Further Redox Reactions of Metal Clusters in the Molybdenum-Iron Protein of *Azotobacter vinelandii* Nitrogenase†

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ABSTRACT: The iron-molybdenum protein of *Azotobacter vinelandii* (AV_1) nitrogenase can be prepared in the $S_2O_4^{2-}$-reduced resting state as well as in oxidation states more oxidized than the resting state. We report the redox properties and spectroscopic characteristics for the interconversion of the protein-bound metal clusters in AV_1 giving rise to these redox states in AV_1. Three-electron-oxidized AV_1 has (1) a fully developed electron paramagnetic resonance (EPR) signal, (2) distinguishable circular dichroic and visible-ultraviolet spectral characteristics, (3) hysteresis in its redox properties, and (4) slow reactivity with $S_2O_4^{2-}$. Six-electron-oxidized AV_1 undergoes polyphasic reduction reactions with $S_2O_4^{2-}$ in which three types of spectroscopically distinct clusters occurring in a 2:1:3 stoichiometric ratio undergo reduction. By correlating the electrochemical, spectroscopic, and kinetic properties of six- and three-electron-oxidized and fully reduced AV_1, we arrive at a model for the metal cluster composition of AV_1 consisting of two EPR centers, three "P-clusters", and a single center that is kinetically and spectroscopically distinct from the EPR and "P-cluster" centers.

Reaction of the as-isolated $S_2O_4^{2-}$-reduced *Azotobacter vinelandii* MoFe protein (AV_1) with various chemical reagents produces oxidized AV_1 with distinct spectroscopic, kinetic, and electrochemical properties. With moderately strong oxidants [methylene blue (MB)[1] and thionine], two redox regions undergo oxidation in which three electrons are removed from each region. This reaction and the resulting oxidized AV_1 species have received considerable study (Watt et al., 1981; Euler et al., 1984; Zimmerman et al., 1978; Stephens et al., 1981). Milder oxidants [indigodisulfonate (IDS)] produce only three-electron-oxidized species (Euler et al., 1984; Stephens et al., 1981) while stronger oxidants [O_2, $Fe(CN)_6^{3-}$, dichlorophenolindophenol (DCPIP)] produce active enzyme species oxidized by 9 and 12 electrons (Wang et al., 1985; Watt et al., 1980a,b). The physiological relevance of these fully active chemically produced oxidized enzyme states remains unclear, although recent considerations by Thorneley et al. (1981) have led to the suggestion that some may function in enzyme turnover. Whether or not part or all of these AV_1 oxidation states are functional in nitrogenase catalysis remains to be further established, but for the present their preparation and study provide useful information regarding the number of redox centers present and the spectroscopic and electrochemical interrelationships among the different types of metal clusters.

While much is known concerning the oxidation behavior of reduced AV_1 by chemical reagents, little is known about the chemical reduction of oxidized AV_1. It would be of interest to know the reduction sequence and reduction characteristics of the various oxidized centers present in AV_1 and to compare their behavior with the corresponding results for oxidation. This comparison would be relevant to the redox reversibility of the redox centers in AV_1 and may relate to the electrochemical hysteresis known to be operative in this protein (Watt et al., 1980a,b) and in the isolated MoFe cofactor (Schultz et al., 1985). In this study we report oxidation reactions involving a wide range of chemical oxidants and reduction of the resulting oxidized AV_1 with $S_2O_4^{2-}$.

Materials and Methods

Oxidized AV_1 Proteins. Two oxidized forms of AV_1 were prepared and studied. The first, from which six electrons were removed, was prepared and characterized as described previously (Watt et al., 1980a,b). The second, from which only three electrons were removed, was prepared by oxidizing the as-isolated AV_1 at pH 8.0 in 0.25 M NaCl and 0.05 M Tris with a 10-fold excess of IDS or other appropriate oxidants listed in Table I. Following oxidation, the protein was passed through an anaerobic Sephadex G-25 column to remove the excess oxidant and oxidant products. The extent of oxidation was determined microcoulometrically (Watt, 1979).

The specific activity of these oxidized AV_1 species remains unchanged from the native protein and possessed activities of 2200-2700 nmol of H_2/(min·mg). The metal content also remained unchanged (26-29 Fe and 1.7-2.0 Mo per 230 000 daltons) after oxidation.

Methods. The controlled potential reduction method using two electrolysis cells for determining which of the two redox regions of the oxidized AV_1 undergoes reduction when limiting reductant is added has been described previously (Watt et al., 1981). The six-electron-oxidized AV_1 protein was reacted for 20-30 min with various equivalents (0-6) of standardized $Na_2S_2O_4$ solution. After incubation, aliquots of the protein solution were transferred to two potentiostats, one controlled at -400 mV vs. NHE and the other controlled at -600 mV

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[1] Abbreviations: MB, methylene blue; MV, methylviologen; FMN, flavin mononucleotide; BAB, brilliant alizarin blue; IDS, indigodisulfonate; Tris, tris(hydroxymethyl)aminomethane; NHE, normal hydrogen electrode; Cp_1 and Kp_1, the MoFe proteins from *Clostridium pasteurianum* and *Klebsiella pneumoniae*, respectively.

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vs. NHE. From these controlled potential reduction measurements, the redox region receiving the electrons from $S_2O_4^{2-}$ was evaluated as previously outlined (Watt et al., 1981).

