More generally, the sensitivity of ^{77}Se NMR spectroscopy to the microenvironment is likely to provide a useful means of monitoring the binding of various ligands, including potential substrates, products, or inhibitors, to the active site.

In summary, ^{77}Se NMR spectroscopy represents a powerful and sensitive tool for examining the subtle interplay between structure and function in the active site of selenoenzymes. Its continued application to the characterization of the artificial peroxidase selenosubtilisin will undoubtedly enhance our understanding of this molecule's detailed catalytic mechanism and may ultimately yield insights that result in improved glutathione peroxidase mimics.

Mössbauer and Integer-Spin EPR of the Oxidized P-Clusters of Nitrogenase: P^{OX} is a Non-Kramers System with a Nearly Degenerate Ground Doublet

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Abstract: The molybdenum–iron protein of nitrogenase contains 2 Mo atoms and ca. 28–30 Fe atoms. Approximately 16 Fe atoms belong to the P-clusters, a novel type of iron–sulfur cluster of unknown structure. Mössbauer studies have established that P-clusters are diamagnetic in the semireduced state, P^N. Upon oxidation of the protein with redox dyes such as thionin the state P^{OX} is attained. Previous studies have revealed that the low-temperature (≤ 4.2 K) Mössbauer spectra of P^{OX} exhibit magnetic hyperfine patterns even in the absence of external magnetic fields. Such behavior is generally characteristic of a Kramers system, i.e., of a system with an odd number of electrons. The spectra had features typical of those observed for a Kramers doublet with extremely anisotropic g-values ($g_1 \gg g_2, g_3$). Recent Mössbauer and EPR studies of integer spin systems in our laboratory have suggested the possibility that P^{OX} may be a very unusual non-Kramers system. Here we report Mössbauer and EPR studies of the proteins from *Azotobacter vinelandii* (Av1), *Clostridium pasteurianum* (Cp1), *Klebsiella pneumoniae* (Kp1), and *Xanthobacter autotrophicus* (Xa1) which prove that the electronic ground doublet of P^{OX} is not a Kramers doublet but rather a nearly degenerate doublet (splitting Δ) of a system with an even number of electrons. Cp1, Av1, and Kp1 have $\Delta \leq 10^{-3}$ cm^{-1}, and the magnetic patterns observed in the zero-field Mössbauer spectra result from mixing of the two electronic levels by ^{57}Fe hyperfine interactions ($|A_z| \approx 10^{-3}$ cm^{-1}). For Av1 and Kp1 we have observed integer-spin EPR transitions, at $g_{eff} = 11.9$, between two excited-state spin levels at 10–15 cm^{-1}. The ground doublet of Xa1 exhibits an integer-spin resonance at $g_{eff} = 15.6$. Analysis of the Xa1 Mössbauer spectra yields $\Delta \approx 0.010$ cm^{-1}. Using this value of Δ, quantitation of the EPR spectra yielded ca. two spins per MoFe protein for P^{OX}. The observed g-values suggested that P^{OX} of Xa1 has $S = 3$ or $S = 4$. However, the ground- and excited-state Δ-values of Kp1 and Av1 indicate that the electronic ground manifold may not consist of an isolated multiplet with definite spin S. Recognition of P^{OX} as a non-Kramers state implies that two electrons are removed from each P-cluster in the transformation $P^N \rightarrow P^{OX}$. Since ca. four electrons per MoFe protein are removed and since ca. 16 Fe atoms are involved, it follows that the $\alpha\beta$ dimer of nitrogenase contains two identical P-clusters and that each cluster has ca. 8 Fe sites. The Mössbauer data are reviewed for a model that considers the P-clusters to consist of two bridged cubanes.

Introduction

In 1978 Zimmermann and co-workers[1] published a Mössbauer study of the MoFe protein of nitrogenase from *Azotobacter vinelandii* (Av1).[2] This study concluded that Av1 contains (30 ± 2) Fe atoms per $\alpha_2\beta_2$ unit. Approximately 12 Fe atoms were found to be associated with the two identical cofactor centers (labelled M-centers), and ca. 16 Fe atoms were assigned to a novel cluster type, called the P-clusters. In the semireduced (i.e., when

kept in excess dithionite) protein the M-centers have cluster spin $S = {}^3/_2$, state M^N, whereas the P-clusters are in the diamagnetic state P^N. When the protein is oxidized with the redox dye thionin, the oxidation proceeds in two phases. In the first oxidation step, approximately four electrons are removed from the protein without

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(2) Abbreviations used: MoFe protein of *Azotobacter vinelandii*, Av1; *Klebsiella pneumoniae*, Kp1; *Clostridium pasteurianum*, Cp1; *Xanthobacter autotrophicus*, Xa1; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism.

any loss of the $S = {}^3/_2$ EPR signal intensity. In the second oxidation step, the two identical M-centers are oxidized. There is general agreement that one electron is removed from each M-center in this step; Mössbauer studies[1,3] have shown that the oxidized state, M^{OX}, is diamagnetic. There is some disagreement about the four-electron oxidation step. While researchers in different fields agree that about four electrons are required, there are questions as to whether all four electrons are removed from the same cluster type. For instance, Watt and co-workers[4-6] have interpreted their coulometric studies as a three-electron process attributed to one-electron oxidations of three P-clusters, and a one-electron step involving a "maverick" cluster. Mössbauer studies, on the other hand, have suggested strongly that only one type of cluster is involved in the four-electron oxidation. Moreover, a 3:1 partitioning is not easy to reconcile with an $\alpha_2\beta_2$ subunit structure.

Oxidation of the P-clusters yields the state P^{OX}. In this state the P-clusters yield at $T \leq 10$ K highly resolved Mössbauer spectra exhibiting magnetic hyperfine structure even in the absence of an applied magnetic field. The observation of magnetic hyperfine interactions in zero field is a characteristic feature of compounds with an odd number of electrons (Kramers systems). If P^{OX} were indeed a Kramers system, it would follow that the oxidation $P^N \rightarrow P^{OX}$ involves the transfer of an odd number of electrons *per cluster*. With this interpretation of P^{OX} Zimmermann et al.[1] proposed that the MoFe protein contains four P-clusters, each containing four iron sites. Zimmermann et al.[1] and Huynh et al.[3] have decomposed the Mössbauer spectra of P^{OX} into eight subcomponents (seven distinct sites). The parameters could roughly be grouped into two similar sets suggesting that the P-clusters occurred in two slightly inequivalent forms, called P and P′. In this view, each $\alpha\beta$ dimer was thought to contain one cofactor center and one pair of P-clusters.

The Mössbauer studies of P^{OX} for Av1, Cp1, and Kp1 suggested that the electronic ground state of P^{OX} is a Kramers doublet with an effective g-tensor such that $g_z \gg g_x, g_y$. Such doublets occur commonly for spin multiplets with $S \geq {}^3/_2$ for $D < 0$ and $E/D \leq 0.1$, where D and E are zero-field splitting parameters. Subsequent low-temperature magnetic circular dichroism studies by Johnson and co-workers showed that $g_z \approx 10.7-12.5$ for Kp1[7] and $g_z \approx 10-14$ for Av1,[8] suggesting a system with $S = {}^5/_2$ or $S = {}^7/_2$.

In the past three years we have studied extensively complexes with integer electronic spin, monomers as well as clusters, that permit observation of an EPR transition. Such signals can originate when two electronic levels of a spin multiplet are separated in energy by an amount Δ which is less than the energy of the microwave quantum ($h\nu \simeq 0.3$ cm^{-1} at X-band). It is of interest to note that the two levels involved have generally uniaxial magnetic properties, i.e., in moderate applied fields, $\Delta \leq \beta H \leq |D|$, the expectation values of the electronic spin are appreciably different from zero only along one molecular direction. If Δ approaches 10^{-3} cm^{-1}, the magnetic hyperfine interaction of the electronic system with the ^{57}Fe nucleus can mix the two electronic states. In the limit $\Delta \rightarrow 0$ the nearly degenerate doublet behaves in many respects like a Kramers doublet.

Analysis of the line shapes of integer-spin EPR signals[9] has shown that all compounds studied thus far exhibit a fairly broad distribution of Δ values. For some of the compounds the distribution extends to $\Delta = 0$. We have studied recently the binuclear

complex [Fe(III)Cu(II)BPMP(O$_2$CCH$_3$)(OCH$_3$)](BPh$_4$)$_2$ where BPMP = 2,6-bis[(bis(2-pyridylmethyl)amino)methyl]-4-methylphenol.[10,11] We have observed that a small fraction of molecules (4%) of this exchange-coupled complex (system spin $S = 3$) exhibited, in zero field, magnetic hyperfine interactions suggesting that these molecules had $\Delta < |A|$ where A is the component of the magnetic hyperfine tensor along the easy axis of magnetization (for the cited complex the mixing of the electronic states is probably dominated by ^{63,65}Cu hyperfine interactions). These observations prompted us to reconsider the nature of the electronic state of P^{OX} and to conduct experiments designed to probe whether P^{OX} is a non-Kramers state. The studies described here were also encouraged by the recent interpretation of preliminary crystallographic results of Cp1.[12] These studies suggested that the $\alpha_2\beta_2$ unit contains two M-centers and two P-clusters. In this paper we find, both by EPR and Mössbauer spectroscopies, that P^{OX} is a state with integer electronic spin.

With the interpretation of P^{OX} as a non-Kramers system it follows that the transformation $P^N \rightarrow P^{OX}$ is a two-electron oxidation rather than a one-electron oxidation. Consequently, the number of Fe atoms per P-cluster is twice as large as suggested by the original interpretation of the Mössbauer spectra and titration data. The spectral decompositions reported,[1,3] and in particular the arguments given to justify the deconvolution procedure, do not require any substantial modifications. The parameter sets quoted by Zimmermann et al.[1] and Huynh et al.[3] rely on the notion that the electronic ground state of P^{OX} is magnetically uniaxial and the parameters quoted are independent of the assumed spin and thus no corrections are necessary.

Experimental Section

The thionin-oxidized Av1 and Cp1 samples used were those described previously.[1,3] Kp1[13] and Xa1[14] were purified, and the thionin oxidation was performed as described for Av1.[1] The Mössbauer spectrometer was run in the constant acceleration mode. The isomer shift is quoted relative to the centroid of Fe-metal at room temperature. Applied fields of 0.4–6 mT were obtained by varying the current passed through a pair of Helmholtz coils mounted around the tail section of the cryostat (Model 8DT from Janis Research Co.) while applied fields of 11 and 45 mT were obtained by mounting permanent magnets outside the tail section of the cryostat. X-band EPR measurements were performed with a Varian E9 spectrometer equipped with an Oxford liquid helium cryostat. A Varian E-236 bimodel cavity was used to generate the microwave fields, H_1, parallel and transverse to the static field, H. The integer-spin EPR signals were quantitated relative to Fe-doped zinc fluorosilicate.[9]

Theoretical Considerations

In this section we wish to discuss the principal features of the EPR and paramagnetic Mössbauer spectra which result from systems with integer electronic spin under conditions where the two lowest levels are nearly degenerate. Throughout this section we will assume that the samples are studied at a temperature where the electronic spin relaxation time is slow on the time scale of Mössbauer spectroscopy ($\tau > 10^{-6}$ s); under these conditions each electronic level produces its own Mössbauer spectrum. For the purpose of keeping the discussion as simple as possible, we will ignore quadrupole interactions.

