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ON THE STRUCTURE OF THE IRON-SULFUR COMPLEX IN THE TWO-IRON FERREDOXINS*

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SUMMARY

Recent spectroscopic and magnetic susceptibility studies of the iron center in the two-iron ferredoxins provide criteria which any model for the iron-sulfur complex in these proteins must satisfy. These criteria are most stringent for parsley and spinach ferredoxin: the reduced proteins contain a high-spin ferric atom antiferromagnetically exchange-coupled (presumably *via* sulfide bridging ligands) to a high-spin ferrous atom. In the oxidized proteins the iron atoms are antiferromagnetically spin-coupled, high-spin ferric atoms. Arguments are given to substantiate the claim that the ferrous atom in the reduced protein is ligated by four sulfur atoms in a distorted tetrahedral configuration: two are the bridging sulfides, two are cysteinyl sulfurs. A treatment of proton contact shifts based upon the above model is pertinent to proton magnetic resonance data already available and provides a means to identify directly the ligands at both iron atoms *via* further PMR experiments.

A recent series of papers have described the results of electron-nuclear double resonance (ENDOR)[1], Mössbauer[2], near-infrared circular dichroism and optical spectroscopy[3] and magnetic susceptibility[4] measurements on spinach ferredoxin and other two-iron ferredoxins. In this paper we show how the results of these experiments allow a precise definition or the active center of these proteins and provide a stringent set of criteria against which any further structural information may be tested. In addition, we show how the anomalous temperature dependence of the contact shifted proton resonances recently reported[5] is a consequence of the properties of this model for the active center of the two-iron ferredoxins.

CHEMICAL AND PHYSICAL PROPERTIES

The term "two-iron ferredoxin" describes any protein which contains only two iron atoms, two labile sulfur atoms and which, in its reduced state, shows an electron paramagnetic resonance (EPR) signal centered at $g = 1.96$. Although this definition

* This is one of a series of papers describing the electronic properties of several of the two-iron-two (labile) sulfur proteins; related publications contain the results of experiments on ENDOR[1] (called I), the Mössbauer[2] (called II), magnetic susceptibility[4] (called III) and infrared, optical and circular dichroism spectra[3] (called IV).

is sufficient to identify a member of this group of proteins, there are many other characteristic properties. All the two-iron ferredoxins function as one-electron oxidation-reduction agents in the oxidative metabolism of their hosts (see for review ref. 6). The reduced proteins are among the strongest reducing agents in biological systems with reduction potentials (E'_0) varying from -250 to -420 mV (ref. 7). They are small proteins[7], (molecular weights ranging from 10600 to 24000) whose preponderance of acidic amino acid residues has facilitated purification procedures. The ratio of absorbances at approx. 280 and 420 nm is a gauge of both the purity and the oxidation state of these proteins since they show a titratable charge transfer band at 420 nm[8]. Several two-iron ferredoxins have been sequenced[9]; in each case, tyrosine and phenylalanine are the only aromatic residues which are common in their amino acid compositions, and the positions of the cysteine residues show remarkable similarities[10]. Because there are no disulfide bridges in two-iron ferredoxins, and because titration of the cysteine with heavy metals always results in denaturation, sulfur becomes a prime candidate for bonding at the iron center of the proteins.

The following lists the important physical data to be considered in any description of the active center.

a. EPR hyperfine interactions

^{57}Fe and ^{77}Se isotopic substitution experiments have demonstrated that both iron atoms and both labile sulfur atoms are involved in bonding at the active center of putidaredoxin and adrenodoxin[11,12]. Similar data on parsley and spinach ferredoxins establishes the role of both sulfur atoms in these proteins also. However from the EPR data no decision on the number of participating iron atoms can be made[13]. Experiments on putidaredoxin grown on ^{33}S-enriched media indicate that at least one cysteine or methionine sulfur is also involved in the active site[14].

b. ENDOR spectroscopy[1]

