Oxidative Titration of the Nitrogenase VFe Protein from *Azotobacter vinelandii*: An Example of Redox-Gated Electron Flow†

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ABSTRACT: The nitrogenase VFe protein of *Azotobacter vinelandii* (Av1′) has been shown to exist in two forms called Av1′$_A$, which has a primary $\alpha\beta_2$ trimeric structure, and Av1′$_B$, which has an $\alpha_2\beta_2$ tetrameric structure [Blanchard, C. Z., & Hales, B. J. (1996) *Biochemistry 35*, 472−478]. Both forms exhibit $S = 5/2$ EPR signals in the as-isolated state that may be assigned to 1-equiv-oxidized P clusters (P^+). These signals are abolished by enzymatic reduction with the component 2 protein (Av2′). Stepwise oxidative titrations of enzymatically reduced Av1′$_B$ result in the restoration of the $S = 5/2$ P^+ signals and the concurrent decrease of the $S = 3/2$ vanadium cofactor signal. Further oxidation results in the appearance of an integer spin signal assigned to the 2-equiv−oxidized P cluster (P^{2+}). Unlike the analogous signal previously observed in Mo nitrogenase component 1 (Av1), which arises from an excited state, the integer spin P^{2+} signal in Av1′$_B$ originates from a ground-state doublet. Similar oxidative titrations of enzymatically reduced Av1′$_A$ show redox behavior dramatically different from that of Av1′$_B$, as monitored by EPR spectroscopy. We observe spectral evidence for a redox-induced intramolecular electron transfer between the reduced P cluster and the oxidized FeV cofactor cluster during the titrations.

Nitrogenase catalyzes the bioreduction of dinitrogen to ammonia. This enzyme consists of two separable metalloproteins (Bulen & LeComte, 1972), called component 1 and component 2. Component 1 is the site of substrate reduction, while component 2 shuttles electrons to component 1 during catalysis. Crystallographic studies (Chan et al., 1993; Georgiadis et al., 1992; Kim & Rees, 1992a,b) of both component proteins of the Mo-containing nitrogenase from *Azotobacter vinelandii* (termed Av1 and Av2) have been performed, and structural models have been proposed. Av2 is a γ_2 dimer with a molecular weight of approximately 63 000 that contains one redox-active [4Fe-4S] cluster that bridges the subunits. Av1 is an $\alpha_2\beta_2$ tetramer with a molecular weight of approximately 230 000 and contains 30 Fe and 2 Mo atoms in two pairs of distinct redox-active metal clusters, called M centers and P clusters. The M centers (also called the FeMo cofactors) are $MoFe_7S_9$ clusters with an octahedrally coordinated Mo ligated to the imidazole nitrogen of histidine, the carboxyl and hydroxyl functional groups of homocitrate, and three cofactor sulfurs. The only other protein ligation to this cluster occurs at the opposite end of the M center where an Fe atom is ligated to cysteine. There is one M center per α subunit. The P clusters are Fe_8S_8 sulfide-bridged double cubanes that are ligated through Fe to cysteine residues in the α and β subunits, forming metal−cluster bridges that span both of the $\alpha\beta$ interfaces. The function of the P clusters is not yet understood, but it is believed that they may play a role in the catalytic process, possibly as electron transfer agents from component 2 to the M centers.

A. vinelandii also expresses an alternative form of nitrogenase (Hales et al., 1986a,b), with V replacing Mo as the M center heteroatom in the component 1 protein (Av1′). The vanadium system expresses a distinct component 2 protein (Av2′), which appears to be very similar in structure to Av2. Although crystallographic data are not available for Av1′, amino acid sequence homology (Bishop et al., 1990), metal analysis (Eady et al., 1987), EXAFS spectroscopy (Arber et al., 1987, 1989; Chen et al., 1993; George et al., 1988), and Mössbauer spectroscopy (Münck et al., 1975; Ravi et al., 1994) indicate striking similarities to Av1. EXAFS and Mössbauer studies of Av1′ have yielded spectra that are very similar to those observed in studies of Av1, strengthening the proposal that Av1 and Av1′ have virtually identical metal cluster composition.

Enzymatic nitrogen reduction is a redox process presumably involving reversible redox changes in the metal clusters of component 1. Obviously, elucidation of the various available redox states of the nitrogenase metal clusters may be fundamental to understanding the mechanism of nitrogenase catalysis. The redox properties of the metal clusters of the Mo-containing Av1 have been studied extensively by EPR (Münck et al., 1975; Zimmermann et al., 1978), MCD (Johnson et al., 1981; Morningstar et al., 1987), and electrochemical methods (Watt, 1980; Watt et al., 1980, 1981). The M centers are EPR-active paramagnetic species (Münck et al., 1975) in the as-isolated state (M), with $S = 3/2$. Each center may be reversibly oxidized (Zimmermann et al., 1978) by 1 equiv to an EPR-silent diamagnetic state (M^+). Enzymatic turnover, with resultant reduction of the M center, yields a redox state that also EPR silent (M^-) (Mortenson et al., 1973; Orme-Johnson et al., 1972; Smith et al., 1973). The P clusters have been shown to be diamagnetic in the as-isolated protein (P), but may be reversibly oxidized by 1 equiv (P^+) to $S = 1/2$ and $S = 5/2$

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states (O'Hara & Hepler, 1961; Orme-Johnson et al., 1981; Smith et al., 1983; Tittsworth & Hales, 1993). A 2-equiv oxidation (Hagen, 1992b; Pierik et al., 1993; Surerus et al., 1992) results in an integer-spin state (P^{2+}), with $S = 3$ or 4. During a stepwise oxidation of Av1 with thionine, the first 4 equiv oxidize the P clusters, after which the M centers become oxidized by the next 2 equiv (Oliver & Hales, 1992; Tittsworth & Hales, 1993; Zimmermann et al., 1978). Further oxidation to the 3-equiv-oxidized P cluster species (P^{3+}) results in a mixed P cluster spin state (Hagen et al., 1987; Pierik et al., 1993), with $S = 1/2$ and $S = 7/2$. The oxidation behavior of the MoFe protein−metal clusters during thionine titrations at or near physiological pH has been well established. However, variation of buffer pH, NaCl concentration, and oxidant type has been reported to result in very different redox behavior (Watt, 1980; Watt et al., 1980, 1981).

