Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 591-595

# SOME STRUCTURAL FEATURES OF CLUSTER-COORDINATING CYSTEINES OF *CLOSTRIDIUM PASTEURIANUM* FERREDOXIN ARE REVEALED BY 2D TOCSY $^1$H NMR ON THE OXIDIZED PROTEIN

Domenico Acquotti[a], Francesco Bonomi[b], Paola Brocca[a], Maria Luisa Ganadu[c,1], and Silvia Pagani[b]

[a]Dipartimento di Chimica e Biochimica Medica, Via Saldini 50, 20133 Milano, Italy

[b]Dipartimento di Scienze Molecolari Agroalimentari and Centro Interuniversitario per lo Studio delle Macromolecole Informazionali, Via Celoria, 2, 20133 Milano, Italy

[c]Dipartimento di Chimica, Via Vienna 2, 07100 Sassari, Italy

Received May 16, 1994

**Summary.** Different sets of geminal J coupling constants for the eight ß-$CH_2$ protons in the iron-coordinating cysteines in *Clostridium pasteurianum* ferredoxin were detected by 2D TOCSY $^1$H NMR experiments on the oxidized protein. Four resonances were characterized by quite similar high values of J, two more resonances had a J value about half of the former ones, while the last two had extremely low J values. These findings suggest that the cysteines required for cubane symmetry around the iron atoms are constrained into different geometries. The simplified model used for fine tuning of $\tau_m$ in these TOCSY experiments is also presented and discussed.


Multidimensional NMR spectroscopy has become the most powerful tool for elucidation of the structure of biomolecules [1], including paramagnetic proteins [2-4]. Several papers focused on the paramagnetically-shifted regions in the 2[4Fe4S] ferredoxin from *Clostridium pasteurianum* (CpFd), elucidating the pattern of resonances arising from the ß-$CH_2$ in iron-coordinating cysteines [5-7]. A fundamental limitation in 2D NMR of paramagnetic proteins is that proton magnetization decays rapidly during the time required for its labelling. To select conditions where the maximum transfer occurs, experimental design aimed at providing new insight into the structure/function relationship has to take $T_1$, $T_{1\rho}$, and $T_2$ values into careful account [4], while standard NMR experiments can provide information on the diamagnetic region [8]. The study of protons in the proximity of the cluster will require modulation of the

[1]To whom correspondence must be addressed at: Dipartimento di Chimica, Via Vienna 2, 07100 Sassari, Italy. Fax: +39-79-212069.

0006-291X/94 $5.00

standard pulse sequences according to the distance of the spin systems from the paramagnetic center(s). In this frame, standard NMR experiments have to be combined with experiments at fast repetition rates and short mixing times [9]. From the biochemical standpoint, estimating the J coupling constants for protons in the proximity of the cluster(s) will help to define the geometry of their environment.

We report here on a 2D TOCSY $^1$H NMR investigation on fully native CpFd, in which standard TOCSY experiments at different mixing times ($\tau_m$) allowed the detection of different sets of J coupling constants for ß-$CH_2$ protons in iron-coordinating cysteines.

## Materials and methods

*Clostridium pasteurianum* was grown and the ferredoxin isolated essentially according to ref. [10]. All manipulations were performed under Ar. For NMR studies the protein was about 1 mM in 1M NaCl, pH 8.0. Solvent exchange was performed on YM3 membranes either in an Amicon cell or in Centricon devices. NMR experiments were performed at 500 MHz on a Bruker AM 500 spectrometer at 300 K. The 2D-TOCSY spectra were recorded in TPPI mode; an MLEV 17 sequence sandwiched between two trim pulses (400 $\mu$sec) was used as mixing period [11]; the frequency carrier was centered at 11.3 ppm; the 90 degree pulse was 20 $\mu$sec.

