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# Detection of EPR Signals Assigned to the 1-equiv-Oxidized P-Clusters of the Nitrogenase MoFe-Protein from *Azotobacter vinelandii*

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**Abstract:** Component 1 (the MoFe protein) of conventional nitrogenase from *Azotobacter vinelandii* contains two types of metal clusters, called M-centers and P-clusters. The M-centers, or FeMo-co, are paramagnetic in the as-isolated form of the protein, exhibiting an EPR signal typical of a rhombic $S = {}^{3}/_{2}$ system. The P-clusters are diamagnetic in the as-isolated form of the protein but become paramagnetic upon chemical or potentiometric oxidation. We have undertaken a controlled oxidative titration of component 1 with thionine solution and observed EPR signals believed to originate from paramagnetic P-clusters. We present EPR spectra associated with half-integer $S = {}^{5}/_{2}$ and $S = {}^{1}/_{2}$ spin states and propose that these spectra arise from the 1-equiv-oxidized form of the P-cluster. Spectral analysis and theoretical models are presented which support the argument that P-cluster oxidation occurs 1 equiv at a time and that the 1-equiv-oxidized P-clusters are stable species in the oxidative titration of Av1.

Nitrogenase catalyzes the biological reduction of dinitrogen to ammonia in the process known as nitrogen fixation. This process is necessary since most species cannot use dinitrogen directly as a nitrogen source but can utilize ammonia.

The nitrogenase studied in this investigation is the Mo-containing form of the enzyme from *Azotobacter vinelandii*, a free-living soil bacterium. This enzyme consists of two separable proteins[1] called component 1 (Av1), an $\alpha_2\beta_2$ tetramer with a molecular weight of about 230 000, and component 2 (Av2), a dimer having a molecular weight of approximately 63 000. It currently is believed that during catalysis Av2 shuttles electrons to Av1, thought to contain the site(s) of substrate reduction.

Both proteins contain metal clusters: Av1 has 30 Fe and two Mo atoms in two different pairs of clusters[2,3] while Av2 possesses only Fe in a single [4Fe–4S] cluster, as demonstrated by recent crystallographic studies.[4] The two different clusters in Av1 have been labeled P-clusters and M-centers,[5,6] or FeMo-co (for FeMo-cofactor),[7] where the M-centers are believed to be at or near the site of substrate reduction. Rees and co-workers[2,3,8] have proposed crystallographic structures for Av1, showing the M-centers to be constructed of a [4Fe–3S] and a [Mo–3Fe–3S] cluster bridged by two S atoms and one unidentified element (possibly S) with a homocitrate molecule ligated to Mo. The P-clusters (also two per Av1) contain two [4Fe–4S] clusters bridged through one S from each cluster as well as by two cysteine thiols. EPR studies[9] of Av1 and X-ray anomalous scattering studies[10] of component 1 from *Clostridium pasteurianum* (Cp1) also support the proposal that there are two P-clusters per protein instead of the earlier predicted four separate [4Fe–4S] clusters. Therefore, for the purposes of this discussion, the term P-cluster will refer to the currently proposed 8Fe double-cubane configuration.

In the as-isolated form of the protein, the M-centers are paramagnetic[5] with $S = {}^{3}/_{2}$, exhibiting an EPR signal with *g* factors of 4.3, 3.6, and 2.0. Upon oxidation,[6] the M-centers become diamagnetic and, therefore, EPR-silent. The P-clusters are diamagnetic in the as-isolated protein, becoming paramagnetic[6] upon oxidation with spin states of $S = {}^{5}/_{2}$–${}^{7}/_{2}$ and $S = {}^{3}/_{2}$–${}^{9}/_{2}$, as indicated by magnetic circular dichroism[11,12] and Mössbauer[6] spectroscopy, respectively. Recent EPR and Mössbauer studies[13–15] of component 1 oxidized with excess thionine solution show the existence of an integer-spin state (proposed $S = 3$) which has been assigned to the P-clusters. Oxidation of component 1 by stirring over solid thionine has been shown[9,15] to result in the formation of a mixed-spin state with $S = {}^{7}/_{2}$ and $S = {}^{1}/_{2}$, which also has been assigned to the P-clusters. This state also can be induced[15] in Av1 by potentiometry or by titration with a large molar excess of thionine solution.