Three methods were used to measure $S_2O_4^{2-}$ uptake by oxidized AV_1. The first method is the polarographic method, which has been described (Watt et al., 1980a,b) and applied to the characterization of oxidized AV_1 species. The second method is simply a variation of the controlled potential reduction method discussed above. Six equivalents of $S_2O_4^{2-}$ was added to oxidized AV_1, the mixture was quickly stirred, and then samples were transferred to the reduction cells controlled at −600 and −300 mV. The cell controlled at −300 mV will not reduce oxidized AV_1 because this cell potential is too positive for reduction to occur. However, at this potential $S_2O_4^{2-}$ is quantitatively oxidized to SO_3^{2-}, and its presence can be detected and the amount quantitated. The cell controlled at −600 mV provides a means for detecting the presence of oxidized or partially oxidized MoFe protein. When the protein–$S_2O_4^{2-}$ mixture is sampled at both potentials as a function of time, the reduction of oxidized AV_1 by $S_2O_4^{2-}$ can be monitored.

The third method is an optical one in which the absorbance decrease of $S_2O_4^{2-}$ contained in an anaerobic quartz cell is measured spectrophotometrically in the presence of a known amount of oxidized AV_1. This method has a minor complication because the protein changes absorbance at the same wavelength that $S_2O_4^{2-}$ is being monitored, 320 or 350 nm, and therefore, corrections must be made to the overall absorbance change for the change in protein absorbance. These corrections were determined separately by adding oxidized and reduced but $S_2O_4^{2-}$-free AV_1 protein to anaerobic buffer and measuring the absorbance differences at the wavelengths of interest.

EPR-silent oxidized AV_1 contained in anaerobic 3-mm quartz EPR cells was titrated stepwise with 0.5-equiv aliquots of standardized $S_2O_4^{2-}$. Standardization of S_2O_4 was accomplished either electrochemically or optically at 315 nm (ϵ = 8000 cm^{-1} M^{-1}). The protein–$S_2O_4^{2-}$ solution was allowed to react for 20–30 min before freezing in liquid nitrogen. EPR spectra were recorded at 11 K with EPR equipment previously described (Watt et al., 1981).

Optical titrations and reduction reactions followed by optical spectroscopy were carried out in anaerobic quartz cells (1.0- or 3.5-mL volume) on a Cary 118 spectrophotometer. Circular dichroism (CD) measurements were made on a Kettering dichrometer (Breeze & Ke, 1972) in the 300–800-nm spectral region.

The protein concentration of $S_2O_4^{2-}$-reduced AV_1 for routine measurements was determined by biuret and Lowry methods, which had been previously calibrated against dry weight and the total amino acid analysis of AV_1. Lowry and biuret methods were also used to measure protein concentration for three- and six-electron-oxidized AV_1. However, in each case, the oxidized protein was reduced with a small excess of solid $Na_2S_2O_4$ and the protein concentration redetermined so that the oxidized protein concentration could be related to the more reliable reduced protein value. The biuret method gives correct protein values for all three oxidation states, and the Lowry method correctly estimates the protein concentration for reduced and three-electron-oxidized AV_1. However, the Lowry method underestimates the protein concentration of six-electron-oxidized AV_1 by 17%.

Results

Spectral Properties of AV_1. Many of the experimental results to be described below monitor the optical or EPR properties or changes in the spectral properties of AV_1 to determine its concentration, its redox state, or the electron-transfer sequence during redox reactions. To aid in discussing the experimental results, a description of the spectral properties of reduced, three-electron-oxidized, and six-electron-oxidized AV_1 will first be presented.

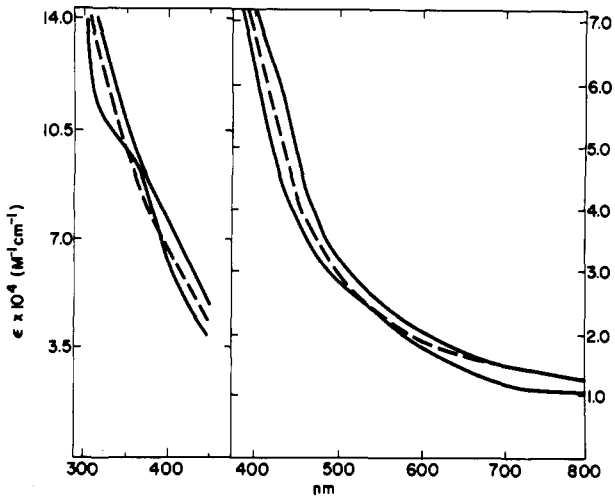

FIGURE 1: Optical spectra of reduced AV_1 (lower solid line), three-electron-oxidized AV_1 (dashed line), and six-electron-oxidized AV_1 (upper solid line). Spectra were recorded in a 1-cm quartz cell with an AV_1 concentration of 2.5 mg/mL or 1.09×10^{-5} M.

Figure 1 displays the optical spectra of fully reduced, three-electron-oxidized, and six-electron-oxidized AV_1, from bottom to top, respectively, and delineates the spectral features of these AV_1 species that clearly distinguish them from one another in certain regions of the spectrum. For example, reduced AV_1 has a small broad shoulder near 360 nm that is absent in three-electron-oxidized AV_1. Isosbestic points develop at 356 and 388 nm as reduced AV_1 is converted into its three-electron-oxidized state. From the isosbestic point at 388 nm to about 500 nm, the optical spectrum of three-electron-oxidized AV_1 is clearly distinguishable from reduced AV_1 by possessing greater absorbance values in this region. Both reduced and three-electron-oxidized AV_1 possess nearly identical optical properties from 500 to 560 nm. At the long-wavelength end of the spectrum, reduced AV_1 has a lower absorbance than three-electron-oxidized AV_1 from 560 to 800 nm, with a maximum difference occurring at 700 nm. Stepwise oxidation results in a uniform spectral increase in this region corresponding to the conversion of reduced to three-electron-oxidized AV_1. The six-electron-oxidized species has an increased absorbance over most of the spectrum, compared to both reduced and three-electron-oxidized AV_1, except from 650 to 800 nm where it is coincident with the latter. The six-electron-oxidized form has a very small shoulder at 430 nm, which is absent in both of the other AV_1 species studied.

