Protein Interaction with and Higher Oxidation States of the Nitrogenase MoFe Protein from *Azotobacter vinelandii*†

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ABSTRACT: The MoFe and Fe proteins from *Azotobacter vinelandii* (Av1 and Av2, respectively) in both their reduced and oxidized states were reacted with small-molecule redox reagents, as well as with various redox proteins. The reactions of Av1 with strong, small-molecule oxidants resulted in oxidation of Av1 by 9 and 12 electrons with only small to moderate losses in specific activity. Spectroscopic titrations of Av1 with these oxidants were complex and indicated that several reaction pathways involving different combinations of the metal clusters present in Av1 were possible. The reaction of heme and copper(II) redox proteins with Av1 is rapid, producing the reduced heme and copper(I) states, respectively, and Av1 oxidized by 9–12 electrons. The cytochromes were generally capable of producing a 12-electron oxidation in Av1 whereas the copper proteins more often limited oxidation to 9 electrons. The reaction of EPR-silent, six-electron-oxidized Av1 with reduced Av flavoprotein, Av ferredoxin I, and bacterial ferredoxin produced EPR signal development in Av1. The EPR intensity reached a maximum at 2–3 equiv of added reduced protein, indicating a quite high specificity for reduction at the MoFe cofactor centers of Av1. Av2 and Av2(MgATP)$_2$ also specifically reduce the oxidized MoFe cofactor centers, giving rise to EPR signal development with 2–3 added equiv of reduced Av2. No EPR signal diminution with reduced but $S_2O_4^{2-}$-free Av1 occurred when Av2(MgATP)$_2$ was added.

The MoFe protein (Av1)[1] is one of two redox-active proteins which comprise the biological nitrogen fixation system from *Azotobacter vinelandii*; the other protein is the MgATP-binding iron protein component (Av2). In combination, Av1 and Av2 couple low-potential electrons, MgATP, protons, and dinitrogen to form ammonia. The slowly emerging mechanism for this overall process (Hageman & Burris, 1978a,b; Thorneley & Lowe, 1983, 1984, 1985) depicts the MgATP-binding Av2 component as the specialized enzymatic reductant of Av1, which in turn carries out substrate reduction. This Av2-mediated electron transfer to and electron accumulation by Av1 is believed to occur by a series of MgATP-promoted electron transfer encounters between Av1 and Av2 (Thorneley & Lowe, 1985). This scheme implies the presence of selective sites on both proteins which orient their interactions for productive electron transfer. We have conducted experiments designed to (1) examine the specificity of Av2 for Av1 and also the specificity of Av1 and Av2 for redox proteins thought to be involved in the in vivo nitrogen reduction process and (2) determine which of the several cluster types known to be present in Av1 is first to receive electrons from reduced Av2, Av flavoprotein (AvFlpH$_2$) or various ferredoxins. Such information is important in understanding the electron flow from reductants through the nitrogenase proteins to the reducible substrates. While the $S_2O_4^{2-}$-reduced or resting state of Av1 would be the most relevant form of Av1 to evaluate in this manner, such studies are limited, because Av2(MgATP)$_2$ is the only known reductant to form "super-reduced" Av1, and complicated, because the nature of the super-reduced state is unknown and might be unstable with respect to turnover on the time scale of our measurements. We have, therefore, chosen to study the EPR-silent, six-electron-oxidized form of Av1 because the reaction of reduced Av2, AvFlp, and ferredoxins to produce resting-state Av1 from oxidized Av1 would still be expected to display reductant specificity but would be more convenient to study without the possibility of enzyme turnover.

If protein recognition and orientation sites play important roles in the formation of various redox states and in the electron transfer reactions of the nitrogenase proteins, then the reaction of redox proteins not associated with *A. vinelandii* or with the nitrogen fixing process would be expected to occur slowly or perhaps not at all. Thus, by examination of the reactivity of various redox proteins toward oxidized and reduced Av1 and Av2, insights might be gained into the nature of the protein interactions, the redox states that form, and the electron transfer reactions occurring within the nitrogenase proteins. In what follows, we report the reactions of various reduced proteins with six-electron-oxidized Av1 and the reaction of selected oxidized proteins with reduced but $S_2O_4^{2-}$-free Av1. For comparison, we also report results on the reaction of Av1 with small-molecule redox reagents possessing redox potentials near those of the redox proteins examined.

Materials and Methods

Av1, Av2, AvFdI, cytochrome c_5 (Campbell et al., 1973), and AvFlp were prepared and purified from *Azotobacter vinelandii* as byproducts of the nitrogenase purification procedure (Burgess et al., 1980). Horse heart cytochrome *c* was purchased from Sigma and further purified (Margoliasch & Schejter, 1966). Plastocyanin was prepared from spinach (Davis et al., 1985). Stellacyanin and laccase were gifts of

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[1] Abbreviations: Av1 and Av2, nitrogenase MoFe and Fe proteins; AvFlpH$_2$, AvFlp*, and AvFlp, reduced, semireduced, and oxidized forms of *Azotobacter vinelandii* flavoprotein; AvFdI ferredoxin I from *Azotobacter vinelandii*; CtFd, 4Fe-4S ferredoxin from *Clostridium thermoaceticum*.

