Oxidized and Reduced $[Fe_2Q_2]$ (Q = S, Se) Cores of Spinach Ferredoxin: a Comparative Study Using ^1H NMR Spectroscopy

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In a continuing effort to better understand the nature of the magnetic coupling in iron−sulfur clusters contained in proteins,[1] we have undertaken a comparative study of the variability of the parameters of the spin Hamiltonian (1) for the $[Fe_2Q_2]^{2+/+}$ (Q = S, Se) cluster core as a function of the bridging ion Q^{2-}, using ^1H NMR spectroscopy.

$$H = JS_1 \cdot S_2 \qquad (1)$$

Proteins containing the $[Fe_2Se_2]$ instead of the $[Fe_2S_2]$ core have been previously prepared,[2−8] and a detailed review has appeared recently.[9] Model compounds containing $[Fe_2Se_2]^{2+}$ cores have also been synthesized.[10−15] These studies have established that upon Se−for−S substitution, the core structure maintains its features, with the Fe−Fe distance increasing by 0.10 Å, and the Fe−Q distance increasing by 0.13 Å.[15,16] These binuclear systems are the simplest cases to study, although the Se-for-S exchange has also been used to characterize a wide range of iron−chalchogenide proteins.[9,17]

Oxidized $[Fe_2S_2]^{2+}$ cores in proteins, constituted by two Fe(III) ions,[18] are characterized by a diamagnetic spin ground state resulting from antiferromagnetic Heisenberg exchange coupling through the S^{2-} bridging ions, (J_{ox} = 290 cm^{-1}).[19,20] The same situation holds for isoelectronic model compounds.[21] Magnetic susceptibility measurements for proteins containing these cores are necessarily inaccurate, because of the large contribution of the protein diamagnetic correction, especially at low temperatures, due to the concurrent tendency of the system to lower its paramagnetism. The reduced $[Fe_2S_2]^+$ core, on the other hand, formally contains a Fe(II) and a Fe(III) ion. For these systems, besides the Heisenberg exchange coupling mechanism, a double exchange mechanism should be allowed,[22−26] but Mössbauer spectroscopy has shown that the valence is mainly localized up to 250 K,[18,20,27−33] limiting the contribution of double exchange to a reduction of the effective value of the magnetic exchange constant J_{red}.[34,35] Magnetic studies on reduced [2Fe−2S] ferredoxins have been carried out,[19,20,36−39] and in the case of spinach ferredoxin, for which both J_{ox}[19] and J_{red}[19,36] have been determined, a decrease of J_{eff} from 290 cm^{-1} to 200 cm^{-1} upon reduction has been observed, that may be partly ascribed to the larger ionic radius of Fe(II), which makes the superexchange mechanism less efficient, and partly to double exchange.

Magnetic susceptibility studies are even more difficult to carry out with the selenium-substituted proteins, and no report has been presented yet. This is probably due to the fact that $[Fe_2Se_2]^{2+/+}$ cores are quite unstable, and large errors would be induced in the magnetic measurements because of the presence of even traces of high spin Fe^{3+} ions in the sample. Furthermore, the Se-for-S substitution may not be complete, with the consequent risk of measuring a mixture of various $[Fe_2S_mSe_{2-m}]$ isomers.[5,8,39] To overcome these problems, we have chosen to investigate these systems using ^1H-NMR. This technique allows the detection of hyperfine shifted proton signals of the cysteine residues bound to the iron ions in the 2Fe core without being influenced by the possible presence of traces of Fe^{3+} ions in solution.[40−45] Moreover, it allows the distinction of various Se/S isomers possibly present in the protein sample.[39]

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NMR spectroscopy thus makes possible a semiquantitative estimate of the magnetic exchange constant from the temperature dependence of the hyperfine shifts.[1,41,46,47]