Thionine titrations of Av1 have demonstrated[6,16] that the first 4 equiv results in oxidation of P-clusters, while an additional 2 equiv of thionine oxidizes the M-centers, which become EPR-silent. Because there are only two P-clusters per component 1, the initial 4 equiv must oxidize each P-cluster by 2 equiv, giving rise to the $S = 3$ state. Recently, using power saturation techniques, we repeated this titration and demonstrated[16] the existence of dipolar interactions between the paramagnetic P-clusters and M-centers. The magnitude of this interaction was nonlinear with oxidation during the addition of the first four oxidizing equivalents, suggesting that the P-clusters are oxidized

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in 1- and not 2-electron steps and that the paramagnetism of the singly oxidized cluster differs from that of the doubly oxidized cluster.

The exact role or roles of the P-clusters are not known, but it is logical to propose that they somehow participate in electron transfer during catalysis. Therefore, elucidation of the possible electronic states of the P-clusters may be helpful in furthering our understanding of their functional role in Av1. As stated above, there are two reversibly oxidized states that have been assigned to the P-clusters by EPR: the $S = 3$ state, which has been suggested[13-15] to arise from the 2-equiv-oxidized state of each cluster, and the $S = {}^{1}/_{2}, {}^{7}/_{2}$ mixed-spin state, which apparently arises[9,15] from further oxidation (one more equivalent per P-cluster) of Av1. We propose the following nomenclature for these states, which is consistent with the experimental evidence to date: P for P-clusters in the as-isolated, diamagnetic state (the same as state $P^N$ in ref 6), $P^{2+}$ for the 2-equiv-oxidized state exhibiting the integer-spin EPR signal from a possible $S = 3$ state, and $P^{3+}$ for the 3-equiv state giving rise to the $S = {}^{1}/_{2}, {}^{7}/_{2}$ EPR signals. As mentioned above, our recent results from power saturation studies suggest that another paramagnetic state exists which may be assigned to the 1-equiv-oxidized form of the P-cluster. We propose to call this state $P^+$ and herein present EPR data and a theoretical analysis that confirm and explain its existence in thionine-oxidized Av1.

## Materials and Methods

The nitrogenase component 1 protein of *A. vinelandii* (Av1) was purified by published methods[17] with final specific activities ranging from 1500 to 1800 nmol of $C_2H_2$ reduced $min^{-1}$ (mg of protein)$^{-1}$. All sample preparation was carried out in a Vacuum Atmospheres glovebox in an argon atmosphere with $[O_2] \leq 2$ ppm. Purified protein was stored in liquid nitrogen. Protein concentrations were determined by the biuret method.

Saturated anaerobic solutions of thionine (ICN) were prepared in 0.025 M Tris-HCl, pH 7.4, with 0.2 M NaCl by dissolving excess oxidant in anaerobic buffer solution. The solutions were filtered with 0.2-μm syringe filters to remove undissolved oxidant. Dithionite was removed from the as-isolated Av1 protein by gel filtration on a 13 cm × 1.5 cm column using Sephadex G-25 (Pharmacia) and the aforementioned buffer. Protein solution eluted from the column was checked for the absence of dithionite with methyl viologen indicator. The dithionite-free protein was subsequently titrated to the end point with thionine solution. The end point was recognized by the appearance of a persistent bluish tint in the protein solution. In the thionine titration, the end point was assumed[18] to occur at the 6-equiv-oxidized state of Av1. Appropriate aliquots of oxidant and protein were then used to prepare samples ranging from 0 to 6 equiv oxidized. A variation of this procedure was to add small aliquots (<1 equiv) of the oxidant to the protein samples and to monitor at the same time, the $S = {}^{3}/_{2}$ EPR signal of Av1, which remains constant until after the fourth equivalent of oxidant has been added and then diminishes in amplitude.[6,16] (Oxidative titration of Av1 with thionine in the 0–10-equiv range has been shown[13-15] to give rise to the integer-spin ($S \geq 3$) $P^{2+}$ state and an EPR-silent M-center electronic state. However, it requires a 10–30-fold molar excess of thionine solution, or stirring over solid thionine, to induce the $P^{3+}$ state with $S = {}^{1}/_{2}, {}^{7}/_{2}$. It therefore follows that titrations performed in the 0–6-equiv range should yield $P^{2+}$, $P^+$, and EPR-silent M-center electronic states but not the $P^{3+}$ state.) The partially oxidized samples were allowed to stand for 30 min to ensure equilibration, loaded into quartz EPR tubes, and frozen in liquid nitrogen under anaerobic conditions. Final sample concentrations ranged from 11 to 30 mg/mL Av1. Thionine oxidation resulted in no significant loss of activity as verified by acetylene reduction assays.