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Dr. E. Solomnon (Stanford University), and the 4Fe-4S bacterial ferredoxin from *Clostridium thermoaceticum* (CtFd; Yang et al., 1977) was prepared by Dr. L. Ljungdahl (University of Georgia). Dichlorophenolindophenol (DCPIP) and $K_3Fe(CN)_6$ were recrystallized and standardized optically or microcoulometrically (Watt, 1979).

Reduced Avl, Av2, AvFlp, AvFdI, and CtFd were prepared by reduction with excess $S_2O_4^{2-}$ followed by anaerobic Sephadex G-25 gel chromatography to remove excess $S_2O_4^{2-}$. The oxidized state of these proteins was first produced either by exposure to air (AvFlp, AvFdI, and CtFd) or by anaerobic reaction with excess methylene blue (Avl and Av2), followed by anaerobic Sephadex G-25 chromatography to remove excess oxidant (Watt et al., 1980, 1986). The copper and heme proteins were isolated in their Cu(II) and Fe(III) states, respectively. The redox states of all proteins used in this study were verified by microcoulometry (Watt, 1979) prior to reaction. All redox reactions involving the nitrogenase proteins and the above-prepared redox proteins were carried out under the strictly anaerobic conditions provided by a Vacuum Atmospheres glovebox (O_2 levels < 0.1 ppm) equipped with dual purifiers for rapid and complete oxygen removal.

Avl Microcoulometry. Coulometric *oxidation* of reduced (free of excess $S_2O_4^{2-}$) or six-electron-oxidized Avl was carried out as a function of potential as previously described (Watt, 1979) with 0.05–0.5 mM thionine as mediator. The number of electrons removed from Avl at a selected potential was obtained by integrating the resulting current–time curves. Coulometric *reduction* was carried out as previously described (Watt, 1979; Watt et al., 1980).

Avl and Av2 Reactivity. Two types of experimental protocol were used to assess the reactivity of Avl and Av2 toward proteins and small-molecule redox reagents examined. The first consisted of reacting either reduced or oxidized Avl or Av2 with ~20-fold *excess* of oxidized or reduced test reagent, respectively, allowing the reaction to proceed 15–30 min, and then separating Avl or Av2 from the mixture. Following these reactions, (1) the Avl or Av2 concentration was determined by Lowry or biuret protein determinations or spectrophotometrically (Anderson & Howard, 1984) at 400 nm (e_{400} = 62.3, 73.5 and 1.11, 1.75 for Avl and Av2, in their reduced and oxidized states, respectively; units = mM^{-1} cm^{-1}), (2) the specific activity was measured by the standard H_2-evolution assay (Wherland et al., 1981), and (3) the redox state was determined microcoulometrically (Watt, 1979) and confirmed by spectral characterization (EPR, vis–UV, CD).

The second approach for reactivity assessment consisted of titrating either reduced or oxidized Avl or Av2 with the appropriate redox state of the test reagent and determining the redox response of Avl or Av2 by spectroscopic or microcoulometric measurements. In most cases, the spectroscopic characteristics of the test reagents were simultaneously followed. For example, the optical properties of the heme proteins and the EPR signal of the copper proteins provided sensitive and unique means for following the extent of their reaction with Avl or Av2. With this procedure, the extent of reaction having occurred with Avl or Av2 was assessed from the amount of limiting reagent used.

Results

Separation of Reaction Components. A complete and unequivocal evaluation of the redox reactivity of a given reagent toward Avl or Av2 requires a complete separation and characterization of the redox state of both reaction partners. This was possible in most reactions studied here, by suitable purification procedures. However, in some instances separation was not easily carried out, and for these cases, spectroscopic uniqueness of one of the components of the reaction mixture was relied upon for reactivity assessment.

The small inorganic or organic redox reagents were completely separated from the Av nitrogenase proteins by anaerobic Sephadex G-25 chromatography. Because of their charge differences, the cytochromes were easily separated from Avl and Av2 by anaerobic DEAE chromatography using either anaerobic water or buffer containing 0.05 M NaCl to elute the cytochromes followed by buffered 0.25 or 0.5 M NaCl to remove Avl or Av2, respectively. Membrane filtration using selected pore diameter membranes separated the ferredoxins, AvFlp, and plastocyanin from Avl and reasonably well from Av2. However, the retained Av2 could not be completely freed from the smaller proteins by this method.

Reaction with Excess Reagents. The reaction of a 15–30-fold excess of $Fe(CN)_6^{3-}$ with reduced Avl during a 15-min reaction interval formed $Fe(CN)_6^{4-}$ and produced Avl oxidized by 12 electrons. Excess DCPIP or O_2 (in the form of O_2-saturated buffer) also reacts with Avl to produce Avl oxidized by 12 electrons, but in a slower and less easily controlled reaction. By variation of the reaction conditions (time of exposure, amount of reagent in excess, etc.), Avl oxidized by nine electrons is obtained. A small loss of Avl specific activity usually occurs during these reactions with 10–25% being common, but with care, the lower value can be routinely obtained. The loss of enzymatic activity seems to arise from additional reactions occurring between excess oxidant and the newly formed 12-electron-oxidized Avl, rather than inherent instability of this latter species, because the activity of Avl remains constant with time after excess oxidant is removed.

The above reagents oxidize Av2 by one electron with little activity loss providing only 1 electron equiv is used. Excess of these reagents completely inactivates Av2.