We have also recorded the CD spectra for these three AV_1 species and find, in agreement with Stephens et al. (1981), that a large CD band develops at 450 nm during the stepwise conversion of reduced AV_1 into three-electron-oxidized AV_1, but subsequent oxidation produces no further CD change during conversion of AV_1 into the six-electron-oxidized state. The EPR spectrum of each species in Figure 1 was concurrently recorded, and the EPR signal was found to be present in reduced AV_1 and undiminished in three-electron-oxidized AV_1 but absent in six-electron-oxidized AV_1. The spectral and electrochemical results presented here are consistent with previous studies (Watt et al., 1981; Euler et al., 1984; Stephens et al., 1981), which suggest that the three-electron-oxidized

Table I: Response of AV_1 to Oxidants[a]

oxidant	$E°$, pH 7	protein reduction at −400 mV	protein reduction at −600 mV	EPR	CD band at 450 nm
$S_2O_4^{2-}$-free AV_1		0	0	present	absent
methylviologen	−400	0	0	present	absent
neutral red	−325	0	~1	present	partially developed
phenosafranine	−250	0	3	present	present
FMN	−250	0	3	present	present
brilliant alizarin blue	−175	0	3	present	present
indigodisulfonate	−125	0	3	present	present
methylene blue	+11	3	6	absent	present
thionine	+64	3	6	absent	present
toluylene blue	+115	3	6	absent	present
DCPIP	+217	3	6	absent	present

[a] AV_1 was first oxidized with a 10-fold excess of the indicated oxidant for 25 min followed by anaerobic Sephadex G-25 chromatography. Coulometric reduction, EPR, and CD measurements were carried out, using this gel-filtered AV_1. Reduction at −600 mV fully reduces oxidized AV_1. The difference in reduction capacity between the −600 and −400 mV yields the number of oxidized "P-clusters" that were initially present (Watt et al., 1981).

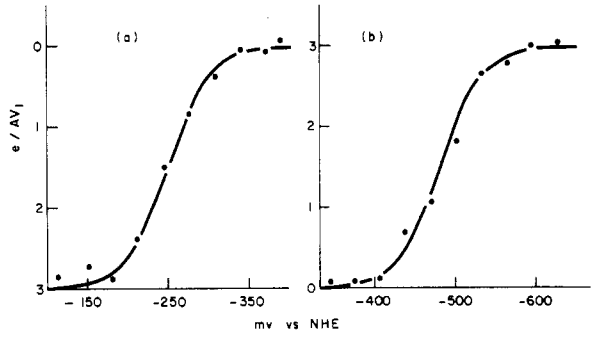

FIGURE 2: Redox reactions of AV_1. (a) The coulometric oxidation of reduced, $S_2O_4^{2-}$-free AV_1 using methylene blue (MB), indigodisulfonate (IDS), and FMN as redox mediators at 5×10^{-5} M in 0.05 M Tris and 0.25 M NaCl, pH 8.0. The ordinate represents the number of electrons removed at the indicated potentials. The solid line is the theoretical fit for an $n = 1$ Nernstian process with $E_{1/2} = -250$ mV (NHE). (b) The coulometric reduction of IDS-oxidized AV_1 using 7.5×10^{-5} M methyl- and benzylviologens at pH 8.0 in 0.05 M Tris and 0.25 M NaCl. The ordinate is the number of electrons added to oxidized AV_1 at the indicated potentials. The solid line is the fit for an $n = 1$ process with $E_{1/2} = -470$ mV.

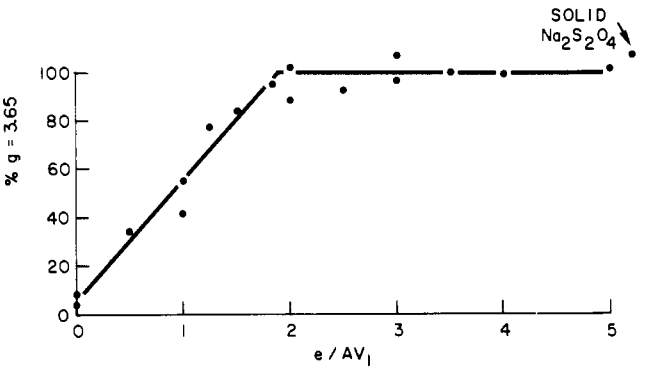

FIGURE 3: EPR titration of six-electron-oxidized AV_1 with standardized $S_2O_4^{2-}$. AV_1 exhaustively oxidized with MB and freed from excess oxidant by anaerobic Sephadex G-25 chromatography was titrated with increments of standardized $S_2O_4^{2-}$. The AV_1 concentration was 10.9 mg/mL. EPR spectra were recorded at 11 K, and the signal height at $g = 3.65$ was used to quantitate AV_1 reduction.

AV_1 species, possessing a fully developed EPR signal, the optical spectral properties shown in Figure 1, and the fully developed CD band at 450 nm, has all "P-cluster" (Zimmerman et al., 1978) oxidized. The results reported here are also consistent with the previously proposed model (Euler et al., 1984) for six-electron-oxidized AV_1 consisting of three "P-clusters", two EPR centers, and an as yet unidentified center different from the other two types.