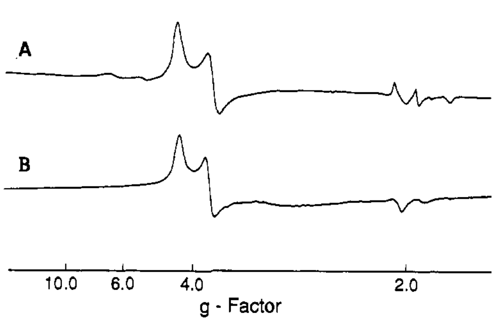

**Figure 1.** EPR spectra of (A) 2-equiv-oxidized and (B) as-isolated Av1. Spectra have been normalized for spectrometer gain and protein concentration. EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; microwave power, 20 mW; temperature, 15 K.

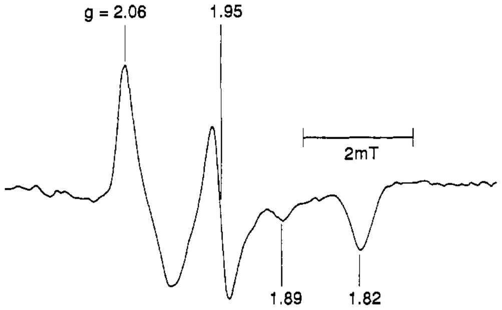

**Figure 2.** EPR spectrum of the $g = 2$ region of 2-equiv-oxidized Av1. The signals at $g = 2.06$, 1.95, and 1.82 are assigned to the same $S = {}^{1}/_{2}$ spin state. The signal at $g = 2.01$ is the high-field inflection of the $S = {}^{3}/_{2}$ FeMo-co signal of Av1. The origin of the small inflection at $g = 1.89$ is unknown (see text). EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; microwave power, 20 mW; temperature, 15 K.

EPR spectra were recorded on a computer-interfaced Bruker ER300D spectrometer with ESP 200 data collection software. The instrument was fitted with a $TE_{102}$ perpendicular-mode cavity resonating at X-band frequencies. An Oxford Instruments ESR-900 helium flow cryostat was used to generate low temperatures (≥2.8 K). Temperature was monitored and controlled with an Oxford Instruments ITC-4 temperature controller connected to an FeAu/chromel thermocouple, which was positioned directly below the sample tube.

## Results

Figure 1 shows EPR spectra of Av1 in the 2-equiv-oxidized (A) and as-isolated (B) states. This figure shows that, in addition to the typical $S = {}^{3}/_{2}$ signal of the as-isolated protein, other signals are visible in the $g = 2$ and $g = 5$–8 regions of the 2-electron-oxidized species. Although small in amplitude compared to the $S = {}^{3}/_{2}$ signal, these signals were reproducible in all samples tested (five different data sets) and have maximal amplitude in the 2-equiv-oxidized state. They are essentially lost by the 4-equiv-oxidized step (see Discussion).

The spectrum of the 2-equiv-oxidized Av1 at $g = 2$ is shown in Figure 2, revealing inflections at $g = 2.06$, 1.95, 1.89, and 1.82. Because of similarities in their relative intensities, the inflections at $g = 2.06$, 1.95, and 1.82 are most likely associated with a single signal and can be assigned to an $S = {}^{1}/_{2}$ system. This signal saturates easily at low temperature (<5 K) and is typical of spectra of reduced ferredoxins.[19] Similar signals have been observed in the spectra of thionine-oxidized Av1[20] and $K_3Fe(CN)_6$-oxidized Kp1[21] (component 1 nitrogenase of *Klebsiella pneumoniae*). The inflection at $g = 1.89$ will be discussed in more detail below.

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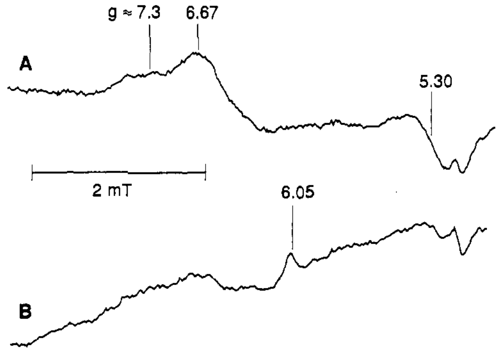