It is assumed that the electronic system has an *orbital* ground singlet well separated from excited orbital states (an assumption justified by the observation of nearly temperature independent quadrupole splittings of P^{OX}). It will be convenient (but not

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Table I. Energy Matrix for the Nuclear Ground State Resulting from Solving Eq 5 with $S = 3$

	$\|\Phi^{+3},+^1/_2\rangle$	$\|\Phi^{-3},+^1/_2\rangle$	$\|\Phi^{+3},-^1/_2\rangle$	$\|\Phi^{-3},-^1/_2\rangle$
$\langle\Phi^{+3},+^1/_2\|$	$+3g_z\beta H_z + {}^3/_2 A_z$	$-^\Delta/_2$	0	0
$\langle\Phi^{-3},+^1/_2\|$	$-^\Delta/_2$	$-3g_z\beta H_z - {}^3/_2 A_z$	0	0
$\langle\Phi^{+3},-^1/_2\|$	0	0	$+3g_z\beta H_z - {}^3/_2 A_z$	$-^\Delta/_2$
$\langle\Phi^{-3},-^1/_2\|$	0	0	$-^\Delta/_2$	$-3g_z\beta H_z + {}^3/_2 A_z$

necessary) to assume that the ground-state manifold is a spin multiplet describable by the spin Hamiltonian

$$H_e = D\left\{S_z^2 - \frac{1}{3}S(S + 1) + \frac{E}{D}(S_x^2 - S_y^2)\right\} + \beta S \cdot g \cdot H \quad (1)$$

where D and E are zero-field splitting parameters and where g is the electronic g-tensor; by suitable choice of a coordinate frame E/D can be restricted to $0 \le E/D \le {}^1/_3$. The spin S may be the spin of a monomeric site or the spin of a cluster of metal atoms; in the latter case the parameters D and E may include a contribution from anisotropic exchange. Although it is not crucial to the argument we will assume for simplicity that S is a good quantum number; we will comment on the possibility of spin-admixed states below.

The spectroscopic features described below result from the circumstance that the electronic ground state of P^{OX} is a nearly degenerate doublet. For the present discussion it will be convenient to describe the system by eq 1, using $S = 3$[15] and choosing D and E such that a nearly degenerate ground doublet results. Throughout this discussion we will assume that $\beta H \ll |D|$. For $D < 0$, $E = 0$ and at zero applied field the $M = \pm 3$ doublet is the ground state. For $E \ne 0$ the two-fold degeneracy of this doublet is removed; for $E/D < 0.1$ the splitting of the two levels[16] is given in good approximation by $\Delta \cong 3|D|(E/D)^3$. Since D-values for iron compounds are typically between 0.5 and 5 cm^{-1} one can, for $E/D < 0.1$, obtain Δ-values of approximately 10^{-3} cm^{-1}. This splitting is 2 orders of magnitude smaller than the microwave quantum of X-band EPR spectroscopy ($h\nu \cong 0.3$ cm^{-1}), and it is comparable to the magnitude of the ^{57}Fe magnetic hyperfine interactions which are typically $0.3-1.0 \times 10^{-3}$ cm^{-1}. In the following we will focus on the properties of this nearly degenerate electronic doublet. A level scheme for an $S = 3$ system for $D < 0$ and $H = 0$ is shown in Figure 1.

In the absence of an applied magnetic field the eigenstates of the doublet are

$$\psi^\pm = \frac{N^\pm}{\sqrt{2}}\{|+3\rangle\pm|-3\rangle\} - \frac{\sqrt{15}\lambda}{a^\pm}(|+1\rangle\pm|-1\rangle)\} \quad (2)$$

where $|S,M\rangle = |3,M\rangle \equiv |M\rangle$, $N^\pm = a^\pm/\sqrt{a^{\pm 2} + 15\lambda^2}$, $a^\pm = -8 \pm 6\lambda + 8\lambda^2/(-4 \pm 3\lambda)$, and $\lambda = E/D$; for $E/D < 0.2$ the normalization factor is $N^\pm \approx 1$. Under conditions where the Zeeman splitting is much larger than Δ and where mixing with higher levels of the spin multiplet can be ignored, i.e., for $\Delta \ll \beta H \ll |D|$, it is advantageous to describe the doublet with the "magnetic" states

$$\Phi^{+3} = |+3\rangle - \frac{\lambda}{2}|+1\rangle$$

$$\Phi^{-3} = |-3\rangle - \frac{\lambda}{2}|-1\rangle \quad (3)$$

For the description of the Mössbauer spectra we augment eq 1 by the magnetic hyperfine interaction

$$H_{hf} = S \cdot A \cdot I \quad (4)$$

where $I = I_g = {}^1/_2$ for the nuclear ground state, and $I = I_e = {}^3/_2$

(15) Although the EPR studies described below have features implicating an $S = 3$ system for P^{OX}, the Mössbauer and EPR data of Av1 and Kp1, taken together, are not strictly compatible with a pure $S = 3$ system described by eq 1. We have chosen to carry out the discussion for a pure $S = 3$ system because this allows us to present the physics of P^{OX} in a more straightforward way.

(16) For a multiplet with spin S the $M = \pm S$ doublet splits through mixing by the $E(S_x^2 - S_y^2)$ term by $\Delta \propto |D|\ (E/D)^S$. Thus, multiplets with large spins are likely to have a nearly degenerate doublet.
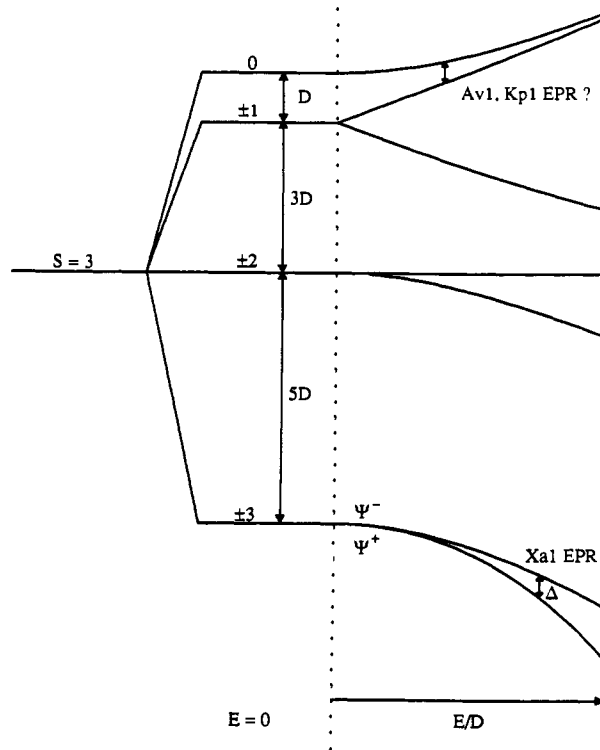

Figure 1. Energy level diagram of an $S = 3$ system according to eq 1 for $D < 0$ and $H = 0$. At the right-hand side the energies of the levels are plotted versus E/D. For clarity of presentation the splitting of the ground doublet is expanded by a factor of 10. In Figure 2 the two lowest levels, Ψ^+ and Ψ^-, are then combined with the ground- and excited-state levels of the ^{57}Fe nucleus. Figure 1 indicates also the levels from which the EPR transition of Xa1 originates and a pair of levels from which the resonances observed for Av1 and Kp1 may originate. Presentation of this diagram does not imply that the electronic ground state of P^{OX} is a pure $S = 3$ multiplet (see text).

for nuclear excited state. Because the nuclear Zeeman interaction is vanishingly small for the situation to be discussed, it need not be considered here. The Mössbauer spectra which result from the two levels of the nearly degenerate spin doublet can be determined by solving

$$H = H_e + H_{hf} \quad (5)$$

For simplicity we will assume that all tensors have the same principal axis system.

The solutions of eq 5 are described by basis states composed of electronic and nuclear spin functions, $|\Phi^{+3},m\rangle$ and $|\Phi^{-3},m\rangle$, where $m = m_g = \pm^1/_2$ for the nuclear ground state and $m = m_e = \pm^3/_2, \pm^1/_2$ for the 14.4-keV level. Inspection of the 4×4 matrix involving the nuclear ground state (Table I) reveals that the nearly degenerate doublet is magnetically uniaxial, i.e., only the z-components of the Zeeman and magnetic hyperfine terms matter; the matrix elements of the x- and y-components are negligibly small and vanish in the limit $E = 0$. The energies resulting from eq 5 are

$$\epsilon = \pm\frac{1}{2}\sqrt{(6g_z\beta H_z + 6A_z m)^2 + \Delta^2} \quad (6)$$

where $m = \pm^1/_2$ for the nuclear ground state, and $m = \pm^1/_2, \pm^3/_2$ for the nuclear excited state. Since the *combined* nuclear and electronic system is a Kramers system, all levels are doubly degenerate for $H_z = 0$. For ^{57}Fe the A-values of the nuclear excited
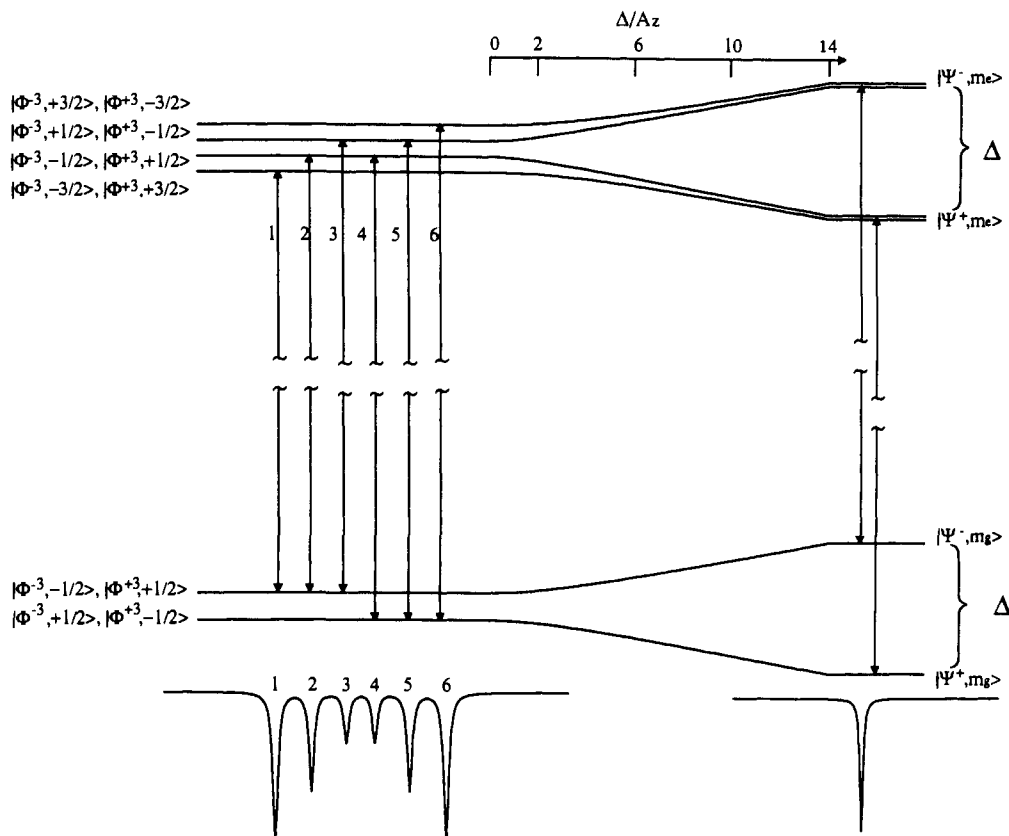
Figure 2. Energy level diagram according to eq 6 for $H_z = 0$. Shown on the left side are the eigenstates for $\Delta/A_z = 0$, with the arrows indicating the allowed nuclear transitions. The corresponding Mössbauer spectrum is shown below the energy levels. Shown on the right side are the eigenstates for $\Delta/A_z = 14$, the allowed nuclear transitions, and the corresponding Mössbauer spectrum. In the absence of quadrupole interactions this spectrum consists of a single line.

state are related to those of the ground state by $A_g/A_e = -1.7$.

