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Proton Magnetic Resonance Study of Ferredoxin from *Clostridium pasteurianum**

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Abstract. Magnetic susceptibilities of both reduced and oxidized ferredoxin from *Clostridium pasteurianum* were obtained in solution. Whereas the reduced form exhibits a Curie law behavior, the magnetic susceptibility of oxidized ferredoxin in fact increases with temperature and suggests extensive antiferromagnetic exchange coupling between the component iron atoms. Contact-shifted resonances are observed for both forms of ferredoxin that are attributed to the β-CH_2 protons of the eight cysteine residues. A model based on these results is presented.

Introduction. Ferredoxin from *Clostridium pasteurianum* is an iron-sulfur protein of low redox potential with important functions in electron transport reactions.[1–3] It contains six[4, 5] to eight[2,3] atoms of nonheme iron per protein molecule with equimolar amounts of "labile" sulfide, and has a molecular weight of 6000.[2, 3, 5] The primary sequence has been established.[6] Clostridial ferredoxin has been the subject of a number of physical studies, including a preliminary X-ray analysis,[7] electron paramagnetic resonance (epr) spectroscopy,[4, 8] Mössbauer spectroscopy,[2] and magnetic susceptibility.[2] However, neither the structure nor the mode of action of this important protein is well understood. We wish to report here results bearing on these problems derived from studies of *C. pasteurianum* ferredoxin by proton magnetic resonance (pmr) spectroscopy.

Materials and Methods. Proton magnetic resonance and solution susceptibility studies: *Clostridium pasteurianum* was grown as described previously.[3] Ferredoxin was isolated by a modification of the Mortenson procedure.[9] The material was maintained in Tris·HCl, pH 7.3, of at least 0.05 molarity throughout the entire purification which was generally completed within one working day. The final ferredoxin solution from a typical preparation was usually in 25 to 30 ml of 0.05 *M* Tris·HCl, pH 7.3, that contained about 120 mg of pure protein. This material was lyophilized to dryness and redissolved in 3 ml of D_2O twice. The final product typically exhibited a 390/280 mμ ratio of 0.81. For pmr studies, ferredoxin was exchanged twice against D_2O. Crystalline dithionite employed in the reductions was purchased from British Drug Houses.

Proton magnetic resonance studies were performed with a Varian 220 MHz pmr spectrometer. Chemical shifts were internally referenced to the sodium salt of 2,2-dimethyl-2-silapentanesulfonic acid (DSS) and are reported in units of Hz or of parts per million (ppm). Positive values correspond to downfield shifts. The signal-to-noise characteristics of some spectra were improved through the use of a Varian C-1024 computer of average transients. The temperature of the spectrometer sample zone was determined

from the resonance frequencies of the OH groups of ethylene glycol and methanol to an estimated accuracy of ±0.5°C.

For the susceptibility and spectral measurements, solutions in 99.77% D_2O containing 7.0–16.5 mM ferredoxin, 0.1 *M* tris(hydroxymethyl)aminomethane hydrochloride at pD 7.7, 2 mM DSS, and 2 mM tetramethylammonium chloride were employed. All ferredoxin concentrations were determined by measuring the difference in absorbance at 425 nm between oxidized and reduced ferredoxin, using an extinction coefficient for the difference of 13,900 cm^{-1} M^{-1}. Methyl proton resonances of both the tetramethylammonium chloride and DSS were used as susceptibility markers to eliminate possible anomalous displacements of resonances of the susceptibility references through specific interactions with the ferredoxin.[11]

Results. Magnetic susceptibility: The paramagnetic contribution to the molar susceptibility (χ^p_M) of the oxidized form of ferredoxin from *C. pasteurianum* was determined by a nmr method.[24] The most striking feature of the temperature dependence of χ^p_M is that the behavior does not follow Curie law. χ^p_M *increases* linearly with temperature from 6° to 65°C rather than exhibiting a $1/T$ temperature dependence. The effective magnetic moment of ferredoxin per iron atom, μ_{eff}, was calculated from

$$\chi^p_M = \frac{N\beta^2}{3kT} n\mu^2_{eff} \qquad (1)$$

and is plotted in Figure 1. In equation (1), N, β, k, and T have their usual significance. n is the number of iron atoms per molecule. There remains some uncertainty as to the number of iron atoms per molecule for the ferredoxin of *C. pasteurianum*, and in Figure 1 temperature dependences of μ_{eff} are plotted for both $n = 6$ and $n = 8$. The assumption implicit in this treatment is that all the iron atoms of the oxidized form of ferredoxin are equivalent with respect to valence state and ligand field environment. Earlier Mössbauer studies[2] indicated equivalence of valence states and at least near equivalence of ligand field environments for the iron atoms of this ferredoxin in the oxidized form.