Three-Electron-Oxidized AV_1. The addition of excess IDS to reduced AV_1 produces AV_1 oxidized by three electrons, but which possess a fully developed EPR signal and a fully developed CD band at 450 nm (Stephens et al., 1981). Table I confirms these results and in addition shows the effect that oxidation potential variation of the chemical oxidant has on the 450-nm CD band, the EPR signal intensity, and the extent of oxidation of AV_1. The table shows that a 10-fold excess of an oxidant with a redox potential more negative than −125 mV has no effect on the EPR signal intensity and limits the extent of AV_1 oxidation to three electrons or less. During this three-electron oxidation, the CD band at 450 nm develops and the optical changes shown in Figure 1 occur. Increasing the oxidation potential to near 0 mV causes the EPR signal to decrease and the degree of AV_1 oxidation to approach 6. A further increase in oxidation potential results in the formation of higher oxidation states of AV_1 as reported by Wang et al. (1985) and to be discussed in more detail in a later report (Watt, unpublished results).

Figure 2b shows the reduction of AV_1 that was oxidized by excess IDS, FMN, or BAB. A clearly established three-electron oxidation of AV_1 occurs with any of the three oxidants. The numerical value of −470 mV for the reduction midpoint potential with an $n = 1$ value is essentially identical with that value for the most negative redox region previously reported (Watt et al., 1980a) for the reduction of six-electron-oxidized AV_1. Figure 2a shows the oxidation potential of reduced AV_1 to be −250 mV and demonstrates that the electrochemical processes are not reversible because the oxidation and reduction midpoint potentials differ from one another by 220 mV.

Reaction of three-electron-oxidized AV_1 with $S_2O_4^{2-}$ is an extremely slow process requiring several hours for complete reduction to occur. The progress of this reaction has been followed by electrochemical and optical (including CD) methods, and kinetic analysis suggests the reaction is zero order in protein. The addition of MV to 0.01 mM or higher in the presence of three equivalents of (or excess) $S_2O_4^{2-}$ causes rapid and complete reduction to occur. This behavior was displayed by three-electron-oxidized AV_1 independent of the oxidant used to produce it.

To summarize, the three-electron oxidation or reduction of AV_1 is accompanied by (1) a large CD change at 450 nm, (2) the optical changes shown in Figure 1, (3) the redox potentials shown in Figure 2, (4) no change in the EPR signal intensity, and (5) slow protein reduction with $S_2O_4^{2-}$ alone but accelerated reduction with $S_2O_4^{2-}$ + MV.

Six-Electron-Oxidized AV_1. The reaction of six-electron-oxidized AV_1 with 6 equiv of $S_2O_4^{2-}$ (or excess) occurs in several steps, as evidenced by EPR, optical, CD, and elec-

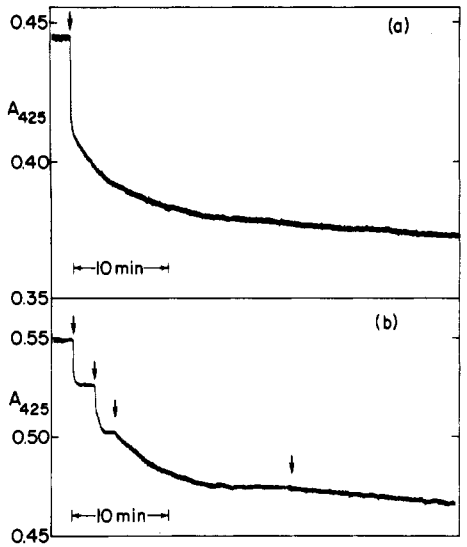

FIGURE 4: Reduction of six-electron-oxidized AV_1 with $S_2O_4^{2-}$: (a) the absorbance change at 425 nm upon addition of excess $S_2O_4^{2-}$ to oxidized AV_1 and (b) the absorbance change at 425 nm corresponding to 1-equiv additions (1 e/AV_1) of $S_2O_4^{2-}$ to oxidized AV_1. The arrows mark the positions of $S_2O_4^{2-}$ addition.

trochemical monitoring of either protein or $S_2O_4^{2-}$ properties. These physical techniques are sufficiently specific that individual cluster types can be monitored, in some cases, independently of others during the reduction reaction.

Figure 3 is an EPR titration of six-electron-oxidized AV_1 with standardized $S_2O_4^{2-}$. Oxidized AV_1 is initially EPR silent, but the EPR signal develops linearly with added increments of standardized $S_2O_4^{2-}$ until at 2 equiv of $S_2O_4^{2-}$ (two added electrons per AV_1) the EPR signal reaches a maximum and remains constant with further addition of $S_2O_4^{2-}$. A time course of EPR signal intensity during reduction of AV_1, using excess $S_2O_4^{2-}$, demonstrated that signal development is complete within less than 1 min of reaction time. No new signals were observed during any of the time course studies or during the titration experiments.