To better understand the analogous oxidation states of the metal clusters in V nitrogenase, this paper describes an EPR spectroscopic study of the oxidative titration of V-containing Av1′. In the as-isolated state, the vanadium cofactor (M center) of Av1′ is similar to the M center of Av1 in that it is a paramagnetic, EPR-active species (Eady et al., 1987; Morningstar & Hales, 1987) with $S = 3/2$. Unlike the spectrum of Av1, the EPR spectrum of as-isolated Av1′ contains an additional axial $S = 1/2$ signal that has not been unequivocally assigned. As-isolated Av1′ also displays $S = 5/2$ EPR signals that are very similar to those associated with the 1-equiv-oxidized P cluster (Tittsworth & Hales, 1993) (P^+) in Av1. These P^+ signals can be removed by enzymatic reduction of Av1′ without change to the $S = 3/2$ vanadium cofactor signal, thus demonstrating that the P clusters may be reduced enzymatically by Av2′. Oxidative titrations of enzymatically reduced Av1′ reveal the concurrent oxidation of both clusters in this protein compared to the consecutive oxidation observed with Av1 during thionine titrations.

A novel form of Av1′, called $Av1'_A$, recently has been isolated and characterized (Blanchard & Hales, 1996) in this laboratory. $Av1'_A$ is an $\alpha\beta_2$ trimer containing only one M center, one complete P cluster, and an additional [4Fe-4S] cluster. In its as-isolated state, $Av1'_A$ exhibits an EPR spectrum very similar to that of the tetrameric form of Av1′ mentioned earlier (now called $Av1'_\beta$) and is similarly enzymatically reducible, resulting in EPR-silent P clusters. However, the oxidation behavior of enzymatically reduced $Av1_A$, as observed by EPR spectroscopy, is strikingly different from that of $Av1'_B$. We believe that we observe a redox gating of electron flow in $Av1'_A$, where a redox-induced conformational change in $Av1'_A$ produces an intramolecular electron transfer from reduced P clusters to oxidized M centers, an event that may be fundamental to the catalytic role of nitrogenase.

MATERIALS AND METHODS

Vanadium-containing nitrogenase was purified from the LS-15 mutant strain of *Azotobacter vinelandii* by using a variation of previously published methods (Burgess et al., 1980) to separate forms $Av1'_A$ and $Av1'_B$ (Blanchard & Hales, 1996). The specific activity of purified VFe protein (Av1′), as determined by acetylene reduction assay, ranged from 200 to 260 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$. All purification steps were conducted under anaerobic conditions in the presence of 2 mM sodium dithionite. Protein concentration was determined by the biuret method. Purified protein was frozen and stored in liquid nitrogen.

The metal clusters of Av1′ were reduced enzymatically to their "native" forms prior to performing oxidative titrations. This reduction was effected by incubating Av1′ with Av2′ in the presence of Mg-ATP and sodium dithionite. The reduction reaction mixtures consisted of Av1′ and Av2′ in a 20:1 molar ratio, Mg-ATP in 20-fold molar excess over Av1′, and a 50-fold molar excess of sodium dithionite in 0.025 M Tris-HCl (pH 7.4) with 0.1 M NaCl. The enzymatic reduction was conducted at ambient temperature in a Vacuum Atmospheres glove box under an Ar atmosphere with $[O_2] < 2$ ppm. The enzymatic reaction was allowed to continue for 20 min from the time of addition of the component 2 protein, after which the excess sodium dithionite and Mg-ADP were removed by gel filtration with Sephadex G-25, using the same buffer (without sodium dithionite) as the eluent. The eluted protein fractions were monitored for residual sodium dithionite with methyl viologen indicator. Final concentration of Av1′ ranged from 13 to 25 mg/mL.

Oxidative titrations were performed using solutions of thionine (Eastman) or indigosulfonate (ICN), which were prepared in anaerobic 0.025 M Tris-HCl (pH 7.4) with 0.1 M NaCl, and filtered with 0.2-μm syringe filters to remove any undissolved oxidant. By using the procedure established for oxidative titration of the MoFe protein (Oliver & Hales, 1992; Tittsworth & Hales, 1993; Zimmermann et al., 1978), reduced Av1′ was divided into equal aliquots that were titrated to the end point, as indicated by the persistent color of the oxidant. After the volume of oxidant necessary to reach the end point was established, separate aliquots of protein were titrated incrementally with smaller volumes of oxidant calculated to ensure that there would be at least six equal steps before reaching the apparent end point. Titrations were carried past the observed end point to ensure maximum oxidation of the metal centers. Titrated samples were allowed to equilibrate for at least 30 min before they were loaded into quartz EPR tubes and frozen in liquid nitrogen. For reference, aliquots of untitrated reduced protein were similarly allowed to stand in the glove box for the duration of the titration procedure, loaded into EPR tubes, and frozen simultaneously with the titrated samples.

EPR spectra were recorded on a computer-interfaced Bruker 300D 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. Temperature was monitored and controlled with an Oxford Instruments ITC-4 temperature controller connected to an FeAu/chromel thermocouple positioned directly below the sample tube.

Values for D (the axial zero-field splitting parameter) were determined by curve fitting Boltzmann distribution expressions for the relative populations of the doublets in question to Curie law-corrected spectral areas recorded in temperature-dependent depopulation experiments. The relative spectral areas were determined by single integration of baseline-corrected absorbance-shaped signals or double integration of derivative-shaped signals, followed by normalization to the maximum obtained signal area (Aasa & Vänngård, 1970,

1975). The expressions used were as follows: $AT = e^{-6D/kT}/(1 + e^{-2D/kT} + e^{-6D/kT})$ and $AT = e^{-3.5D/kT}/(1 + e^{-3.5D/kT} + e^{-7D/kT})$ for the $|\pm 1/2\rangle$ doublet of the inverted $S = 5/2$ system and the middle $|\pm 3/2\rangle$ doublet of the rhombic $S = 5/2$ spin system, respectively, where A is the integrated signal area, T is the absolute temperature, and k is the Boltzmann constant. EPR spectral assignments for half-integer spin systems were effected by numerical determination of possible g-factors in the weak-field limit by using the computer program "RHOMBO" (Hagen, 1992b), which was generously furnished by Professor W. R. Hagen.

RESULTS

Prior to performing a controlled oxidative titration of a multicluster protein, it is imperative that all of the clusters be fully reduced. However, recent Mössbauer studies (Ravi et al., 1994) of as-isolated Av1′ (both forms $Av1'_A$ and $Av1'_B$) have indicated the presence of oxidized P clusters. This situation can be contrasted to that of the MoFe protein (Av1) (Münck et al., 1975) under the same conditions where both cluster types exist only in their reduced native (P and M) forms. Mössbauer spectroscopy (Ravi et al., 1994) has also demonstrated that full reduction of the Av1′ clusters to their P and M states occurs following limited enzymatic reduction (described earlier) without any of the M centers existing in the "superreduced" M^- state.