| SIGNAL | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| $T_1$ | 7.0 | 9.3 | 8.1 | 10.7 | 13.1 | 10.7 |
| $T_2$ | 3.7 | 3.2 | 3.3 | 5.05 | 6.24 | 6.1 |

| SIGNAL | G | H | K | K' | L | M | V |
|---|---|---|---|---|---|---|---|
| $T_1$ | 6.0 | 4.4 | 14.5 | 14.5 | 16.8 | 11.8 | |
| $T_2$ | 3.1 | 3.5 | 7.07 | 7.07 | 6.92 | 6.62 | 7.58 |

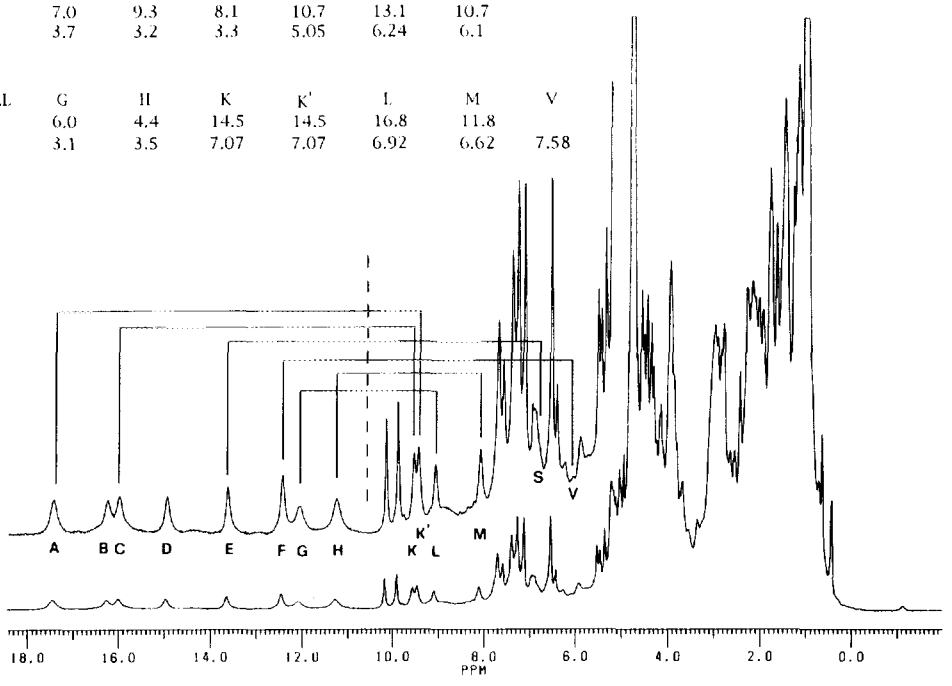


**Figure 1. 500 MHz $^1$H NMR spectrum of oxidized *Clostridium pasteurianum* ferredoxin.** Signals stemming from ß-$CH_2$ of ligand cysteines are labeled with capital letters. Values of $T_1$ and $T_2$ for individual lines are also given.

## Results and discussion

The $^1H$ NMR spectrum of oxidized CpFd (Figure 1) allows the identification of different sets of isotropically shifted ß-$CH_2$ resonances and the determination of individual $T_2$ values for lines of resonances (for example, A-M) in the range 18-8 ppm.

Figure 2 shows the 2D-TOCSY spectra of less than 1 mM oxidized CpFd, taken at 4, 9, and 15 msec mixing time. At $\tau_m$=4 msec, four cross-peaks arising from A/K', C/K, G/L, H/M can be clearly recognized after 192 scans (Fig. 2a). These cross-peaks were evident in spite of the very low values of $T_1$ (4-8 msec) and $T_2$ (3.1-3.7 msec) for signals A, C, G, H . The same cross-peaks were evident at 15 ms even if only 48 scans were recorded. Cross-peaks arising from E and F became evident after 1088 scans for $\tau_m$=15 ms and $\tau_m$=9 ms (Fig. 2b, c), although with a low intensity. Further increase of $\tau_m$ to 20 ms results in loss of these signals, likely because of their rapid relaxation rates. To explain the easy detection of cross-peaks arising