The EPR resonance condition for Δ values that are small compared with the Zeeman energy is derived from eq 6 to give

$$\Delta E = h\nu \simeq 6g_z\beta H_z + 6A_z m + \frac{\Delta^2}{12g_z\beta H_z} \qquad (7)$$

with spin eigenfunctions

$$\Psi_1 \simeq \Phi^{+3} - c\Phi^{-3}$$

$$\Psi_2 \simeq c\Phi^{+3} + \Phi^{-3} \qquad (8)$$

where $c = \Delta/(12g_z\beta H_z)$. For $A_z = 0$ the EPR resonance has an effective g-value of $g_{eff} \simeq 6g_z$, but finite values of Δ will shift the resonance downfield.

An EPR transition between Ψ_1 and Ψ_2 is induced by the magnetic component of the microwave field that oscillates *parallel* to the molecular z-axis. The transition probability is proportional to

$$(g_z)^2|\langle \Psi_1|S_z|\Psi_2\rangle|^2 = (6g_z c)^2 \qquad (9)$$

Since $c^2 \propto \Delta^2 \propto (E/D)^6$ for the states under consideration, no signal can be observed for the axial case $E = 0$. This is readily understood, since in the axial limit the eigenstates are $|\pm 3\rangle$, and $\Delta M = 6$ transitions are strictly forbidden.

Equation 9 can be generalized for a spin doublet within a spin manifold S having the eigenstates $\frac{1}{\sqrt{2}}(|S,+M\rangle \pm |S,-M\rangle)$, to

$$\text{transition probability} \sim \frac{(g_z M \Delta)^2}{(2g_z M \beta H_z)^2} = \frac{\Delta^2}{(2\beta H_z)^2} \qquad (10)$$

For a given Δ and H_z, the transition probability is independent of the spin S of the multiplet. This is an important result, because the spin concentration is thus also independent of the value of the (perhaps unknown) electronic spin S. For the Xa1 P-clusters discussed below the resonance field H_z is determined from the EPR
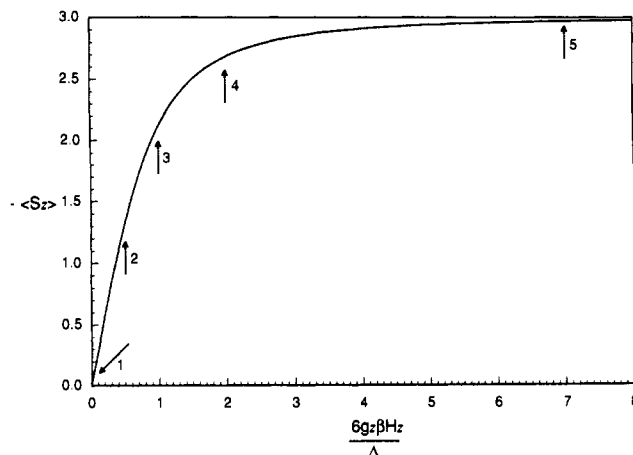

Figure 3. Spin expectation value of the lower level of a nearly degenerate spin doublet of an $S = 3$ system in the limit $\beta H \ll |D|$. Indicated by the arrows are the ratios $(6g_z\beta H_z)/\Delta$ for which the spectra of Figure 4 were calculated. The corresponding curve for the upper level of the doublet is identical to that of the lower level, except that the sign of $\langle S_z \rangle$ is reversed.

spectrum and Δ from Mössbauer spectroscopy. Thus, the concentration of the cluster giving the signal can be determined after accounting for powder averaging.

Finally, it should be pointed out that resonances of integer spin systems exhibit increased signal amplitude when the samples are studied in parallel mode (magnetic component of the microwave field, H_1, parallel to the static field, H); under these conditions the signals originating from Kramers doublets are suppressed by more than a factor of 100 relative to the signal observed in standard, transverse mode. This fact provides a unique identification of integer spin signals.
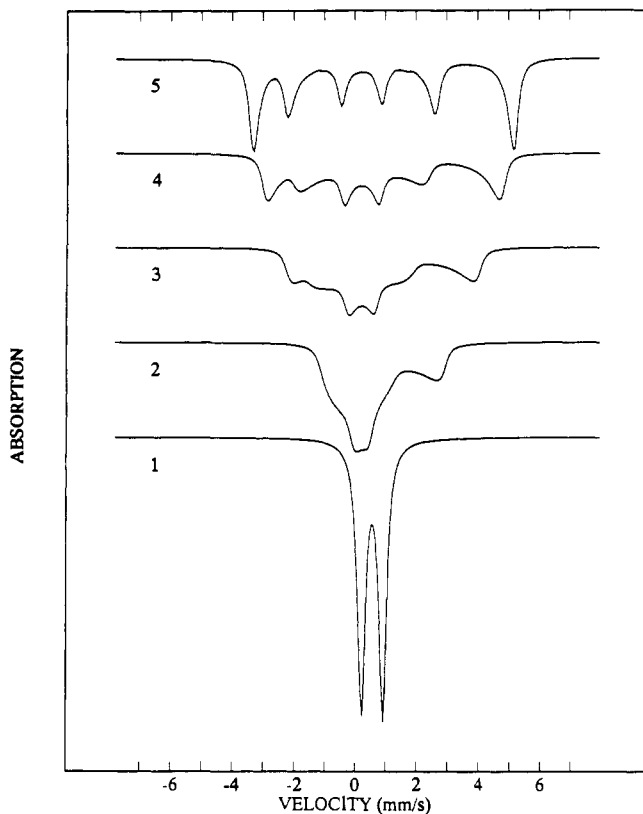
Figure 4. Mössbauer spectra resulting from the nearly degenerate doublet of an $S = 3$ system for the $(6g_z\beta H_z)/\Delta$ ratios indicated in Figure 3. The spectra were computed by diagonalization of the full $S = 3$ spin Hamiltonian and powder averaging. By adjusting g_z and A_z very similar spectra are obtained for other spin multiplets. In fact, any integer-spin system with a nearly degenerate spin doublet may produce spectra as these shown; it is not necessary that the doublet belongs to a system with definite spin S.

The character of the solutions of the matrix of Table I depends crucially on the relative magnitudes of the quantities Δ, βH, and A_z. Therefore, we wish to discuss three limiting cases.

Case 1. $\Delta \gg |A_z|$ **and** $H = 0$. This case, illustrated on the right side of Figure 2, pertains to the situation generally encountered in Mössbauer spectroscopy of paramagnetic non-Kramers systems. Equation 6 yields two electronic states, Ψ^+ and Ψ^-, separated in energy by Δ. The Mössbauer transitions are nuclear dipole transitions occurring between $|\Psi^+,m_g\rangle \leftrightarrow |\Psi^+,m_e\rangle$ and $|\Psi^-,m_g\rangle \leftrightarrow |\Psi^-,m_e\rangle$. Cross transitions $|\Psi^+,m_g\rangle \leftrightarrow |\Psi^-,m_e\rangle$ or $|\Psi^-,m_g\rangle \leftrightarrow |\Psi^+,m_e\rangle$ are forbidden because they connect orthogonal electronic states. Thus, each level of the electronic doublet produces the same Mössbauer spectrum, consisting of a single absorption line (or a quadrupole doublet when nuclear quadrupole interactions are included; see spectrum 1 of Figure 4).

Case 2. $\Delta \gg |A_z|$ **and** $H \neq 0$. In the presence of an applied magnetic field the diagonal terms of the matrix of Table I compete with the off-diagonal terms. This causes mixing of the zero-field states Ψ^+ and Ψ^- and leads to the development of the "magnetic" states Φ^{+3} and Φ^{-3}. For $\beta H \gg |A_z|$ we can replace the spin operator S in eq 4 by the expectation value $\langle S \rangle$. For the $S = 3$ system we have $\langle S_z \rangle \approx \pm 3$ and $\langle S_x \rangle \approx \langle S_y \rangle \approx 0$ for the two lowest electronic levels, and eq 4 becomes an effective nuclear Hamiltonian

$$H_n = \langle S_z \rangle A_z I_z = -g_n\beta_n H_{int}I_z \qquad (11)$$

where the internal magnetic field is $H_{int} = -\langle S_z \rangle A_z/(g_n\beta_n)$. Figure 3 shows a plot of $\langle S_z \rangle$ versus $6g_z\beta H_z/\Delta$ for the $S = 3$ system considered here. By studying the Mössbauer spectra along this "magnetization" curve one can determine A_z at saturation ($\beta H/\Delta$ large) and Δ. Once the magnetization curve has reached saturation the Mössbauer spectra are essentially independent of the applied field, and they consist of sharp six-line patterns (spectrum

5 in Figure 4). This situation has been discussed in some detail in the literature.[17] For applied fields $H \leq 0.1$ T the nuclear Zeeman term has negligible effects on the splittings of the nuclear levels, and since the internal fields produced by the two electronic states are essentially equal, but opposite, both states yield the same Mössbauer spectrum. In strong applied fields, $\beta H \geq |D|$, the electronic ground doublet has a considerable Zeeman splitting, and thus only the lowest level is appreciably populated at $T \leq 4.2$ K. Moreover, in strong fields the electronic Zeeman term mixes the $M_s = \pm 2$ levels into the ground doublet. This yields finite values for $\langle S_x \rangle$ and $\langle S_y \rangle$, and the Mössbauer spectrum becomes sensitive to A_x and A_y (for a discussion of this point see ref 17).

Case 3: $\Delta < |A_z|$. For very small values of Δ the diagonal elements of the matrix of Table I dominate and the "magnetic" states $|\Phi^{+3},m\rangle$ and $|\Phi^{-3},m\rangle$ are the approximate eigenstates. In the limit $E/D = 0$, $\Delta \to 0$ and eq 6 yields

$$\epsilon(m) = \pm(3g_z\beta H_z + 3A_zm) \qquad (12)$$

and the doublet behaves in most respects like a Kramers doublet. Note that for $\Delta \ll |A_z|$ the eigenstates are independent of the applied field (as long as $\beta H \ll |D|$) and thus the zero-field Mössbauer spectrum exhibits magnetic hyperfine interactions (see Figure 2), a feature generally attributed to Kramers systems (S = half-integral). In our previous analyses[1,3] we had assumed that the state P^{OX}, which yields field-independent Mössbauer spectra (for $H < 0.3$ T) and zero-field spectra with magnetic features, was a Kramers system with $S \geq 3/2$ and $D < 0$, $E/D < 0.1$. For these parameters a system with half-integral spin yields a ground Kramers doublet for which

$$\epsilon(m) = \pm(Sg_z\beta H + SA_z'm) \qquad (13)$$

where the signs refer to the two electronic levels. The energy levels described by eqs 12 and 13 yield the same type of Mössbauer spectra. For $SA_z' = 3A_z$ the spectra are identical.