In this paper we report, for the first time, the ^{1}H NMR spectra of the reduced and oxidized [2Fe−2Se] cores in ferredoxins (Fd). Parts A and B of Figure 1 show, for comparison, the 298 K ^{1}H NMR spectra of oxidized spinach Fd $[Fe_2S_2]^{2+}$ (similar to the spectra previously reported,[40,41] except for the higher resolution of the broad absorption around 35 ppm, corresponding to the β-CH_2 protons of cysteine residues bound to the two formally Fe^{3+} ions) and $[Fe_2Se_2]^{2+}$ cores, respectively. The lower signal-to-noise ratio observed for the spectrum of Se-substituted Fd is due to the smaller amount of protein. Two observations are nevertheless obvious: the signals in the Se-substituted protein, corresponding to the α-CH and β-CH_2 protons of cysteine residues bound to the formally Fe^{3+} ion, are shifted about 4 ppm more downfield than in the native protein, but the increase in shift is not so large to be justified by a change in the ground spin state from $S = 0$. The presence of higher spin ground states has been shown to cause a large increase of hyperfine shifts in homologous protons of $[Fe_4S_4]^+$ and $[Fe_4Se_4]^+$.[48] Moreover, the hyperfine shifted signals of the ^{1}H NMR spectrum of $[Fe_2Se_2]^{2+}$ Fd shows a slight increase of their downfield shift upon passing from 281 to 298 K (not shown). This characteristic behavior, opposite to what expected from Curie law and thus termed anti-Curie, has also been observed for the native $[Fe_2S_2]^{2+}$ oxidized ferredoxin,[40] and is interpreted in terms of antiferromagnetic coupling between the two Fe^{3+} ions present in the Se-substituted protein, generating an $S = 0$ spin ground state. The larger downfield shift observed for the Se-substituted protein is thus interpreted, in the absence of other effects, as a consequence of a decrease of the magnetic exchange constant for the oxidized protein, J_{ox}, upon passing from S to Se.

The variation of J_{ox} can be estimated using a simple Heisenberg approach.[1,41,47] Hamiltonian (1) involves the presence of eigenstates characterized by energy values given by eq 2 with S', the spin states of the pair, ranging from $S_1 + S_2$ to $|S_1 - S_2|$.

$$E_i = {}^1/_2 JS'_i(S'_i + 1) \tag{2}$$

The contribution of, e.g., metal 1 to the contact shift of a proton is given by eq 3,

$$\left(\frac{\Delta\nu}{\nu_0}\right)_j^{con} = \frac{2\pi g\mu_B}{3\gamma_N kT}\left(\frac{A_1}{h}\right)\sum_i C_{1i}S'_i(S'_i+1)\frac{(2S'_i+1)\exp(-E_i/kT)}{\sum_i(2S'_i+1)\exp(-E_i/kT)} \tag{3}$$

where A_1 is the hyperfine coupling between metal 1 and the proton nucleus in the case of an uncoupled system. The C_{1i} and C_{2i} coefficients are given by eqs 4 and 5,

$$C_{1i} = \frac{\langle S_1\rangle_i}{\langle S'\rangle i} \tag{4}$$

$$C_{2i} = \frac{\langle S_2\rangle_i}{\langle S'\rangle i} \tag{5}$$

and they reflect the contribution of S_1 and S_2 to the total S' of each i level.[1] When the two metals are alike, $C_{1i} = C_{2i} = {}^1/_2$.