The magnitudes of the principal components of the ^{57}Fe magnetic hyperfine tensors have been measured by ENDOR experiments on proteins which were chemically substituted with ^{57}Fe nuclei[1]. These experiments give effective A-values for two non-equivalent iron atoms in the reduced proteins: one iron has an almost isotropic effective A-tensor of magnitude about 46 (± 4) MHz (17 electron gauss), the other iron has a highly anisotropic effective A-tensor with principal values of about 17(± 4), 24(± 4) and 35(± 2) MHz in adrenodoxin and putidaredoxin.

c. Mössbauer spectroscopy[2]

The Mössbauer spectra of the oxidized proteins show a slightly broadened, single quadrupole pair which is temperature independent from 4.2° to 77°K. The Mössbauer spectra of the reduced proteins are strongly temperature dependent with the spectrum obtained at 4.2° K in an applied magnetic field exhibiting well-resolved magnetic hyperfine splittings given by hyperfine tensors which are in agreement with the ENDOR results. Two non-equivalent, spin-coupled iron sites are observed for the proteins: one with the same isomer shift and quadrupole splitting exhibited in the oxidized protein spectra and with a slightly anisotropic effective A-tensor for the ground ($I = 1/2$) state of ^{57}Fe at around -46 MHz; the other iron is a high spin ferrous ion (large isomer shift and quadrupole splitting), and has a highly anisotropic A-tensor. In the case of the high spin ferrous atom, the identity of the orbital ground

state is contained in the electric field gradient tensor and the hyperfine tensor at this site. This ground state has d_{z^2} symmetry in the case of parsley and spinach ferredoxin, with the symmetry in the other proteins as yet undetermined.

d. Magnetic susceptibility measurements[4, 15–18]

These studies indicate antiferromagnetic coupling of the iron atoms in both the oxidized and reduced forms of the proteins. These couplings result in molecular diamagnetism at temperatures below 50°K for the oxidized protein, and a molecular paramagnetism corresponding to that of a single unpaired electron for the reduced protein. The most precise studies (ref. 4 and A. EHRENBERG, personal communication) of the magnetic susceptibility show the existence of higher magnetic states which become populated as the temperature is increased. The factor J in the spin-Hamiltonian term, $-2JS_1 \cdot S_2$, where S_1 and S_2 are the spins of the individual iron atoms, is measured to be -182 cm^{-1} in oxidized spinach ferredoxin and -80 to -100 cm^{-1} in reduced spinach ferredoxin[4].

e. Near infrared spectroscopy[3]

The infrared spectra of reduced parsley and spinach ferredoxin and adrenodoxin show absorption bands at about 1.6 and 2.5 μm which coincide almost exactly with those found in rubredoxin. These bands are broad, of low intensity (ε approx. 50) and are very optically active ($|\varepsilon_L - \varepsilon_R|/\varepsilon$ approx. 0.03), which characterizes them as being the electric-dipole forbidden, magnetic-dipole allowed d–d transitions of the ferrous ion.

These data lead to a number of important conclusions: in large part the conclusions obtained from a given technique rest on comparison with results provided by one or more of the other methods. However in the following we will include an indication of the principal support for each conclusion.

(1) The active center contains two iron atoms, two labile sulfur atoms and at least one cysteine or methionine sulfur atom (mainly from EPR). (2) In the reduced protein, one of the iron atoms is high-spin ferric (from ENDOR and Mössbauer) and the other is high-spin ferrous (from ENDOR, Mössbauer and infrared). (3) The iron atoms interact *via* an antiferromagnetic exchange coupling mechanism (from Mössbauer and magnetic susceptibility). (4) In the reduced protein, the ferrous iron is coordinated by a distorted tetrahedral array of ligands (from Mössbauer and infrared) and these ligands are probably four sulfur atoms (from infrared). (5) In the reduced protein, the ground state of the ferrous atom is an orbital singlet with d_{z^2} symmetry (from Mössbauer). (6) Both iron atoms participate in strongly covalent bonding with their ligands (from ENDOR and Mössbauer). (7) The differences in the active sites from protein to protein must allow variations in the EPR g-values, magnitudes of the exchange coupling and spin relaxation times, but must not allow differences in the magnetic hyperfine coupling constants, electric field gradients and isomer shifts at the iron atoms.