The presence of oxidized P clusters in as-isolated Av1′ can also be detected in the EPR spectrum of this protein. Previous oxidative titrations of Av1 strongly suggest that singly oxidized P clusters (Tittsworth & Hales, 1993) (P^+) give rise to $S = 1/2$ and $S = 5/2$ EPR signals that reach maximal amplitude at 2 oxidizing equiv per protein. Figure 1 compares the EPR spectra in the $g = 3-12$ region of as-isolated $Av1'_B$, enzymatically reduced $Av1'_B$, and 2-equiv-thionine-oxidized Av1. The difference spectrum (Figure 1C) of as-isolated (Figure 1A) minus enzymatically reduced $Av1'_B$ (Figure 1B) reveals a broad inflection at $g = 4.3$ and inflections at $g = 6.67$ and 5.3. We have observed these inflections in numerous difference spectra and do not feel that they are subtraction artifacts. The spin state of the broad absorbance-shaped inflection centered at $g = 4.3$ is unknown, but this signal presumably arises from an oxidized cluster of $Av1'_B$ (see Discussion). A similar signal is not observed during the oxidation of Av1; however, its presence may be masked by the intense $g = 4.34$ inflection of the paramagnetic M center (FeMoco). The remaining inflections at $g = 6.67$ and 5.3 are associated with an excited state, as determined by temperature studies. These signals may be tentatively assigned to the excited $|\pm 1/2\rangle$ doublet of an inverted $S = 5/2$ spin system, with $D = -1.6$ cm^{-1} and rhombicity $E/D = 0.029$. Partially oxidized Av1 protein ($P \rightarrow P^+$, Figure 1D) shows analogous signals (Tittsworth & Hales, 1993) at $g = 6.67$ and 5.30, similarly assigned to the $|\pm 1/2\rangle$ doublet of an $S = 5/2$ spin system with $D = -3.2$ cm^{-1} and $E/D = 0.029$. The slightly broader $g = 6.67$ signal in $Av1'_B$ compared to that in Av1 may indicate a greater heterogeneity in the environment of the P clusters in the former protein.

The spectrum of as-isolated $Av1'_B$ (Figure 1A) also shows the $S = 3/2$ signal of the M centers (FeVco), which arises from the ground and excited doublets of an inverted ($D = -0.74$ cm^{-1}) spin system with g-factors of 5.68 and 5.45

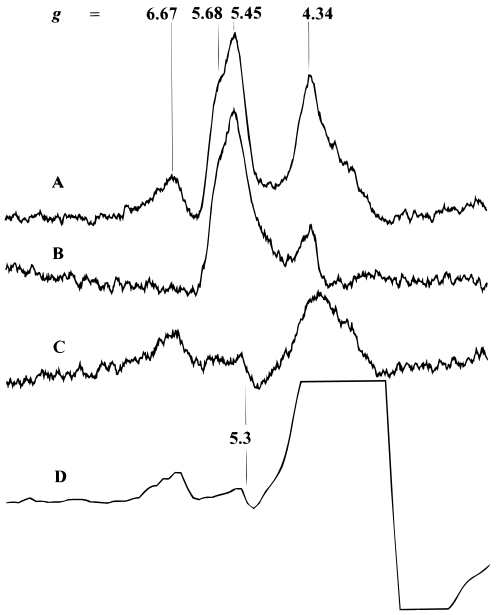

FIGURE 1: EPR spectra of the $g = 3-12$ region of (A) as-isolated $Av1'_B$, (B) enzymatically reduced $Av1'_B$, (C) a difference spectrum (C = A − B), and (D) 2-equiv-oxidized Av1. An absorbance-shaped inflection at $g = 6.67$ is visible in the spectrum of as-isolated $Av1'_B$ (A) and is removed by enzymatic reduction (B). A broad inflection at $g \approx 4$ visible in (A) is not present in (B). The difference spectrum (C) shows inflections at $g = 6.67$ and $g \approx 4$ and also reveals a derivative-shaped inflection at $g = 5.2$. Two-equiv-oxidized Av1 (D) exhibits an $S = 5/2$ EPR signal with $g = 6.67$ and 5.3, which have been assigned to 1-equiv-oxidized P clusters (P^+). The large off-scale features in (D) are the $S = 3/2$ FeMo cofactor signals at $g = 4.3$ and 3.7. The spectra are normalized for protein concentration and instrument gain. EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; microwave power, 20 mW; temperature, 12 (A−C) and 16 K (D).

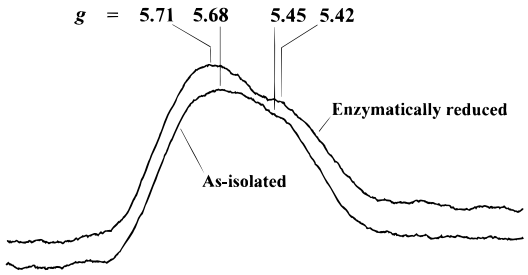

FIGURE 2: Low-temperature EPR spectra of the cofactor in as-isolated and enzymatically reduced $Av1'_B$. As-isolated $Av1'_B$ exhibits g-factors of 5.68 and 5.45. Enzymatic reduction results in a change in rhombicity and with an observed shift to $g = 5.71$ and 5.42. The spectra are normalized for protein concentration and instrument gain. EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.6 mT; microwave power, 20 mW; temperature, 3.2 K.

($E/D = 0.29$). Previously observed g-factors were 5.80 and 5.40 (Hales et al., 1986a, 1989). We observe these g-factors to change to 5.71 and 5.42 ($E/D = 0.28$) upon enzymatic reduction (Figure 2). It is possible that this change arises from a change in paramagnetic interaction between the oxidized P clusters and the vanadium cofactor in as-isolated $Av1'_B$, where enzymatic reduction of the P clusters to their native diamagnetic state would remove this interaction. However, previous studies of Av1 (Oliver & Hales, 1992) have shown that oxidation of the P clusters to the P^+ state does not change the rhombicity of the cofactor (FeMoco),