When added in a 15–30-fold excess, horse heart cytochrome *c* undergoes rapid reduction with Avl, as evidenced by formation of the reduced heme spectrum, forming Avl oxidized by 12 electrons. A similar reaction occurs with *A. vinelandii* cytochrome c_5 as oxidant, but in this case Avl undergoes only a nine-electron oxidation. No reaction was observed when excess oxidized AvFdI or oxidized AvFlp react with reduced Avl. Both proteins oxidize reduced Av2.

Figure 1 describes the reduction characteristics of 9- and 12-electron-oxidized Avl obtained from either small-molecule or cytochrome *c* oxidation of Avl. The controlled potential reduction (Watt, 1979; Watt et al., 1980) of the former occurs at two separate potentials, of which the more positive ($E_{1/2}$ = −290 mV) requires a total of six electrons with an $n = 2$ value and the more negative ($E_{1/2}$ = −460 mV) requires three electrons with an $n = 1$ value. The 12-electron-oxidized Avl also undergoes reduction at these same two potentials ($E_{1/2}$ = −290 mV and $E_{1/2}$ = −460 mV) but with $n = 2$ values in each case. Very minor vis–UV spectral changes occur upon formation of 9-electron- and 12-electron-oxidized Avl compared to 6-electron-oxidized Avl.

Oxidative EPR Titrations of Avl. In order to assess more clearly the reactivity patterns of the various metal clusters present in Avl and to determine their oxidation sequence, titrations of Avl with both small-molecule and protein oxidants were undertaken. The titrations monitored the change in EPR, vis–UV, or CD characteristics of the metal clusters in Avl as a function of added small-molecule or protein oxidant. A typical titration of reduced but $S_2O_4^{2-}$-free Avl with standardized $Fe(CN)_6^{3-}$ is shown in Figure 2a as a monotonic decrease of the EPR signal of Avl as the $Fe(CN)_6^{3-}$ concen-

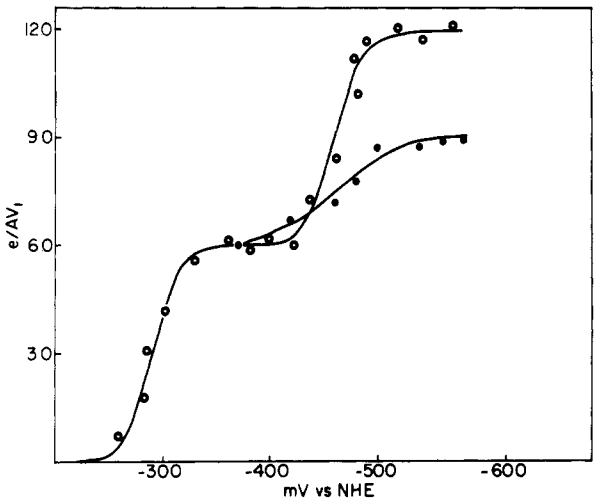

FIGURE 1: Reduction potentials of oxidized Av1. Oxidation of Av1 by 9 and 12 electrons was carried out as outlined in the text. Oxidized Av1 accepted the number of electrons at the indicated potentials with 75 μM methyl and benzyl viologen as mediators in 0.05 M Tris–0.25 M NaCl at pH 8.0. The solid lines are Nernst curves drawn for a two-step 12-electron reduction reaction with $E_{1/2}$ = −290 mV and $E_{1/2}$ = −460 mV (○) and n = 2 for each six-electron step. For the nine-electron reduction (●), the Nernst curve is for $E_{1/2}$ = −290 mV with n = 2 for the six-electron reduction step and for $E_{1/2}$ = −460 mV with n = 1 for the three-electron reduction step. All potentials are with reference to the normal hydrogen electrode (NHE).

tration is increased. The EPR signal of Av1 is eliminated at 6 equiv of $Fe(CN)_6^{3-}$, but a new signal begins to weakly develop at a $Fe(CN)_6^{3-}$/Av1 ratio greater than 6. This new signal has EPR resonances at g = 1.99 and g = 1.89 and is similar to that reported for O_2 oxidation of Av1 (Wang et al., 1986). Similar results were obtained for titration of Av1 with DCPIP. In some cases, titrations of Av1 with $Fe(CN)_6^{3-}$ and DCPIP have a smaller slope than that shown in Figure 2a, which causes the titration line to intersect the x axis near 9 electron equiv. This behavior is not surprising in view of the formation of 9-electron- and 12-electron-oxidized Av1 described in the previous section. Because these higher oxidation states of Av1 occur, it is reasonable to expect that variation in reaction conditions (efficiency of mixing, time of reaction, concentration of Av1 or oxidant, temperature, etc.) during the titration could cause them to be variably produced (see later sections for further details).

Figure 2a is an EPR titration of Av1 with cytochrome *c* which shows a monotonic decrease of the characteristic EPR signal of Av1 with increasing cytochrome *c* concentration. The EPR signal of Av1 disappears near 10 equiv of added cytochrome *c*. No new signals were observed during this titration. Figure 2b is an EPR titration of Av1 with plastocyanin which demonstrates that 6 equiv of the Cu protein are required to eliminate the Av1 EPR signal. Further addition of plastocyanin does not produce the distinctive g = 2.0 EPR signal typical of this species until a total of 9 equiv has been added, suggesting that Av1 undergoes a nine-electron-oxidation with plastocyanin. Stellacyanin and laccase give similar results except the laccase reaction occurs more slowly.