In addition, the following chemical data are important. The mercurial titer of the two-iron ferredoxins can be interpreted in terms of the number of reacting cysteine and sulfide anions present in the denatured protein. In spinach ferredoxin, nine mercurial equivalents are necessary to titrate the protein[18]. The five cysteine residues and two labile sulfur atoms are accounted for exactly by assigning the following valences to

the sulfur atoms: RS^- for the cysteines and S^{2-} for the labile sulfur. Therefore, to propose a persulfide structure at the iron site, one must postulate that mercurials can promote a reductive scission of the persulfide bond to satisfy the stoichiometry of the above data. Because of the unlikelihood of this reaction we feel the mercurial titer data for the two-iron ferredoxins is an argument against a structure at their iron centers which involves persulfides. Likewise, the titration of these proteins by oxidizing agents (potassium ferricyanide) gives a stoichiometry consistent with the oxidation of sulfur to the zero-valent state[19], again supporting the formal assignment of -1 to the mercaptide and -2 to the sulfide in the intact protein structure.

STRUCTURE OF THE ACTIVE SITE

Without X-ray crystallographic information on these proteins, choices between the models proposed for the active site has previously relied on very limited physical data. We are now in a position to set stringent criteria for a structural model of this active site, and, in so doing, we shall argue against many of the previously proposed models.

Models which explain the EPR signal of the "two-iron ferredoxins" in terms of a single paramagnetic iron atom[20,21] or a two-iron system in which a single electron is postulated as resonating quickly between the iron atoms[21,22] are completely incompatible with the data on these proteins. The EPR spectrum results from an $S = 1/2$ ground state of a spin-coupled two-iron system. Proton magnetic resonance (PMR) spectra show that the rate of electron transfer between the two iron atoms at 300°K is much slower than 10^4 sec^{-1} (ref. 5).

Although the assignment of definite valencies to the iron atoms in the active site is presumptuous in view of the covalent bonding present, any assignment of electron configuration other than two high-spin d^5 ions in the oxidized proteins and one each of high-spin d^5 and d^6 in the reduced proteins is much more misleading, since the electric field gradient tensor at the reduced protein ferrous iron is characteristic of the high-spin ferrous ion. Thus, we reject all models based on low-spin[24] and d^7 configurations[23].

In 1966, GIBSON *et al.*[25,26] proposed a model for the active site of spinach ferredoxin in which two high-spin ferric atoms are spin-coupled in the oxidized state and a high-spin ferric atom is coupled to a high-spin ferrous atom in the reduced state. Further, they explained the EPR g-values in terms of a ligand field treatment of the ferrous atom. While we agree with the broad conclusions drawn in these papers, we point out that the high degree of covalency present at the iron atom coupled with the known presence of sulfur ligands necessitates the explicit inclusion of the sulfur spin-orbit contribution in any calculation of the g-values for the complex. The spin-orbit coupling constant of mercaptide sulfur[27] is 80 % that of ferrous ion; thus, one must go beyond the inclusion of an iron spin-orbital reduction factor if one is to make a calculation of the g-values at the iron center of the two-iron ferredoxins.

While we are not in a position to choose between square planar and tetrahedral symmetry in any of the two-iron ferredoxins except in spinach and parsley ferredoxin, we can refuse octahedral symmetry at the ferrous iron for any of the proteins. Neither the temperature dependence of the electric gradient, the sign of its major components, the g-values of the proteins nor the infrared and CD results on spinach and parsley

ferredoxin are compatible with a *d*-electron configuration with the *t*-orbitals low[2,3].

In spinach and parsley ferredoxin the Mössbauer spectroscopic experiments show that a *d*-orbital (presumably $d_{x^2-y^2}$) lies around 400 cm^{-1} above the d_{z^2} ground state[1] of the ferrous atom. Although this is evidence that the ligand symmetry at the ferrous ion is "distorted tetrahedral" it does not, for example, exclude the possibility of a weakly bound fifth ligand at this iron atom. Further, although the only ligands which have been positively identified are the labile sulfur atoms, the similarities of the infrared data of the plant ferredoxin with that of rubredoxin (whose structure is known) is strong support for a tetrahedral arrangement of sulfurs at the ferrous atom. Although such an arrangement is compatible with all of the existing data on spinach and parsley ferredoxin, it remains to be seen whether or not additional ligands are present.