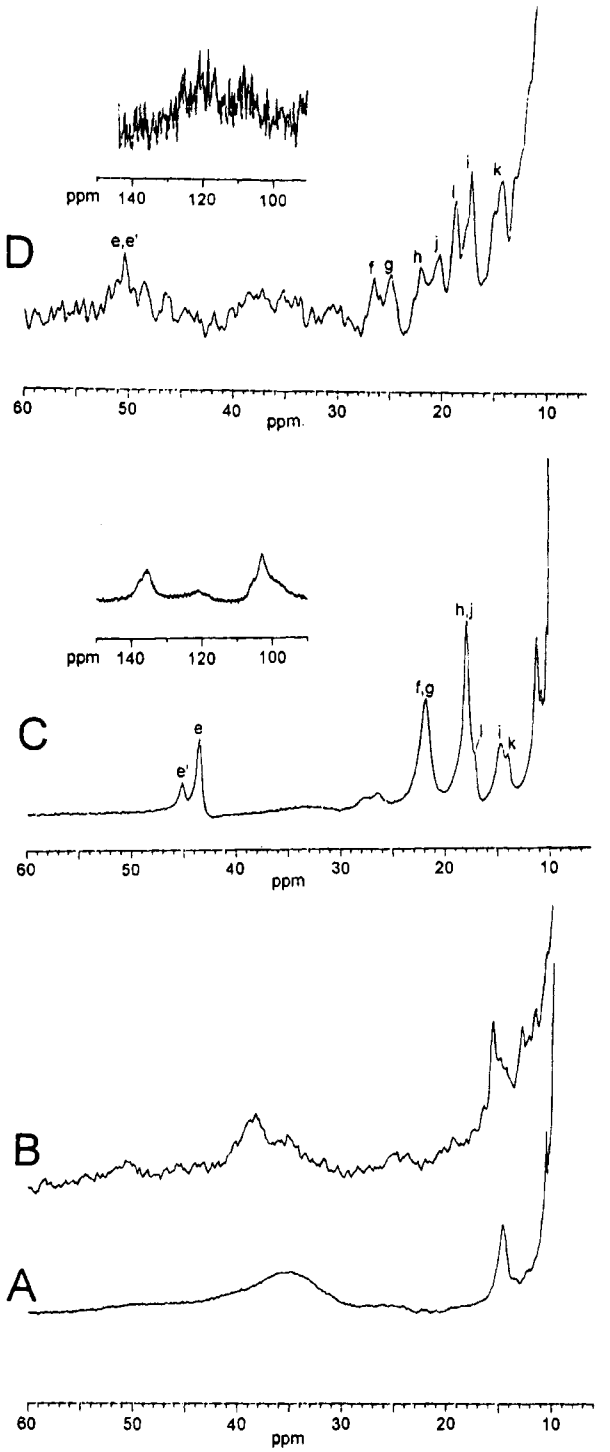

Figure 1. 300 MHz, ^{1}H NMR spectra of oxidized native (A) and Se-substituted (B) spinach ferredoxin (298 K), and of reduced native (C) and Se-substituted (D) spinach ferredoxin (285 K). The 90−150 ppm spectral regions are shown in the insets.

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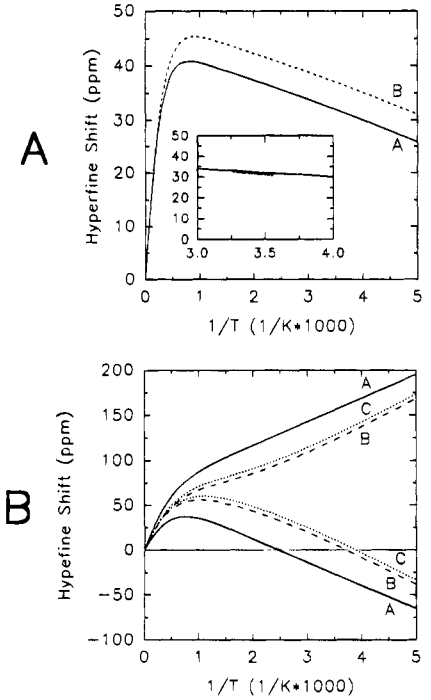

Figure 2. (A) Calculated hyperfine shifts for β-CH_2 protons of oxidized $[Fe_2S_2]^{2+}$ (curve A, solid line) and $[Fe_2Se_2]^{2+}$ (curve B, dashed line) spinach ferredoxins. Curve A was obtained using J_{ox} = 290 cm^{-1}; curve B was obtained using J_{ox} = 270 cm^{-1}. In the inset, the experimentally observed behavior (filled circles) is compared with the theoretical curve A. (B) Calculated hyperfine shifts for β-CH_2 protons of reduced $[Fe_2S_2]^{+}$ (curves A, solid line, and B, dashed line) and $[Fe_2Se_2]^{+}$ (curve C, dotted line). Curve A was calculated using J_{red} = 200 cm^{-1}, Δ = 0 cm^{-1}; curve B was calculated using J_{red} = 200 cm^{-1}, Δ = 300 cm^{-1}; curve C was calculated using J_{red} = 185 cm^{-1}, Δ = 300 cm^{-1}.