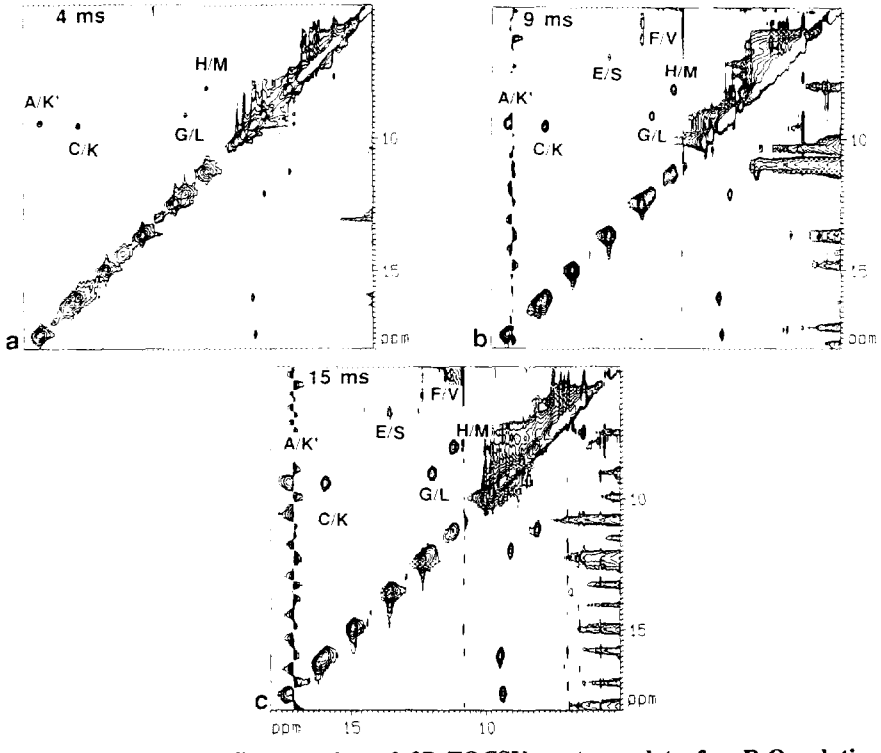


**Figure 2. Low field portion of 2D-TOCSY contour plot of a $D_2O$ solution of *Clostridium pasteurianum* ferredoxin.** The TOCSY experiments with 4 (a), 9 (b) or 15 ms (c) MLEV-17 spin lock as mixing time were acquired with 256 $t_1$ points and 2048 $t_2$ points. A relaxation delay of 100 ms was used. For every value of $t_2$, 192 (a) and 1088 (b, c) scans were accumulated with a sweep width of 20 kHz and acquisition time 88 (a) and 52 ms (b, c). Data were processed with a 90° shifted squared-sine-bell function in both dimensions and zero filled to a 2048x1024 matrix.

from A, C, G, H signals in spite of their very low $T_1$ and $T_2$ (Fig. 1), we analyzed our results by using the approximate transfer function for the TOCSY experiment $T=\sin^2(\pi Jt)e^{(-t/T2eff)}$ [12,13]. In these calculations, the contributions of the terms involving the cross relaxation were neglected and the value for $T_{2eff}$ was inferred from the linewidth extrapolated from all signals but S and V. The term $T_{2eff}$ obviously depends on the distance of the proton from the iron atom.

The dependence of the TOCSY transfer magnetization on $\tau_m$, shown in Fig. 3, indicates that the range of the position shift of maximum transfer is regulated by $T_2$, while the intensity of the TOCSY transfer is regulated by the J value. Even if the plots of Fig. 3 were obtained by neglecting the cross relaxation term and by considering only one effective transverse relaxation time $T_{2eff}$ in the asymmetrical doublet, they rationalize the behavior of signals like E/S and F/V (requiring more transients to become clearly visible) as due to a lower J value. This conclusion holds true for other values of $T_{2eff}$. Thus, cysteine ß-$CH_2$ signals A, C, G and H have similar J values (≈14 Hz), while signals E and F have a definitely smaller J coupling constant (<5 Hz).