Results

Mössbauer Spectroscopy. The Mössbauer spectra of Av1, Kp1, Cp1, and Xa1 distinguish between two classes of iron sites; namely, those belonging to the M-centers (cofactors) and those assigned to the P-clusters.[1] In the semireduced protein the two (spectroscopically identical) cofactor clusters are paramagnetic (state M^N; $S = 3/2$) whereas the P-clusters are diamagnetic (state P^N; $S = 0$). In the state P^N we distinguish two types of sites: those with a large quadrupole splitting (component Fe^{2+}; $\Delta E_Q \approx 3$ mm/s, $\delta \approx 0.66$ mm/s, 25% of the P-cluster iron) and those with a small splitting (component D; $\Delta E_Q \approx 0.8$ and ≈ 1.3 mm/s, $\delta \approx 0.63$ mm/s, 75% of the P-cluster iron). In our initial work[1] we had labeled the $\Delta E_Q \approx 1.3$ mm/s sites as component S. Our subsequent work with isotopic hybrids (P-cluster enriched in ^{57}Fe, M-centers with natural abundance ^{57}Fe) showed, however, that component S belonged to the P-clusters and we have therefore relabeled it as D'.[18] After a six-electron oxidation with a dye such as thionin the two M-centers are observed in the diamagnetic state M^{OX} whereas the P-clusters are in the paramagnetic state P^{OX}. There is universal agreement that the oxidation $M^N \to M^{OX}$ is a one-electron process.

We wish to focus first on the spectra of thionin-oxidized Xa1. For this protein we have collected a large set of Mössbauer spectra in both oxidation states. We will provide a detailed analysis of these spectra elsewhere. Here we will discuss those spectral features which are relevant to the identification of the electronic state P^{OX}.

The Mössbauer spectra of semireduced Xa1 (data not shown here; see ref 14) are virtually identical to those reported for Av1, Cp1, and Kp1. Figure 5A and B shows high-temperature Mössbauer spectra of thionin-oxidized samples of Xa1 and Av1,

(17) (a) Zimmermann, R.; Huynh, B. H.; Münck, E.; Lipscomb, J. D. *J. Chem. Phys.* **1978**, *69*, 5463–5467. (b) Papaefthymiou, V.; Girerd, J.-J.; Moura, I.; Moura, J. J. G.; Münck, E. *J. Am. Chem. Soc.* **1987**, *109*, 4703–4710.
(18) McLean, P. A.; Papaefthymiou, V.; Orme-Johnson, W. H.; Münck, E. *J. Biol. Chem.* **1987**, *262*, 12900–12903.
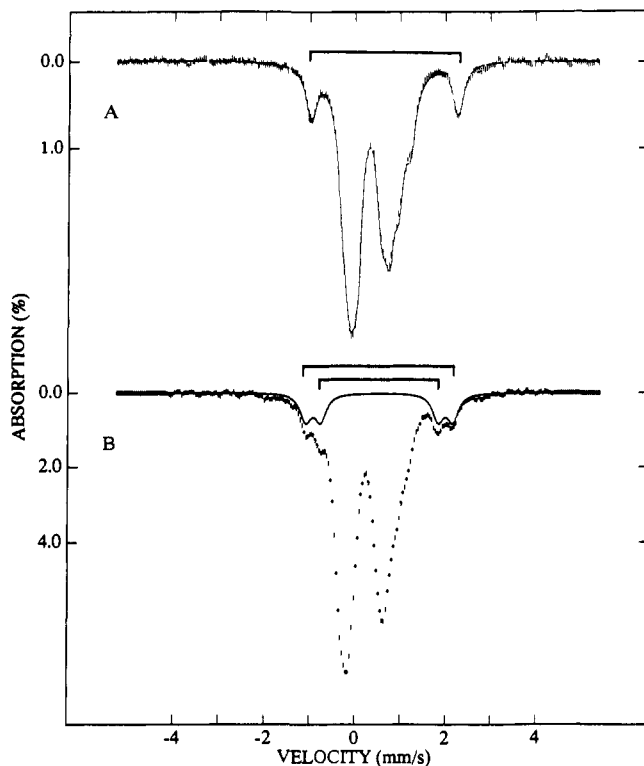
Figure 5. Mössbauer spectra of thionin-oxidized samples of (A) Xa1 and (B) Av1 recorded in zero-field at $T = 170$ K. The Av1 spectrum was obtained from the same sample as used by Zimmermann et al.[1] Components Fe^{2+} of the P-clusters are indicated by the brackets. The solid line in A is the result of fitting five quadrupole doublets to the data, with the purpose to determine the position and intensity of component Fe^{2+}. Component Fe^{2+} accounts for 13% of the total iron and has $\Delta E_Q = 3.28$ mm/s and $\delta = 0.67$ mm/s.

respectively. At the temperature of the experiments the relaxation rate of the electronic spin of the P-clusters in the state P^{OX} is fast on the time scale of Mössbauer spectroscopy and, consequently, the magnetic hyperfine interactions are averaged out. There is only one noteworthy difference between the spectra of Av1, Kp1, and Cp1 and that of Xa1; namely, the Fe^{2+} sites (four sites per $\alpha_2\beta_2$) of Xa1 contribute one quadrupole doublet (bracket in Figure 5A) whereas those of the other proteins contribute two doublets of equal intensity (brackets above the spectrum of Figure 5B). Component Fe^{2+} of Figure 5A accounts for 13% of the total absorption and has thus the same percentage as the sum of the two doublets indicated in Figure 5B (the Av1 spectrum has been discussed in detail by Zimmermann et al.[1]).

Figure 6 shows a 4.2 K Mössbauer spectrum of thionin-oxidized Xa1 recorded in a field of 45 mT applied parallel to the observed γ-radiation. In this spectrum the diamagnetic state M^{OX} contributes the intense doublet in the center of the spectrum. This doublet is essentially the same in all four proteins studied thus far. Its shape can be reasonably well synthesized by a superposition of quadrupole doublets, and for clarity, we have subtracted the contribution of M^{OX} from the spectra shown in Figures 7 and 8.[19]

(19) To prepare the P-cluster spectra of Figure 7 we have subtracted the doublet of M^{OX} at 48% of the total absorption. We do not wish to claim that 48% is the correct fraction. If the theoretical decomposition of P^{OX} is used as a guide the spectra of Figure 7 suggest that this fraction is somewhat too high for Av1 (and Kp1) and about right for Cp1. However, given the complexity of the spectra and given that two large clusters of unknown stoichiometry and substructure are involved, and given the possibility that both clusters may not be 100% in their presumed oxidation state, it would be foolish to claim an accuracy of better than one iron site per M-center. However, our studies suggest that a P-cluster has at least one, but not more than two, iron site more than an M-center. Overall, our Mössbauer data are best fitted to a model that considers a P-cluster with an Fe_8 stoichiometry and an M-center with $MoFe_6$ metal composition. However, an $MoFe_7$ stoichiometry would be compatible with the Mössbauer data if one of the iron sites would have unusually small magnetic hyperfine interactions in the $S = {}^3/_2$ state.
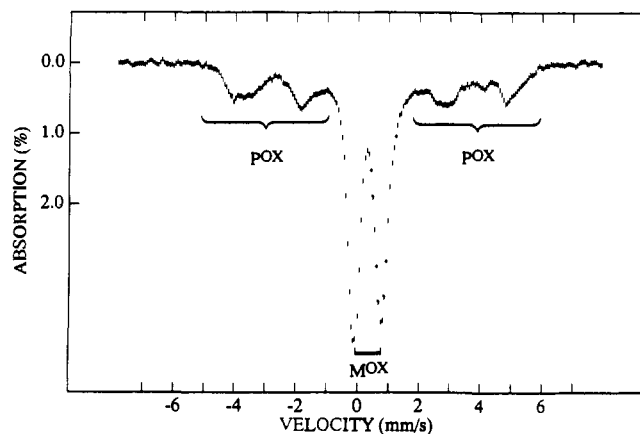

Figure 6. 4.2 K Mössbauer spectrum of ^{57}Fe-enriched thionin-oxidized Xa1 recorded in a parallel field at 45 mT. The spectral contributions of M^{OX} and P^{OX} are marked by the brackets (P^{OX} has contributions masked by M^{OX}).
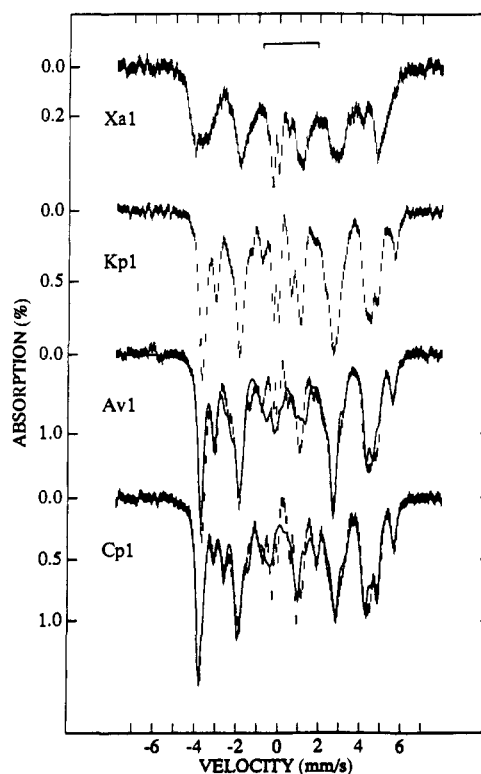

Figure 7. 4.2 K Mössbauer spectra of thionin-oxidized samples of Xa1, Kp1, Av1, and Cp1. The samples were oxidized by the addition of 6–7 equiv of thionin as described previously.[1,3] The contribution of M^{OX} has been subtracted from the data; the spectral region indicated by the bracket has therefore a distorted shape. The solid lines drawn through the Av1 and Cp1 spectra are the spectral simulations reported by Zimmermann et al.[1] and Huynh et al.[3]

Thus, the spectra of Figures 7 and 8 are those contributed by the P-clusters in the state P^{OX}.