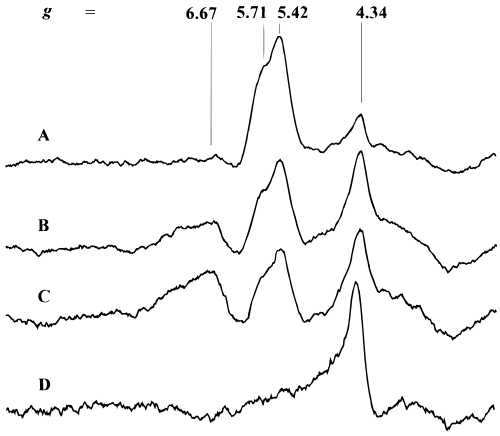

FIGURE 3: EPR spectra of thionine-titrated enzymatically reduced Av1′$_B$: (A) enzymatically reduced Av1′$_B$; (B) reduced Av1′$_B$ titrated with 1.5 equiv of thionine, in which the inflections at $g = 6.67$ and 4.3 have reappeared; (C), 3-equiv-oxidized Av1′$_B$; and (D) 6-equiv-oxidized Av1′$_B$. EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; microwave power, 20 mW; temperature, 12 K.

but does produce changes in its spin relaxation behavior. Furthermore, paramagnetic interactions usually result in line broadening, which is opposite what is observed with Av1′$_B$. While it is questionable that spectral broadening occurred, it is clear that the rhombicity (E/D) of the cofactor signal in Av1′$_B$ decreases as the P clusters become oxidized. This change in rhombicity implies a structural change about the cofactor upon oxidation of the P clusters.

Oxidative titrations of enzymatically reduced Av1′$_B$ were performed and monitored by EPR spectroscopy. Changes in the EPR spectrum (recorded at 12 K) of the $g = 5-8$ region during a thionine titration are shown in Figures 3 and 4. In this region of the spectrum, enzymatically reduced Av1′$_B$ (Figure 3A) exhibits EPR signals associated with the vanadium cofactor ($g = 5.71$ and 5.42), along with a small, sharp inflection at $g = 4.34$. A 1.5 equiv/protein oxidation (Figure 3B) restores both the $g = 6.67$ and the broad $g = 4.3$ EPR signals previously observed in as-isolated Av1′$_B$ (Figure 1A), while slightly diminishing the vanadium cofactor signal. Superimposed on the broad $g = 4.3$ signal is a sharp inflection at $g = 4.34$, which increases in intensity upon further oxidation (Figure 3C,D). Oxidation by 3 equiv (Figure 3C) results in a further attenuation of the Av1′ cofactor signal by approximately 50%, yet enhances the $g = 6.67$ and 4.3 signals to their maximal amplitudes. Finally, except for the sharp line at $g = 4.34$, all of the spectral signals of Av1′$_B$ decrease in amplitude upon further oxidation (Figure 3D and 4A).

The as-isolated VFe protein also exhibits an axial $S = 1/2$ EPR signal (Hales et al., 1986a, 1989), with inflections at $g = 2.04$ and 1.93 (Figure 5A). The origin of this signal is currently unknown, but it is presumed to be associated with either the P clusters or M centers. During enzymatic reduction, this signal was occasionally, but not always, removed. In the cases where it was not removed by enzymatic reduction (Figure 5B), it disappeared upon introduction of the first aliquot (<1 equiv) of oxidant (Figure 5C). On the other hand, in those cases where this signal was removed by enzymatic reduction (Figure 5D), it reappeared upon introduction of the first aliquot of titrant (Figure 5E) and was subsequently abolished by the addition of the second aliquot (similar to Figure 5C), suggesting that it may

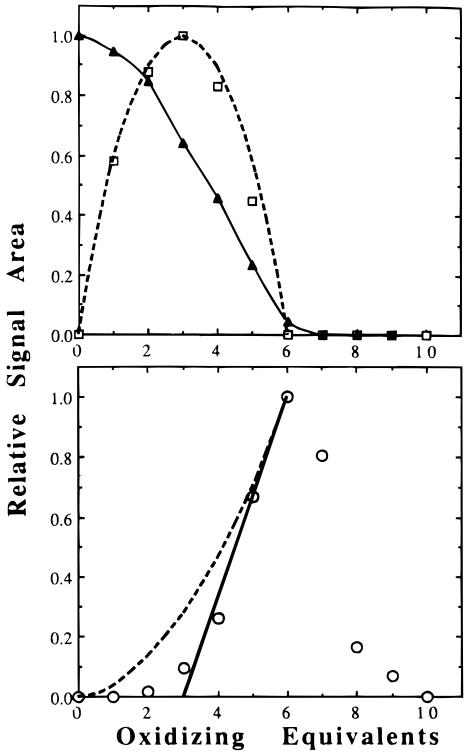

FIGURE 4: (Top) Relative EPR spectral areas of the $S = 5/2$ (P^+) signal at $g = 6.67$ and the $S = 3/2$ VFe cofactor signal of Av1′$_B$ as a function of oxidizing equivalents. Experimental data (□) for the $g = 6.67$ (P^+) signal and a theoretical line (- - -) calculated with the random probability P cluster oxidation model (see text) are shown. Also, the area of the $S = 3/2$ cofactor signal (▲) is plotted along with a line (—) interpolated through the data points. (Bottom) Experimental data (○) for the relative signal area of the $S = 3$ (P^{2+}) signal with $g = 11.5$ plotted along with theoretical models. The random oxidation model is represented by (- - -) and the consecutive model by (—).

represent a minor component of the protein. A similar $S = 1/2$ EPR signal is believed to be associated with a low-activity form of the vanadium nitrogenase of *Azotobacter chroococcum* (Ac1*) (Eady, 1991; Eady et al., 1990). Ion exchange chromatography has been used to isolate an early eluting form of Ac1* that exhibits the $S = 1/2$ EPR signal. The early eluting protein fractions exhibiting the $S = 1/2$ signal were shown to have a lower Fe content and a lower specific activity than the later eluting protein, which exhibits only the $S = 3/2$ EPR signal. However, Av1′ does not exhibit similar chromatographic behavior, and we have not observed forms that exhibit only the $S = 3/2$ signal (Blanchard & Hales, 1996).

