Redox Reactions of and Nucleotide Binding to the Iron Protein of *Azotobacter vinelandii*†

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ABSTRACT: The reaction of IDS (indigodisulfonate) with *Azotobacter vinelandii* iron protein (Av_2) produces fully active Av_2(ox). The reduction stoichiometry was found to be one electron per Av_2, and the reduction potential, measured by spectroelectrochemical and coulometric methods, was found to be −300 mV at pH 7–9. Reduction potentials of −385, −430, and −490 mV were measured for Av_2(ox) in the presence of CH_2-substituted MgATP, MgATP, and MgADP, respectively. Equilibrium binding measurements of MgATP and MgADP to Av_2(ox) were carried out by equilibrium dialysis (5.40×10^7 M^{-2}) and vis–UV and circular dichroism (CD) spectroscopy (6.67×10^7 M^{-2}), yielding the indicated binding constants for the MgATP interaction. For both MgATP and MgADP, the binding stoichiometry was measured to be two molecules of nucleotide bound to each Av_2(ox). Thermodynamic cycles were constructed from the data discussed above to yield a binding constant of 4.5×10^5 M^{-2} for two molecules of MgATP binding to Av_2(red). This indirect method for measuring MgATP binding to Av_2(red) was required because Av_2(red) (free of $S_2O_4^{2-}$) is too O_2 sensitive to yield reproducible binding data. The reaction of excess Av_2 ($S_2O_4^{2-}$ free) with MgATP in the presence of catalytic amounts of Av_1 (the MoFe protein) produced 0.5 mol of H_2/Av_2 and Av_2(ox), establishing that a complete utilization of all electrons present in Av_2 occurs in the formation of H_2. The irreversible binding of ATP (no Mg^{2+}) by Av_2(ox) and its inactivation in the presence of ATP were studied. The addition of Mg^{2+} during the inactivation process prevents further inactivation.

The Fe protein component (Av_2) of the *Azotobacter vinelandii* nitrogenase system is a redox-active MgATP binding protein that is essential in the biological dinitrogen reduction process. Because such a large amount (Watt et al., 1975; Mortenson & Thorneley, 1979) of MgATP is hydrolyzed during the reduction of N_2 to ammonia and because Av_2 is recognized as the site of MgATP binding, much effort has been directed toward studying the interaction of MgATP and the influence that nucleotide binding has on the redox properties of Av_2. It is clear that both aspects are related, and this relationship is considered in some details herein.

Several equilibrium studies have been reported for MgATP binding to the reduced Fe protein from various organisms. These data have recently been reviewed (Burgess, 1984). The most recent data for MgATP binding to Av_2 have been reported by Stephens et al. (1983) and Cordewener et al. (1983). The former authors, using the circular dichroism (CD) change that occurs when either MgATP or MgADP binds to Av_2(ox), demonstrated that two molecules of either nucleotide are bound with no decrease in protein activity with a dissociation constant (K_D) of <5 μM. The latter authors using equilibrium dialysis and flow dialysis methods also report the binding of two MgATP molecules to Av_2(ox) but with a significantly different K_D value of 290 μM. The severe activity losses and problems associated with Av_2 oxidation reported for these dialysis experiments raise some questions about the values obtained.

Kinetic methods have also been used to estimate the number of MgATP molecules bound and the associated K_D values. Silverstein and Bulen (1970) found that Av nitrogenase activity responds to MgATP concentration in a predictable way if two MgATP molecules were assumed bound with a combined dissociation constant (K_D) of 3.2×10^{-9} M^2. Watt and Burns (1977) confirmed that two MgATP molecules bind with $K_D = 2.8 \times 10^{-9}$, but because of more extensive steady-state kinetic data, they were able to conclude that MgATP binding occurred with Av_2(ox). Hageman et al. (1980), using the bathophenanthrolinedisulfonate (BPS) method of Ljones and Burris (1978), reported separate K_D values of 430 μM and 230 μM for the binding of two MgATP molecules to reduced Av_2. All reported binding results are consistent with two MgATP molecules binding to Av_2, but the magnitude of this binding interaction is still uncertain as is the reliability of MgATP binding to reduced Av_2, given the problems of "self-oxidation" noted by Stephens et al. (1981), Cordewener et al. (1983), and Burns et al. (1985).

The redox properties of Av_2 and other Fe proteins from other organisms have been reviewed recently by Burgess (1984). It is generally accepted that the Fe protein is a one-electron-transfer protein and that the midpoint potential is shifted negatively in the presence of MgATP, MgADP, and certain ATP analogues. This shift in midpoint potential is probably a consequence of a differential binding of the nucleotide to the two redox states of the Fe protein. A clear understanding of this process and its consequences is important in defining the role of the iron protein and MgATP in the dinitrogen reduction process. In this paper we describe detailed nucleotide binding studies to Av_2 as well as redox measurements of Av_2 in the presence and absence of nucleotides.