Oxidative Optical Titrations with Redox Proteins. The development of the 550- and 530-nm absorption peaks of the reduced cytochromes demonstrates the reducibility of the heme proteins by reduced Av1 and provides a convenient spectroscopic marker for assessing and quantitating protein redox reactivity. Figure 3a is the reaction of limiting Av1 with excess cytochrome *c*, conditions under which Av1 is oxidized by 12 electrons. After each addition of Av1 to the cytochrome *c* solution, the reaction was monitored for 20 min before the

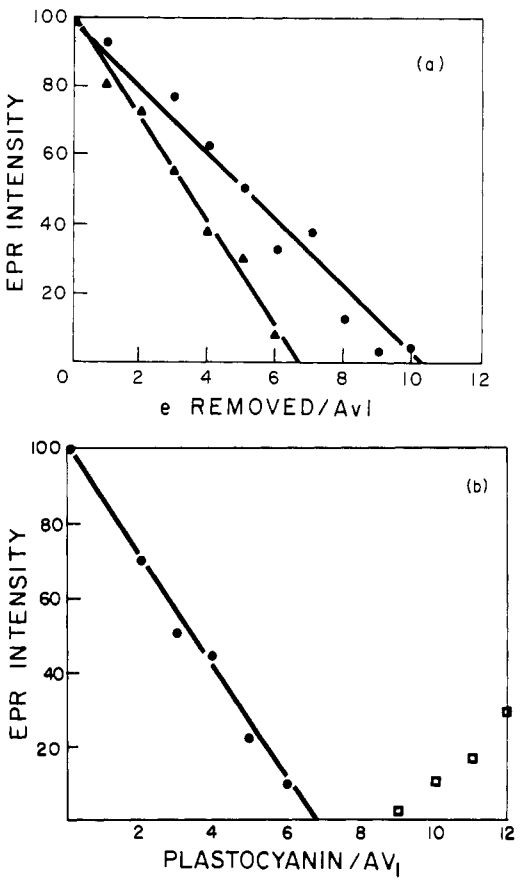

FIGURE 2: Oxidative titrations of Av1. (a) The EPR intensity at g = 3.65 of Av1 as a function of added $Fe(CN)_6^{3-}$ (▲) and horse heart cytochrome *c* (●). (b) The EPR intensity of Av1 at g = 3.65 (●) as a function of added plastocyanin. The EPR intensity at g = 2.0 of plastocyanin (□).

spectrum was recorded. This procedure was followed because under these conditions (at longer exposure times) the excess oxidized cytochrome *c* began reacting further with the newly formed 12-electron-oxidized Av1, causing protein inactivation. The development of reduced heme was monitored as a function of time, allowing us to observe a biphasic oxidation reaction of Av1 consisting of the rapid formation (<30 s) followed by a much slower (<15 min) linear production of reduced cytochrome *c*.

Figure 3b is an optical titration in which limiting *Euglena* cytochrome *f* ($E_{1/2}$ = +380 mV; Mitsui, 1971) reacts with excess, reduced Av1. Rapid reaction is evidenced by the development of the reduced heme bands. The production of reduced cytochrome *f* is accompanied by absorption increases in the 600–800-nm region of the Av1 spectrum, changes indicative of "p-cluster" oxidation. The spectrum of Av1 remains constant at 600–800 nm after >3 equiv of cytochrome *f* has reacted. Subsequent addition results in no easily recognized Av1 spectral changes but continues to produce reduced cytochrome *f*, indicating that continued oxidation of Av1 is occurring. The reaction of cytochrome *f* at ratios >3 equiv occurs at a rate about one-third that of the first 3 equiv.

The reaction of limiting stellacyanin with excess Av1 was also studied. Reduced stellacyanin has no bands in the optical region investigated, so that observed optical changes are those due only to Av1. Several isosbestic points develop during the titration suggesting that Av1 passes through different oxidized states during the reaction. One isosbestic point forms near 383 nm during the addition of the first 3 equiv of stellacyanin, but then, new isosbestic points develop at 330 and 525 nm when >3 equiv is added. The reaction of Av1 with laccase