The position of the labile sulfur atoms in the complex can be inferred by two means. Firstly, since the mercurial titer data argue against persulfide, we find that the only structures we can draw, which find precedents in the literature, have both labile sulfur atoms bridging the two iron atoms (Fig. 1)[24].

Secondly, this structure is consistent with the value of the exchange-coupling constant (-182 cm^{-1}) of oxidized spinach ferredoxin which is around twice that of oxygen-bridged, two -iron compounds[28] in accordance with predictions of the effect of substituting sulfur for oxygen in antiferromagnetically coupled systems[29]. However we emphasize that we have no data on the coordination number or the symmetry at the ferric ion, nor do we know whether there are any changes in coordination number or symmetry during the oxidation-reduction process.

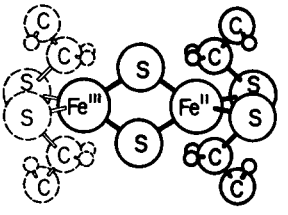

Fig. 1. Indicated structure of the iron-sulfur complex in the two-iron ferredoxins (*cf.* ref. 24).

The test of any model is its ability to predict the outcome of additional measurements. We, therefore, apply this model, to interpret the existing PMR experiments on these proteins and to predict the results of future PMR experiments.

INTERPRETATION OF PROTON MAGNETIC RESONANCE DATA

Recently, POE *et al.*[5] have described proton resonances contact-shifted to low field in the PMR spectra of reduced spinach and parsley ferredoxin and other iron-sulfur proteins. These resonances are tentatively ascribed to the β-CH_2 protons of cysteinyl residues. A very puzzling feature of that work is the observation that the contact shifted resonances exhibited temperature dependences which are inexplicable in terms of the contact shifts of a single paramagnetic center in the protein. As we now show, these shifts are consistent with the above model for the two iron proteins.

The pertinent spin-Hamiltonian for the active site follows:

$$\mathcal{H} = V_1 + V_2 - 2J\tilde{S}_1 \cdot \tilde{S}_2 + D_1[S^2_{1z'} - \tfrac{1}{3}S_1(S_1+1)] + D_2[S^2_{2z'} - \tfrac{1}{3}S_2(S_2+1)] + g\beta H_z S_z + \sum_{i=1}^{M} a_i S_1 \cdot I_i + \sum_{j=1}^{N} a_j S_2 \cdot I_j$$

The first two terms are ligand field terms which result in orbitally non-degenerate ground states at iron No. 1 (Fe^{III}) and No. 2 (Fe^{II}). This approximation is justified by the Mössbauer spectra of these proteins which show, that at the ferrous atom, the lowest level with symmetry other than d_{z^2} is 400 cm^{-1} above the ground state. For the moment we shall neglect this excited state: and consider only the ground d_{z^2} state.

The third term describes spin coupling between the spin $S_1 = 5/2$ and $S_2 = 2$ states. This term results in states with the quantum numbers S_1, S_2, S and M, where S is the resultant spin (1/2, 3/2, 5/2, 7/2 or 9/2) and M is the projection of S on the applied field direction. The fourth and fifth terms are zero-field splitting contributions, where S_{1z}, and S_{2z}, are the projections of S_1 and S_2, respectively, along the symmetry axis of the complex; D_1 (Fe^{III}) is presumably 1.5 cm^{-1} (ref. 30) and D_2 (Fe^{II}) is constrained to be between 0 to 20 cm^{-1} (ref. 31). The next term is the electronic Zeeman contribution and is included here as the spin relaxation time is short compared to the time of measurement in PMR so that a measurement of $\langle S_z \rangle$ yields the average over the Boltzmann populations of all the M states. The rest of the terms in the spin-Hamiltonian are contact and pseudo-contact[32] shifts of the protons I_i and I_j coupled to iron sites No. 1 and No. 2, respectively. In the following, we assume that the constants, a_i and a_j are isotropic with the same sign but not necessarily the same magnitudes.