**Figure 3.** EPR spectra of the $g$ = 5–8 region of 2-equiv-oxidized Av1 at (A) 15 K and (B) 8 K. The signals at $g$ = 6.67 and 5.30 are assigned to the same $S = {}^5/_2$ spin system, while the signal at $g \approx 7.3$ may arise from another $S = {}^5/_2$ spin state. The increased amplitudes of these proposed $S = {}^5/_2$ signals at 15 K indicate that they are excited states. The small inflection at $g$ = 6.05 in part B is probably the excited-state transition ($|\pm{}^3/_2\rangle$) of the $S = {}^3/_2$ FeMo-co spin manifold. This signal similarly arises from an excited state, since it increases in amplitude in the temperature range 4–8 K (spectra not shown). Similar signals have been observed[28] at $g$ = 6.11 for Av1 and Kp1 (the component 1 protein of *K. pneumoniae*) and at $g$ = 6.06 for Cp1.[29] The inflection at $g$ = 5.1 was not consistently observed for all samples and is suspected to arise from a cryostat or sample impurity. EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; microwave power, 20 mW.

The $g$ = 5–8 region EPR spectra of the 2-equiv-oxidized species at 15 K (A) and 8 K (B) are shown in Figure 3. These spectra show inflections at $g$ = 7.3, 6.67, 6.05, and 5.30. Using "RHOMBO"[13] (kindly supplied to us by W. R. Hagen) and a real $g$ factor of 2, the inflections at $g$ = 6.67 and 5.30 may be assigned to low-field lines from the $|\pm{}^1/_2\rangle$ doublet of an $S = {}^5/_2$ system with $\lambda = |E/D| \approx 0.029$, where $D$ and $E$ are the axial and rhombic zero-field splitting parameters, respectively. The third inflection predicted at $g \approx 1.97$ is probably obscured by the $g$ = 1.95 line of the $S = {}^1/_2$ signal. Predicted lines at $g$ = 5.97, 0.68, and 0.70 for the semiforbidden $\Delta m_s = 3$ transition in the $|\pm{}^3/_2\rangle$ doublet were not observed at any temperature. This is not surprising, considering the low rhombicity ($E/D$ = 0.029) and significant $g$ factor anisotropy. Similarly, the transition probability within the $|\pm{}^5/_2\rangle$ doublet, with predicted $g$ = 0.006, 0.006, and 9.99, is essentially zero and no observable spectrum would be expected.

The inflection at $g \approx 7.3$ (Figure 3A) will be tentatively assigned to the $|\pm{}^1/_2\rangle$ doublet of another $S = {}^5/_2$ spin system with $\lambda \approx 0.059$. The second inflection predicted at $g \approx 4.6$ may be obscured by the dominant $g$ = 4.3 line of the FeMo-co signal. A high-field inflection at $g \approx 1.9$ is also predicted and may correspond to the signal at $g$ = 1.89 (Figure 2). However, because the $g$ = 1.89 inflection is narrower than that at $g \approx 7.3$ and since the EPR spectra of the high-field inflections of high-spin metal centers and clusters often experience $g$-strain broadening,[22] the assignment of the $g$ = 1.89 inflection to the $g \approx 7.3$ signal is suspect. Predicted inflections from the $|\pm{}^3/_2\rangle$ doublet with $g \approx 5.9$, 1.3, and 1.4 and the $|\pm{}^5/_2\rangle$ doublet with $g \approx 10$, 0.023, and 0.024 were not observed at any temperature. This can be rationalized in terms of low transition probabilities as was described above for the $g \approx 6.67$, 5.30 signal.

Assignment of $S$ is problematical when only one spectral line is observed. The line at $g \approx 7.3$ is absorbance shaped and, thus, is the low-field inflection arising from a doublet in a spin system with $S > {}^3/_2$. We have tentatively assigned this signal to the $|\pm{}^1/_2\rangle$ doublet of an $S = {}^5/_2$ system because we feel it is the most rational assignment, given the spectral data. It is possible that this signal could arise from an $S = {}^7/_2$ or $S = {}^9/_2$ spin system, although this is doubtful because such assignments would require relatively high rhombicity, and one would expect to observe lines from other doublets within the spin manifold with $g > 7.3$. It must be stressed that unequivocal assignments to specific spin states are impossible with limited observable spectral data. However, exact assignment of $S$ is not an absolute requirement for the proposal of a reasonable model for P-cluster oxidation, which follows in the Discussion.