Materials and Methods

Av_2 Protein. Av_2 prepared as described (Burgess et al., 1980) produces no measurable H_2 when assayed by itself under the normal assay conditions and gives only one discernible band on sodium dodecyl sulfate (SDS) gel electrophoresis. However,

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when freed from excess $S_2O_4^{2-}$ and monitored at 420 nm in an anaerobic, optical cell, Av_2 undergoes oxidation over a 20–30-min period upon addition of anaerobic MgATP (5.0 mM). No reaction occurs in the absence of MgATP. Oxidation occurs because of the presence of undetected amounts of contaminating MoFe protein (Av_1) at $\sim$<0.01 mg/mL, the presence of which completes the requirements for MgATP-required H_2 evolution. Several preparations of Av_2 obtained by the procedure of Burgess et al. (1980) were pooled, concentrated, and run through a 200 × 2.5 cm ultrogel ACA 54 (LKB) column until Av_2, in the presence of 5 mM MgATP, failed to undergo oxidation. This Av_2 was used in the experiments reported here.

Protein concentration was determined either from amino acid analysis or optical absorbance measurements with molar absorptivity values of 18.1 mM^{-1} and 11.1 mM^{-1} for the oxidized protein at 365 nm and the reduced protein at 400 nm, respectively (Anderson & Howard, 1984). Samples 1–4, 9, and 11 described in Watt and McDonald (1985) were used for the studies reported here and possessed activities of 2367–3312 nmol of H_2/(min·mg) and contained 3.72–4.79 Fe/Av_2.

Oxidized Av_2 was prepared in a Vacuum Atmospheres glovebox (O_2 < 0.1 ppm) by reaction of dithionite-reduced Av_2 with indigodisulfonate (IDS) or methylene blue (MB) followed by removal of the dyes on an anaerobic G-25 column. The extent of oxidation was determined microcoulometrically (Watt, 1979) or by titration with standardized methyl viologen (MV) or $S_2O_4^{2-}$. Reductant-free, reduced Av_2 was prepared in a similar way from $S_2O_4^{2-}$-reduced Av_2 and the extent of reduction verified by microcoulometry or titration with standardized IDS and MB.

MgATP Solutions. Most nucleotide binding results were obtained on stock 0.02–0.1 M ATP or MgATP solutions at pH 7.4 (prepared from Sigma grade I ATP) appropriately diluted for the described experimental techniques. Extensive degassing procedures failed to removal all coulometrically reducible material from these ATP solutions, in contrast to degassed buffer control solutions which gave no reduction response. This unidentified redox contaminant, amounting to 0.03% in some samples, is sufficient to oxidize significant portions of Av_2(red) to Av_2(ox) when MgATP titrations or dialysis experiments are conducted. To avoid this complication, one of the following procedures was used to eliminate this contaminating oxidant: (1) the use of Pharmacia ultrapure ATP, (2) Sigma grade I ATP reacted under argon with metallic zinc for several hours, or (3) as in (2) except the ATP solutions were titrated with $S_2O_4^{2-}$ until zero coulometric reduction was attained.

Redox Measurements. Midpoint potentials of Av_2 were determined by controlled potential microcoulometry or controlled potential spectrophotometry (Watt, 1979). Changes in the redox state of Av_2 during redox titrations or reagent addition were monitored in 1.0- or 2.5-mL quartz cuvettes (1-cm path length) equipped with tapered, ground joints. A Cary 118 spectrophotometer was used for optical measurements, a Kettering CD instrument (Breeze & Ke, 1972) for circular dichroism measurements (300–800 nm), and an Aminco fluorometer for fluorescence measurements. The EPR equipment and procedures used have been described (Watt & McDonald, 1985).

Equilibrium Binding of Nucleotides. Equilibrium dialysis of Av_2(ox) and Av_2(red) against MgATP, MgADP, and ATP was carried out in duplicate in two eight-chambered dialysis blocks at 27 °C in a Vacuum Atmospheres glovebox. Each dialysis chamber contained 150 μL of Av_2 and 150 μL of the nucleotide, separated by a semipermeable membrane. Each dialysis chamber contained a small glass ball, which facilitated mixing and expedited dialysis as the dialysis blocks were mechanically rotated. Equilibrium was attained in about 4 h, but equilibrium times of 6 and 12 h were chosen for the experiments reported here. Following the attainment of equilibrium, protein samples were removed for redox state determination by microcoulometry and for activity measurements. Free MgATP was determined in the non-protein compartment by absorbance at 260 nm or by fire fly luminescence, and bound MgATP was determined by total MgATP − free MgATP = bound MgATP. Av_2 concentration was varied from 2 mg/mL (31.7 μM) to 12 mg/mL (190 μM), and MgATP was varied from 0.05 to 1.0 mM. Dialysis experiments in which the $MgCl_2$ concentration was held constant at 1.0 mM and various amounts of ATP were added gave the same results as those in which various levels of premixed MgATP were added. The addition of MgATP to the protein compartment to initiate the experiment gave the same results as addition of MgATP to the non-protein compartment.