Reduction of six-electron-oxidized AV_1 with excess $S_2O_4^{2-}$ was monitored by following the decrease of absorbance of AV_1 with optical spectroscopy at 425 and 750 nm. At these wavelengths $S_2O_4^{2-}$ absorption is negligible, and absorption decreases result from the combined contributions from all six reactive clusters when monitored at 425 nm but only three of the clusters when monitored at 750 nm (see Figure 1). Figure 4a shows the absorbance decrease at 425 nm as oxidized AV_1 undergoes $S_2O_4^{2-}$ reduction and suggests that three types of reactive centers are present in the protein. The first undergoes essentially instantaneous reduction during the time of $S_2O_4^{2-}$ addition, the second is a rapid exponential decrease in absorbance, while the third is a slow, linear decrease of absorbance with time. Kinetic analysis of these two latter curves indicates a first-order and zero-order reaction in protein, respectively. The presence of three separate types of centers was confirmed with greater precision by the optical titration of six-electron-oxidized AV_1 with 1-equiv aliquots of standardized $S_2O_4^{2-}$ shown in Figure 4b. The first two additions caused essentially instantaneous protein reduction as monitored at 425 nm. The third resulted in an exponential absorbance decrease lasting $\sim$10 min, and then subsequent additions produced only very slow optical decreases.

When monitored at 750 nm, the $S_2O_4^{2-}$ titration of six-electron-oxidized AV_1 produced no change with the first two added electrons but with the third a slow exponential change was noted. This change at 750 nm corresponds to the slow

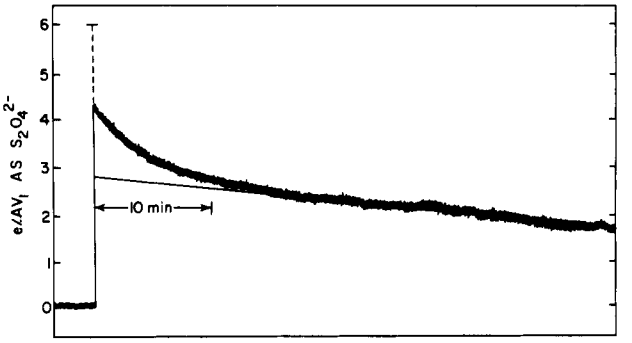

FIGURE 5: Stoichiometric reduction of six-electron-oxidized AV_1 with $S_2O_4^{2-}$ as monitored at 320 nm, the absorption maximum of $S_2O_4^{2-}$. Six equivalents of $S_2O_4^{2-}$ (6 e/AV_1) was added initially, and the amount remaining at a given time is indicated by the ordinate axis. Extrapolation of the slow, linear reaction to the time of $S_2O_4^{2-}$ addition suggests three slowly reacting centers.

exponential change observed in Figure 4 occurring with addition of the third electron to AV_1 and suggests the third added electron reduces a "P-cluster". The optical spectrum of this three-electron, $S_2O_4^{2-}$-reduced AV_1 species differs in several ways from the three-electron-oxidized AV_1 species (in which all three "P-clusters" are oxidized) shown in Figure 1, a result that suggests a different distribution of reduced centers in the two AV_1 forms, each having three of the possible six centers reduced. The CD spectrum of this three-electron, $S_2O_4^{2-}$-reduced AV_1 species shows the band at 450 nm has decreased by about 30% upon addition of the third electron compared to the original six-electron-oxidized state in which all three "P-clusters" were oxidized. This CD result is consistent with a single "P-clusters" reduction because "P-clusters" redox reactions are the only metal cluster reactions in AV_1 that give rise to the 450-nm CD band. Subsequent $S_2O_4^{2-}$ additions cause only very slow reduction in AV_1 as monitored at 425 and 750 nm in the optical spectrum and 450 nm in the CD spectrum. This slow reaction corresponds to reduction of the two remaining "P-clusters" and the single non-EPR center.

The addition of 0.01 mM methylviologen has no significant effect on the rapid or first-order reductions but rapidly accelerates the slower reaction to completion in a matter of minutes. The addition of methylviologen causes the reaction order to change from zero- to first-order in protein while catalyzing the reduction reaction. Comparison of the optical titration results in Figure 4 with those of Figure 3 establishes that the rapid optical changes occurring with the first 2 equiv of $S_2O_4^{2-}$ correspond to the reduction of the EPR centers. The CD spectrum supports this conclusion by showing the lack of reduction of any "P-clusters" after a two-electron addition.

Figure 5 displays the reaction of six-electron-oxidized AV_1 with a stoichiometric amount of added $S_2O_4^{2-}$ as monitored at 320 nm, an absorbance maximum for $S_2O_4^{2-}$. In this figure, 6 equiv of $S_2O_4^{2-}$ is initially reacted with six-electron-oxidized AV_1, and the amount of $S_2O_4^{2-}$ consumed is followed as a function of time. The results indicate that an initially rapid $S_2O_4^{2-}$ loss occurs followed by a slow linear decrease in $S_2O_4^{2-}$ concentration over a period of several hours, results similar to that shown in Figure 4. As shown by the ordinate in Figure 5, the rapidly reacting equivalents of $S_2O_4^{2-}$ can be resolved into two sets in which nearly 2 equiv reacts essentially instantaneously and 1 equiv reacts more slowly. The linear decrease corresponds to the slow $S_2O_4^{2-}$ reduction of the three remaining centers. The stoichiometric reduction of AV_1 by $S_2O_4^{2-}$ clearly reveals a reduction sequence involving two, one, and three types of centers as discussed above. The reduction of three-electron-oxidized AV_1 with a stoichiometric amount