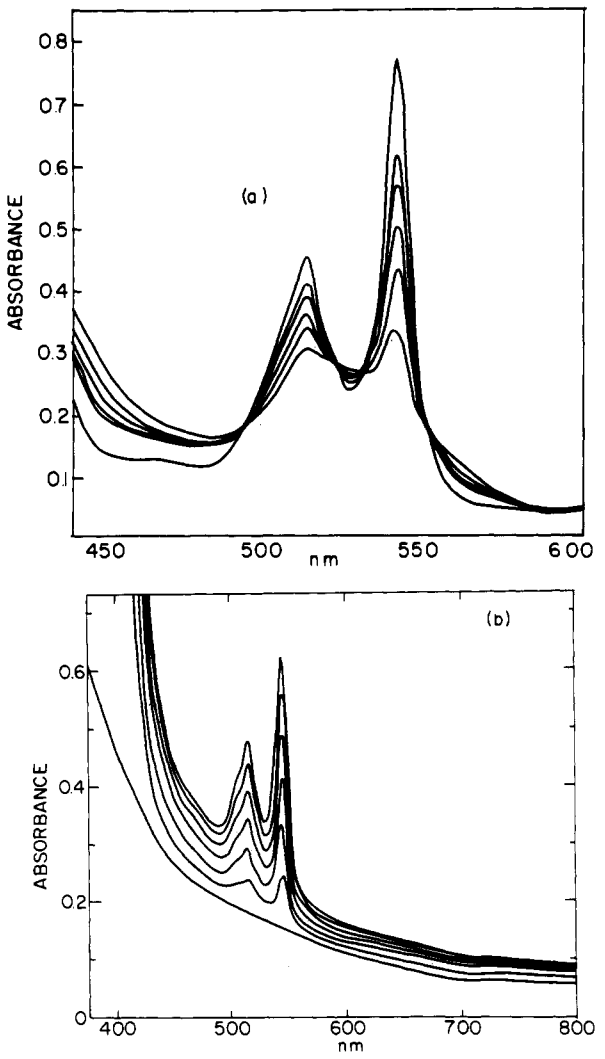

FIGURE 3: Protein oxidation of reduced Av1. (a) The addition of four separate aliquots of Av1 (0.6 nmol each) to excess oxidized cytochrome *c* (68 nmol). Complete reduction was accomplished by $Na_2S_2O_4$ (top spectrum). From the absorbance changes in cytochrome *c* at 550 nm after each Av1 addition, a 10.9-electron oxidation of Av1 was calculated. (b) The addition of 1.5-equiv aliquots of *Euglena* cytochrome *f* to Av1 (bottom spectrum).

occurs at a rate about 10 times slower than that with stellacyanin. Optical spectroscopy gave no indication of reaction between Av1 and beef heart cytochrome oxidase, a protein containing two copper atoms and two heme groups per molecule.

Oxidative Optical Titrations with Small Molecules. The stepwise oxidation of Av1 using mild, small-molecule oxidants has been reported (Euler et al., 1984; Watt & Wang, 1986) to produce only three- or six-electron-oxidized Av1, depending on the redox potential of the oxidant. Depending on the molar excess and the reaction time, strong oxidants ($E_{1/2} > 0$) were reported to produce either six-electron-oxidized Av1 (Euler et al., 1984) or Av1 oxidized by 9 or 12 electrons (Watt et al., 1980). We have confirmed the formation of 9- and 12-electron-oxidized Av1 (see Figure 1 and preceding sections) by reaction with excess small-molecule oxidants but have been unable to obtain consistent and reproducible optical titration curves. The probable reason for this behavior is discussed in the next section where two types of Av1 oxidative behavior are reported. The small molecules [$Fe(CN)_6^{3-}$, O_2, and DCPIP] used as titrants react quite randomly with the various clusters present in Av1, giving irreproducible titration curves. These results are different from those in the previous section

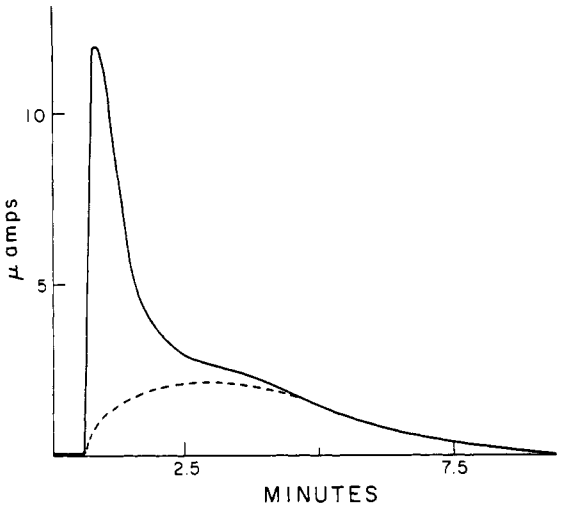

FIGURE 4: Microcoulometric oxidation of Av1. The addition of 1.13 μmol of reduced but $S_2O_4^{2-}$-free Av1 to the microcoulometric cell controlled at +90 mV (NHE) containing 1×10^{-4} M thionine in 0.05 M Tris–0.25 M NaCl, pH 8.0. The dotted line resulted from oxidation of six-electron-oxidized Av1.

where proteins with similar redox potentials to the titrants used here produced quite uniform titration curves. The proteins apparently possess some specificity toward reduction which is absent in small-molecule titrants.

Microcoulometric Oxidation of Av1. The mediated oxidation of Av1 by thionine ($E_{1/2}$ = +64 mV) at selected, controlled potentials is shown in Figure 4. The addition of Av1 to the cell controlled at +100 mV (NHE) produces a rapid anodic (oxidative) current flow as shown in Figure 4, which peaks and then declines rapidly to approach a smaller, decreasing anodic current resulting from a secondary oxidation process. This second reaction slowly continues until the current returns to the initial level prior to Av1 addition. The area enclosed by the curve in Figure 4 (i.e., the current–time integral) represents the total charge removed from Av1 by the two oxidative processes. The dotted line is the controlled potential oxidation of Av1 which had been oxidized previously by six electrons (Watt et al., 1980) prior to addition to the coulometric cell. The two-phase oxidation of reduced Av1, thus, results from a rapid six-electron oxidation accompanied by oxidation of this latter species by an additional six electrons in a slower step. Oxidation of reduced Av1 at potentials more negative than 0 mV produces curves with only the rapid reaction present, but no oxidation of six-electron-oxidized Av1 is observed. These results establish that the slower step has a potential threshold value near 0 mV above which Av1 is oxidized by more than six electrons (>12 e/Av1) and below which only a six-electron oxidation occurs. Using mediators such as toluylene blue ($E_{1/2}$ = +115 mV) or DCPIP ($E_{1/2}$ = +250 mV) in place of thionine increases the rate of the slower oxidation reaction, causing the two processes to occur at comparable rates. It is clear, then, that titrations of Av1 using these reagents and also $Fe(CN)_6^{3-}$ or O_2 will produce variable titration curves depending on the relative rates of the two Av1 reactions, consistent with results observed in the previous section.