Oxidation of Av1 has been shown to induce a 2-equiv-oxidized P cluster species (P^{2+}), which gives rise to an integer spin EPR signal (Hagen, 1992b; Pierik et al., 1993; Surerus et al., 1992). This signal has been assigned to an excited-state doublet of an $S = 3$ or 4 spin system and, typical of integer spin EPR signals, exhibits absorptions in both parallel and perpendicular mode EPR with g-factors of 11.9 and 11.6, respectively. A similar EPR signal, at $g = 11.5$ in perpendicular mode (Figure 6) and $g = 12.8$ in parallel mode (not shown), is generated during thionine oxidation of Av1′$_B$. This signal attains maximal amplitude at the 6-equiv-oxidized step in the titration and may be assigned to the 2-equiv-oxidized P cluster (P^{2+}). Temperature studies indicate that it arises from a ground-state transition of a probable $S = 3$ spin system, in contrast to the integer spin signal observed

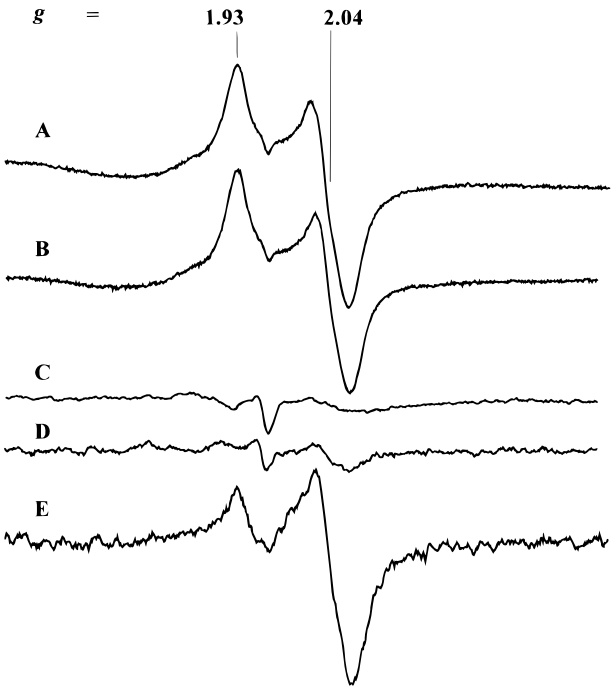

FIGURE 5: EPR spectra of the $g = 2$ region of Av1′$_B$: (A) as-isolated Av1′$_B$ showing the axial $S = 1/2$ signal with $g = 1.93$ and 2.04; (B) enzymatically reduced Av1′$_B$ in which the $S = 1/2$ signal was not removed; (C) thionine-oxidized Av1′$_B$ (<1 equiv), in which the $S = 1/2$ signal has disappeared; (D) enzymatically reduced Av1′$_B$ in which the $S = 1/2$ signal is not present; and (E) thionine-titrated Av1′$_B$ in which the $S = 1/2$ signal was restored by the first aliquot (<1 equiv) of titrant. The last signal was removed by the addition of the next aliquot of titrant. EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; microwave power, 20 mW; temperature, 12 K.

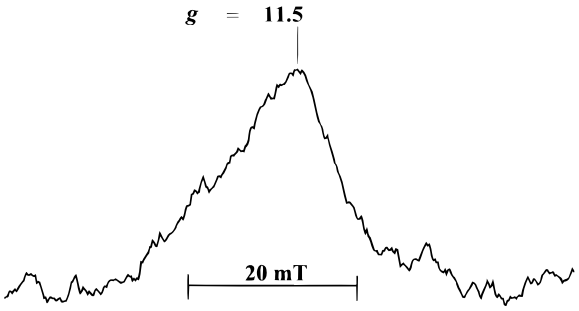

FIGURE 6: Perpendicular mode EPR spectrum of 6-equiv-oxidized Av1′$_B$ showing a signal at $g = 11.5$, which arises from the integer spin state ($S = 3$) of P^{2+}. EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; microwave power, 20 mW; temperature, 3.2 K.

in Av1 mentioned earlier, which arises from an excited-state doublet.

We recently have shown that there is a second form of Av1′ (Blanchard & Hales, 1996) called Av1′$_A$, which has a smaller $\alpha\beta_2$ trimeric structure. Metal, sulfide, and EPR analyses of this form suggest that it contains only one cofactor and one P cluster along with an additional [4Fe-4S]-like cluster. In spite of its smaller subunit structure, Av1′$_A$ is still enzymatically active, exhibiting approximately 75% of the specific activity of form Av1′$_B$ in acetylene reduction assays. However, oxidative titrations of Av1′$_A$ yield very different spectral data from those described earlier for Av1′$_B$.

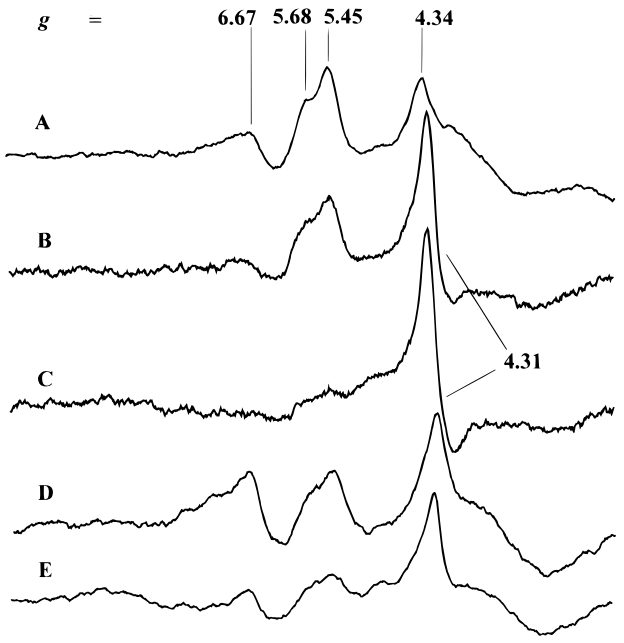

FIGURE 7: EPR spectra of Av1′$_A$: (A) as-isolated Av1′$_A$ showing the $S = 3/2$ cofactor signal, the $S = 5/2$ (P^+) signal at $g = 6.67$, and the broad inflection at $g = 4$; (B) enzymatically reduced Av1′$_A$ showing a sharp inflection at $g = 4.31$ and loss of both the P^+ signal at $g = 6.67$ and the broad inflection at $g = 4$; (C) 1-equiv-oxidized Av1′$_A$ showing the $g = 5.31$ signal at maximal amplitude, while the $S = 3/2$ cofactor signal has been abolished; (D) 1.2-equiv-oxidized Av1′$_A$ showing the $S = 5/2$ (P^+) and $S = 3/2$ cofactor signals, which have returned to full amplitude; (E) 2.5-equiv-oxidized Av1′$_A$ in which the P^+ and cofactor signals have diminished. EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; microwave power, 20 mW; temperature, 12 K.