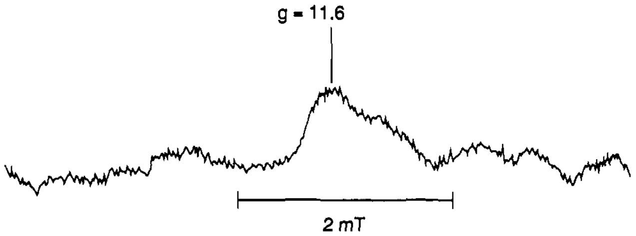

**Figure 4.** EPR spectrum of the $g$ = 11 region of 4-equiv-oxidized Av1. The spectrum has been corrected by subtraction of a sloping baseline. EPR spectrometer conditions: frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; microwave power, 50 mW; temperature, 15 K.

Temperature-dependent, depopulation experiments indicate that the proposed $S = {}^5/_2$ signals arise from excited states with $D < 0$. Experimental data were fitted to a Boltzmann distribution expression for the intensity of the signal arising from the $|\pm{}^1/_2\rangle$ doublet, which is the upper excited state of the inverted spin system. The equation used was $AT = e^{-6D/kT}/(1 + e^{-2D/kT} + e^{-6D/kT})$, where $A$ is the signal area, $T$ is the absolute temperature, $D$ is the axial zero-field-splitting parameter, and $k$ is the Boltzmann constant. Signal areas were multiplied by the absolute temperature for Curie law correction. Curve fitting of the experimental data yielded $D = -3.2 \pm 0.2$ cm$^{-1}$ for the $g$ = 6.67 signal and $D = -3.2 \pm 0.2$ cm$^{-1}$ for the $g$ = 7.3 signal. The uncertainties expressed are calculated standard deviations for the curve fits. Fits were done in the 0–25 K temperature range. At temperatures above 25 K, signal broadening made accurate integration impossible.

During the oxidative titration of Av1, a low-field signal at $g$ = 11.6 appeared (Figure 4), gaining maximal amplitude at the point of approximately 4-equiv oxidation. This signal remains after ≥6 electrons are removed by thionine titration, a region where the $S = {}^3/_2$ Av1 signal has completely disappeared. A signal at $g$ = 11.9 in thionine-oxidized Av1 has been observed[13–15] in parallel-mode EPR and assigned to an excited state of an integer-spin system with suggested $S$ = 3. Perpendicular-mode EPR observations[15] of thionine-oxidized Av1 have shown a weak inflection at $g = 11.6 \pm 0.4$ that is very similar to the one that we observe. The $g$ = 11.6 signal observed by us in perpendicular mode is extremely small compared to the $S = {}^3/_2$ signal of Av1 and arises from an excited-state transition, as verified by temperature studies. Titration with excess indigosulfonate results in the appearance of a similar signal at $g$ = 11.6, while the $S = {}^3/_2$ cofactor signal is still present.

The $S = {}^7/_2$ and $S = {}^1/_2$ signals assigned[9,15] to triply oxidized P-clusters (P$^{3+}$) were not observed in the spectra of our samples. This is an expected result since our titrations were not in the range (10–30 molar excess of oxidant) in which these signals appear.

Spin integrations of the proposed $S = {}^5/_2$ and $S = {}^1/_2$ signals at maximal amplitude were performed by the method of Aasa and Vänngård.[23] Integration of the $S = {}^1/_2$ signal was straightforward, but the $S = {}^5/_2$ signals were problematical because of signal overlap as well as the lack of information on the exact $g$ factors for the predicted (but unobserved) inflections. To integrate the observed $S = {}^5/_2$ signals, a perpendicular line was constructed from the spectral baseline to the minimum between the peaks at

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**Table I.** Parameters for Oxidized P-Clusters

| identity | $S$ | $g_x$ | $g_y$ | $g_z$ | $D$ (cm$^{-1}$) | $\|E/D\|$ | max %/M-center |
|---|---|---|---|---|---|---|---|
| P$^+$ | $^1/_2$ | 2.06 | 1.95 | 1.82 | | | 8 ± 2$^a$ |
| P$^+$ | $^5/_2$ | 6.67$^b$ | 5.30 | (1.97)$^c$ | −3.2 ± 0.2$^a$ | 0.029 | 29 ± 6 |
| P$^+$ | $^5/_2$ | 7.3$^b$ | (4.6) | (1.9)$^d$ | −3.2 ± 0.2 | 0.059 | 34 ± 5 |
| M-center | $^3/_2$ | 6.05 | (<0.5)$^e$ | (<0.5)$^e$ | | | |
| P$^{2+\,f}$ | 3 | | | 11.6 | | | |
| P$^{3+\,g}$ | $^7/_2$ | 10.4$^b$ | 5.5 | (1.8) | −3.7 | 0.043 | 65 ± 10 |
| P$^{3+\,g}$ | $^1/_2$ | 1.97 | 1.88 | 1.68 | | | 12.5 ± 1.5 |

[a] Uncertainties reported are calculated standard deviations among data sets. [b] $g$ factors listed correspond only to the $|\pm 1/2\rangle$ level. [c] $g$ factors predicted but not observed are in parentheses. [d] See text. [e] Out of range of spectrometer. [f] References 12–14. [g] Data taken from refs 14 and 15.