The flow dialysis method of Colowick and Womack (1969) was used for equilibrium dialysis measurements of short time duration (5–15 min). The apparatus was housed in a Vacuum Atmospheres glovebox where strictly anaerobic conditions were maintained. Protein and MgATP concentrations were similar to those described above for the longer term dialysis experiments. Activity and redox-state measurements of Av_2 were carried out during the dialysis experiments by periodically removing Av_2 samples from the protein compartment.

The binding of MgATP and MgADP to Av_2(ox) was determined from the CD and optical changes induced in Av_2(ox) as increasing amounts of nucleotide were added. The experiments were conducted in 2.5-mL (1.0-cm path-length) quartz cuvettes at 25 °C. The optical changes occurring with added nucleotide were converted into the fraction of total Av_2(ox) in the nucleotide-bound form. This fraction was plotted against $1/[MgATP]^2$, yielding a straight line over the entire concentration range of MgATP studied from which the combined binding constant K_1K_2 was determined. No significant CD or optical changes were observed when Av_2 in the presence of $S_2O_4^{2-}$ was reacted with MgATP.

Results

Redox Measurements. The Av_2 used in this study was highly active and contained near four Fe atoms per molecule, consistent with a single [Fe_4S_4] redox center being present. The previously reported EPR titrations (Watt & McDonald, 1985), the H_2/Av_2 ratio of 0.5 obtained by the oxidation of Av_2 in the absence of additional reductant, and the results presented in Figures 1 and 2, as well as CD and electron paramagnetic resonance (EPR) data not shown, all demonstrated that Av_2 engages in a single electron redox reaction.

Figure 1 displays the spectral changes occurring in Av_2 during controlled potential electrolysis conducted in a spectrophotometric cell. From the charge transferred to the cell at the potentials indicated in Figure 1, it was determined that a single electron redox process occurs.

Figure 2 summarizes the results of coulometric reduction measurements of IDS-oxidized Av_2 in the presence and absence of Mg^{2+} nucleotides. A midpoint potential of −300 mV for (1) is obtained from both the spectroelectrochemical results

$$Av_2(ox) + e^- \rightleftharpoons Av_2(red) \qquad \epsilon^0 = -300 \text{ mV} \qquad (1)$$

of Figure 1 and the controlled potential coulometry results as

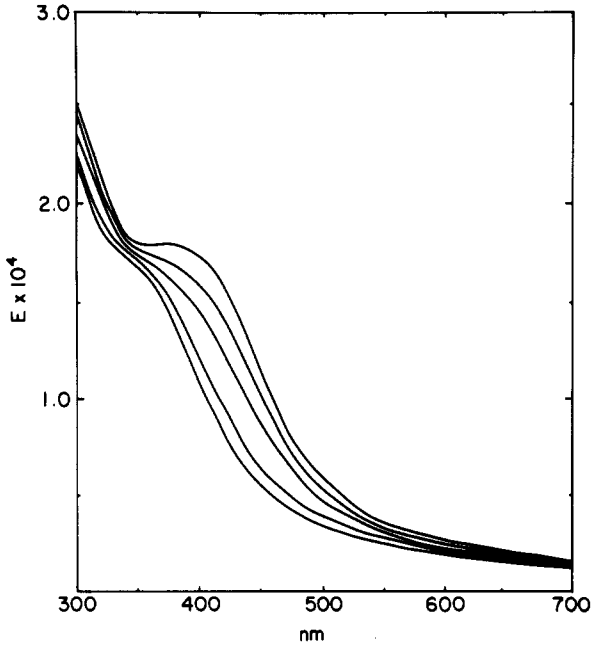

FIGURE 1: Spectroelectrochemical reduction of $Av_2(ox)$. Spectra resulting from 2.5 mL of $Av_2(ox)$ at 2.95 mg/mL contained in a stirred electrochemical cell reduced as a function of applied reduction potential. From top to bottom: as prepared, reduced at −260, −290, −330, and −450 mV. All potentials are relative to the normal hydrogen electrode.