The 12 K EPR spectrum ($g = 4-12$ region) of as-isolated Av1′$_A$ (Figure 7A) is indistinguishable from that of Av1′$_B$ (Figure 1A), showing an inflection at $g = 6.67$ (the P^+ state of the P clusters), two signals with $g = 5.68$ and 5.45 (the M state of the VFe cofactor), and a broad inflection at $g = 4.3$ with a sharper superimposed signal at $g = 4.34$. Enzymatic reduction of this species (Figure 7B) is also similar to that of Av1′$_B$, resulting in the attenuation of both the $g = 6.67$ and the broad $g = 4.3$ signals. The only new feature in the spectrum of enzymatically reduced Av1′$_A$ that is not observed in the spectrum of Av1′$_B$ is a minor sharp first-derivative-shaped signal at $g = 4.31$ (Figure 7B). Surprisingly, the initial thionine oxidation of Av1′$_A$ results in the oxidation of *only* the cofactor ($M \rightarrow M^+$) without any detectable oxidation of the P clusters to P^+. This is exactly opposite of what occurs during the oxidation of Av1, where the P clusters oxidize prior to the cofactor (Oliver & Hales, 1992; Tittsworth & Hales, 1993; Zimmermann et al., 1978). Also, during the initial oxidation of Av1′$_A$, the new inflection at $g = 4.31$ (Figure 7B) increases in amplitude, reaching a maximum at approximately 1 oxidizing equiv (Figures 7C and 8), at which point the cofactor signal has disappeared. Addition of the next aliquot (approximately 0.2 equiv) of oxidant produces several sudden and previously unobserved spectral changes, i.e., the $g = 4.31$ signal disappears while the signals of P^+ ($g = 6.67$), the reduced cofactor M ($g = 5.68$, 4.45), and the broad absorbance ($g = 4.3$) all suddenly appear (Figures 7D and 8). This sudden change suggests that the cofactor, oxidized during the initial titration, is rereduced with concurrent oxidation of the P cluster ($P \rightarrow$

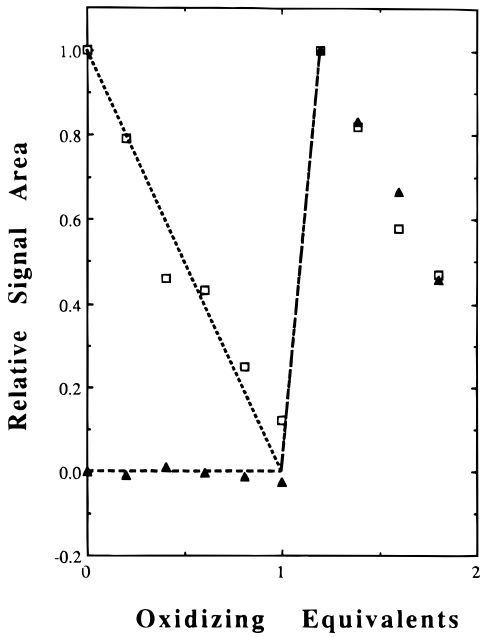

FIGURE 8: Relative EPR signal areas of the $S = 5/2$ (P+) (▲) and $S = 3/2$ cofactor signals (□) in Av1′$_A$ plotted as a function of oxidizing equivalents. The dashed and dotted lines represent predicted oxidation behavior for the P cluster and cofactor, respectively, for the coupled ($P \rightarrow P^+$) and ($M^+ \rightarrow M$) redox-gated electron flow between these clusters (see text).

P^+ is coupled to $M^+ \rightarrow M$). All of these new spectral changes (Figure 7D) appear upon addition of a single aliquot (0.2 equiv) of oxidant past the 1-equiv-oxidized point in the titration, where the $S = 3/2$ M center EPR signal has been removed ($M \rightarrow M^+$). After this point in the titration, the oxidative behavior of Av1′$_A$ mimics that of Av1′$_B$, with a simultaneous decrease of both the cofactor ($M \rightarrow M^+$) and P^+ EPR signals ($P^+ \rightarrow P^{2+}$) as well as a loss of the broad inflection at $g = 4.3$ (Figures 7E and 8). During this final oxidation, a different first-derivative-shaped inflection at $g = 4.3$ appears (Figure 7E). This signal exhibits the same temperature dependence ($|D| = 2.47$ cm^{-1}) as the $g = 4.3$ signal observed in the later stages of the oxidative titration of enzymatically reduced Av1′$_B$ (Figure 3C), but differs from the signal (Figure 7C) at $g = 4.31$ observed in the early stages of the oxidation of Av1′$_A$ ($|D| = 3.24$ cm^{-1}).

The EPR spectrum of the P^{2+} state in Av1′$_A$ was investigated at 3.2 K (Figure 9). At this temperature, the spectrum of the 1-equiv-oxidized species (Figure 9A) exhibits the same derivative-shaped inflection at $g = 4.31$ and attenuated vanadium cofactor signal observed at 12 K (Figure 7). Following a 1.2-equiv oxidation of Av1′$_A$ (Figure 9B), the EPR spectrum of the reduced cofactor reappears along with a broad inflection at $g = 4.3$ (as observed at 12 K) and the first indication of a broad inflection at $g = 11.5$, which is identical to that of the integer spin species previously assigned to P^{2+} (Figure 6). This latter signal gains maximum amplitude with approximately 3 equiv (Figure 9C), while the amplitude of the $S = 3/2$ cofactor signal has diminished and the inflection at $g = 4.3$ has increased slightly. Finally, it should be noted that the $g = 6.67$ signal assigned to P^+ (Figure 7D) is not visible in these low-temperature spectra (Figure 9) since it arises from an excited-state doublet.

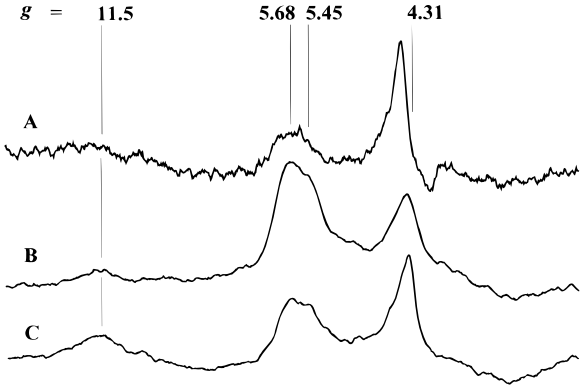

FIGURE 9: EPR spectra of thionine-oxidized Av1′$_A$: (A) 1-equiv-oxidized Av1′$_A$ showing the attenuated $S = 3/2$ cofactor signal and an inflection at $g = 4.31$; (B) 1.2-equiv-oxidized Av1′$_A$ showing the restored cofactor signal and the disappearance of the inflection at $g = 4.31$; (C) 2.5-equiv-oxidized Av1′$_A$ showing the proposed $S = 3$ (P^{2+}) signal and the diminished cofactor signal, along with a slightly increased first-derivative-shaped inflection at $g = 4.34$. EPR spectrometer conditions: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; microwave power, 20 mW; temperature, 3.2 K.