Since the resultant spin, S, is quantized about the applied field, the contact shift terms should be rewritten as:

$$\mathcal{H}_{con} = \sum_{i=1}^{M} a_i \langle S_{1z} \rangle I_{iz} + \sum_{j=1}^{N} a_j \langle S_{2z} \rangle I_{jz}$$

where $\langle S_{1z} \rangle$ and $\langle S_{2z} \rangle$ include averaging over all the states. In order to calculate the temperature dependence of the contact and pseudo-contact shifts, we apply perturbation theory to the zeroth-order spin functions and calculate $\langle S_{1z} \rangle$ and $\langle S_{2z} \rangle$.

The zeroth-order functions, $|SM\rangle$, are given as:

$$|SM\rangle = \sum_{M_1=-S_1}^{S_1} \langle S_1 S_2 M_1 M_2 | SM \rangle |S_1 S_2 M_1 M_2\rangle$$

where $\langle S_1 S_2 M_1 M_2 \,|\, SM \rangle$ are the relevant Clebsch–Gordon coeffcients[33] and $|S_1 S_2 M_1 M_2\rangle$ are spin functions with the quantum numbers S_1, S_2, M_1 and M_2. The following expressions are for $\langle S_{1z} \rangle$; the expressions for $\langle S_{2z} \rangle$ are identical with the obvious subscript interchanges.

$$\langle S_{1z} \rangle = \frac{\sum_{S,M} \exp\left(-\frac{\langle \mathcal{H}_{S,M} \rangle}{kT}\right) \langle SM | S_{1z} | SM \rangle}{Z}$$

where the sum is taken over all states of different S and M and Z is the partition

function of the states. Since $g\beta HM/kT \ll 1$, we may simplify the above expression and find that

$$\langle S_{1z}\rangle = \frac{-\frac{g\beta H}{kT}\sum_{S,M^2}\exp\left(\frac{\langle \varkappa' S,M\rangle}{kT}\right)\frac{\tilde{S}_1\cdot\tilde{S}}{\tilde{S}\cdot\tilde{S}}M^2}{\sum_{S,M^2}\exp\left(-\frac{\langle \varkappa' S,M\rangle}{kT}\right)}$$

where the sums are taken over states of difference S and M^2 and $\varkappa'$ refers to a Hamiltonian which contains only the exchange and zero-field splitting terms.

Next we include the effect of the states with $d_{x^2-y^2}$ symmetry at the ferrous atom (Fig. 2) by forming the Hamiltonian for these states and adding their contribution to that from the states with d_{z^2} symmetry at the ferrous iron. We assume that the zero-field splittings are the same for both symmetries since the spin-orbit terms are similar in first order for high-spin d^6 configurations with d_{z^2} or $d_{x^2-y^2}$ lying lowest[34]. The expression:

$$\langle S_{1z}\rangle = \frac{-\frac{g\beta H}{kT}\sum_{S,M2}\{\exp[-E^{dz2}_{S,M}/kT] + \exp[-(E^{dx2-y2}_{S,M}+\Delta)/kT]\}\frac{\tilde{S}_1\cdot\tilde{S}}{\tilde{S}\cdot\tilde{S}}M^2}{Z'}$$

includes $E^{d_{z^2}}_{S,M}$ and $E^{d_{x^2-y^2}}_{S,M}$ which are the energies of the levels in Fig. 2 normalized to the energy of the $S = 1/2$ states of the respective symmetries; Δ is the difference energy which is implied by the temperature dependence of the ferrous electric field gradient tensor, and not an energy resulting directly from the crystal field (see Fig. 2).