Thus, the contact shift of protons sensing each individual iron ion (eq 3) depends only on the A and E_i values, the latter being proportional to J. We have therefore redetermined the temperature dependence (in the range 282−306 K) of the hyperfine shifts for $[Fe_2S_2]^{2+}$ Fd,[40] to obtain a set of reliable data. In Figure 2A we report the experimental points together with the theoretical curve obtained using eq 3, with J_{ox}(S) = 290 cm^{-1} (curve A), as estimated from magnetic susceptibility measurements,[19] and A/h = 1.8 MHz. Using the same A/h value and J_{ox}(Se) = 270 cm^{-1} for $[Fe_2Se_2]^{2+}$ (curve B), the average β-CH_2 shift changes, upon passing from native to Se−Fd, from ca. 35 to ca. 39 ppm in correspondence to room temperature, as observed experimentally. The inaccuracy of J_{ox} from magnetic susceptibility measurements of the [2Fe−2S] protein limits also the accuracy of the present estimate of J_{ox} for the [2Fe−2Se] protein. However, and more importantly, their *relative* values as estimated by NMR are more reliable than the absolute values.

In parts C and D of Figure 1, the 285 K, ^1H NMR spectra of $[Fe_2S_2]^{+}$ and $[Fe_2Se_2]^{+}$ ferredoxins are reported, respectively. Again, the signal-to-noise ratio of the Se-substituted protein is low, both because of the lower amount of protein, and because of the intrinsic instability of the $[Fe_2Se_2]^{+}$ cluster, which leads to a rapid loss of intensity of the hyperfine-shifted signals. The quality of the spectrum is sufficient, however, to allow us to make the following observations: (i) The signal observed around 44 ppm in the native protein, and previously assigned to the α-CH proton of a cysteine bound to the formally Fe^{3+} in the reduced core of the protein[43,44] is clearly detectable in the Se-substituted protein around 52 ppm, with a Curie behavior in the range 281−285 K (not shown); the fractional signal around 45 ppm in the native protein, previously assigned to a less abundant isomer,[49,50] is not observed in the Se-substituted derivative probably because of the lower signal-to-noise ratio. (ii) It is possible to observe anti-Curie behavior, in the temperature range 281−285 K, of the signals labeled *f*, *g*, *h*, and *i* for both native and Se-substituted protein, whereas Curie behavior, in the same temperature range, has been observed for signals labeled *j* and *l* in both cases. For all these resonances, the signals for the Se-substituted protein are more downfield shifted than the corresponding signals of the native protein (Figure 1). Curie and anti-Curie behaviors of the signals corresponding to the protons of the cysteine residues bound to the iron ions of the binuclear core have been previously and thoroughly interpreted[41,43,51] as indicating coupling of the protons of the Fe^{3+} and to the Fe^{2+} ions, respectively. Thus, the first conclusion is that the valence distribution has not changed upon passing from S^{2-} to Se^{2-} as the bridging atom. (iii) The group of broad signals observed between 90 and 150 ppm in the native protein, and previously assigned to the β-CH_2 of cysteines bound to the iron ion which largely maintains the Fe^{3+} character[43,51] in the one-electron reduced core, is also observed, roughly in the same region of the spectrum, for the Se-substituted protein, and can be assigned to the same type of protons on the basis of the magnitude of the shifts. The signals of the native protein show a Curie behavior, and we assume the same behavior for the signals of the Se-derivative, because the low signal-to-noise ratio and the instability of the protein do not allow a precise definition of their temperature dependence. In summary, the $[Fe_2Se_2]^{+}$ system shows larger downfield shifts for both Curie and anti-Curie signals (excluding the broad signals further downfield, whose exact behavior and position is not determined because of the very low signal-to-noise ratio).