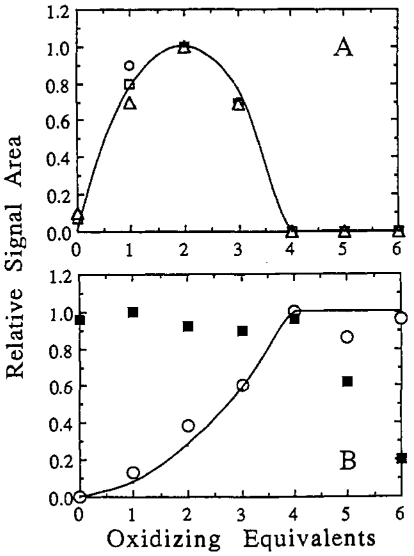

**Figure 5.** Relative EPR spectral areas for (A) half-integer-spin ($S = {}^5/_2$, $^1/_2$) and (B) integer-spin ($S \geq 3$) states. In part A, relative spectral areas of the $S = {}^5/_2$ signal with $g = 7.3$ (△), the $S = {}^5/_2$ signal with $g = 6.67$ (○), and the $S = {}^1/_2$ signal (□) are plotted as a function of oxidizing equivalents. The relative spectral areas of the $S = {}^3/_2$ FeMo-co signal (■) and the $S \geq 3$ signal (○) with $g = 11.6$ are plotted in part B. Theoretical plots are also shown (—) predicting the relative spectral amplitdues of both the half-integer-spin ($S = {}^5/_2, {}^1/_2$, arising from P$^+$) and integer-spin ($S \geq 3$, arising from P$^{2+}$) signals.

$g = 7.3$ and $g = 6.67$ to separate the two areas, and each was integrated. This has the obvious effect of cutting off the high-field wing of the $g = 7.3$ signal and the low-field wing of the $g = 6.67$ signal, yielding somewhat inaccurate integrations of these two signals. The signal areas were normalized by direct comparison, after population correction, to the $S = {}^3/_2$ cofactor signal of the Av1 protein in the same sample tube. $D$ values, used for population correction, were those experimentally determined by us ($D = -3.2 \pm 0.2$ cm$^{-1}$ for both $S = {}^5/_2$ systems) and the published value of $D = 5.1$ cm$^{-1}$ for the $S = {}^3/_2$ cofactor signal.[5] The results are expressed on Table I as a percentage of the area of the cofactor signal. The relative intensity of the absorbance-shaped integer-spin signal at $g = 11.6$ was obtained by single integration but could not be compared directly to the $S = {}^3/_2$ cofactor signal since, in this case, we are observing an integer-spin system in perpendicular-mode EPR.

## Discussion

The relative integrated areas of the $S = {}^1/_2$, $S = {}^5/_2$, $S = 3$, and $S = {}^3/_2$ cofactor signals, normalized to maximum area for each particular signal, are plotted as a function of oxidizing equivalents in Figure 5. The $S = {}^1/_2$ (as well as the inflection at $g = 1.89$) and both $S = {}^5/_2$ signals have maximum areas (Figure 5A) at 2-equiv oxidation, while the $S = 3$ signal attains a maximum area (Figure 5B) at about 4-equiv oxidation. The $S = {}^1/_2$ and $^5/_2$ signals have essentially disappeared in the 4-equiv-oxidized samples. In agreement with previous thionine titrations of Av1,[6,16] the $S = {}^3/_2$ cofactor signal of Av1 does not decrease in area until more than 4 electrons are removed, as is expected if the first 4 electrons removed are taken from P-clusters, as has been suggested by Mössbauer results.[6]

The intensity variation of our spectroscopic data (Figure 5) suggests that the P-clusters oxidize 1 electron at a time, where the first oxidizing equivalent gives rise to the $S = {}^1/_2$ and $S = {}^5/_2$ signals while the second equivalent produces the $S = 3$ signal. This corroborates our earlier results[16] which show that the relaxation times of the $S = {}^3/_2$ cofactor signal, in response to magnetic interaction with the paramagnetic P-clusters, change in a nonlinear fashion during the initial 0–4-equiv oxidation of the P-clusters, thus suggesting that the P-clusters oxidize 1 equiv at a time. In fact, the spectral data (Figure 5A) follow a pattern very similar to that observed for the relaxation times (Figure 3 in ref 16).