Figure 7 shows the P-cluster spectra of Xa1, Kp1, Av1, and Cp1 recorded at 4.2 K in an applied magnetic field of 45 mT. The intensities of the absorption bands of these spectra are the same whether the field is applied parallel or transverse to the observed Mössbauer radiation, implying that the spectra result from an electronic doublet with uniaxial magnetic properties. The spectra of all four proteins are remarkably similar; those of Kp1 and Av1 are virtually indistinguishable under all conditions studied. The spectra shown for Av1 and Cp1 are those reported by Zimmermann et al.[1] and Huynh et al.,[3] respectively. The solid lines are theoretical curves obtained by decomposing the spectra of P^{OX}
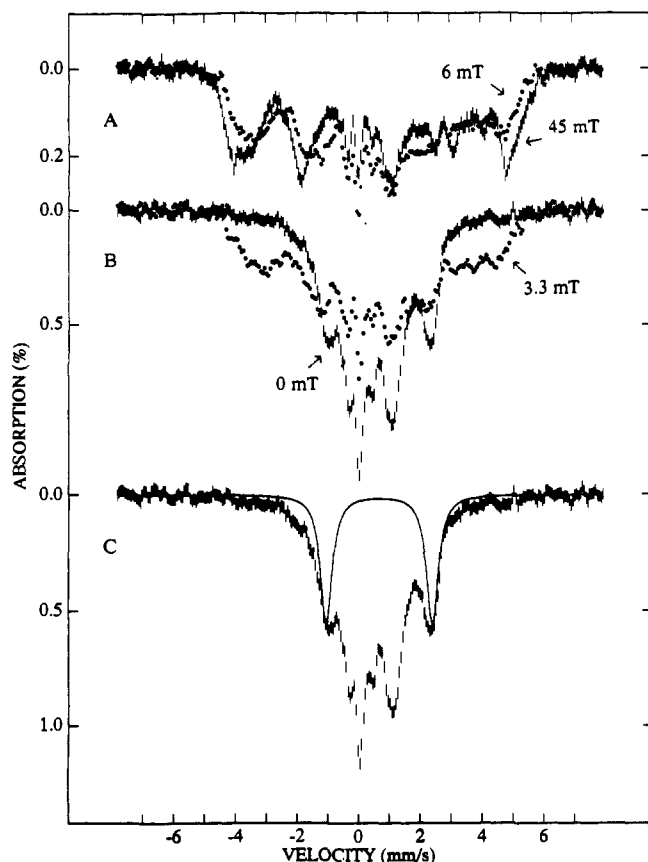
Figure 8. 4.2 K Mössbauer spectra of thionin-oxidized Xa1 with the contribution due to M^{OX} removed from the data (48% of total ^{57}Fe absorption) recorded at (A) 45 mT (hash marks) and 6 mT (circles), (B) zero field (hash marks) and 3.3 mT (circles), and (C) zero field. The solid line in (C) outlines the Fe^{2+} component.

into eight subspectra, each subspectrum consisting of a six-line pattern. Although the theoretical curves were generated[1,3] under the assumption that the state P^{OX} is a Kramers system (with a ground doublet of uniaxial magnetic properties), the magnetic hyperfine interactions were quoted as internal magnetic fields, H_{int}, and they are thus not affected by the reinterpretation of P^{OX} as a non-Kramers system.

The low-temperature ($T \leq 4.2$ K), low-field ($H < 0.1$ T) spectra of Cp1 are independent of the strength of the applied field. The 4.2 K zero-field spectrum of Cp1, in fact, is identical to the spectrum shown in Figure 7. The 4.2 K spectra of Av1 and Kp1 are independent of the strength of the applied field for 2 mT $< H < 50$ mT. At fields smaller than 2 mT the absorption bands broaden slightly. Figure 8 shows a series of Xa1 P-cluster spectra recorded at 4.2 K in zero-field (C) and in applied fields of 3.3, 6, and 45 mT. Virtually identical spectra were observed at 1.5 K. The spectra shown are typical of those originating from a system with integer electronic spin as discussed above for case 2 ($\Delta \gg |A_z|$). The zero-field spectrum of Figure 8C consists of a sum of quadrupole doublets. The solid line, drawn to represent 13% of the total Fe absorption of the sample, outlines the doublet of component Fe^{2+}. Its absorption lines are about 50% broader than those observed at 170 K. A weak applied field induces a magnetic hyperfine field which, for $H \leq 7$ mT, increases with the strength of the applied field. Since eight subsites contribute to the spectrum the magnetic pattern is, of course, quite complicated. However, one can recognize the typical features of spectra collected along a magnetization curve of a system with $\Delta \gg |A_z|$. For example, the spectra of Figure 8A show an increase of the internal fields and a transfer of intensity toward higher absolute velocities with increasing field. The transfer of intensity can be understood with reference to Figures 3 and 4. Spectrum 5 of Figure 4 obtained for $6g_z\beta H_z/\Delta = 7$ consists of six fairly sharp lines. For

comparison consider spectrum 4 for which $6g_z\beta H_z/\Delta = 2$. For a sample containing randomly oriented molecules the z-component of the applied field is $H_z = H \cos \theta$ where θ is the angle between the applied field and the *molecular* z-direction. For a system that is uniaxial along z only H_z is effective. Thus, molecules for which $\theta > 0$ have smaller values of $\langle S_z \rangle$ and exhibit therefore smaller magnetic splittings. In a powder average one obtains then a distribution of internal fields. In contrast, for $6g_z\beta H \gg \Delta$ the component H_z is large enough to saturate $\langle S_z \rangle$ for most molecular orientations and thus most molecules exhibit the full magnetic hyperfine splittings; the resulting powder pattern consists then of six sharp lines. The observation of quadrupole doublets in zero-field, and the characteristic development of magnetic splittings with increasing applied field are typical for integer spin systems and incompatible with a Kramers system. In order to estimate Δ for Xa1 we have simulated Mössbauer spectra for single sites and compared the splittings with those observed in Figure 8.[20] These simulations suggest that $\Delta \approx (9 \pm 3) \times 10^{-3}$ cm^{-1}. This value is large enough to provide sufficient EPR intensity for a transition within the nearly degenerate ground doublet (see below).

The states P^{OX} of Av1 and Kp1 have substantially smaller values of Δ as witnessed by the observation that a field as small as 1 mT will induce a fully developed magnetic pattern. This suggests that $\Delta \approx 1 \times 10^{-3}$ cm^{-1} for these two proteins. In zero applied field we observed a magnetic hyperfine pattern similar to those shown in Figure 7, except that the absorption lines were about twice as broad. This observation suggests that $\Delta \approx |A_z|$ in the matrix of Table I. As can be seen from an inspection of the energy levels of Figure 2 the energy of line 1 decreases and that of line 6 increases as the ratio of $\Delta/|A_z|$ increases from 0 to 1. In this region the electronic states are mixed by the hyperfine term and transitions are possible between virtually all levels. A further increase of Δ leads to a rapid decrease of the intensity of lines 1 and 6 because the transitions connect then different electronic states. Of course, the situation is more complex for the P-clusters because one has to consider the magnetic hyperfine interactions of all (probably eight) subsites. If the local A-tensors have the same principal axis system as the zero-field splitting tensor, or if the A-tensors are reasonably isotropic, the quantity A_z in Table I has to be replaced by $\sum^n_{i=1} A_z(i)$ where n is the number of subsites.[21]

We recognize now that the broadening of the zero-field spectra of Kp1 and Av1 is an indication that P^{OX} is a non-Kramers system. Such broadening should not occur for an uniaxial doublet of a Kramers system. A uniaxial Kramers doublet yields a matrix similar to that of Table I except that $\Delta = 0$. Thus, any A_z will force the system into the same eigenstate as that produced by a weak applied field. When we noticed the broadening first in 1977 we suspected that spin–lattice relaxation was the cause for the broadening, although we were not aware of any mechanism that leads to faster relaxation for decreasing applied field. (The electronic spin relaxation rate of P^{OX} becomes fast for $T > 10$ K for all four proteins studied here, and we have therefore studied the spectra carefully at different applied fields and temperatures down to 1.5 K. The spectra shown in Figures 6–8 were all recorded in the slow-fluctuation limit.)

(20) Although we have obtained a spectral decomposition of the Xa1 spectrum of Figure 7, the fits are of poorer quality than those shown for Av1 and Kp1. Moreover, there is no assurance that this decomposition is unique. Using Kp1 samples with P-clusters selectively enriched in ^{57}Fe, we have embarked on a program to obtain improved fits of the Mössbauer spectra of P^{OX}. Improved fits should guide us to better simulations of the Xa1 spectra. It is very difficult, even for a mononuclear iron complex, to obtain good fits to the shapes of Mössbauer spectra recorded in the region corresponding to the steep rise (for $6g_z\beta H/\Delta < 1$) of the magnetization curve of Figure 3. In this region distributions in Δ tend to broaden the absorption lines substantially. The simulations mentioned in the text show the correct movement of the lines as the function of the applied field. However, given the large number of unknowns and the heterogeneity problem it is unrealistic to expect a close match of the theoretical curves with the data in the rising portion of the magnetization curve.

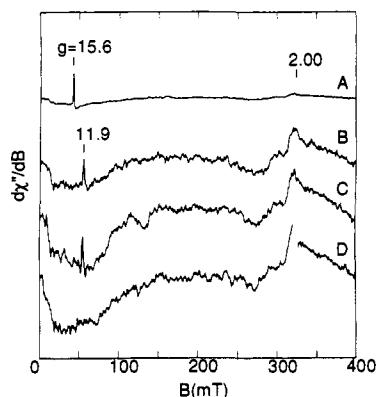(21) Rotated A-tensors do not change the argument qualitatively.

Figure 9. X-band EPR spectra of thionin-oxidized (A) Xa1, (B) Av1, (C) Kp1, and (D) Cp1 recorded with H_1 parallel to H. Instrumental conditions: (A, D) T = 2 K, (B, C) T = 20 K; 2 mW microwave power at 9.08 GHz; 1 mT$_{pp}$ modulation amplitude at 100 KHz; gain (A) 10^3, (B, C, D) 10^4; dH/dt = 1.7 mT/s.
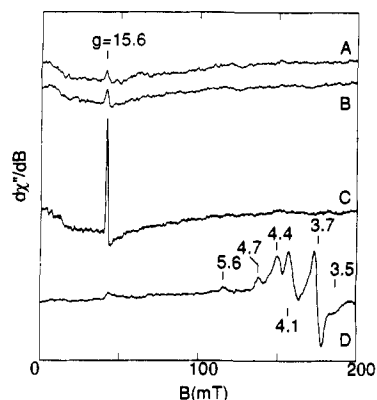

Figure 10. EPR spectra of thionin-oxidized Xa1 recorded with H_1 parallel to H at (A) 20 K, (B) 10 K, (C) 3 K and (D) with $H_1 \perp$ H at 3 K. Instrumental conditions: 2 mW microwave power at (A, B, C) 9.08 GHz and (D) 9.14 GHz; 1 mT$_{pp}$ modulation amplitude at 100 KHz; 1.25 $\times 10^4$ gain; dH/dt = 0.8 mT/s.

As pointed out above, the 4.2 K zero-field spectrum of Cp1 is identical to those recorded in weak applied fields. This suggests that Δ is small compared to the magnitude of $\sum^n_{i=1}A_z(i)$. Assuming S = 3 (but see below) we can sum the internal magnetic fields, $H_{int} = 3A_z(i)/g_n\beta_n$, listed in Table II of Huynh et al.,[3] to obtain $\sum^n_{i=1}A_z(i)$ = 1.49 $\times 10^{-3}$ cm^{-1}. It appears that $\Delta \leq 0.5 \times 10^{-3}$ cm^{-1} for Cp1.

Finally, we wish to comment on the widths of the lines of Figure 8C, focusing on the resolved features of component Fe^{2+}. The sharpness of the Fe^{2+} component at 170 K rules out heterogeneities as the cause for the broad lines observed at 4.2 K. Further, the broadening cannot be caused by intermediate spin relaxation rates; the applied field spectra prove that the relaxation rate is slow at 4.2 K. Most probably, the line width at 4.2 K reflects the fact that the ratio $\Delta/|\sum^n_{i=1}A_z(i)|$ is not infinite but ca. 4–8, and the lines thus are broadened by incipient magnetic interactions.