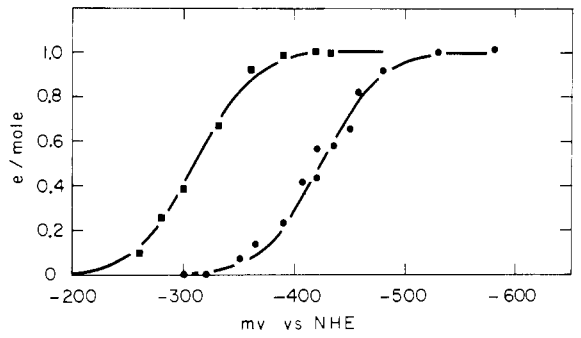

FIGURE 2: Reduction curves for $Av_2(ox)$. Coulometric or spectroelectrochemical reduction of $Av_2(ox)$ in the absence of MgATP (■) and coulometric reduction of $Av_2(ox)$ in the presence of 3 mM MgATP (○). Midpoint potentials of −300 and −430 mV with $\eta = 1$ values in each case were determined from each curve, respectively.

shown in Figure 2. A midpoint potential of −430 mV for $Av_2(ox)$ in the presence of 1–5 mM MgATP is reproducibly obtained as shown in Figure 2. This same result is obtained whether $Av_2(ox)$ is added to the coulometry cell containing MgATP at the desired concentration or $Av_2(ox)$ is premixed with MgATP and then added to the coulometry cell. The stoichiometry of MgATP binding to Av_2 shown in (2) can be

$$Av_2(ox)(MgATP)_2 + e^- \rightleftharpoons Av_2(red)(MgATP)_2 \qquad \epsilon^0 = -430 \text{ mV} \quad (2)$$

inferred from the redox data alone but was more accurately determined from the direct binding studies discussed below.

The negative shift of the midpoint potential of $Av_2(red)$ upon addition of MgATP was confirmed operationally and quantitatively by the following series of reactions. $Av_2(ox)$ was first stoichiometrically reduced in the absence of MgATP with standardized MV, forming $Av_2(red)$ and a stoichiometric amount of oxidized MV, as evidenced by optical spectroscopy. Anaerobic MgATP was then added to give a final concentration of 2.5×10^{-3} M, shifting the midpoint potential of $Av_2(red)$ in a negative direction and driving the redox equilibrium in favor of reduced MV and $Av_2(ox)(MgATP)_2$. Quantitation of the resulting spectral changes indicates the formation of 49% $Av_2(ox)$ and 53% reduced MV, giving a midpoint potential −440 mV for $Av_2(red)$ in the presence of MgATP, in good agreement with the results given in Figure 2.

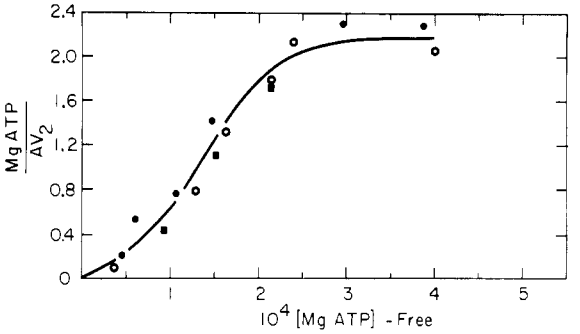

FIGURE 3: Equilibrium binding of MgATP to $Av_2(ox)$. Equilibrium dialysis of MgATP with $Av_2(ox)$ was conducted for 6 (○) and 12 h (●) at 27 °C in a Vacuum Atmospheres glove chamber. Rapid flow dialysis (■) was carried out under the same conditions, except with equilibration periods of 10–15 min. The solid line is calculated for a binding constant $K_1K_2 = 5.40 \times 10^7$ M^{-2}. The vertical axis is the ratio of MgATP bound to Av_2 at the molar (M) concentration of free MgATP indicated on the horizontal axis.

Midpoint potentials for (1) and (2) carried out at pH 7–9 were invariant with pH. The reduction of $Av_2(ox)$ in the presence of buffer containing 1–5 mM $MgCl_2$ or in buffer containing 1–4 mM ATP gave midpoint potentials of −330 and −340 mV, respectively. These shifts are significant compared to $Av_2(ox)$ reduction in buffer but much smaller than the shift that occurs when the $MgCl_2$ and ATP are mixed. Midpoint potentials of −490 and −385 mV were measured for the reduction of $Av_2(ox)$ in the presence of 2.5 mM MgADP and Mg^{2+}/adenosine 5′-(β,γ-methylenetriphosphate).

