredoxin; COSY, correlation spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; FID, free induction decay; NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; RD, recycle delay; τ_m, mixing time

Correspondence address: I. Bertini, Department of Chemistry, University of Florence, via Gino Capponi 7, 50121 Florence, Italy.

2. MATERIALS AND METHODS

Clostridium pasteurianum was grown and ferredoxin isolated and purified according to the method of Rabinowitz [18,19]. The purity of the sample was checked by absorption spectroscopy by monitoring the A_{390}/A_{280} absorbance ratio. For ^{1}H NMR experiments the protein was dissolved in 50 mM NaP_i buffer, pH 8.0. Deuteration of the sample was achieved by utilizing an ultrafiltration Amicon cell, equipped with a YM1 membrane. At least five changes of deuterated buffer were performed to ensure satisfactory solvent exchange. The pH values are reported as uncorrected pH meter readings.

All the ^{1}H NMR spectra were recorded on an AMX 600 Bruker spectrometer. Chemical shift values are referred to DSS. T_1 values were determined by the inversion recovery method [20]; T_2 values were obtained from analysis of the linewidths.

2D COSY spectra were recorded in the magnitude mode [21]. Experiments were recorded with 180 t_1 values and 4096 scans per t_1 value, 1K in f_2. Repetition rate was 100 ms. Prior to Fourier transformation, the 2D data matrix was multiplied with an unshifted sine-squared bell window function in both dimensions.

2D NOESY spectra were recorded in the phase sensitive mode, with the sequence RD-90-t_1-90-τ_m-90-AQ [22]. Experiments were recorded with 1K data points in t_2, and 128–256 t_1 values with 1024–4096 scans per FID. Several experiments were performed with mixing times rang-

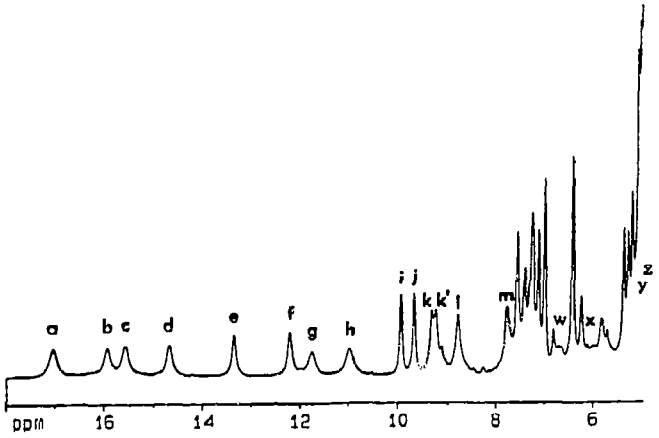

Fig. 1. 600 MHz ^{1}H NMR spectrum of oxidized Cp Fd at 300 K. The chemical shifts and the nuclear relaxation times of the labelled signals are reported in Table I. Conditions: 5 mM protein, 50 mM phosphate buffer, D_2O, pH 8.0.

ing from 5 to 50 ms. Prior to Fourier transformation, the data matrix was multiplied with a phase shifted sine-squared bell window function in both dimensions.

3. RESULTS AND DISCUSSION

The downfield region of the 600 MHz ^{1}H NMR spectrum of a very concentrated sample (5 mM) of ferredoxin from *Clostridium pasteurianum*, pH 8, phosphate buffer 50 mM, D_2O, is shown in Fig. 1. The spectrum corresponds to that previously reported at 300 MHz [23] and exhibits eight well-resolved isotropically shifted signals above 10 ppm which were previously assigned to β-CH_2 protons of the metal coordinated cysteines (signals a through h); the other eight resonances from Cys β-CH_2 protons are expected to fall into the diamagnetic envelope [23]. The T_1 and T_2 values have been determined again on the present sample at 600 MHz for the isolated resonances (Table I).

In order to proceed with the assignment of the isotropically shifted lines, we performed a series of 2D experiments. The results of a COSY magnitude experiment and three NOESY experiments recorded with mixing times of 20, 10 and 5 ms, are shown in Fig. 2 and Fig. 3A,B,C, respectively. Extensive search for the best experimental parameters was performed in order to optimize the intensity of cross peaks with respect to diagonal peaks.