In Fig. 3, we plot $\langle S_{1z}\rangle$ (FeIII) and $\langle S_{2z}\rangle$ (FeII) *vs.* temperature for various values of $J_{d_{z^2}}$, $J_{x^2-y^2}$, and Δ. By comparing many such curves we found that $\langle S_{1z}\rangle$ and $\langle S_{2z}\rangle$ are relatively insensitive to variations in D_1 and D_2 in the range expected for ^6S and ^5D configurations; therefore, we set $D_1=D_2=0$ for the curves in Fig. 3. We have also calculated the temperature dependences of the magnetic susceptibility and ferrous quadrupole splitting within the framework of the energy level diagram in Fig. 2. By simultaneously fitting the data from susceptibility, Mössbauer, and PMR experiments we were able to obtain a value for Δ (400 cm^{-1}) which is more precise than the previously reported value[2]. Thus, the curves in Fig. 3 effectively cover the range of combination of parameters expected for the plant ferredoxins. By comparing the curves in Fig. 3 with the PMR data of POE *et al.*[5], we find that the contact shifted lines which move away from 0 ppm as the temperature is increased have the same temperature dependence as $\langle S_z\rangle$ for the ferrous atom. We therefore assume that these protons are contact shifted *via* covalent bonding to the ferrous atom. Since there are four lines of this type in the PMR data of reduced spinach and reduced parsley ferredoxins, we attribute these lines, as did POE *et al.*[5] to β-CH_2 protons of cysteine and thus place two cysteine sulfurs as ligands at the ferrous atom in the reduced proteins. If we assume that the contact shift at the ferrous β-CH_2 cysteinyl protons is due solely to spin density originating at the ferrous atom and that their chemical shift is 3 ppm downfield[35], we find that $J_{d_{z^2}} = -55$ cm^{-1}.

By assuming that two additional cysteinyl sulfurs are ferric ligands in the reduced proteins, we calculate the contact shifts shown in Fig. 4 for the reduced plant ferredoxins and adrenodoxin. In constructing the curves shown in Fig. 4, we have calibrated the curves in Fig. 3 to the average of the four ferrous PMR resonances in

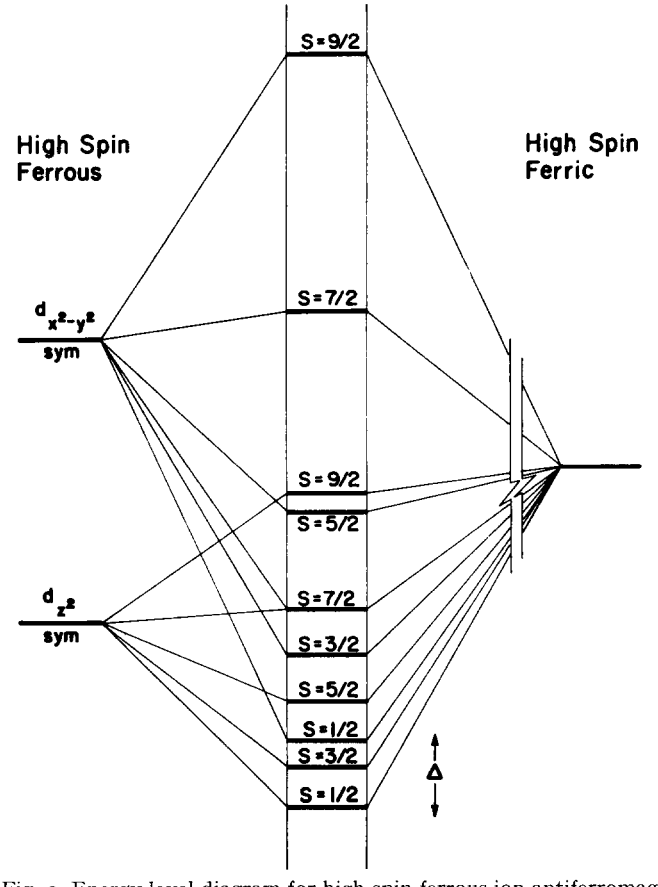

Fig. 2. Energy level diagram for high-spin ferrous ion antiferromagnetically spin-coupled to high-spin ferric ion. Ferrous atom is in a distorted tetrahedral ligand field. Figure drawn to scale for $J_{d_{z^2}} = -80$ cm^{-1}, $J_{d_{x^2-y^2}} = -175$ cm^{-1}, $\Delta = 400$ cm^{-1}. Energy of ferric ion not to scale. Zero-field and Zeeman splittings are omitted.