The reduction properties of two other states of Av_2 were studied by controlled potential coulometry. The first is the oxidized form of Av_2, which results from the spontaneous oxidation of Av_2 solutions initially containing $S_2O_4^{2-}$ (Stephens et al., 1983; Cordewener et al., 1983; Watt et al., 1985). Av_2 solutions containing excess $S_2O_4^{2-}$ were allowed to stand in the anaerobic Vacuum Atmospheres glovebox until coulometry demonstrated the absence of $S_2O_4^{2-}$ and the presence of reducible $Av_2(ox)$. The time required for this reaction to take place varied somewhat with the source of Av_2 and the amount of excess $S_2O_4^{2-}$ but generally was 1–3 h. Once the presence of $Av_2(ox)$ was confirmed by coulometry and EPR, optical, and/or CD spectroscopy, the sample was then reduced in the coulometry cell as a function of potential. Activity measurements carried out during the course of the spontaneous oxidation and for 2 h following complete oxidation demonstrated no activity loss. Control reaction vials containing only buffer showed the complete absence of oxidant accumulation (i.e., O_2) during the course of the experiment. A midpoint potential of −385 mV was found for one-electron, self-oxidized Av_2.

The second Av_2 species that was examined results from the oxidation of $Av_2(red)$ ($S_2O_4^{2-}$ free) by addition of MgATP and catalytic amounts of MoFe. Coulometric reduction of the $Av_2(ox)(MgATP)_2$ formed in this reaction as a function of controlled potential gave a midpoint potential of −425 mV in agreement with Figure 2. This reaction and other observations of the self-oxidation reaction will be discussed under Nucleotide Reactions with Av_2.

Nucleotide Binding to Av_2. The binding of MgATP to $Av_2(ox)$ as determined by static equilibrium dialysis of 6- and

12- duration is shown in Figure 3. This figure shows a sigmoidal binding curve with 2.1 ± 0.2 MgATP molecules ultimately binding to each $Av_2(ox)$ at MgATP concentrations exceeding 0.5 mM. Analysis of this curve gives a combined binding constant K_1K_2 of 5.40×10^7 for reaction 3. Activity

$$Av_2(ox) + 2MgATP \rightleftharpoons Av_2(ox)(MgATP)_2 \quad (3)$$

measurements after 6 and 12 h of dialysis showed no significant activity loss after 6 h and 0–15% loss after 12 h. These times assure that equilibrium was attained and further demonstrate that $Av_2(ox)(MgATP)_2$ is stable with time. Long-term, static dialysis of reduced Av_2 in the presence of $S_2O_4^{2-}$ was not possible because the time necessary for the attainment of equilibrium exceeded the time required to suppress spontaneous oxidation, and consequently, only MgATP binding to $Av_2(ox)$ was measured under these conditions.

Figure 3 shows that binding of MgATP to $Av_2(ox)$ as measured by the rapid flow dialysis method is completely consistent with the longer term, static dialysis results. The binding data measured by this method were acquired with an elapsed time of less than 10 min for each MgATP concentration examined. This rapid, flow dialysis procedure was also applied to the binding of MgATP to $Av_2(red)$ in order to conduct the experiments rapidly and thereby avoid the spontaneous oxidation problem encountered during longer term static dialysis experiments discussed above. Values ranging from 4×10^7 to 7×10^7 M^{-2} for the binding of two MgATP molecules to $Av_2(red)$ were obtained with no loss of Av_2 activity or change in redox status, as evidenced by microcoulometric monitoring. Attempts to confirm this value by using the change in the EPR signal of Av_2 resulting from MgATP addition gave a value of 4×10^6 M^{-2}. As discussed later, these values may not be meaningful because of the heterogeneous nature (Lindahl et al., 1985; Hagen et al., 1985; Watt & McDonald, 1985; Howard et al., 1985) of $S_2O_4^{2-}$-containing Av_2 solutions.

The binding of MgATP and MgADP to $Av_2(ox)$ was also investigated by vis–UV and CD spectroscopy. Figure 4 displays the optical spectrum of $Av_2(ox)$ in the presence and absence of 3 mM MgATP. Details of the stepwise optical change resulting from MgATP addition are shown in the insert. In Figure 4 is plotted the absorbance change at 365 nm against the concentration of free MgATP from which an equilibrium binding constant of 6.67×10^7 M^{-2} was determined for reaction 3, in quite good agreement with that from the equilibrium dialysis methods. An identical vis–UV spectral change (data not shown) was observed when MgADP was added except the stronger binding of MgADP to $Av_2(ox)$ caused the optical changes to vary linearly with added MgADP. Because of this strong binding, equilibrium constants were not determined, and only the stoichiometry of 2MgADP/$Av_2(ox)$ was obtained with the estimate that $Av_2(ox)(MgADP)_2$ formed with $K_1K_2 > 10^9$.

Figure 5 shows the CD spectrum before and after addition of 3 mM MgADP to $Av_2(ox)$ with details of how the CD change varies with added MgADP shown as the insert. A value of two MgADP per $Av_2(ox)$ is obtained from the lower curve in Figure 5 for the binding stoichiometry, when the CD change is plotted against the MgADP/$Av_2(ox)$ ratio. Similar CD spectral changes were observed during MgATP additions (data not shown) and when plotted against free MgATP concentration yielded a plot nearly identical with that shown in Figure 4 for the analogous vis–UV experiment. The binding curves thus obtained for either MgATP or MgADP binding to $Av_2(ox)$ were found to be identical when monitored either by CD or by optical spectral changes.