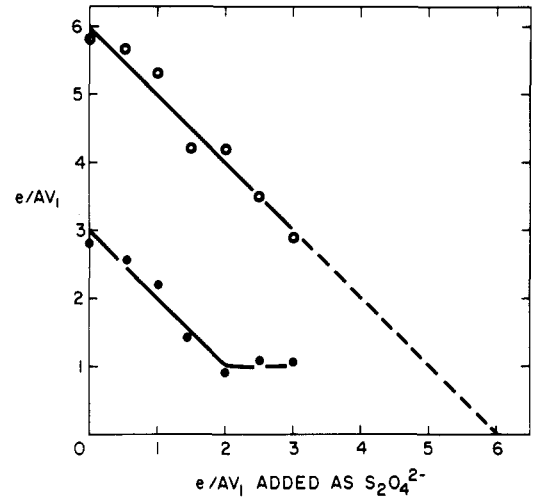

FIGURE 6: Reduction of six-electron-oxidized AV_I with $S_2O_4^{2-}$ monitored electrochemically. The ordinate is the electrons transferred electrochemically to oxidized AV_I that was previously reacted with the aliquots of $S_2O_4^{2-}$ indicated on the abscissa. The reduction potential of the cell was −600 mV so that complete reduction of oxidized or partially oxidized AV_I occurred (○, upper curve). The potential for the lower curve (●) was set at 400 nm, a potential too positive for "P-cluster" reduction. The lack of reduction at −400 mV, shown in the lower curve, after addition of the third electron, indicates that the third added electron was transferred to a center more negative than −400 mV, a "P-cluster".

of $S_2O_4^{2-}$ produces only the slow, linearly decreasing curve shown in Figure 5.

Electrochemical monitoring of $S_2O_4^{2-}$ consumption and of the measurement of the number of unreduced redox centers and their midpoint potentials remaining in oxidized AV_I following sequential reduction by limiting $S_2O_4^{2-}$ was found to be concordant with the optical results just described. Figure 6 shows that stepwise reduction of six-electron-oxidized AV_I with $S_2O_4^{2-}$ causes rapid (within 10 min or less) reduction to occur exclusively in the most positive ($E_{1/2}$ = −290 mV) redox region [see Figure 2, Watt et al. (1980a,b)] of AV_I, until a total of two electrons has been added. The lower curve in Figure 6 shows that the addition of the third electron then produces reduction in the most negative redox region ($E_{1/2}$ = −480 nM) of AV_I, indicative of "P-cluster" reduction. Subsequent addition of $S_2O_4^{2-}$ results in the initial appearance of unreacted $S_2O_4^{2-}$ (the presence of unreacted $S_2O_4^{2-}$ is a consequence of the slow reduction of the remaining unreduced centers), which only very slowly disappears. These results are consistent with the first two electrons reducing the EPR centers, the third added electron reducing a "P-cluster", followed by a slow, random reduction of the remaining two "P-clusters" and the one remaining unknown center.

The results just described for $S_2O_4^{2-}$ reduction of six-electron-oxidized AV_I are the most often encountered mode of reaction. From a total of 16 separate $S_2O_4^{2-}$-reduction experiments, 11 followed the reaction pattern described above. The remaining 5 followed the reaction course in which three centers reacted exponentially within 5 min, followed by a very slow linear reaction requiring several hours for completion. The optical spectrum of this three-electron-reduced AV_I was identical with that of the three-electron-oxidized AV_I species shown in Figure 1. The EPR signal is fully developed but there is no decrease in the 450-nm CD band, indicating that the three-electron transfer does not involve any "P-cluster" reduction. Determination of the reduction potential of AV_I reduced by 3 equiv of $S_2O_4^{2-}$ gives a reduction curve identical with the three-electron-oxidized AV_I curve shown in Figure 2. These results are all consistent with a three-electron reaction of AV_I in which the two EPR centers and the unknown center are reduced quickly as a group, followed by a very slow reduction of the remaining "P-clusters" as a group [this reactivity pattern is that displayed by methylviologen; see Watt et al. (1980a,b)]. The unknown center behaves similarly to an EPR center during this reduction pattern but behaves as a "P-cluster" in the most common reductive reaction pattern discussed above. We have not yet identified the cause of this variability of reduction behavior.

The polarographic method for measuring $S_2O_4^{2-}$ decrease during the reaction with oxidized AV_I has been described (Watt et al., 1980a,b). At $S_2O_4^{2-}$ concentrations below 0.1 mM both the three- and six-electron-oxidized AV_I accept three and six electrons, respectively, when $S_2O_4^{2-}$ consumption is measured by this technique. We report here that above 0.1 mM $S_2O_4^{2-}$ the number of electrons transferred to AV_I in either oxidation state apparently increases to about eight or more at 1 mM $S_2O_4^{2-}$. Reduced but $S_2O_4^{2-}$-free AV_I also appears to accept electrons at $S_2O_4^{2-}$ concentrations above 0.1 mM but not below. This unusual result may be due to reduced AV_I having an affinity for and weakly binding $S_2O_4^{2-}$. Thus, as the $S_2O_4^{2-}$ concentration exceeds 0.1 mM, the binding of $S_2O_4^{2-}$ by AV_I becomes significant and the apparent loss of $S_2O_4^{2-}$ from solution upon AV_I addition, which the polarographic method detects, is not just a consequence of electron transfer to oxidized AV_I but also includes binding of $S_2O_4^{2-}$ to AV_I. Caution should therefore be exercised when using this method for quantitative electron-transfer measurements to the nitrogenase proteins by keeping $S_2O_4^{2-}$ concentration below 0.1 mM.

Discussion

Table I presents data that show that a number of oxidants with redox potentials more negative than −125 mV react with AV_I but limit the extent of oxidation to three electrons or less. The EPR signal intensity is unaffected by reaction with excess of these oxidants but both optical (see Figure 1) and CD spectral changes at 450 nm accompany the three-electron oxidation of AV_I. The oxidation potential of AV_I for this three-electron oxidation was measured to be −220 mV (Figure 2), a result that the data in Table I empirically support.