Figure 2B (curve A) reports the calculated hyperfine shift for the β-CH_2 protons bound to the formally Fe^{3+} and Fe^{2+} ions in the reduced core of native spinach Fd, obtained using the simple Heisenberg approach described above, and the estimated value of J_{red}(S) for spinach Fd of about 200 cm^{-1}.[19,36] At about room temperature the signals corresponding to Fe^{2+} are in this way calculated to have averaging negative shifts, whereas the signals corresponding to Fe^{3+} have average positive shifts of about 150 ppm. These calculations, then, do not reproduce well the experimental behavior. It has been pointed out, since the first theoretical account of the NMR behavior of these systems,[51] that, at variance with Fe(III), the electronic structure of Fe(II) certainly entails low-lying excited states due to low symmetry removal of the orbital degeneracy of the ground state and to spin−orbit coupling effects. Inclusion of a low-lying excited state with a different Heisenberg coupling constant J may better account for the experimental NMR behavior.[51] Another factor which should be taken into consideration is the existence of an excited state with reversed localization of the extra electron, which can be populated following a Boltzmann distribution.[1] Such a state is separated from the ground state by an amount of energy Δ which depends on the difference in microscopic reduction potential for the two Fe(III) ions in the oxidized form. A $\Delta E°$ of 100 mV translates into an energy separation of ca. 800 cm^{-1}. Therefore, even modest $\Delta E°$ values may account for energy separations of the order of J. It has been recently shown that fast exchange, on

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the NMR time scale, between two states

$$Fe^{III}{}_1Fe^{II}{}_2 \rightleftharpoons Fe^{II}{}_1Fe^{III}{}_2$$

differing by about 300 cm^{-1} in energy (Figure 2B, curve B) can by itself account for the experimental behavior.[1]

Using the latter as a working model, and maintaining the same energy difference between the two electronic configurations, we can achieve the modest increase of the downfield shifts of both Curie and anti-Curie signals, as observed experimentally, by simply decreasing the magnetic exchange constant from 200 (J_{red}(S)) to 185 cm^{-1} (J_{red}(Se)) (Figure 2B, curve C).

Thus, both in the oxidized and in the reduced case, a *similar* decrease of J_{ox} and J_{red}, of less than 10%, reproduces the experimental behavior. This conclusion can be achieved independently of the detailed model for the excited states of the $[Fe_2Q_2]^+$ systems.[1,51] This modest decrease is consistent with the increase in the Fe−Q distance upon passing from S to Se, observed in model compounds, and with a small contribution of double exchange to J_{eff} in $[Fe_2Q_2]^+$ cores. In these systems, apparently, magnetic exchange coupling utilizes mainly a superexchange mechanism. Similar modest reductions of Heisenberg exchange coupling constants have been proposed to occur on passing from $[Fe_4S_4]^{3+/2+}$ to $[Fe_4Se_4]^{3+/2+}$ clusters.[17] Double exchange contributes significantly to the magnetic coupling in tris-μ-oxo bridged Fe(II)−Fe(III) dimers, probably because of occurrence of partial metal-metal bonding.[52]

The protein was isolated from spinach,[8] and the Se-for-S substitution was carried out as previously described.[8] The absence of sulfide ions after native cluster disruption was obtained performing a gel filtration chromatography on Sephadex G-25 of the apo-protein, and it was confirmed by UV−vis spectroscopy.[8] The ^1H NMR experiments were carried out on samples dissolved in D_2O buffer (P_i 30 mM, pH = 7.5) using a Bruker AC-P spectrometer operating at 300 MHz, with a short recycle time (16 ms) to speed up the data accumulation and to partially reduce the intensity of the residual water signal. Shifts are measured assuming the signal of residual HDO at 5.01 ppm at 281 K and 4.81 ppm at 298 K.

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