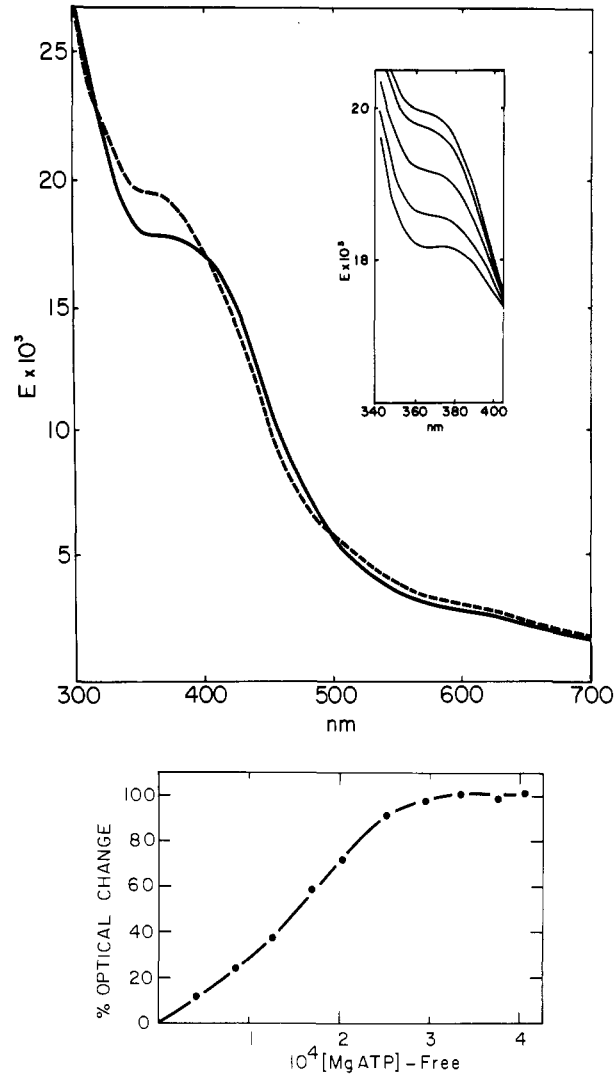

FIGURE 4: Spectroscopic measurement of MgATP binding to $Av_2(ox)$. The upper curve is $Av_2(ox)$ in the presence (dashed line) and absence (solid line) of 3 mM MgATP. $E \times 10^3$ is the molar extinction coefficient (M/cm) of Av_2 at the indicated wavelengths. The insert shows the spectra of $Av_2(ox)$ in the presence of 0, 8.35×10^{-5}, 1.67×10^{-4}, 2.50×10^{-4}, and 5.0×10^{-4} M MgATP from bottom to top. The lower curve is the binding profile of MgATP to $Av_2(ox)$ resulting from the spectral change at 365 nm. The solid line is calculated for a binding constant $K_1K_2 = 6.67 \times 10^7$ M^{-2}. The horizontal axis is the molar concentration of free MgATP.

Attempts to measure the binding of ATP (no Mg^{2+} present) to $Av_2(ox)$ by anaerobic equilibrium dialysis resulted in a linear increase of ATP bound as a function of increasing ATP concentration up to >7 ATP/Av_2 (see Figure 6) at the upper concentration limit of 1 mM ATP and a concomitant loss in Av_2 activity. An irreversible reaction between ATP and $Av_2(ox)$ was suspected from this behavior and subsequently demonstrated by changes in the vis–UV and CD spectra of $Av_2(ox)$ upon addition of ATP. The reaction occurs over a period of 1–2 h during which complete loss in activity occurs (see Nucleotide Reactions with Av_2).

Both $Av_2(red)$ and $Av_2(ox)$ exhibit fluorescence at 340 nm when excited at 290 nm, but the fluorescence is not changed by the addition of either MgATP or MgADP, and consequently, no information about nucleotide binding could be obtained.