μ_{eff} for the iron atoms of oxidized ferredoxin are seen from Figure 1 to increase with increasing temperature, again reflecting a dependence on temperature of magnetic properties other than Curie law. The values for μ_{eff} for either $n = 6$ or

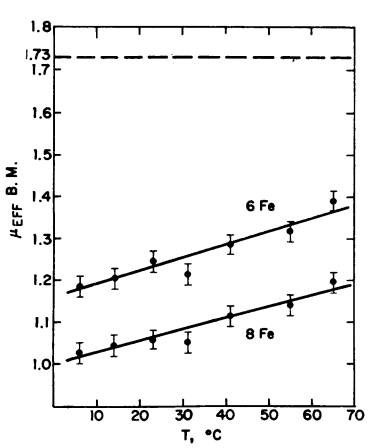

FIG. 1.—Temperature dependence of the effective magnetic moment per iron atom of oxidized ferredoxin from *C. pasteurianum*. These results are calculated from the susceptibilities of oxidized ferredoxin for both six and eight atoms of iron per molecule of ferredoxin. The susceptibility data were obtained upon a ferredoxin solution which contained 7.02 mM ferredoxin, buffer, and references, and had an absorbance ratio A_{390}/A_{280} of 0.805.

$n = 8$ are less than the expected moment of 1.73 Bohr magnetons for low-spin Fe^{+3} with $g = 2.0$. The results suggest a high degree of antiferromagnetic exchange coupling between iron atoms in oxidized ferredoxin with a diamagnetic or weakly paramagnetic ground state and a thermally accessible state (or states) more paramagnetic than the ground state. Unfortunately, in solution the 60° range in temperature over which the susceptibility can be measured is too narrow to permit determination of the high temperature asymptotic approach value. A value of 1.73 Bohr magnetons, i.e., that of low-spin Fe^{+3} with $g = 2.0$, is, however, not unreasonable for the high temperature value of μ_{eff}.

Ferredoxin from *C. pasteurianum* undergoes a two-electron reduction with dithionite.[8, 12–14] In contrast to oxidized ferredoxin, the reduced form exhibits Curie law behavior over to range 5° to 35°C.

Proton magnetic resonance of ferredoxin: Since the ferredoxin of *C. pasteurianum* is paramagnetic in both oxidized and reduced forms, it was expected that contact shifts of the isotropic hyperfine or pseudocontact variety might be observed that would reflect local environments about paramagnetic centers. Contact interaction shifts have been observed in a variety of paramagnetic transition metal coordination compounds of nonbiological origin[15] and in cytochrome c, hemoglobins, and myoglobins,[16] and have been of very great value in elucidating the electronic and geometrical structures of these systems.

The pmr spectrum of oxidized ferredoxin is shown in Figure 2. Diamagnetic proteins normally exhibit resonances in the −2 to +8 ppm range when referenced internally to DSS.[17] Intense, relatively well-resolved resonances are observed for ferredoxin in this range. In addition, a number of additional weak resonances appear in the low-field 8- to 18-ppm region of resonance absorption. These are best seen in the computer-averaged, expanded scale inset of Figure 2.