EPR Spectroscopy. EPR spectra of thionin-oxidized Xa1, Av1, Kp1, and Cp1 are shown in Figure 9. These spectra were recorded in "parallel mode", i.e., under conditions where the magnetic component of the microwave field, H_1, fluctuates parallel to the static field, H. The broad feature at g = 2 in all spectra is a cavity impurity. The sharp feature at g = 2 in Figure 9D is from a radical species; this resonance was not fully attenuated in *parallel* mode. All low-field resonances are significantly attenuated in *transverse* mode, $H_1 \perp$ H, showing that these features originate from nearly degenerate doublets of a system with integer spin.

The 3 K EPR spectrum of Xa1 exhibits two low-field features, namely, a sharp resonance at g_{eff} = 15.6 and a broad underlying feature in the range g = 12 to 16; the latter is assigned to a minority species (see below). As shown in Figure 10 the intensity
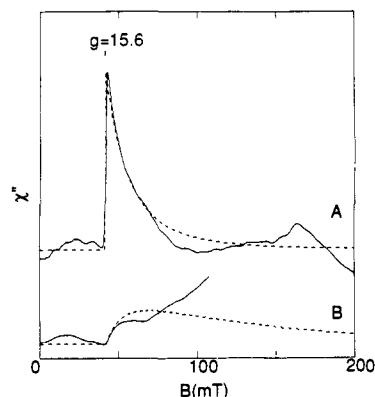

Figure 11. Integrated X-band EPR spectra (solid line) and simulations (dashed line) of thionin-oxidized Xa1 recorded with (A) H_1 parallel to H and (B) $H_1 \perp$ H. Simulation parameters: S = 3, Δ = 0.009 cm^{-1}, and g_z = 2.26. Simulations are normalized relative to Fe-doped zinc fluorosilicate[9]. Instrumental parameters are given in Figure 10 for T = 3 K. The experimental spectrum in B of the integer-spin signal at g = 15.6 is partially obscured by minority signals which dominate the spectrum above 70 mT.

of the sharp feature is at maximum at the lowest temperature indicating that the g_{eff} = 15.6 resonance originates from a ground doublet. In transverse mode the amplitude of the g_{eff} = 15.6 resonance is reduced approximately 16-fold (Figure 10D). This reduction is much larger than the factor of 3 typically observed,[9] and thus these signals are difficult to observe with standard transverse mode cavities. The transverse-mode spectrum of Figure 10D shows also a number of resonances in the g = 3.6 to 6 region. The resonances at g = 4.1 and 3.7 belong to a very small remnant of the semireduced form (S = $^3/_2$) of the M-center. The signals at g = 5.6, 4.7, 4.4, and 3.5 are minority Kramers species of unknown nature; they were present already in the native, semireduced state of this particular protein. The species were not observed with Mössbauer spectroscopy (probably because of their low concentration).

Quantitative analyses have been described in previous papers for a variety of iron-containing proteins and synthetic complexes.[9,22,23] For the spectra of Figure 9 the method of analysis must be modified, primarily because the value Δ of the doublet splitting is so small. The analyses cited above have shown that the line shapes of the spectra were dominated by a distribution of the zero-field parameters (D, E). There is no evidence that the nitrogenase resonances are broadened by this mechanism. In fact, there is surprisingly little broadening from sources such as g-strain or unresolved hyperfine interactions. The shape of the spectrum is nearly equal to that of a pure powder average, as demonstrated in Figure 11 which shows simulations overlayed on integrated spectra of Figure 10C and D. The simulations were performed for the Ψ_1 and Ψ_2 states of an S = 3 manifold, using Δ = 0.009 cm^{-1} and g_z = 2.26. The simulations predict reasonably well the relative intensities of the parallel and transverse mode spectra with no adjustable parameters.

The intensity of the g_{eff} = 15.6 resonance depends on Δ^2 and the cluster concentration. If the P-cluster concentration were known the value of Δ could be determined from EPR. Conversely, if Δ is known from an independent measurement, the cluster concentration can be determined. For the simulations of Figure 11 we have used the value Δ = 0.009 cm^{-1} estimated from analysis of the Mössbauer spectra. Comparison of the simulations of Figure 11 with those obtained for an appropriate spin standard (see Experimental Section) gave a normalization factor which allowed an estimate of the P-cluster concentration; we obtained a spin concentration of 210 μM which is within experimental error equal

(22) Hendrich, M. P.; Münck, E.; Fox, B. G.; Lipscomb, J. D. *J. Am. Chem. Soc.* **1990**, *112*, 5861–5865.

(23) Juarez-Garcia, C.; Hendrich, M. P.; Holman, T. R.; Que, L.; Münck, E. *J. Am. Chem. Soc.* **1991**, *113*, 518–525.
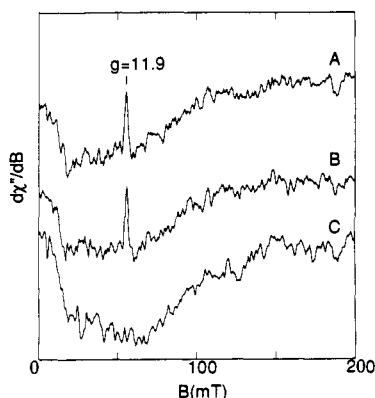
Figure 12. EPR spectra of thionin-oxidized Av1 recorded with H_1 parallel to H at (A) 20 K, (B) 10 K, and (C) 3 K. Instrumental conditions: 2 mW microwave power at 9.08 GHz; 1 mT_{pp} modulation amplitude at 100 KHz; 2.5×10^4 gain; $dH/dt = 1.7$ mT/s.

to the M-center concentration (≈ 200 μM) of the Xa1 sample.[24] Arguments presented below as well as the preliminary X-ray results[12] suggest that the MoFe protein contains two M-centers and two P-clusters. Thus, the spin quantitation of the Xa1 signal shows that the $g_{eff} = 15.6$ resonance belongs to a cluster which is present in stoichiometric concentrations, i.e., about two per MoFe protein. From our discussions of the Mössbauer and EPR spectra of integer spin systems it follows that the $g_{eff} = 15.6$ resonance originates from the same doublet as the Mössbauer spectra of Figure 8. This completes the proof that the state P^{OX} of Xa1 has integer electronic spin.

The spectral simulations of Figure 11 were performed in the framework of an $S = 3$ spin Hamiltonian. Equation 10 shows that the transition probability depends on Δ and H_z but not on the values of S. Thus, the same simulations could be obtained by choosing a different value for S. In fact, the Mössbauer and EPR results do not require that the states Ψ_1 and Ψ_2 of the nearly degenerate doublet are members of a pure spin multiplet. The only model-dependent quantity of the simulations is g_z. For a chosen spin the value of g_z is determined by the position of the resonance. Choosing a multiplet with $S \neq 3$ will yield a different value for g_z but the same values for Δ and the P-cluster concentration. (For a multiplet with spin S the effective g-value would be $g_{eff} \approx 2Sg_z$.)

The Xa1 spectra exhibit a broad underlying feature between $g = 12$ and 16. Its origin is uncertain, but its shape is typical of spectra analyzed in previous studies.[9] Spin quantitation suggests that it represents <5% of a minority species of the protein.

The EPR spectra of thionin-oxidized Av1 and Kp1 are essentially identical, as are the corresponding Mössbauer spectra. As shown in Figure 9B and C both proteins exhibit a sharp resonance at $g_{eff} = 11.9$. For Av1, the sharp resonance is not observed at the lowest temperatures (see Figure 12) and we thus conclude that it originates from transitions between excited states of the spin manifold. The same observations apply to Kp1. Our variable temperature EPR data do not yet have the desired precision to allow a more detailed analysis of the system. However, we can make a rough estimate of the energy of the EPR-active doublets of Av1 and Kp1 by assuming the $g = 11.9$ resonances result from

transitions between the highest two levels of an $S = 3$ system. With this quite arbitrary (see below) assumption we can place the EPR-active doublet at 10–15 cm^{-1} above the ground state.

The absence of an EPR signal for Cp1 can be rationalized in various ways. The simplest assumption is that E/D of eq 1 is very small. For $E/D = 0$ all doublets of multiplets with integer electronic spin will be EPR-silent; for $E/D = 0$ the splitting Δ is zero for all doublets and thus the EPR transition probability is zero according to eq 10. By amending eq 1 by fourth and sixth order terms one can concoct situations where the Δ-value of the ground state is vanishingly small and excited-state splittings are too large to be amenable to X-band EPR spectroscopy.

Discussion

Electronic Structure. We have shown above that the state P^{OX} is a non-Kramers system and that the 4.2 K Mössbauer spectra reflect a nearly degenerate doublet with uniaxial magnetic properties. The electronic splitting of the ground doublet, Δ, varies by about 1 order of magnitude between Cp1 ($\Delta < 0.5 \times 10^{-3}$ cm^{-1}) and Xa1 ($\sim 10^{-2}$ cm^{-1}). Nevertheless, Δ is remarkably small for all four proteins. The quadrupole splittings of the iron sites of the P-clusters in the state P^{OX} are essentially independent of temperature, indicating that the ground-state manifold is an orbital singlet well isolated from excited orbital states. The lowest energy levels thus consist of a manifold of spin levels. The remarkable Mössbauer spectroscopic features of the Cp1, Av1, and Kp1 P-clusters result from the circumstance that the two lowest electronic spin levels are so close in energy that they are effectively mixed by ^{57}Fe hyperfine interactions. We are not aware of any other iron compound with integer electronic spin that has similar properties. The Δ-values of the clusters of Cp1, Av1, and Kp1 have Δ-values so small that the ground doublet behaves like a Kramers doublet under standard conditions for magnetic susceptibility studies and Mössbauer and MCD spectroscopy. EPR spectroscopy, however, recognizes the non-Kramers nature of the system.

One of the enigmatic features of P^{OX} has been the fact that no EPR signals were observed during the previous 15 years of study. Zimmermann et al.[1] have argued from the Mössbauer properties of the Av1 and Cp1 clusters that the electronic ground doublet was not expected to exhibit an EPR signal. The arguments advanced by these authors, with slight modifications for the fact that P^{OX} is a non-Kramers state, are still valid. It required the development of quantitative integer-spin EPR[9] to bring EPR to bear on the problem. For Δ-values of the order of 0.1 cm^{-1}, integer-spin EPR signals can be quantitated quite well[23,25] to yield the spin concentration of the species of interest. On the other hand, for $\Delta \ll h\nu$ the resonance condition is $h\nu = (\Delta^2 + (2Sg_z\beta H)^2)^{1/2}$ $\approx 2Sg_z\beta H$ and thus the value of Δ, required for evaluating the intensity, cannot be determined directly from EPR unless g_z is known with precision. We have used here Mössbauer spectroscopy to estimate Δ for Xa1, and we were thus able to determine the EPR spin concentration. The value obtained is in good agreement with the concentration of P^{OX} estimated by Mössbauer spectroscopy. This links the integer-spin EPR signal of Xa1 to spectral component P^{OX} defined by Mössbauer spectroscopy, and thus the integer-spin character of P^{OX} is proven by both spectroscopies.