Nucleotide Reactions with Av_2. During attempts to measure ATP binding (no Mg^{2+} present) to $Av_2(ox)$, the unexpected and nontypical upper curve shown in Figure 6 was obtained after a 12-h dialysis period. The protein had undergone

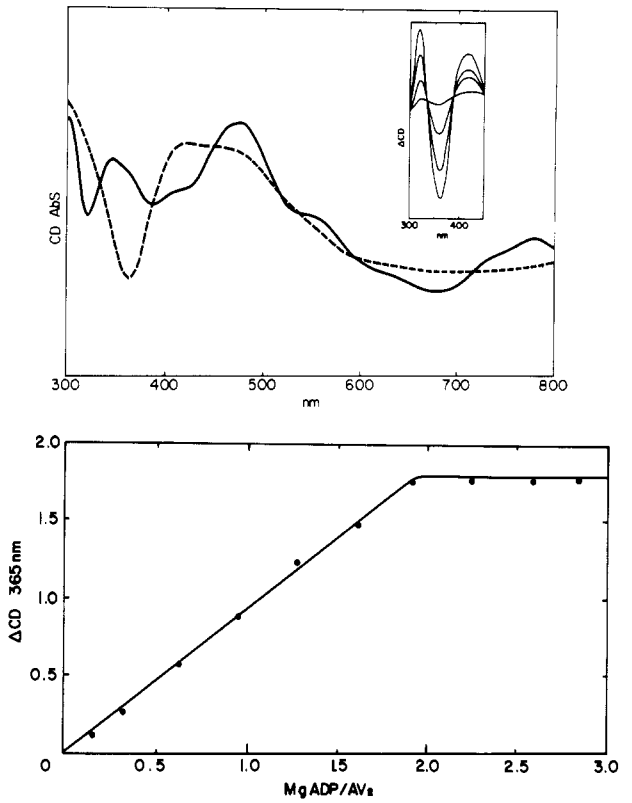

FIGURE 5: CD changes due to MgADP binding to $Av_2(ox)$. The upper curve is the CD spectrum of $Av_2(ox)$ in the absence (dashed line) and presence (solid line) of 3 mM MgADP. The insert is difference CD spectra [$Av_2(ox)$ – ($Av_2(ox)$ + MgADP)] at MgADP concentrations of 0, 3.83×10^{-5}, 7.66×10^{-5}, and 1.15×10^{-4} M MgADP, corresponding to MgADP/$Av_2(ox)$ ratios of 0, 0.59, 1.19, and 1.79, respectively. The lower curve is the binding profile of MgADP to $Av_2(ox)$ resulting from the spectral change at 365 nm. The binding stoichiometry was found to be 1.85 MgADP/$Av_2(ox)$.

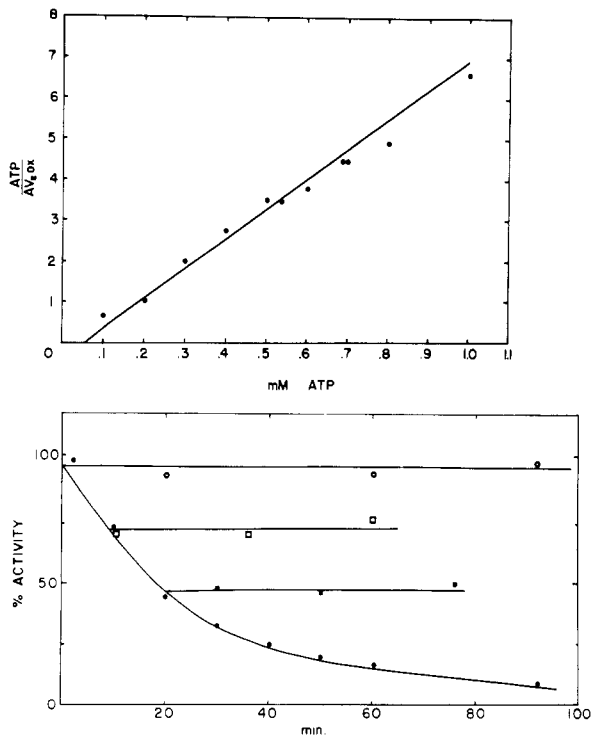

FIGURE 6: ATP interaction with $Av_2(ox)$. The upper curve is the amount of ATP (no Mg^{2+} present) bound to $Av_2(ox)$ as a function of ATP concentration after a 12-h anaerobic dialysis. The lower curve is the response of $Av_2(ox)$ activity to the presence of 3 mM ATP (○) compared to the presence of 3 mM MgATP (●) as a function of time. After an activity decrease to 73% (10 min) (□) and 46% (20 min) (■), $MgCl_2$ was added to a final concentration of 5 mM.

complete loss of activity but still was capable of undergoing reduction as evidenced by microcoulometric measurements. Figure 6 further shows that a concentration exceeding 0.05 mM in ATP is required before the interaction between ATP and $Av_2(ox)$ becomes significant. Figure 6 (lower curve) shows the change in activity that occurs when $Av_2(ox)$ is reacted with excess ATP. An exponential decrease in activity occurs over a 2-h time period, after which complete loss of activity was found. The absorption spectrum in the 300–400-nm range decreases in parallel with the decrease in activity. If $MgCl_2$ is added anytime during the inactivation period, the decrease in activity and optical absorbance change is halted and the activity and spectral properties present at that time remain unchanged as shown in Figure 6. We have not found conditions that will reverse the activity lost during ATP treatment of $Av_2(ox)$, nor have we fully determined the cause of activity loss. The reaction of ADP with $Av_2(ox)$ is about 5 times slower than that with ATP as monitored by the decrease in absorbance at 365 nm.