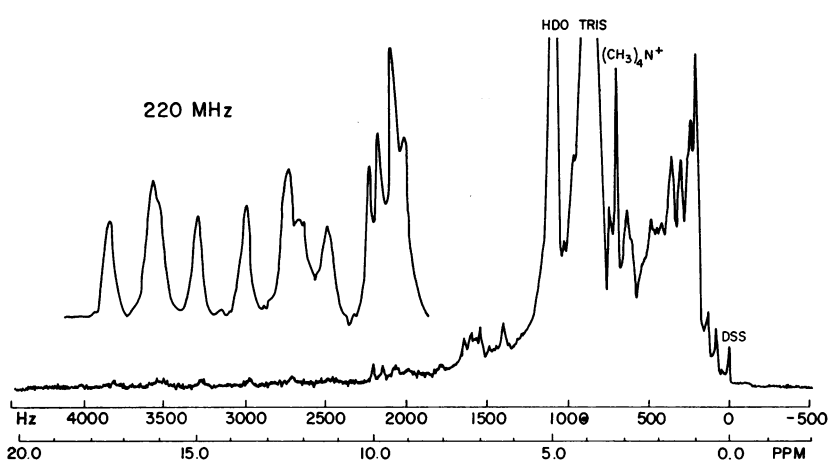

FIG. 2.—Proton magnetic resonance spectrum at 220 MHz of oxidized ferredoxin from *C. pasteurianum*. The ferredoxin is dissolved in a solution of D_2O containing Tris buffer with tetramethylammonium chloride and DSS as internal references; the ferredoxin concentration is 16.5 mM. The lower spectrum in the figure is a single trace, the insert on the upper left is spectrum computer averaged over 75 scans. Temperature, 23°.

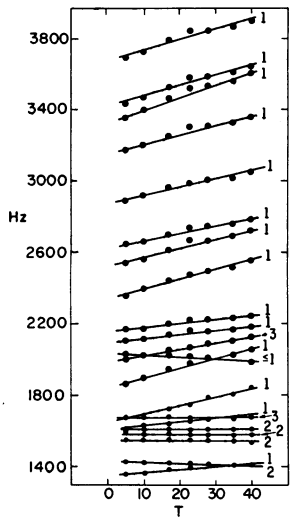

FIG. 3.—Temperature dependences of the low-field resonances in oxidized *C. pasteurianum* ferredoxin. Numbers of protons giving rise to each resonance are indicated on the figure.

In the absence of contact interaction effects and conformational changes, resonances of proteins are expected to be temperature independent. The temperature dependences of contact-shifted resonances of paramagnetic proteins, on the other hand, are expected to parallel that of the magnetic susceptibility. The temperature dependence of the 8- to 18-ppm region of resonance absorption of oxidized ferredoxin is shown in Figure 3. It is seen that there is indeed a marked temperature dependence over this fairly narrow temperature range and that the resonances uniformly trend towards *increasing* values of chemical shift with increasing temperature. Recalling the temperature dependence of the magnetic susceptibility, those resonances which exhibit positive temperature dependences may be attributed to protons perturbed by contact shift interactions and those which are essentially temperature invariant may be assigned to protons unaffected by the paramagnetic center or centers. An additional reason for attributing contact shift characteristics to resonances of the 8- to 18-ppm region of resonance absorption is, of course, their unusual low-field positions.

We next proceed to assignment of the contact-shifted resonances of oxidized ferredoxin. From the temperature dependence of the low-field region of resonance absorption of ferredoxin (Fig. 3), it is clear that the region from 10.4 to 19.0 ppm consists of eight resolvable resonances. Careful intensity determinations employing internal intensity standards reveal that each of these eight resonances corresponds, within an experimental error of ± 10 per cent, to *one* proton per molecule of ferredoxin. Ferredoxin contains eight cysteine residues distributed into two closely spaced groups of four along the single polypeptide chain.[6] These cysteine residues have been implicated in the binding of iron to the protein,[12] as has cysteine been implicated in iron binding in other nonheme iron proteins.

If iron is so bound to ferredoxin via coordinate linkage to the sulfur of cysteine residues, spin density could be transferred to a nonbonding p-orbital of sulfur by spin polarization. Such spin polarization or superexchange interactions may in fact be at least partially responsible for the antiferromagnetic coupling between iron atoms in ferredoxin and other nonheme iron proteins. Spin density transferred from iron to sulfur by these mechanisms then could be manifested in contact shifts of the β-CH_2 protons of cysteine by a hyperconjugative process. Manifestations of spin densities by hyperfine splittings in electron spin resonance or isotropic hyperfine contact shifts by hyperconjugative mechanisms are well known in organic radicals and coordination compounds based on carbon and nitrogen,[15] but are less well characterized in paramagnetic species where sulfur is involved. Nevertheless, the effects should be similar.