These different sets of geminal J coupling constants suggest that the eight cysteine moieties required for a cubane symmetry around the iron atoms are constrained into different geometries by steric hindrance or by protein folding. The dependence of the TOCSY transfer function on $T_{2eff}$ could explain the observed persistence of signals E/S and F/V with increasing $\tau_m$, despite their low J values. The same observation supports the hypothesis based on quite high values of $T_2$ for E and F.

In conclusion, in studies of paramagnetic proteins fine tuning of the NMR parameters (in this case the $\tau_m$ for TOCSY measurements run at low protein concentration) makes it possible

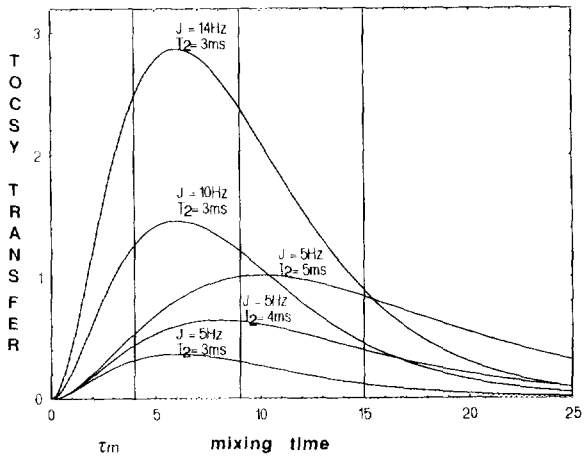


**Figure 3. Influence of the J and $T_2$ parameters on the shape of the transfer function.** The vertical lines indicate the experimentally investigated values of $\tau_m$. A decrease in intensity is caused by a change of J for the same $T_2$. A shift of the maximum is observed on an increase of $T_2$ in the absence of changes in J.

to evidentiate different geometries in the cysteinyl groups bound to the Fe-S clusters. Informative experiments can be performed by using standard sequences and by taking into account the relationships between the signal intensity (as dictated by magnetization transfer) and the relaxation behavior of the system under investigation, without using particular spectral manipulations, "ad hoc" sequences, or very high protein concentrations.

**Acknowledgments**

Financial support was obtained from Regione Autonoma Sardegna and from MURST, Rome, Italy, 40% National Project "Liveprotein".

**References**

1. Griesinger, C., Sorensen, O. W., and Ernst , R. R., (1989) J. Magn. Reson. 84, 14-63.
2. Gaillard, J., Albrand, J. P., Moulis, J. M., Wemmer, D., (1992) Biochemistry 31, 5632-5639.
3. De Ropp, J. S., Yu, L. P., La Mar, G. N., (1991) J. Biomolecular NMR 1, 175-190.
4. Marion, D., Guerlesquin, F. (1992) Biochemistry 31, 8171-8179.
5. Bertini, I., Briganti, F., Luchinat, C., Messori, L., Monanni, R., Scozzafava, A., Vallini, G. (1992) Eur. J. Biochem. 204, 831-836.
6. Busse, S. C., La Mar, G., Howard, G. B., (1991) J. Biol. Chem. 266, 23714-23723.
7. Bertini, I., Briganti, F., Luchinat, C., Scozzafava, A., (1990) Inorg. Chem. 29, 1874-1880.
8. Ganadu, M. L., Bonomi, F., Pagani, S., Boelens, R., (1992) Biochemistry International 26, 577-585.
9. Sadek, M., Brownlee, R. T. C., Scrofani, S. D. R., Wedd, A. G. (1993) J. Magn. Reson. Series B 101, 309-314.
10. Lovenberg, W., Buchanan, B. B., Rabinowitz, J.C. (1963) J. Biol. Chem., 238, 3899-3912.
11. Bax, A., Davis, D. G. (1985) J. Magn. Reson. 65, 355-360.
12. Ernst, R. R., Bodenhausen, G., Wokaun, A., (1987) in: One and Two Dimensions, Principles of Nuclear Magnetic Resonance, Clarendon Press, Oxford.
13. Norwood, T. J., (1992) J. Magn. Reson. Series A 101, 109-112.