The immediate consequence of assigning an integer spin state to P^{OX} is that the number of iron atoms per P-cluster doubles. Since both P^N and P^{OX} are now cluster states with an even number of electrons, the oxidation $P^N \rightarrow P^{OX}$ must involve the transfer of two rather than one electron per cluster. Since four electrons are required to oxidize the P-clusters[1,3] the spectroscopic data suggest that the MoFe protein contains two, rather than four, P-clusters per $\alpha_2\beta_2$. Moreover, since approximately 16 Fe atoms per $\alpha_2\beta_2$ contribute to the P-clusters Mössbauer spectra,[1,3] a cluster with eight iron atoms is indicated. This interpretation is compatible with the previous decompositions of the P^{OX} Mössbauer spectra into eight subcomponents.[1,3] We suggest that the iron sites

(24) The Xa1 sample was prepared in 1985 and transferred into a Mössbauer cuvette. To our knowledge this is the only existing ^{57}Fe-enriched Xa1 sample. After some initial studies in 1985 the sample was stored in liquid N_2. After recording the spectra of Figures 6–8 in November 1991 we transferred the material into an EPR tube. In principle, we could relate the spin concentration to the protein and/or iron concentration by removing aliquots for appropriate analysis. We are hesitant to deplete this valuable sample and subject it to further manipulations and freeze–thaw cycles. We have, therefore, estimated the M-cluster concentration from the observed Mössbauer absorption by comparing the spectra with those of the well characterized Av1 protein. The estimated M-center concentration of ≈ 200 μM has an uncertainty of $\pm 20\%$ and since the EPR quantitations has a larger uncertainty, at least $\pm 50\%$, a precise protein and iron determination will not significantly alter our conclusions.

(25) Hendrich, M. P.; Pearce, L. L.; Que, L., Jr.; Chasteen, N. D.; Day, E. P. *J. Am. Chem. Soc.* **1991**, *113*, 3039–3044.

formerly attributed to the two slightly different P and P' clusters[18] belong to one P-supercluster. With this interpretation the Mössbauer spectra of the four MoFe proteins studied reveal two pairs of identical P-clusters and two pairs of identical M-centers, in accord with the dimeric nature of the protein. This interpretation is in agreement with recent X-ray crystallographic studies of Cp1 which suggest that the MoFe protein contains a total of four metal clusters.[12]

What is the value of the electronic spin of the cluster state P^{OX}? For Xa1 we have only information for the ground doublet. Assuming that orbital contributions to g in eq 1 are small, the g_{eff} = 15.6 resonance can either be assigned to a multiplet with S = 3 and g_z = 2.26 or to an S = 4 spin system with g_z = 1.95. If the spin manifold is describable by eq 1 the zero-field splitting parameter D must be negative in order to produce a ground doublet with a sufficiently small Δ. According to our EPR studies the ground doublet is approximately 20% depopulated at T = 20 K which yields $D \approx -4$ cm^{-1}, regardless of whether S = 3 or 4. For $D = -4$ cm^{-1} the required $\Delta \approx 0.01$ cm^{-1} results when E/D = 0.09 for S = 3 or E/D = 0.18 for S = 4. Interestingly, the next higher pair of energy levels, for either choice of spin, is predicted to have a splitting $\Delta \approx 0.45$ cm^{-1} and could thus yield an EPR resonance at Q-band. The Xa1 data are incompatible with a positive D-value because a ground doublet splitting of $\Delta \approx 0.01$ cm^{-1} would require $D < 0.1$ cm^{-1}, resulting in a multiplet splitting of less than 1 cm^{-1}. The Mössbauer studies rule out this possibility; the data show that no levels other than the ground doublet are measurably populated at 4.2 K. In conclusion, the Mössbauer and EPR data of Xa1 are compatible with an isolated S = 3 or 4 multiplet with $D \approx -4$ cm^{-1}.

For Av1 and Kp1 the observed integer-spin resonance results from transitions between excited spin levels. As discussed above, the states Ψ_1 and Ψ_2 of eq 8 produce for small values of Δ a resonance slightly below $g_{eff} \approx 6g_z \approx 12$ if we assume that there is negligible orbital moment in the system, i.e., if $g_z \approx 2$. Johnson and co-workers[7] have studied thionin-oxidized Kp1 with low-temperature MCD spectroscopy and have concluded that the two *lowest* electronic levels split in a magnetic field corresponding to an effective g-value between 10.7 and 12.5. The ground- and excited-state g-values of Kp1 and Av1 are compatible with an S = 3 system describable by eq 1. For instance, for $D < 0$, E/D = 0.19, g_y = 2.1, and g_z = 2.0 eq 1 yields g_z = 11.9 for the ground doublet (compatible with MCD) and g_y = 11.9 for the uppermost pair of levels (compatible with EPR). However, when the Δ-values are taken into account as well it is difficult to reconcile the EPR and Mössbauer data with the simple S = 3 spin Hamiltonian of eq 1, for the following reason. The temperature dependence of the g_{eff} = 11.9 resonance of Kp1 and Av1 suggests that the EPR-active doublet is approximately 10–15 cm^{-1} above the ground state. This would suggest that $|D| \approx 1.0$–1.5 cm^{-1}. The intensity of the resonance depends on the population of the doublet, the Δ-value of the excited-state doublet (Δ_e), and the concentration of the species giving rise to the resonance. Assuming two P-clusters per MoFe protein, and assuming that >95% of the P-clusters are in the state P^{OX} (as indicated by the Mössbauer spectra), the observed intensity of the g_{eff} = 11.9 resonance suggests $\Delta_e \approx$ 0.02–0.04 cm^{-1} for the EPR-active doublet. For the quoted range of $|D|$ only the $M = \pm 3$ doublet (Ψ_1 and Ψ_2) can yield a sufficiently small Δ_e. For example, $D = +1$ cm^{-1} and E/D = 0.22 yield Δ_e = 0.03 cm^{-1}. However, these values for D and E yield Δ = 0.23 cm^{-1} for the ground doublet, a value that is more than 200-fold larger than the value obtained from Mössbauer spectroscopy.[26]

There are at least two possible explanations for this (perhaps minor) discrepancy. First, the $g_{eff} \approx 11.9$ resonances in Kp1 and Av1 could represent a minority species not recognized with Mössbauer spectroscopy. With regard to this possibility we offer the following considerations. As shown above the Mössbauer spectra of Av1 and Kp1 are virtually indistinguishable, and interestingly, both proteins exhibit identical integer-spin resonances. Since the EPR intensity is proportional to the concentration of the EPR-active species and to Δ_e^2, assignment of the $g_{eff} \approx 11.9$ resonances to a minority species (for example, with <0.2 spins per MoFe protein) would require a Δ_e of at least 0.1 cm^{-1}. In our experience (see refs 9, 22, and 23) the integer-spin signals of doublets with $\Delta \geq 0.1$ cm^{-1} have line shapes substantially different from those observed here and thus we consider it unlikely that the g_{eff} = 11.9 resonances belong to a minority species.

Alternatively, and most likely, the discrepancy between the ground-state Δ-values inferred from Mössbauer and EPR spectroscopy may suggest that eq 1 is inadequate for describing the low-lying spin levels of P^{OX}. We wish to stress that the analysis of the EPR and Mössbauer data shows the presence of an integer-spin system with nearly degenerate pairs of spin levels; the analysis does not require that these levels belong to an isolated multiplet with well defined spin S. Thus, although it is clear that the iron sites of the P-clusters are exchange coupled, little is known about the details of the coupling. If the coupling is such that the resultant spin multiplets of P^{OX} are well separated in energy, eq 1 should be a good approximation for describing the magnetic properties of the low-lying spin levels. On the other hand, if the cluster has closely spaced spin multiplets, the states will be mixed by zero-field splitting terms (in the language of the spin Hamiltonian approximation) and the system spin S will not be a good quantum number.[27] As a consequence, eq 1 would be a poor approximation to describe the resulting mixed-spin states.

The parameters of eq 1 can generally be adjusted to describe the behavior of one nearly degenerate doublet. However, for mixed-spin states eq 1 would fail to predict the properties of excited-state EPR resonances from the Δ and g_{eff} values obtained for the ground doublet. It can be quite a task to establish the character of a family of low-lying spin states in even much simpler systems. For instance, for the diferrous form of the binuclear cluster of methane monooxygenase it has not yet been possible to deduce from EPR and Mössbauer studies whether the two iron sites are coupled by weak or by strong ferromagnetic interactions, i.e., it was not possible to decide whether the low-lying spin states form an S = 4 multiplet or whether these states are best described by two weakly interacting S = 2 systems (see Figures 3 and 4 of ref 22). The coupling scheme of the P-clusters is certainly much more difficult to unravel. Regardless of whether the cluster contains six ferrous and two ferric sites or four ferrous and four ferric sites the spin coupling scheme involves >5 × 10^5 microstates organized into many spin multiplets. Conceptually, a simplification may arise if a P-cluster would be a supercluster consisting of two bridged Fe$_4$S$_4$ cubanes (see below). In this case the coupling might involve two very similar cubanes with strong intrinsic coupling coupled by (perhaps weaker) exchange interactions mediated by the bridging ligand(s). Such a situation could give rise to a high density of low-lying spin levels, resulting in a ground multiplet insufficiently separated in energy from other multiplets to be describable in all details by eq 1.

Although the Mössbauer spectra of Xa1 are very similar to those of Av1, Kp1, and Cp1, the state P^{OX} of Xa1 differs from that of the other proteins in two respects. First, all "Fe^{2+}" sites of P^{OX} of Xa1 have the same ΔE_Q values whereas the other proteins have two types of similar Fe^{2+} sites. This implies that the 4.2 K spectrum of Xa1 shown in Figure 7 has to differ somewhat from those of Av1, Kp1, and Cp1; this is indeed observed. Second, the ground-state Δ-value is substantially bigger for Xa1 but still very small compared to all other cases we have found in the literature. Although the differences between the P^{OX} state of Xa1 on one hand and Av1, Kp1, and Cp1 on the other hand seem to be minor, one might ask whether P^{OX} of Xa1 is fundamentally different.

(26) In order to obtain a better agreement between the Mössbauer and EPR results of Av1 and Kp1 one could keep $D < 0$ and decrease Δ_e by adding fourth and sixth order terms to the spin Hamiltonian of eq 1, with the goal of maintaining a small Δ but decreasing Δ_e. Inclusion of fourth order terms improved the theoretical description somewhat but as yet we have found no parameter set which matches the data to our satisfaction.

(27) Debrunner and co-workers have analyzed mixing of spin multiplets by zero-field splitting terms for the binuclear iron center of uteroferrin: (a) Sage, J. T.; Debrunner, P. G. *Hyperfine Interact.* **1986**, *29*, 1399–1402. (b) Sage, J. T.; Xia, Y.-M.; Debrunner, P. G.; Keough, D. T.; de Jersey, J.; Zerner, B. *J. Am. Chem. Soc.* **1989**, *111*, 7239–7247.

We do not have as much experience with Xa1 as we have with Av1 and Kp1, but all evidence supports that we are studying fundamentally the same state. Thus, the Mössbauer spectra of P^N are virtually identical in all proteins, and the same treatment, namely addition of ca. 6 oxidizing equiv of thionin, elicits the spectra we refer to as P^{OX}. Moreover, as shown by EPR the state P^{OX} of Xa1, Kp1, and Av1 is a non-Kramer system. Finally, the EPR and Mössbauer data suggest that the state P^{OX} of Xa1 is homogeneous, i.e., the spectra reflect one spin system. It is conceivable that small differences in the exchange coupling among the eight iron sites of a P-cluster yield a different integer-spin state (for example, $S = 4$ vs $S = 3$) for Xa1; however, we believe that the state P^{OX} is fundamentally the same for all proteins studied. It is clear further studies of the electronic structure of P^{OX} are warranted.