DISCUSSION

Both Mössbauer (Ravi et al., 1994) and EPR (Figure 1A) spectroscopic techniques clearly demonstrate that as-isolated Av1′ (i.e., in the presence of 2 mM dithionite) contains a mixture of oxidized and reduced P clusters and M centers. Increase of the dithionite concentration to 10 mM and/or addition of the mediators methyl viologen and benzyl viologen to Av1′ are ineffective in completely reducing all of the clusters. Full reduction (Figure 2B) of the clusters in Av1′ to their P and M forms (as determined by Mössbauer spectroscopy) has only been achieved by us through enzymatic reduction using limiting Av2′ and Mg-ATP. These results are significant for two reasons. First, they suggest that the clusters of Av1′ have redox midpoint potentials more negative than those in clusters of Av1 (since all of the clusters in the latter protein are fully reduced in the presence of 2 mM dithionite and methyl viologen). Second, these results demonstrate that enzymatic reduction is able to reduce oxidized P clusters. Although the enzymatic role of P clusters is unknown, it has been assumed that they serve to mediate electron transfer from component 2 to the M centers (as opposed to component 2 reducing the M centers directly). Our enzymatic reduction experiment clearly demonstrates that oxidized P clusters can be reduced by component 2, thus strengthening this assumption.

Previous oxidative titrations of Av1 showed the presence of a single oxidation step for each P cluster pair from the native state to the 1-equiv-oxidized state ($P \rightarrow P^+$) (Oliver & Hales, 1992; Tittsworth & Hales, 1993). Further oxidation converted the P clusters into the 2-equiv-oxidized state ($P^+ \rightarrow P^{2+}$) with an apparent midpoint potential approximately identical to that of the first oxidation step. The EPR spectrum of P^+ in Av1 revealed both $S = 5/2$ and $S = 1/2$ spin states. The presence of nearly identical $S = 5/2$ inflections in the spectra in partially oxidized Av1′ (Figures 1C and 3B) clearly establishes the existence of similar P^+ states in this protein. On the other hand, the $S = 1/2$ signal (Orme-Johnson et al., 1981; Smith et al., 1983) assigned to the P^+ state in Av1 is absent from the spectrum of oxidized Av1′. Apparently, the $S = 5/2$ signals represent the presence

of the P^+ state in both proteins, while the $S = 1/2$ signal appears to be a minor component (~10%) unique to the MoFe protein. It has been suggested that the $S = 1/2$ and 5/2 spin states assigned to P^+ in Av1 may represent the 1-equiv oxidation of separate [4Fe-4S] units at opposite halves of the P cluster pair (Oliver & Hales, 1992; Tittsworth & Hales, 1993). In this model, if the oxidation midpoint potentials ($P \rightarrow P^+$) for the two units are similar, then the units are randomly oxidized and both P^+ spin states will be observed during the oxidative titration of the protein, as is the case for Av1. However, as the difference between the midpoint potentials of each unit increases, the oxidation of one half will be preferred and the P^+ spin state of that half (in the case of Av1′, $S = 5/2$) will be the dominant signal observed. This model also predicts (Tittsworth & Hales, 1993) that the profile for the oxidation to P^{2+} during the titration should differ for Av1 and Av1′. During the titration of Av1, the oxidation of each [4Fe-4S] unit of the P clusters to P^+ is random and, therefore, should simultaneously generate a statistical amount of P^{2+}. However, the generation of P^{2+} during the oxidation of Av1′ should only occur *after* all of the P^+ state ($S = 5/2$) has been produced. In other words, if this model is correct, the production of P^{2+} during the oxidation of Av1′ should lag that observed for Av1. The theoretically predicted formations of P^{2+} for both of these situations are shown in Figure 4 (bottom), where it can be seen that there is a lag in the formation of P^{2+}, thus strengthening our hypothesis that the $S = 1/2$ and 5/2 spin states of Av1 P^+ represent separate oxidations of the two [4Fe-4S] units of the P cluster. It should be noted that the oxidative titrations described here are not evidence of reversible redox equilibria for the metal cluster states described. Although we have demonstrated the existence of stable metal cluster redox states in Av1′ during oxidative titration, and we have shown that the oxidized P clusters in Av1′ may be reduced enzymatically, we present no evidence of electrochemical reversibility for any of the possible redox states (i.e., P and P^+) that may exist in Av1′.

In addition to the $S = 5/2$ signals in the $g = 5-8$ region, oxidation of Av1′ also generates a broad absorption-shaped signal (Figures 1, 3, and 7) centered at $g = 4.3$ that was not observed in the oxidative titration of Av1, possibly being obscured by the dominant $g = 4.32$ FeMoco signal in that protein. This broad $g = 4.3$ signal obviously arises from an oxidized metal cluster. EPR spectral evidence strongly suggests that the $g = 4.3$ signal is associated with P^+. First, the amplitude variation of the $g = 4.3$ signal during the oxidative titration of either $Av1'_B$ (Figure 3) or $Av1'_A$ (Figure 7D) mimics that of the $S = 5/2$ P^+ signals. Second, in the initial oxidation of $Av1'_A$, during which only the M centers are oxidized ($M \rightarrow M^+$) while the P clusters remain reduced, the $g = 4.3$ signal is not generated (Figure 7). Therefore, this signal is only observed when the state P^+ is also present. Other than the fact that this broad $g = 4.3$ signal obviously arises from a state with $S > 1/2$, the observation of only one inflection makes the identification of the spin state of this signal ambiguous.

During the oxidation of Av1′, a sharp inflection appears at $g = 4.34$ (Figures 3D and 7E), which increases in intensity as more oxidant is added. This inflection is still present following the oxidation of both metal clusters in Av1′ (i.e., $M \rightarrow M^+$ and $P \rightarrow P^{2+}$). Addition of oxidant beyond this 6-equiv point results in a decrease in the intensity of the P^{2+} signal, with a further increase in the amplitude of the sharp $g = 4.34$ inflection. At this point in the titration it was also noted that Av1′ was starting to become irreversibly inactivated. Signals in the region of $g = 4.34$ often reflect the presence of adventitiously bound iron. Therefore, these results suggest that the $g = 4.34$ signal may arise from the oxidative destruction of one or both of the metal clusters in Av1′. By comparison, Av1 has been oxidized to the P^{3+} ($S = 7/2$) state (a state not observed by us in Av1′) without detectable inactivation.