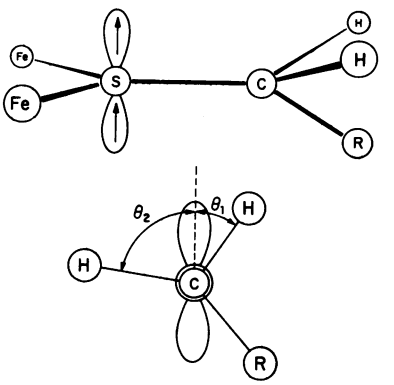

FIG. 4.—Proposed mode of cysteine-iron bonding in ferredoxin. Spin density is transferred from iron to the p-orbital of the sulfur atom as a result of coordinative binding and the β-CH_2 protons sense this spin density via a hyperconjugative mechanism.

A possible mode of binding of iron to the polypeptide chain via the sulfur of cysteine residues is depicted in Figure 4. Sulfur is considered to exhibit sp^2 hybridization and, as a result of coordination to iron, spin density is transferred to the nonbonding p-orbital perpendicular to the plane of the sp^2 orbitals. The β-CH_2 protons of cysteine can sense this spin density centered on sulfur by hyperconjugation and manifest the spin density by isotropic hyperfine contact-interaction shifts. If free rotation about the C-S bond existed, the two β-CH_2 protons would be shifted equally since on the average their positions relative to the spin density centered on sulfur would be equivalent. However, for the above-depicted bonding situation in the folded protein, the two β-CH_2 protons of a cysteine residue presumably would be fixed in space relative to the axially symmetrical p-orbital of sulfur and would, in general, be positioned nonequivalently with respect to the p-orbital of sulfur. The angular dependence of the isotropic hyperfine interaction in this situation has been given[18] as

$$A = A_0 \cos^2\theta, \tag{2}$$

where A and A_0 are contact-interaction constants and θ is the angle between the axis of the p-orbital of sulfur and the vector obtained by projecting a C-H bond on the plane containing the β-CH_2 protons and the R group. Thus, for a given cysteine residue bonded to iron, the two nonequivalent β-CH_2 protons would be expected to reflect by different contact shifts the spin density centered on the sulfur atom.

Returning now to Figure 2, a marked symmetry is exhibited by the eight resonances, each of unit intensity, between 10.4 and 19.0 ppm. Numbering the eight resonances from the left, the following resonance position averages yield almost identical results: 1-8, 2-7, 3-6, and 4-5. We associate these four combinations with the β-CH_2 protons of four of the eight cysteine residues of ferredoxin. Observed unequal contact shifts of the β-CH_2 protons for each of these combinations are attributed to fixed orientations about the C-S bonds in the native protein that result in nonequivalent isotropic hyperfine contact interactions.

A detailed treatment of the above will be presented in a forthcoming publication.[10] Based on this model and observed β-CH_2 resonance nonequivalences, estimates of the orientations about the C-S bonds of cysteine residues of fer-

redoxin are possible. However, for purposes of the present study, the important conclusion from the above analysis is as follows: *As the result of coordinative binding to iron, sulfur atoms of four of the eight cysteine residues of ferredoxin experience essentially identical paramagnetic environments.*

In the 7- to 10-ppm region of resonance absorption of oxidized ferredoxin, six other resonances exhibit positive temperature dependences (Fig. 3). Five of these are of unit intensity and one corresponds to three protons. These six contact-shifted resonances are attributed to the β-CH_2 protons of the remaining four cysteine residues of ferredoxin. The resonances whose intensity corresponds to three and one protons (the 9.0 and 9.4 ppm resonances of Fig. 2) are attributed to the β-CH_2 protons of two cysteine residues. Another cysteine is manifested in the pmr spectrum of Figure 2 by the two relatively sharp resonances at 9.8 and 10.1 ppm. A pair of contact-shifted resonances further upfield is assigned to the eighth cysteine residue. Positions of the contact-shifted resonances of oxidized ferredoxin and suggested assignments are given in Table 1. The im-

TABLE 1. *Contact-shifted resonances of oxidized ferredoxin.*[a]

Number[b]	Number of protons	Position[c] (Hz)	Contact shift[d] (Hz)	Suggested pair assignment	Average pair contact shift[e] (Hz)
1	1	3840	3169	—	—
2	1	3577	2906	—	—
3	1	3516	2845	—	—
4	1	3299	2628	—	—
5	1	2996	2325	4–5	2476
6	1	2736	2065	3–6	2455
7	1	2664	1993	2–7	2450
8	1	2473	1802	1–8	2486
9	1	2222	1551	—	—
10	1	2161	1490	9–10	1520
11	3	2072	1401	2 Pair	1401
12	1	1974	1303	11–12	1352
13	1	1744	1073	—	—
14	1	1654	983	13–14	1028