The interpretation of P^{OX} as an integer-spin system sheds also a new light on the nature of the $S = {}^7/_2$ state observed when MoFe protein is oxidized by solid thionin. Resonances at $g = 10.4$, 5.8, and 5.5 were first reported by Hagen and co-workers and convincingly assigned to an $S = {}^7/_2$ system.[28] On the basis of their EPR studies these authors have proposed two distinct models for the P-clusters. Their first model considers two P-clusters, each containing eight iron atoms; in this model the $S = {}^7/_2$ state is attained by a three-electron oxidation of the state P^N. Although we previously rejected their model on the basis of a combined Mössbauer and EPR study,[29] the identification of P^{OX} as an integer-spin state lets us conclude that the proposal by Hagen and co-workers is probably correct, i.e., the eight-iron P-cluster undergoes the oxidation $P^N \xrightarrow{-2e^-} P^{OX}$ (integer spin) $\xrightarrow{-1e^-} P$ ($S = {}^7/_2$).

Structural Implications. The spectroscopic and chemical data reported to date do not give sufficient information to define a structure for the P-clusters. However, the chemical evidence[30-32] is that the P-clusters yield Fe_4S_4 cores upon displacement from the protein with added thiophenol. It is thus not unreasonable to try to explain the spectroscopic and (low-resolution) crystallographic data at hand in terms of linked Fe_4S_4 cores. In this light it is useful to discuss some of the features which have emerged from the Mössbauer studies of the P-clusters. The spectral analyses reported for Av1[1] and Cp1[3] suggest that the spectra of P^{OX} consist of a superposition of eight subspectra, suggesting an Fe_8S_x supercluster.

One of the distinctive spectroscopic signatures of the P-clusters is Mössbauer component "Fe^{2+}". This component has a quadrupole splitting and an isomer shift very similar to that observed for the high-spin ($S = 2$) ferrous site of rubredoxin. Since the "Fe^{2+}" is a member of a spin-coupled cluster its magnetic hyperfine interactions depend on the exchange couplings among the cluster sites. In the state P^N the "Fe^{2+}" site resides in a diamagnetic cluster and, therefore, does not exhibit magnetic hyperfine interactions. Since our first observations of the P-cluster Mössbauer spectra we have been intrigued by component "Fe^{2+}" and we have wondered what structural features would cause the formation of such a trapped-valence site. During the past few years we have realized that the features of component "Fe^{2+}" are entirely consistent with the presence of a tetrahedral site of sulfur ligands as commonly observed in iron-sulfur clusters; no special coordination features seem to be required. This conclusion is strongly supported by Mössbauer studies of the $[ZnFe_3S_4]^{1+}$ cluster of *Desulfovibrio gigas* ferredoxin II.[33-35] X-ray crystallographic studies of fer-

redoxin II have shown[36] that the native protein contains a cubane Fe_3S_4 cluster where each of the three Fe atoms is connected to the protein by coordination to a single cysteine sulfur, providing a tetrahedral FeS_3S-cys environment. $[ZnFe_3S_4]^{1+}$ is produced by incorporation of Zn^{2+} into the empty site of the cubane cluster. Mössbauer studies of $[ZnFe_3S_4]^{1+}$ have revealed a delocalized $Fe^{2+}-Fe^{3+}$ pair and a trapped-valence Fe^{2+} site; the latter had $\Delta E_Q \approx 3.1$ mm/s and $\delta = 0.65$ mm/s, parameters virtually identical to those of component "Fe^{2+}". Bioinorganic chemists may note that one does not necessarily have to design P-cluster model complexes such that the D:"Fe^{2+}" = 3:1 ratio is built into the ligand structure. The spectrosopic features of component Fe^{2+} may be the result of electronic effects similar to that giving rise to the Fe^{2+} trapped-valence of $[ZnFe_3S_4]^{1+}$.

Each P-cluster has two trapped-valence "Fe^{2+}" sites. These two sites are indistinguishable in the reduced state of the cluster for the four proteins studied. It is noteworthy that none of the "Fe^{2+}" sites becomes oxidized when two electrons are removed from the cluster in the transformation $P^N \rightarrow P^{OX}$.[37] This lends support to the notion that the D-sites have ferrous character and, interestingly, the D-sites have nearly the same isomer shift as component "Fe^{2+}". Thus, if the D-sites were a member of a regular Fe-S cluster, an "all-ferrous" cluster state would be indicated for P^N.

Let us assume, in the absence of direct experimental evidence, that P-clusters are a supercluster consisting of two bridged cubanes. When we viewed the P-clusters as some form of a modified (to account for the unusual spectroscopic properties) Fe_4S_4 cubane we had to reject the $[Fe_4S_4]^{1+}$ oxidation state for P^N because P^N is a non-Kramers system whereas $[Fe_4S_4]^{1+}$ is a Kramers state. However, if two $[Fe_4S_4]^{1+}$ cubanes are bridged the resultant supercluster is a non-Kramers system. Are the observed isomer shifts, $\delta(Fe^{2+}) \simeq 0.66$ mm/s and δ(three D-sites) $\simeq 0.63$ mm/s (using the average of the four proteins studied) compatible with this proposal? From a Mössbauer spectroscopic standpoint the $[Fe_4S_4]^{1+}$ core contains a mixed-valence Fe(II)-Fe(III) pair and a pair of Fe(II) sites. There is considerable electron delocalization between the sites as evidenced by the observation that ΔE_Q and δ of the ferrous sites are considerably reduced relative to the values observed for ferrous rubredoxin and the Fe(II) site of Fe_2S_2 clusters. The isomer shifts of the ferrous pair of protein-bound $[Fe_4S_4]^{1+}$ range from 0.55-0.64 mm/s (average $\delta = 0.60$ mm/s), while the δ-values of the delocalized Fe(II)-Fe(III) pair range from 0.49-0.55 mm/s (average $\delta = 0.51$ mm/s). The average shift for all four sites, $\delta_{av} \approx 0.55$ mm/s, is distinctly lower than the 0.64 mm/s observed for P^N. (For the Fe_4S_4 cubanes the average δ increases by about 0.1 mm/s per electron added.) If the P-clusters are considered as bridged $[Fe_4S_4]^{1+}$ cubes some modification in the ligand structure of some D-sites should be considered.

Alternatively, one might view P^N as a supercluster consisting of two bridged $[Fe_4S_4]^0$ cubanes. This assumption would be in agreement with the observed isomer shifts, but one might expect a very low potential for an "all-ferrous" cubane unless the bridged system would have chemical or structural features which facilitate stabilization of an "all-ferrous" configuration. One way to stabilize a ferrous core would be to reduce the negative charge of the complex, perhaps by protonization of sulfur ligands or by sharing thiolates as bridging ligands. In this respect, the redox chemistry of the *D. gigas* ferredoxin II $[ZnFe_3S_4]$ cluster is instructive: by incorporation of a Zn^{2+} into the core of an $[Fe_3S_4]^0$ cluster the negative charge of the cluster is reduced by one unit (the Zn^{2+} seems to bind to cys-11) allowing the Fe_3S_4 fragment of the core to accommodate one more electron.[33-35] We wish to stress, however, that although the Mössbauer data are compatible with

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(37) Interestingly, the ferrous character of Xa1 component Fe^{2+} increases slightly upon oxidation of the P-clusters, as witnessed by an increase of the isomer shift from $\delta(P^N) = 0.65$ mm/s to $\delta(P^{OX}) = 0.72$ mm/s.

suitably modified bridged cubanes, we have no evidence to reject structures which are not based on the assembly of cubanes.

Finally, if P^{OX} is the two-electron oxidized form of P^N, what evidence is there for a cluster state oxidized by one electron relative to P^N? This state has to be a Kramers system, and we suspect that the g = 1.94 EPR signals observed in the early phase of thionin titrations of Av1 represent this state.[38] More than 10 years ago we had obtained evidence from Mössbauer spectroscopy that these signals result from the P-clusters. However, since only a small fraction of the clusters were present in the g = 1.94 form, the relevant Mössbauer spectrum was masked by the magnetic features of M^N, and for that reason a convincing argument could not be presented for the general reader. However, the availability of isotopic hybrids of the MoFe protein (P-clusters enriched in ^{57}Fe; M-centers enriched with the Mössbauer-silent ^{56}Fe) will allow

us to study the g = 1.94 state with reasonable resolution. It is clear that the new developments in P-cluster research offer a broad spectrum for biophysical, bioinorganic, and biochemical inquiries.

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Note Added in Proof. Based on crystallographic analysis of nitrogenase from *A. vinelandii* at 2.8-Å resolution, Kim and Rees have recently proposed that the P-clusters consist of two cubane Fe$_4$S$_4$ clusters bridged by two cysteine residues; Kim, J.; Rees, D. C. *Science.* In press.

Crystal Structure of Cd$_5$(CN)$_{10}$(H$_2$O)$_4$·4C$_6$H$_{11}$OH Studied by X-ray Diffraction and Solid-State ^{113}Cd NMR. A New Type of Cristobalite-like Framework Host with a Site Interacting with Cyclohexanol by Hydrogen Bonding[†]

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Abstract: The structure of the title clathrate represents the first example of a metal cyanide structural analysis which takes into account explicitly the order/disorder of the metal-linking cyanide units. The structure, which has some unique features, is tetragonal $I4_1/a$, with a = 20.070 (2) Å, c = 12.733 (3) Å, V = 5129 (2) Å^3, and Z = 4, and was refined to R = 0.049 for 2483 independent reflections. ^{113}Cd NMR was used to determine local order around the metal centers. The two distinct Cd atoms are coordinated by bridging cyanides in tetrahedral positions, one Cd also having a coordinating water molecule. The distorted β-cristobalite framework has two cavities, one containing a cyclohexanol molecule and the other the four coordinating waters. These form a near regular tetrahedron, with four cyclohexanols in neighboring cages coordinating to the four faces of the tetrahedron by hydrogen bonding. ^{2}H NMR indicates that the four water molecules in a cage rapidly interchange positions at ~250 K, whereas the cyclohexanol hydroxyl is undergoing an anisotropic motion at this temperature. ^{13}C NMR results indicate that the whole cyclohexanol molecule is in motion at room temperature.

Introduction

The structure of Cd(CN)$_2$ consists of two interpenetrating, but nonbonded, cristobalite-like lattices built from Cd atoms linked tetrahedrally by CN bridges. Removal of one of the interpenetrating lattices leaves a structure which has adamantane-shaped cavities, which are large enough to accommodate guest molecules and thus form a clathrate Cd(CN)$_2$·G. A number of such clathrates are now known with guests G = CCl$_4$, CHCl$_3$, CH$_3$-CCl$_3$, (CH$_3$)$_3$CCl, C$_6$H$_{12}$, and so on.[1,2] Recent systematic studies of cadmium cyanide clathrates and related compounds, carried out with a view to developing new materials (having three-dimensional frameworks and large void spaces for accommodating guest molecules), have led to the discovery of a number of different

cadmium cyanide frameworks. Robson et al. reported a new cadmium cyanide clathrate Cd(CN)$_2$·^{2}/$_3$H$_2$O·Bu^{t}OH with a honeycomb-like framework,[3,4] and Kitazawa et al. have been developing other cadmium cyanide type clathrates of the general formula [onium][Cd$_3$(CN)$_7$]·G.[5] Although these new clathrates

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