Reductive EPR Titrations of Av1. Av1, oxidized by six electrons, is EPR silent. An EPR titration of oxidized Av1 with fully reduced Av flavoprotein ($AvFlpH_2$) is shown in Figure 5a. The g = 4.3 and g = 3.65 components of the reduced Av1 EPR signal develop early upon addition of $AvFlpH_2$, reaching a maximum near 2.0 equiv. Because of the difficulties in preparing, standardizing, and handling these

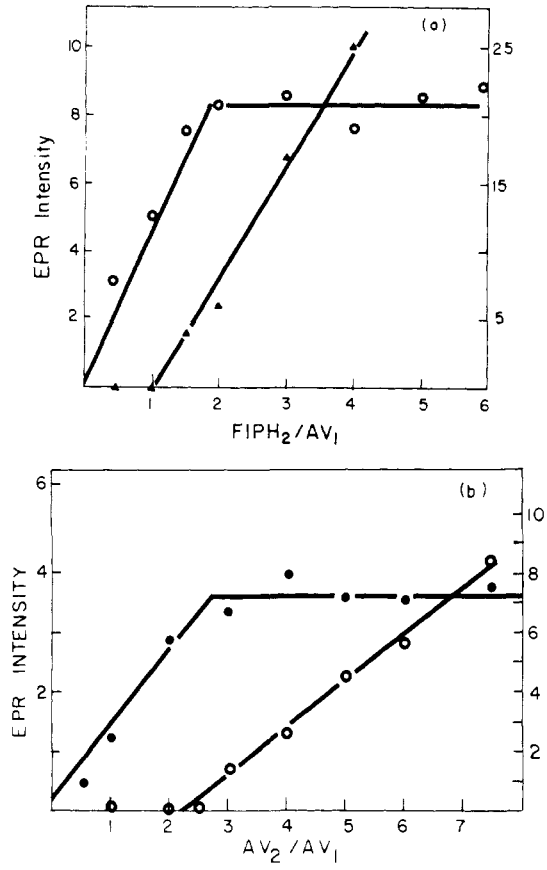

FIGURE 5: Reductive titrations of Av1. (a) The left axis is the development of the EPR intensity of Av1 at $g = 3.65$ (○), and the right axis is the development of the EPR intensity of AvFlp• at $g = 2.0$ (▲) as aliquots of $AvFlpH_2$ are added to six-electron-oxidized Av1. (b) The development of the Av1 $g = 3.65$ signal (●) on the left axis as a function of added Av2. The EPR signal of unreacted Av2 at $g = 1.94$ (○) begins to appear at >2 Av2/Av1, right axis.

two air-sensitive proteins, duplicate and repeat experiments gave a stoichiometric range of values from as low as two to, in some cases, as high as three $FlpH_2$ per Av1 (with two being most commonly found), for complete development of the Av1 EPR signal. During these titrations, the blue color of the resulting free-radical semiquinone form of the oxidized flavoprotein (AvFlp•) is evident as is the formation of its $g = 2$ EPR signal. However, as Figure 5a shows, no signal from AvFlp• is observed until after 1 equiv of $AvFlpH_2$ has reacted. This may be an experimental anomaly resulting from low levels of formation of AvFlp• in the early stage of the titration combined with the development of the $g = 2.01$ "dip" in the Av1 EPR signal. The further addition of $AvFlpH_2$ to Av1 clearly results in a uniform increase in AvFlp•, indicating that further reduction of Av1 occurs, most likely, at the p-cluster centers. However, there is a noticeably slower reaction rate with the non-EPR centers of Av1 toward this reductant. The results in Figure 5a show that $AvFlpH_2$ acts only as a single electron reductant forming AvFlp• as the major product. The redox couple for this process ($Flp^{\bullet} + e = FlpH_2$) occurs at a midpoint potential of −515 mV (Watt, 1979) versus the normal hydrogen electrode (NHE), a value consistent with the required reduction potentials of Av1 (Watt et al., 1981).

Reduced but $S_2O_4^{2-}$-free Fe_4-S_4 ferredoxin from *Clostridium thermoaceticum* also causes maximum development of the Av1 EPR signal at $g = 4.3$ and 3.65 with 2–3 equiv of added ferredoxin. Addition of reduced ferredoxin to 3 equiv or higher results in the appearance of an EPR signal characteristic of unreacted, reduced ferredoxin.

Figure 5b is an EPR titration of oxidized Av1 with reduced but $S_2O_4^{2-}$-free Av2. EPR signal development in Av1 at $g = 4.3$ and $g = 3.65$ begins immediately upon addition of Av2, increases with added Av2, and reaches a maximum between two and three Av2 per Av1, with two being the most often obtained value. A more precise value for the Av2 reaction stoichiometry for interaction with oxidized Av1 was not possible because of difficulties in preparing, handling, and standardizing the two proteins. The extent of Av2 interaction with Av1 is limited to about two Av2 per Av1 because, as Figure 5b shows, at ratios of Av2/Av1 greater than 2 the EPR signal due to unreacted Av2 is evident and increases linearly with added Av2. The reaction of Av2 with Av1 occurs specifically at the EPR centers, a result consistent with optical titrations (data not shown) which show no p-cluster reduction with added, reduced Av2. Identical results were obtained when $Av2(MgATP)_2$ (Av2 in the presence of 1 mM MgATP) was reacted with oxidized Av1, also indicating reaction specificity of $Av2(MgATP)_2$ for the EPR centers of Av1.