A theoretical model for P-cluster oxidation, similar to that earlier proposed,[16] can be created to simulate the spectral data. The assumptions of our model are as follows: (1) The first 4 electrons removed by oxidation are taken from P-clusters. (2) There are four equivalent sites (two on each P-cluster) from which 1 electron can be removed during the initial oxidation. (3) In a titration with *limiting* thionine solution, each site can only be oxidized by 1 equiv; i.e., you can generate P$^+$ (one site) or P$^{2+}$ (both sites) but not P$^{3+}$. (4) The two sites on the same P-cluster (when both are oxidized) magnetically interact.[16] (5) Oxidation occurs randomly; i.e., any of the four sites is just as likely to be oxidized as any other, even if the other site on the same P-cluster is already oxidized. (6) When only one site on a P-cluster is oxidized (P$^+$), it becomes paramagnetic with half-integer spin. (7) When both sites on a P-cluster are oxidized (P$^{2+}$), the magnetic interaction between these sites[16] gives rise to an integer spin.[13-15] Although our model cannot distinguish between different $S = {}^n/_2$ spin states, it does predict the occurrence of half-integer-spin and integer-spin states.

Using these assumptions, the intensity variation of the P$^+$ and P$^{2+}$ states can be predicted. If $n$ equals the number of equivalents oxidized ($0 \leq n \leq 4$), then (Ox) = $n/4$ represents the fraction of sites oxidized and (Red) = $(1 - n/4)$ is the fraction of sites still reduced. The equation

$$(\mathrm{Ox})^4 + 4(\mathrm{Ox})^3(\mathrm{Red}) + 6(\mathrm{Ox})^2(\mathrm{Red})^2 + 4(\mathrm{Ox})(\mathrm{Red})^3 + (\mathrm{Red})^4 = 1 \quad (1)$$

can be used to calculate the fraction of Av1 proteins in different states of oxidation; i.e., (Ox)$^4$ is the fraction of proteins with all four sites on the P-clusters oxidized, 4(Ox)$^3$(Red) is the fraction with one site reduced while the remaining three are oxidized, etc. In this equation, the binomial coefficients of each term represent the degeneracy of each state, assuming four oxidation sites (in the *P*-clusters) per Av1 molecule. This equation may be used to calculate the populations of integer-spin (P$^{2+}$) and half-integer-spin (P$^+$) states as the titration proceeds. This calculation was performed for each step of the titration, extracting the contribution from each term to integer-spin and/or half-integer-spin states. The results, normalized to maximal amplitude, are plotted in Figure 5. As can be seen in this figure, our model gives a reasonable fit to the experimental data and accurately predicts the relative amplitudes of the integer-spin and half-integer-spin states.

Our theoretical model, however, is not as successful in predicting the absolute areas of the half-integer-spin signals. According to our model, the areas of the half-integer-spin P-cluster signals should be maximal in the 2-electron-oxidized species but should only attain 44% of the area of the $S = {}^{3}/_{2}$ cofactor signal. The sum of the percentages of the areas of the $S = {}^{1}/_{2}$ and the two $S = {}^{5}/_{2}$ signals at maximal amplitude is 71 ± 8% (the uncertainty expressed is the calculated standard deviation of the value obtained in the integrations), which is greater than the predicted percentage. There are several possible explanations for the source of this disagreement. First of all, spin quantitation of very small signals can be difficult, and it is possible that significantly greater inaccuracy than the 8% standard deviation calculated was introduced into the integration of the $S = {}^{5}/_{2}$ signals, as discussed above. Secondly, the $g \approx 7.3$ may not arise from an $S = {}^{5}/_{2}$ state. Our rationale for assigning this inflection to an $S = {}^{5}/_{2}$ state is that no other inflections at higher $g$ factors, normally indicative of spin states $> {}^{5}/_{2}$, were observed at any temperature investigated. However, if the $g = 7.3$ inflection arises from a spin state different from ${}^{5}/_{2}$, errors would have been introduced into the calculations of both the magnitude of $D$ and the spin integration. Finally, our model assumes random statistics for P-cluster oxidation. If this is not the case and the oxidation of $P \rightarrow P^+$ is favored over $P^+ \rightarrow P^{2+}$, our statistical equation should be modified (by changing the coefficients) to more exactly represent the relative percentage of the half-integer-spin signals. We have performed this calculation using this assumption and have found that the relative percentage of $P^+$ can be made to approach the experimental value of 71%. However, as this occurs, the theoretical prediction of the relative amplitude of the $P^{2+}$ state (giving rise to the $S = 3$) signal becomes worse.