Inspection of the spectra allows the identification of through bond and through space connectivities. All the far isotropically shifted signals (a through h), with the only exception of e and f, exhibit a single scalar cross peak which probably represents the geminal connectivity. Signals e and f show two cross peaks each; probably the larger ones represent the geminal connectivity whereas the smaller ones (9 and 10) correspond to the β-to-α connectivity.

The COSY pattern is confirmed by the NOESY dipolar maps. The NOESY experiments reported in Fig. 3, show a number of dipolar connectivities from the eight far shifted signals to signals in the range 10–0 ppm. A correct interpretation of the NOESY maps could be obtained by comparison of spectra recorded at progressively shorter mixing times. Indeed, the experiment re-

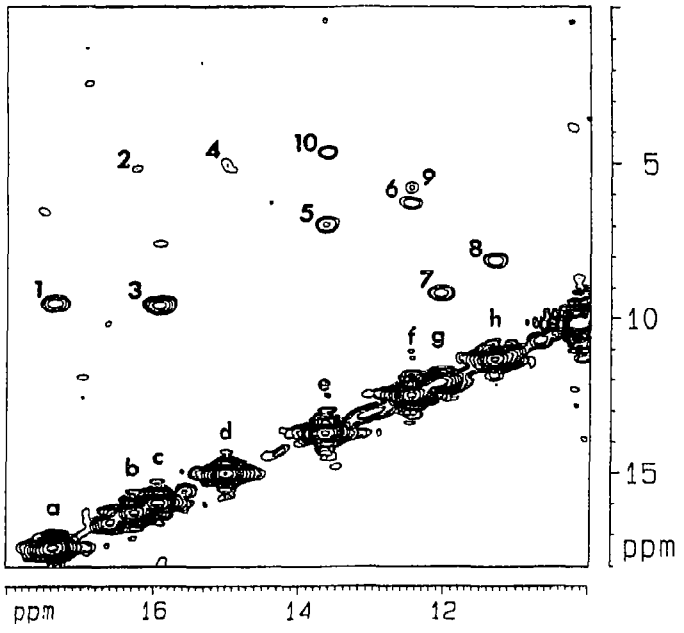

Fig. 2. 600 MHz magnitude COSY spectrum of oxidized Cp Fd; 300 K. Conditions as in Fig. 1.

Table I
T_1 and T_2 values for the isotropically shifted resonances of the oxidized ferredoxin from *Clostridium pasteurianum*[a]

Signal[b]	Shift (ppm)	T_1 (ms)	T_2 (ms)
a (k')	17.4	6.2	3.2
b (y)	16.3	7.0	4.6
c (k)	16.0	7.2	4.1
d (z)	15.0	8.3	4.9
e (w)	13.7	11	6.8
f (x)	12.5	9.8	6.4
g (l)	12.1	4.9	3.5
h (m)	11.3	5.1	3.5
i	10.1	29	18
j	9.9	29	14
k (c)	9.6	13	6.4
k' (a)	9.5	14	6.4
l (g)	9.1	11	6.8
m (h)	8.1	14	5.9
w (e)	6.9	–	–
x (f)	6.1	–	–
y (b)	5.1	–	–
z (z)	4.9	–	

[a]Measured at 600 MHz, 300 K, pH 8.0. The errors on T_1 and T_2 measurements are always below 10%.
[b]The corresponding geminal β-CH_2 protons, assigned by means of COSY and NOESY connectivities, are reported in parentheses.