When any of the oxidants in Table I are used to exhaustively oxidize AV_I to its fully three-electron-oxidized state and then excess oxidant is removed by anaerobic Sephadex G-25 chromatography, followed by electrochemical reduction of the resulting oxidized AV_I (Watt, 1979), a reduction potential of −470 mV results. This value is significantly different from the results suggested in Table I and from the directly measured oxidation potential shown in Figure 2. This electrochemical hysteresis has been noted previously (Watt et al., 1980a,b), but its cause still remains unexplained. It is of significance that $S_2O_4^{2-}$ reduces this three-electron-oxidized AV_I only very slowly even though its reduction potential (Mayhew, 1978) is more than sufficient for complete reduction to occur. The addition of a catalytic amount of MV (a cationic redox mediator) rapidly accelerates reduction and suggests that these three oxidized centers in AV_I might reside in an anionic protein environment. A previous observation of MV action in accelerating $S_2O_4^{2-}$ reduction of AV_I was made by Zimmerman et al. (1978) as determined by the much more rapid appearance in the Mössbauer spectrum of reduced "P-clusters" during AV_I reduction in the presence of MV than in its absence. The spectroscopic and electrochemical data presented here show these three centers to be closely related during chemical and electrochemical oxidation but to be clearly distinct from the

other three centers present in AV_1, which similarly behave as a related group. During reduction, however, the most common reactivity pattern encountered is one in which two EPR centers are first reduced, followed by a "P-cluster" that undergoes reduction separately from the others. The less commonly encountered reduction sequence is the thermodynamically expected one in which the two EPR centers and the single unknown center are first reduced, followed by the three "P-clusters". It seems that a maverick "P-cluster" and the unknown center are similarly poised thermodynamically so that with minor perturbations either can undergo reduction following the two EPR centers, but a different reduction pattern is dictated depending upon which cluster type first undergoes reduction.

From oxidative studies of AV_1, Euler et al. (1984) concluded that six-electron-oxidized AV_1 resulted from a one-electron oxidation of three separate types of redox centers occurring in a 3:2:1 ratio. Those centers appearing in duplicate give rise to the EPR signal in reduced AV_1 (Zimmerman et al., 1978; Euler et al., 1984) and can be separated from the protein and isolated as the MoFe cofactor (Shah & Brill, 1977). The remaining centers, related by a 3:1 stoichiometry, were shown to have very distinct spectroscopic and redox properties (Watt et al., 1980a,b, 1981; Euler, 1984; Stephens et al., 1981). The diversity of spectroscopic and electrochemical techniques used in this study and the inherent kinetic and thermodynamic properties displayed by the oxidized centers present in AV_1 during $S_2O_4^{2-}$ reduction make possible a distinction among and the quantitation of the various cluster types present. The results of this study are consistent with the oxidative results just mentioned but show more clearly the 3:2:1 stoichiometry. Although the most commonly encountered reduction sequence differs from that expected from the oxidation sequence, the results presented here are nevertheless consistent with the 3:2:1 stoichiometry obtained from the oxidation study (Euler et al., 1984). Figure 3 shows that two EPR centers first undergo reduction and Figures 1, 4, and 5 and the CD titration results (not shown) taken together indicate a single "P-cluster" is next reduced, followed by the unknown center and the remaining two "P-clusters". The alternate $S_2O_4^{2-}$-reduction pathway is interpretable in terms of the two EPR centers and the unknown center reacting together, followed by a much slower reduction of three "P-clusters". The measured reduction potentials and the results of Figure 6 also strongly support the presence of three "P-clusters" ($E_{1/2}$ = −470 mV), two EPR centers ($E_{1/2}$ = −290 mV), and a single unknown center ($E_{1/2}$ = −290 mV).

Mössbauer spectra of reduced AV_1 (Zimmerman et al., 1978; Dunham et al., 1985), Cp_1 (Huynh et al., 1980), and Kp_1 (Smith & Lang, 1974) have been reported and analyzed in considerable detail. A recent quantitative reinvestigation (Dunham et al., 1985) of AV_1 has raised several questions regarding previously drawn conclusions concerning iron distribution within the metal clusters of AV_1. Earlier interpretations (Zimmerman et al., 1978) of Mössbauer data suggested iron ratios of 4/2/12/12, while the newer data indicate 4/2/16/12 for the spectroscopically unique iron environments designated as Fe^{2+}, S, M, and D, respectively. The 3/1 ratio of D/Fe^{2+} persists in both sets of measurements and was initially interpreted (Zimmerman et al., 1978; Orme-Johnson et al., 1977) in terms of four identical iron clusters, each containing three distinct D-type iron atoms and one Fe^{2+}-type iron atom. While this interpretation explained many of their spectroscopic results and seemed consistent with other data, Zimmerman et al. (1978) were careful to discuss the novelty of this proposal and even suggested other possible, though less likely, alternatives. Because of the number of iron atoms involved, the number of diverse metal clusters present, and the overlapping of several features, Mössbauer spectroscopy in and of itself may not be sufficient to give a complete and unique solution to the iron distribution within the MoFe protein component of nitrogenase. The results of this study and other related studies (Euler et al., 1984; Watt et al., 1980a,b, 1981; Stephens et al., 1981) are not fully consistent with the proposed number of "P-clusters" as discerned by the Mössbauer results. We feel that a further analysis of the Mössbauer data would be appropriate, perhaps including additional spectra on carefully prepared samples of intermediate levels of oxidation. This proposal is quite feasible now in view of the results presented here and elsewhere (Watt et al., 1980a,b, 1981) detailing methods and techniques for characterizing redox intermediates of AV_1 appropriate for these studies.