From the above discussion, the 1-equiv-oxidized species, $P^+$, gives rise to two $S = {}^{5}/_{2}$ and one $S = {}^{1}/_{2}$ spin states. The occurrence of mixed-spin states in paramagnetic Fe–S clusters is not unprecedented and, in fact, was first observed[24–26] in the EPR spectrum of reduced nitrogenase component 2. The P-cluster is now believed to be composed of two bridged [4Fe–4S] cubanes. According to our theoretical model, oxidation of this cluster, to give rise to multiple spin states, can be viewed in different ways. In one, oxidation of the P-cluster generates a delocalized paramagnetism over the entire double-cubane structure. In this situation, the presence of mixed-spin states would possibly arise from an equilibrium of conformational differences about the cluster, similar to the equilibrium previously observed in simpler Fe–S clusters.[24–26] Another way of viewing P-cluster oxidation is to assume that the paramagnetism is not delocalized over the 8Fe structure but that oxidation occurs only on each [4Fe–4S] half. In this situation, oxidation of each half could give rise to its own set of spin states, and when both halves are oxidized, spin coupling between them would generate the observed ground-state integer-spin signal at $g = 11.6$. If this second view of P-cluster oxidation is correct, then double oxidation would actually correspond to two single oxidations of each half of the cluster, which might explain why the oxidations of the steps $P \rightarrow P^+$ and $P^+ \rightarrow P^{2+}$ appear to be equally probable.

Our model states that double oxidation of the P-clusters to $P^{2+}$ gives rise to the EPR inflection at $g = 11.6$. A similar inflection has been previously observed[13–15] in parallel- and perpendicular-mode EPR spectroscopy and assigned to an integer-spin state, possibly $S = 3$. The shape and $g$ factor of the signal that we observe in perpendicular mode are consistent with those observed by other investigators.[15]

The assignment[13–15] of the $g = 11.6$ signal to a transition within the $|\pm 3\rangle$ doublet of an $S = 3$ state with $D > 0$ is not consistent with all of the experimental data. Specifically, both Mössbauer[6] and MCD spectra[11,12] suggest that the doubly oxidized P-cluster, $P^{2+}$, possesses a paramagnetic ground state. This would not be the case for $D > 0$ where $|m_s = 0\rangle$ is the lowest level. Another possibility which has been suggested[14] is that this inflection corresponds to a transition induced between excited states by state mixing. Finally, it is also possible[27] that the $g = 11.6$ signal is associated with the $|\pm 3\rangle$ doublet of an $S = 4$ state with $D < 0$. In this situation, the lowest level would be the paramagnetic $|\pm 4\rangle$ doublet and the EPR-observable transition in the $|\pm 3\rangle$ doublet would still be from an excited state. While it may be argued[15] that the $|\pm 4\rangle$ ground state should be EPR observable with an inflection even sharper than that observed for the $g = 11.6$ ($|\pm 3\rangle$) transition, the ability to observe a transition within this $|\pm 4\rangle$ doublet, especially in the parallel mode, may be restricted due to lack of the proper level mixing necessary to satisfy the $\Delta m_s = 0$ selection rule as well as a decreased transition probability due to the much greater spectral width of this signal compared to the observable signal associated with the excited-state $|\pm 3\rangle$ doublet. Therefore, it may be more correct to assign the $g = 11.6$ transition to a state with $S \geq 3$.

In conclusion, we have shown that there are paramagnetic and EPR-active $S = {}^{1}/_{2}$ and proposed $S = {}^{5}/_{2}$ species formed by a 1-equiv oxidation of the P-clusters, $P^+$. Further oxidation generates an integer-spin state with $S \geq 3$ associated with the 2-equiv-oxidized state, $P^{2+}$. Using a theoretical model assuming equal probability for each oxidation step, we have been able to satisfactorily simulate the amplitude variation of both $P^+$ and $P^{2+}$ during the oxidative titration. The close match of the theoretical model with the spectral data (Figure 5B) further strengthens our basic assumption that the integer-spin species is obtained by a 2-equiv-oxidation of the P-clusters, 1 equiv at a time. It remains to be shown if either of these redox states is relevant to the physiological function of Av1.

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