Discussion

In general, the MoFe proteins isolated from different organisms have been shown (Orme-Johnson et al., 1972; Smith et al., 1973; Mortenson et al., 1973) to exist in at least three differentiable redox states, as summarized by Scheme I. The center species is the typical "as isolated" MoFe protein usually prepared in the presence of $S_2O_4^{2-}$ and characterized by the biologically unique $S = {}^3/_2$ EPR signal with the indicated g values. Upon reduction by the Fe protein component of nitrogenase, in the presence of MgATP and $S_2O_4^{2-}$, specific reduction of MoFe occurs, yielding the EPR-silent species on the right, thought to be responsible for substrate reduction. Of relevance to this study, however, is the species on the left which is produced by reaction of the MoFe protein with excess small-molecule oxidants.

Scheme I

$$\underset{\text{EPR silent}}{MoFe_o} \xleftarrow{\text{oxidation}} \underset{g = 4.3,\ 3.6,\ 2.01}{MoFe(S_2O_4^{2-})} \xrightarrow{\text{reduction}} \underset{\text{EPR silent}}{MoFe_r}$$

In this study and in previous reports (Watt et al., 1981; Euler et al., 1984; Watt & Wang, 1986) we have examined in some detail the nature and characteristics of the EPR-silent species represented by $MoFe_o$ in Scheme I for the specific case of *A. vinelandii* MoFe protein, Av1. Previous studies have shown (Watt & Wang, 1986) that weak oxidants ($E_{1/2} < -125$ mV) produce Av1 oxidized by three electrons, while mild oxidants ($E_{1/2} > 0$ mV) produce Av1 oxidized by six electrons. The results presented here and reported earlier (Watt & Bulen, 1975) indicate that with strong oxidants ($E_{1/2} = 200$–300 mV), under carefully controlled conditions, up to 12 electrons can be removed from Av1 with little loss in activity. Figure 1, Figure 2b, and previous results (Watt, 1980) along with a recent report by Stephens (1985) all show that a nine-electron oxidation of Av1 also occurs. Thus, instead of a single oxidized species as shown in Scheme I, there exists a family of oxidized Av1 species oxidized by 3, 6, 9, and 12 electrons which are formed by oxidants of increasing oxidation potential.

Attempts to follow spectroscopically the stepwise formation of the 9- and 12-electron-oxidized Av1 with strong, small-molecule oxidants, as was conveniently done (Zimmerman et al., 1978; Watt et al., 1980; Stephens et al., 1981, 1985) for sequential oxidation of Av1 to form 6-electron-oxidized Av1, resulted in a varied spectroscopic response of Av1 to added oxidant. Figures 1 and 4 show the nature of these oxidation states in Av1 which give rise to +9 and +12 oxidized Av1. Apparently, the kinetic and thermodynamic barriers which

exist between the various cluster types in Av1 which allow for an orderly and sequential oxidation with mild oxidants are insignificant with respect to the strong oxidants used here, resulting in nonsequential nearly random oxidation of the clusters present in Av1. Thus, it has not been possible to reproducibly and sequentially oxidize Av1 by 9 and 12 electrons and cleanly characterize the resulting spectroscopic states with the strong oxidants that we have studied.

Reactions of Av1 with strongly oxidizing proteins are more reproducible than those with the strong, small-molecule oxidants but are still quite complex. The Av–cytochrome c_5 complex, plastocyanin (see Figure 2b), and stellacyanin reproducibly react to form nine-electron-oxidized Av1. *Euglena* cytochrome *f* first oxidizes the p-centers in Av1, as evidenced by the absorbance increase in the 600–800-nm range (Figure 3b), on its way to forming nine-electron-oxidized Av1. This reaction closely parallels the reaction shown as Figure 3 in the report by Stephens (1985). Cytochrome *c* produces only 12-electron-oxidized Av1 with no apparent selectivity toward reaction with certain cluster types found in Av1 as shown in Figure 2a.

The physiological relevance of these higher oxidation states is not clear, but we believe it is highly questionable because their rate of formation is quite slow and they are only formed by reagents possessing $E_{1/2}$ values more positive than 0 mV. However, they do demonstrate a more extensive redox capacity of Av1 than had been previously appreciated. Because only minor optical changes are seen and because of the presence of EPR signals near $g = 2$ (Wang et al., 1985; Stephens, 1985) in these higher oxidation states, we suspect that nonmetal centers (i.e., S, S^{2-}, etc.) are implicated. Further study of these oxidation states is important in understanding the electron transfer network in Av1.

The rapid, semispecific reactivity between Av1 and proteins not associated with the nitrogen fixation process or with proteins not found in *A. vinelandii* suggests that accidental recognition sites are present on both proteins which facilitate electron transfer, or, more likely, that the redox centers on Av1 are quite accessible to external reagents. Little is known regarding the placement of the metal clusters in Av1, and until a better understanding is available, we can only identify the relatively large thermodynamic driving force (0.7 V) as one of the possibly many factors (Mayo et al., 1986) involved in the long-distance electron transfer observed in these Av1–protein reactions. Further study seems appropriate in order to uncover what other factors may be involved in this facile reactivity.