The polarographic method for measuring $S_2O_4^{2-}$ changes during protein reduction has been applied to AV_1 (Watt et al., 1980a,b) and subsequently to other proteins with reliable success. However, the results reported here indicate that at $S_2O_4^{2-}$ concentrations higher than those previously used, more $S_2O_4^{2-}$ apparently disappears than can be accounted for by electron-transfer reactions. Control reactions utilizing $S_2O_4^{2-}$-reduced AV_1, which should be incapable of $S_2O_4^{2-}$ reaction, at $S_2O_4^{2-}$ concentrations >0.1 mM clearly demonstrate $S_2O_4^{2-}$ uptake even though the protein is fully reduced as monitored spectroscopically or microcoulometrically. Apparent $S_2O_4^{2-}$ loss in this system increases with increasing $S_2O_4^{2-}$ concentration, reaching 3–5 $S_2O_4^{2-}/AV_1$ at $S_2O_4^{2-}$ concentration ≥1.0 mM. Other redox protein reactions do not display this behavior, a result that tends to eliminate technique and instrumental artifacts as an explanation. These results suggest that AV_1 is capable of binding several unreacted $S_2O_4^{2-}$ ions, thereby causing an apparent $S_2O_4^{2-}$ decrease. The recently reported (Burns et al., 1985) inhibition of nitrogenase activity by large anions may be a further manifestation of this effect.

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Bacillus subtilis Mutant Succinate Dehydrogenase Lacking Covalently Bound Flavin: Identification of the Primary Defect and Studies on the Iron–Sulfur Clusters in Mutated and Wild-Type Enzyme†

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ABSTRACT: Succinate dehydrogenase consists of two protein subunits and contains one FAD and three iron–sulfur clusters. The flavin is covalently bound to a histidine in the larger, Fp, subunit. The reduction oxidation midpoint potentials of the clusters designated S-1, S-2, and S-3 in *Bacillus subtilis* wild-type membrane-bound enzyme were determined as +80, −240, and −25 mV, respectively. Magnetic spin interactions between clusters S-1 and S-2 and between S-1 and S-3 were detected by using EPR spectroscopy. The point mutations of four *B. subtilis* mutants with defective Fp subunits were mapped. The gene of the mutant specifically lacking covalently bound flavin in the enzyme was cloned. The mutation was determined from the DNA sequence as a glycine to aspartate substitution at a conserved site seven residues downstream from the hisitidine that binds the flavin in wild-type enzyme. The redox midpoint potential of the iron–sulfur clusters and the magnetic spin interactions in mutated succinate dehdyrogenases were indistinguishable from the those of the wild type. This shows that flavin has no role in the measured magnetic spin interactions or in the structure and stability of the iron–sulfur clusters. It is concluded from sequence and mutant studies that conserved amino acid residues around the histidyl-FAD are important for FAD binding; however, amino acids located more than 100 residues downstream from the histidyl in the Fp subunit can also effect flavinylation.

Succinate dehydrogenase is a membrane-bound iron–sulfur flavoenzyme found in aerobic cells. It catalyzes the oxidation of succinate to fumarate and is located on the matrix side of the inner membrane of mitochondria and on the inner side of the cytoplasmic membrane in procaryotic cells. The catalytic portion of the enzyme is composed of two different protein subunits (Hederstedt & Rutberg, 1981; Hatefi, 1985; Condon et al., 1985). The larger subunit (M_r 64 000–72 000) is designated as Fp and contains a covalently bound flavin adenine dinucleotide (FAD)[1] in 8α-*N*(3)-histidyl linkage to the protein (Salach et al., 1979). The smaller subunit (M_r 25 000–30 000) is designated as Ip.

The structural genes for the Fp and the Ip subunit of *Escherichia coli* (*sdhA* and *sdhB*) (Wood et al., 1984; Darlinson & Guest, 1984) and *B. subtilis* (*sdhB* and *sdhC*) (Philips, Magnusson, Rutberg and Guest, unpublished observations) have been sequenced. The predicted amino acid sequences of the two bacterial enzymes show extensive homologies, and the sequence around the putative FAD binding histidyl in the Fp protein is almost identical with that in bovine heart succinate dehydrogenase (Kenney et al., 1972).

Studies, principally with mitochondrial succinate dehydrogenase, have shown that there are three iron–sulfur clusters, designated as S-1, S-2, and S-3 in the enzyme (Ohnishi & Salerno, 1982; Beinert & Albracht, 1982; Johnson et al., 1985). Clusters S-1 and S-3 can be reduced with succinate, but cluster S-2 can only be reduced with a strong reductant such as dithionite. Cluster S-1 is a binuclear $[2Fe\text{-}2S]^{2+,1+}$ (Salerno et al., 1977; Albracht & Subramanian, 1977) and S-3 is a trinuclear [3Fe-*X*S] type cluster (*X* is 3 or 4) (Ackrell et al., 1984; Johnson et al., 1985). Recently cluster S-2 has been identified and shown to be a tetranuclear $[4Fe\text{-}4S]^{2+,1+}$ type cluster (Johnson et al., 1985; Maguire et al., 1985). Where the clusters are located in relation to the Fp and Ip subunits has not yet been established (Ohnishi & Salerno, 1982; Beinert & Albracht, 1982; Hederstedt et al., 1985).

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[1] Abbreviations: CAP, chloramphenicol; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; Fp, flavoprotein subunit of succinate dehydrogenase; Ip, smaller protein subunit of succinate dehydrogenase; MCD, magnetic circular dichroism; PA, purification agar; Tc, tetracycline.

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