the data of POE *et al.* for reduced spinach ferredoxin assuming that $J_{d_{z^2}} = -55$ cm^{-1}. The predicted position of the β-CH_2 protons at the ferric atoms result from assuming that proton hyperfine coupling constants are the same at both iron atoms, an assumption which is probably in error by around 20 %. For adrenodoxin, we assume that the proton coupling constants are the same as those in spinach and parsley. The contact shifts of α-CH protons are obtained from the shifts of the β-CH_2 protons of the same cysteine residue by scaling down by a factor of ten in all cases to account for the reduction in spin density across the additional C–C bond. We also assume that the proton coupling constants are the same in the oxidized proteins as they are in the reduced forms. Consequently the curves in Fig. 4 are only semi-quantitative for, although the slopes of these curves are correct relative to their positions on the ordinate, the ordinates themselves are only accurate to 50 %. However, there are many conclusions to be drawn from Fig. 4. If cysteine is indeed a ligand at the ferric site, then the β-CH_2 protons from the cysteines at this iron atom were probably not observed in the work of POE *et al.*[5] since they occur very far downfield and not in the range investi-

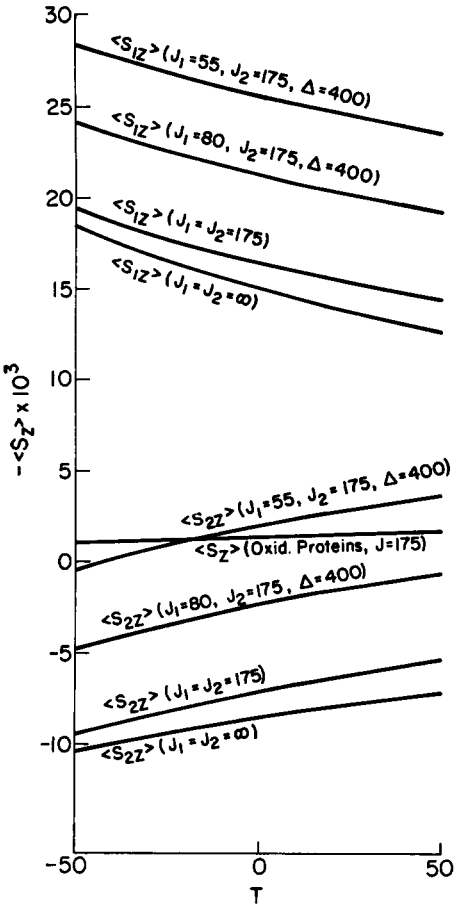

Fig. 3. Temperature dependence of $\langle S_z \rangle$ at the two irons in a high-spin ferric antiferromagnetically coupled to high-spin ferrous system. $\langle S_{1z} \rangle$ = ferric, $\langle S_{2z} \rangle$ = ferrous, $\langle S_z \rangle$ = ferric in oxidized proteins. Applied magnetic field is 51.5 kGauss.

gated by these workers. The prediction for oxidized ferredoxin in Fig. 4 is in complete agreement with the data[5] except there is only a single proton in the PMR spectra of oxidized parsley and spinach ferredoxins at around +15 ppm. The protons from cysteine side chains bound to either iron in oxidized ferredoxin should exhibit positive temperature dependences in their PMR spectra. In the data of POE *et al.*, a resonance of this type is found at +15 ppm. We would expect to find as many as eight resonances with shifts within a factor of two of +15 ppm: one for each of the β-CH_2 protons*. The approximations we have made for the size of the a-values for these proteins are crude and any assignment of this +15 ppm resonance requires further PMR experiments on deuterated proteins with only specific amino acids protonated. If these experiments are successful, the oxidized proteins probably will yield more information than the reduced proteins since resonances due to β-CH_2 protons near the ferric iron in the reduced proteins are probably too broad to be seen.

* See NOTE ADDED IN PROOF.