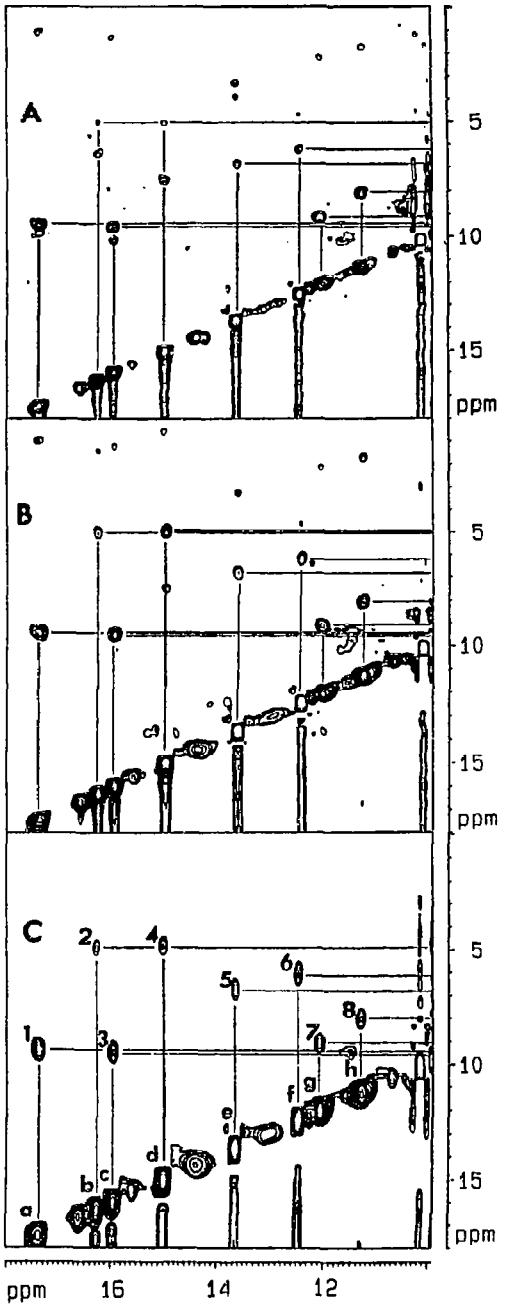

Fig. 3. 600 MHz phase sensitive NOESY spectra of oxidized Cp Fd at different mixing times. (A) τ_m = 20 ms; (B) τ_m = 10 ms; (C) τ_m = 5 ms. Conditions as in Fig. 1.

corded with mixing time of 5 ms allows detection of only eight cross peaks, representing short-range dipolar connectivities; these peaks are nicely superimposable to the above detected scalar cross peaks. We consider this finding as an unambiguous evidence for the complete assignment of the Cys β-CH_2 geminal protons. It must be noted that in the NOESY spectra some geminal connectivities (see signal **b** and **d**) are very hard to detect because of the short nuclear relaxation times; the respective cross peaks already disappear at mixing times as long as 20 ms. The full assignment of the 16 β-CH_2 protons of the eight coordinated cysteines is reported in Table I (geminal connectivities: **a-k'**, **b-y**, **c-k**, **d-z**, **e-w**, **f-x**, **g-l**, **h-m**). Beyond the geminal connectivities of the β-CH_2 protons, inspection of the 2D NOESY spectra also permits the identification of further cross peaks among Cys β-CH_2 protons and other protons which probably represent β to α connectivities. Tentatively, we assign signals **i** and **j** as α-CH protons of pairs **c-k** and **a-k'**, respectively. It is worthwhile to note that these β to α connectivities are not observed in the COSY spectra, probably because of the values of the J coupling constants; in principle, information on the J values and the H-C-C-H dihedral angles can be obtained.

The present assignment differs from that previously published [23] in the case of the geminal partners of signals **b** and **d**, i.e. signals **y** and **z**; indeed, the latter signals are hardly detected since they are broad and very close to the solvent signal. In the previous monodimensional experiments, NOE's had been detected between signal **b** and a signal at 7.7 ppm, and between signal **d** and a signal at 6.5 ppm. These cross peaks are still present in the NOESY spectra but the COSY map rules out that they are β-CH_2 geminal partners.

Finally, we like to outline the importance of a 2D approach to obtain firm assignments of isotropically shifted signals in paramagnetic metalloproteins. Monodimensional NOE experiments, largely utilized in recent years for the assignment of isotropically shifted resonances [24–27], give information only on dipolar effects and, as a consequence, on the spatial proximity between protons. It often happens that a proton located in the active cavity of a metalloprotein lies near to several other protons; under these conditions the assignments based exclusively on the observation of NOE's are ambiguous, in particular if they are referred to ill defined crystallographic structures. In these cases it is essential to establish whether two spatially proximal protons are connected through bond. The simultaneous observation of 2D dipolar and scalar maps offers this additional criterion and makes resonance assignment in paramagnetic systems more reliable.