[a] Derived from 220 MHz pmr spectra at 23°C.
[b] Arbitrarily assigned numerical designation in low to high field order.
[c] Chemical shift relative to internal DSS.
[d] Observed resonance position, as given in column 3, minus the chemical shift of the β-CH_2 protons of cysteine (671 Hz),[22] the proposed origin of these resonances in the absence of contact interactions.
[e] The average contact shifts for the pair assignments of column 5.

portant aspects of the positions of resonance of these latter four cysteine residues are (1) their contact shifts are only 40–60 per cent of those of the four extreme low-field-shifted residues discussed above and (2) they are not all equivalently shifted as a class as appears to be the case for the four low-field-shifted residues. A tentative structural conclusion to be drawn from these results is that each of the four cysteine residues that exhibit β-CH_2 resonances in the 10.4 to 19.0 ppm region binds two iron atoms as depicted in Figure 4, but that the remaining four cysteine residues that experience smaller contact shift interactions bind only single iron atoms with somewhat more variable coordinative environments.

Partially reduced ferredoxin exhibits very broad contact-shifted resonances, due to electron exchange between oxidized and reduced forms. Electron ex-

change between oxidized and reduced ferredoxin, and contact shifts and the magnetic susceptibility of the reduced form will be elaborated in a subsequent publication.[10]

Discussion. A number of lines of evidence have a bearing upon the structure of ferredoxin from *C. pasteurianum.* (a) The eight cysteine residues of ferredoxin are found in two short segments of the polypeptide chain with the following sequences:[6]

Cys-Val-Ser-Cys-Gly-Ala-Cys-Ala-Ser-Glu-Cys
Cys-Val-Asp-Ala-Cys-Asp-Gly-Cys-Asp-Ileu-Cys

Four cysteine residues bind the single iron atom to the polypeptide chain of the nonheme iron protein rubredoxin from *C. pasteurianum.*[19] It seems likely that cysteine also plays a major role in the binding of iron to ferredoxin. If so, the above grouping of cysteine residues suggests that the six to eight iron atoms of ferredoxin may be found concentrated in one or two compact clusters.

(b) Earlier Mössbauer studies on oxidized ferredoxin indicated that the component iron atoms exhibited similar valence states and resided in similar ligand field environments.[2]

(c) The magnetic susceptibility results of the present study suggest extensive antiferromagnetic coupling between the iron atoms of ferredoxin, again suggesting proximity of iron atoms with exchange being mediated by commonly coordinated ligands such as inorganic sulfide and the sulfur of cysteine as well, probably through direct iron-iron interactions.

(d) The contact-shifted resonances observed for ferredoxin are attributed to the β-CH_2 protons of the eight cysteine residues. Sulfur of cysteine is taken as coordinated to iron and the β-CH_2 protons sense by hyperconjugation the spin density transferred to sulfur as a result of such coordination. From the analysis presented, four of the cysteine residues experience about twice the spin density of the other four. The latter are assigned to cysteine residues coordinated to single iron atoms, whereas the former are assigned to cysteine residues whose sulfur atoms are shared by two iron atoms.

A structure for clostridial ferredoxin which is compatible with above restrictions, and containing six iron atoms, is presented in Figure 5. This model contains features found in inorganic iron-sulfur coordination complexes.[20, 21] The model can accommodate eight as well as six iron atoms and will be elaborated in a forthcoming publication.[10]

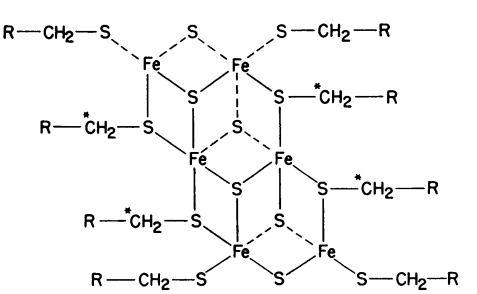

FIG. 5.—Model for iron-sulfur complex in *C. pasteurianum* ferredoxin.

* Contribution no. 1632 from Central Research Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware.

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