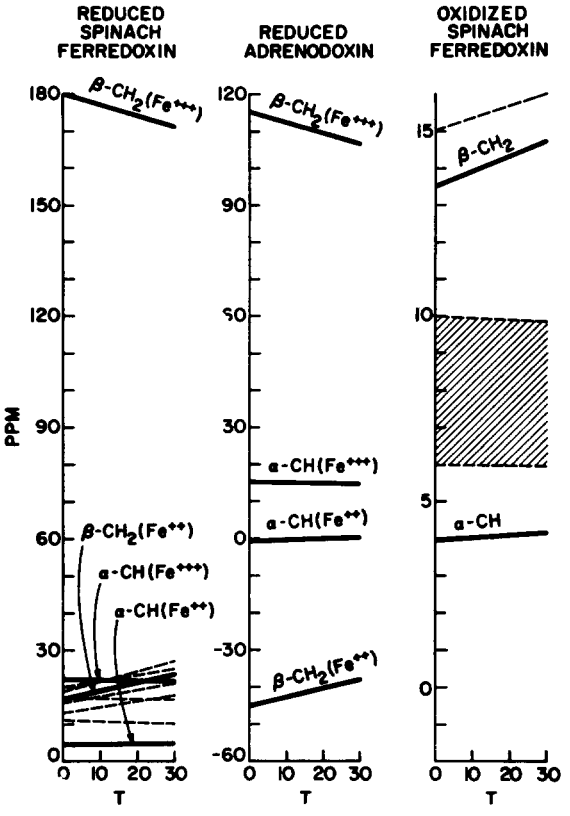

Fig. 4. PMR energies *vs.* temperature for structure in Fig. 1. Ordinate given in parts per million; positive values of ppm refer to resonances downfield relative to 2,2-dimethylsilapentane 5-sulfonic acid. Predicted line positions are shown in solid lines; dashed lines and shaded areas indicate the position of measured lines[5].

If the structure of the ferrous site (Fig. 1) of the reduced protein is correct, then there is an inconsistency in the above argument when compared to the results of magnetic susceptibility measurements on spinach ferredoxin. The susceptibility measurements yield a value of about -80 cm^{-1} for $J_{d_{z^2}}$ in the reduced protein. A value of -80 cm^{-1} will produce contact shifts upfield relative to 0 ppm (Fig. 2). Thus, either all the magnetic susceptibility measurements are in error or there is a "ferric" contact shift at the "ferrous" β-CH_2 protons. Close inspection of the susceptibility data has led us to believe that J is indeed -80 cm^{-1}. To resolve this inconsistency we propose that the β-CH_2 protons from the cysteines at the ferrous atoms sense some spin density from the ferric site so that the sign of the $\langle S_z \rangle$ at these protons is that of the ferric ion whereas the temperature dependence of the spin density is dictated by the ferrous ion. A possible mechanism would be that the ferric spin density is sensed by contact to the bridging sulfur as shown in Fig. 1. In adrenodoxin the much larger absolute value of J should result in much smaller downfield shifts for the ferric protons while the ferrous protons may well be shifted upfield.

The data we have summarized in this paper provide strong support for the structure shown in Fig. 1 and in particular for the electronic configuration and coordi-

nation geometry at the ferrous ion. The electronic configuration at the ferric ion is also established but we are unable to deduce the coordination geometry at this site. For this reason we indicate the ferric ligands in broken lines. Our analysis of the PMR of this system provides a way to directly associate specific amino acid ligands with a particular iron atom and should lead to substantial clarification of this problem.

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We are indebted to the many colleagues whose expertise facilitated the success of various aspects of this investigation and who, through many discussions, have shaped the conclusions and ideas presented here. In particular, we would like to record our appreciation of the encouragement provided by Dr. H. Beinert as one of the pioneers in these endeavors. We also wish to express our gratitude for the research support provided by the National Institutes of Health (GM12176, GM12394 and RR00417-01, the National Science Foundation (GB13585) and the U.S. Atomic Energy Commission through Donner Laboratory. G. P. (1-K3-GM 21,213) and A. J. B. (1-K4-GM 24,496) were the recipients of Career Development Awards from the National Institutes of Health.

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Dr. Irving T. Salmeen of the Ford Scientific Laboratory, Dearborn, Michigan has observed a broad proton line centered at 37 ppm in oxidized spinach ferredoxin. The line is ca. 8 ppm wide and the integrated area corresponds to 5 (± 2) protons. If this is due to the β-CH_2 protons then this would indicate that the hyperfine coupling constant is larger for the protons coupled to the ferric atoms in the oxidized protein than for the protons coupled to the ferrous atom in the reduced protein and all of our predictions should be corrected accordingly.

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