Of more relevance to the nitrogen fixation process and the required electron transfer reactions are the interactions of Av1 with its companion protein, Av2, and with other low-potential electron transfer proteins.

Figure 5b summarizes the reaction between oxidized Av1 and reduced Av2. It is seen that reduced Av2 has a high specificity for reaction with the EPR centers of oxidized Av1, reducing them with a stoichiometry of one electron per MoFe cofactor center (2 Av2/Av1). Further reaction with the other oxidized centers present in Av1 does not occur or does so only slowly as evidenced by the presence of unreacted Av2. Av2-$(MgATP)_2$ also reacts rapidly with oxidized Av1, restoring the EPR signal completely with 2 equiv. This specific reactivity of reduced Av2 (with or without MgATP) toward the Av1 EPR centers is of interest because the first discernible event to occur during nitrogenase turnover (Hageman & Burris, 1978a,b, 1979) is the Av2-promoted diminishment of the Av1 EPR signal (presumably to form $MoFe_r$ in Scheme I) prior to H_2 evolution. The results reported here lead us to conclude that under the conditions of our experiments we are observing the same electron transfer events that occur during nitrogenase turnover, except that the electron transfer reaction is proceeding from the extreme left species to the center species of Scheme I. Interestingly, the reaction of reduced Av2-$(MgATP)_2$ with *reduced* Av1 (both in the absence of $S_2O_4^{2-}$) does not result in the decrease of EPR intensity of reduced Av1, as would be expected if Av2$(MgATP)_2$ is the specific reductant of the EPR centers of Av1. This interesting result is presently being investigated in more detail and suggests the intervention of additional reduction is required.

The uniqueness of the reaction between reduced Av2 and the EPR centers of Av1 is somewhat lessened by the equally specific formation of an EPR signal in Av1 by addition of reduced CtFd and AvFdI ferredoxins. These reactions are essentially identical with that between Av2 and Av1 in terms of rate and stoichiometry and indicate that both Fe_3-S_3 and Fe_4-S_4-containing proteins are also capable of productive electron transfer interaction at the MoFe cofactor sites in Av1.

Figure 5a shows that $AvFlPH_2$ also reacts specifically with EPR-silent Av1, fully developing the EPR signal characteristic of reduced Av1 with 2 added equiv. Further addition of $AvFlPH_2$, beyond 2 equiv, continues to reduce the oxidized centers present in Av1, as evidenced by the further production of AvFlP•; however, the reaction rate is noticeably slower. The operational couple ($AvFlp^{\bullet} + e = AvFlPH_2$) occurs at −515 mV (Watt, 1979) and demonstrates that strongly reducing conditions are required to produce reduced EPR and p-cluster centers from oxidized Av1. The uniqueness of the Av flavoprotein existing in three stable, easily discernible, and quite widely separated redox states (Watt, 1979) ($AvFlPH_2$, AvFlP•, and AvFlP) is useful in demonstrating the unusual redox character of the clusters in Av1. If the Av1 reactions were reversible, the prediction can be made, from the relative redox potentials of Av1 and AvFlp, that the reaction between AvFlP and reduced Av1 should produce oxidized Av1 and AvFlP•. However, no reaction is observed to form the EPR-detectable and optically detectable AvFlP• even with a 10-fold excess of AvFlP. This lack of reactivity further substantiates the hysteretic character of Av1 (Watt et al., 1980).

The redox behavior described for Av1 stands in contrast to that displayed by the reactions of reduced Av2 with AvFlP and oxidized Av2 with $AvFlPH_2$. In both cases, AvFlP• is rapidly formed as evidenced by both EPR and optical monitoring of $AvFlp^{\bullet}$ formation. These reactions indicate that all protein forms successfully, and rapidly, interact to undergo successful electron transfer and demonstrate an electrochemical reversibility of Av2. These experiments with both Av1 and Av2 clearly indicate that $AvFlPH_2$ recognizes and successfully interacts with both proteins, but which of these processes occurs during nitrogenase function remains unknown.

The conclusions we draw from the studies reported here are that Av1 can undergo extensive oxidation (up to 12 electrons), depending on the strength of the oxidant, and that the intermediate three-, six-, and nine-electron-oxidized Av1 states appear to possess some inherent kinetic or thermodynamic stability allowing them to be formed with a wide variety of reagents. The reaction of a variety of proteins not related to the nitrogen fixation process is interpreted in terms of facile accessibility of the clusters in Av1 to external electron transfer agents. Finally, those proteins that are implicated and/or suspected of being involved in the nitrogenase reaction undergo facile reaction with both Av1 and Av2. Interaction at the MoFe cofactor site of Av1 is quite specific for Av2, ferredoxins,

and AvFlPH$_2$, although the latter protein also reacts slowly to reduce the p-clusters present in Av1.

Registry No. DCPIP, 956-48-9; $Fe(CN)_6^{3-}$, 13408-62-3; O_2, 7782-44-7; cytochrome *c*, 9007-43-6; laccase, 80498-15-3; thionine, 581-64-6; nitrogenase, 9013-04-1.

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