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REFERENCES

[1] Thompson, A.J. (1985) in: Metalloproteins (Harrison, P., ed.) part I, pp. 79–120, Verlag Chemie, Weinheim.
[2] Bezkorovainy, A. (1980) in: Biochemistry of Nonheme Iron, chapter 8, Plenum, New York.
[3] Backes, G., Mino, Y., Loehr, T.M., Meyer, T.E., Cusanovich, M.A., Sweeney, W.V., Adman, E.T. and Sanders-Loehr, J. (1991) J. Am. Chem. Soc. 113, 2055-2064, and references therein.
[4] Antanaitis, B.C. and Moss, T.H. (1975) Biochim. Biophys. Acta, 405, 262–279.

[5] Thompson, C.L., Johnson, C.E., Dickson, D.P.E., Cammack, R., Hall, D.O., Weser, U. and Rao, K.K. (1974) Biochem. J. 139, 97–103.
[6] Poe, M., Phillips, W.D., McDonald, C.C. and Lovenberg, W. (1970) Proc. Natl. Acad. Sci. USA 65, 797–804.
[7] Wuethrich, K. (1986) NMR of Proteins and Nucleic Acids, Wiley Interscience, New York.
[8] Ernst, R.R., Bodenhausen, G. and Wokaun, A. (1987) Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Claredon Press, Oxford.
[9] Bertini, I. and Luchinat, C. (1986) NMR of Paramagnetic Molecules in Biological Systems, Benjamin-Cummings, Menlo Park, CA.
[10] Banci, L., Bertini, I. and Luchinat, C. (1991) Electronic and Nuclear Relaxation, VCH, Weinheim.
[11] Banci, L., Bertini, I., Luchinat, C. and Piccioli, M. (1991) in: NMR and Biomolecular Structure (Bertini, I., Molinari, H. and Niccolai, N., eds.) pp. 31–60, VCH, Weinheim.
[12] Oh, B.-H. and Markley, J.L. (1990) Biochemistry 29, 3993–4004.
[13] Yu, L.P., La Mar, G.N. and Rajarathnam, K. (1990) J. Am. Chem. Soc. 112, 9527–9534.
[14] Emerson, S.D. and La Mar, G.N. (1990) Biochemistry 29, 1545–1556.
[15] Veich, N.C., Concar, D.W., Williams, R.J.P. and Whitford, D. (1988) FEBS Lett. 238, 90.
[16] Yamamoto, Y., Osawa, Y., Inoue, Y., Chujo, R. and Suzuki, T. (1989) FEBS Lett. 247, 263.
[17] Feng, Y., Roder, H. and Englander, S.W. (1990) Biochemistry 29, 3494–4004.
[18] Rabinowitz, J. (1972) Methods Enzymol. 24, 431–446.
[19] Miller, T.L. and Wolin, M.J. (1974) Appl. Microbiol. 27, 985–987.
[20] Vold, R.L., Waugh, J.S., Klein, M.P. and Phelps, D.E. (1968) J. Chem. Phys. 48, 3831.
[21] Bax, A., Freeman, R. and Morris, G. (1981) J. Magn. Reson. 42, 164–168.
[22] Macura, S.R. and Ernst, R.R. (1980) Mol. Phys. 40, 95–117.
[23] Bertini, I., Briganti, F., Luchinat, C. and Scozzafava, A. (1990) Inorg. Chem. 29, 1874–1880.
[24] Banci, L., Bertini, I., Luchinat, C., Piccioli, M., Scozzafava, A. and Turano, P. (1989) Inorg. Chem. 28, 4650–4656.
[25] Unger, S.W., Lecompte, J.T.J. and La Mar, G.N. (1985) J. Magn. Reson. 64, 521–526.
[26] Thanabal, V., de Ropp, J.S. and La Mar, G.N. (1987) J. Am. Chem. Soc. 109, 7516–7525.
[27] Dugad, L.B., La Mar, G.N. and Unger, S.W. (1990) J. Am. Chem. Soc. 112, 1386–1392.