The oxidative titration of $Av1'_B$ (Figures 3 and 4) illustrates the concurrent oxidation of both the P clusters and M centers. This situation differs from the analogous oxidation of Av1 using thionine, which shows a 2-equiv oxidation of the P clusters to the P^{2+} state preceding the oxidation of the M centers (Oliver & Hales, 1992; Tittsworth & Hales, 1993; Zimmermann et al., 1978) and suggests similar redox midpoint potentials for both metal clusters in the $Av1'_B$ protein. At present it is unknown whether the similar redox potentials of the clusters in $Av1'_B$ or the fact that both of these potentials are more negative than those of the corresponding clusters in Av1 has any correlation with the apparent lower substrate reduction efficiency (Eady et al., 1987; Hales et al., 1986a) of the vanadium form of the enzyme.

The 2-equiv oxidation of the P clusters ($P \rightarrow P^{2+}$) in various MoFe proteins has been shown to yield an integer spin state (Hagen, 1992a; Pierik et al., 1993; Surerus et al., 1992). Except for the protein isolated from *Xanthobacter autotrophicus* (Xa1) (Surerus et al., 1992), where P^{2+} exhibits a ground-state EPR signal with $g = 15.6$, all previously observed P^{2+} EPR signals have occurred in the $g = 12$ region and are associated with excited states. The P^{2+} state in both $Av1'_A$ and $Av1'_B$ similarly (Figures 6 and 9) exhibits an EPR signal in the $g = 12$ region associated with an integer spin state, as verified by parallel mode EPR spectroscopy (data not shown). However, unlike the analogous signal in the MoFe proteins, the signal in the VFe protein arises from a ground state and, therefore, most likely represents the transition $\Delta m_s = 2S$ of an integer spin system with negative zero-field splitting ($D < 0$) and the restriction $g \geq 4S$. By using these restrictions with $g = 12.8$, we can assign this inflection in oxidized Av1′ to an $S = 3$ state.

The behavior of $Av1'_A$ during an oxidative titration (Figure 7) is highly unusual. To explain this behavior, a model will be proposed that presumes the existence of two different redox forms of this protein, called $Av1'_{A1}$ and $Av1'_{A2}$. Form $Av1'_{A1}$ is generated during the initial enzymatic reduction of the protein. In this protein, the M centers have a redox midpoint potential more negative than that of the P clusters and, therefore, oxidize first upon the addition of thionine. Following oxidation of the M centers, $Av1'_{A1}$ converts to form $Av1'_{A2}$ upon the addition of more oxidant to the medium. To trigger this conversion, $Av1'_{A1}$ must possess a redox receptor that responds to the environmental potential. The only known redox-active sites in component 1 are the M centers, P clusters, and [4Fe-4S] cluster. Since the M centers have already been oxidized, it is doubtful that they will respond to further oxidation. The same is true for the [4Fe-4S] cluster, which is also oxidized (and EPR silent) at this point in the oxidation. It is also difficult to see how the [4Fe-4S] cluster could act as an effective redox receptor since it is probably over 67 Å from both the cofactor and the P

cluster. Therefore, the most likely candidate for the redox receptor is the reduced P cluster. In other words, it must be the establishment of the equilibrium $P \leftrightarrow P^+ + e^-$ in $Av1'_{A1}$ that triggers its conversion into $Av1'_{A2}$. Form $Av1'_{A2}$ has a redox profile like that of $Av1'_B$ discussed earlier, where the midpoint potential of the M center is slightly more positive than that of the P clusters. Because of this, the initial oxidation of the P clusters induces the conversion of $Av1'_{A1}$ to $Av1'_{A2}$, inverting the relative redox potentials of the two metal clusters and promoting a redistribution of the electrons in the protein. Electrons flow from of the P clusters to reduce the previously oxidized M centers.

Why is this phenomenon only observed in $Av1'_A$ and not in $Av1_B$? The major structural difference between $Av1'_A$ and $Av1'_B$ is the absence of an α subunit in the former protein. The absence of this subunit appears to render $Av1'_A$ susceptible to a P cluster oxidation-induced change from $Av1'_{A1}$ to $Av1'_{A2}$, which gates the oxidation changes ($P \rightarrow P^+$ and $M^+ \rightarrow M$). A similar coupling of the oxidation of the P clusters with a concurrent reduction of the M center may also take place in $Av1'_B$ or the MoFe protein, but may occur at a different, possibly more negative, redox level outside the range of our oxidative titration. Obviously, this coupling would be mechanistically important because it would force the cofactor into a more reduced state, possibly necessary for N_2 reduction. Even though redox gating is not observed during the oxidative titration of $Av1'_B$, the observed change in rhombicity (Figure 2) of the $S = 3/2$ cofactor signal upon P cluster oxidation does suggest a coupling between P cluster oxidation and the structure about the cofactor. Similar couplings have already been proposed to occur during catalysis (Chan et al., 1993).

How does the oxidation of the P cluster induce a redox and rhombicity change detected at the cofactor? The structure of component 1 may suggest a possible mechanism for the coupling of redox and conformational changes. An aspect of the P cluster structure not typically observed in FeS clusters is the bridging of the cluster between different subunits. An analogous situation exists in the component 2 protein, where a single [4Fe-4S] cluster is bridged between identical subunits. In component 2, Mg-ATP binding, which has been proposed to occur about 20 Å from the [4Fe-4S] cluster, induces a dramatic lowering of the midpoint potential of the cluster and changes the rhombicity of the EPR spectrum of the reduced cluster (Orme-Johnson & Davis, 1978; Zumft et al., 1973, 1974). Small angle X-ray scattering studies have shown that Mg-ATP binding induces a conformational change in the protein (Hodgson et al., 1994). Also, Mg-ATP binding to component 2 greatly enhances the chelation of the [4Fe-4S] cluster by bathophenanthrolinedisulfonate (Ljones & Burris, 1978). In other words, Mg-ATP binding to component 2 induces both a conformational change in the protein as well as a change in the redox midpoint and spectral rhombicity of the cluster, i.e., conformational and redox changes are coupled in component 2. It is possible that a similar situation exists in component 1, where a redox change in the P cluster induces a conformational shift from $Av1'_{A1}$ to $Av1'_{A2}$. While these are only two isolated examples, it may be found that the bridging of a metal cluster between different subunits of a protein is an effective way of coupling a redox change in the cluster with a conformational change in the protein.

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