$Av_2(red)$ can be conveniently prepared by passing $S_2O_4^{2-}$-reduced Av_2 through an anaerobic Sephadex G-25 column, a procedure that provides $S_2O_4^{2-}$-free $Av_2(red)$ useful for redox studies. $Av_2(red)$ thus prepared is stable with respect to activity loss as well as the process of self-oxidation for at least 6 h, when kept completely anaerobic. The addition of $S_2O_4^{2-}$ to $Av_2(red)$ provides conditions for $S_2O_4^{2-}$ destruction and the self-oxidation reaction to occur. A possibly related phenomenon that we have consistently observed is that when $S_2O_4^{2-}$ containing Av_2 is passed through the Sephadex G-25 column, the emerging $Av_2(red)$ is slightly oxidized by 5–10%. Control reactions involving such redox-sensitive proteins as Av_1, Av–flavoprotein, and various ferredoxins, all initially containing excess $S_2O_4^{2-}$, emerge in the fully reduced state, <2% oxidized. It thus appears that Av_2 undergoes partial self-oxidation (5–10%) during the transit time of the column or that there is a certain fraction (5–10%) of the sample that is nonreducible or at least is very susceptible to spontaneous oxidation. The nature of this partial oxidation process is not clear at this time, but the use of microcoulometry to detect and quantitate it has been indispensable.

Once a solution of $Av_2(red)$ is obtained and the small amount of $Av_2(ox)$ is determined, there is no change in the redox status of $Av_2(red)$ even after 5–6-h storage under anaerobic conditions. However, as shown in Figure 7 as monitored by optical spectroscopy (CD, EPR, and coulometry gave the same result), oxidation of $Av_2(red)$ occurs in the presence of catalytic amounts of MoFe upon addition of anaerobic MgATP. No reaction occurs with the addition of an equal volume of anaerobic buffer, Mg^{2+}, ATP, or ADP as evidenced by CD, optical, or EPR spectroscopy or by microcoulometry. Total oxidation of all Av_2 occurs, yielding a limiting H_2/Av_2 value of 0.5 and demonstrating that Av_2 functions exclusively as a one-electron reductant in this MgATP-driven, H_2-evolution reaction.

DISCUSSION

The Av_2 used in the studies reported here contained 3.7–4.5 Fe per Av_2 and possessed activities of 2400–3300 nmol of H_2/(min·mg), values most consistent with the presence of a single [Fe_4S_4] cluster. The results in Figures 1 and 2 and the EPR titration results of Figure 1 in Watt and McDonald (1985) clearly establish that Av_2 undergoes only a single electron redox reaction, a result also consistent with the presence of a single cluster alternating between the [Fe_4S_4-

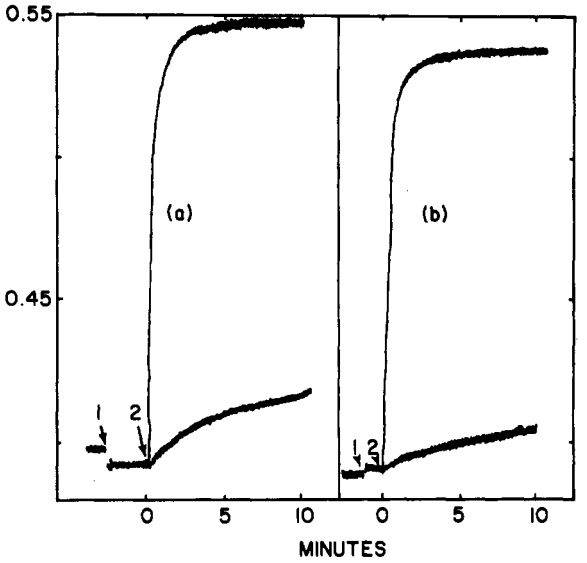

FIGURE 7: Reaction of Av_2(red) with MgATP. (a) Absorbance of Av_2(red) containing 0.05 mg of Av_1 monitored at 420 nm after addition of $MgCl_2$ to 5 mM (arrow 1) followed by the addition of ATP to 5 mM (arrow 2). (b) Same as (a) except ATP was added at arrow 1 and $MgCl_2$ added at arrow 2. The lower curves in (a) and (b) result from MgADP addition to Av_2(red) under the same conditions as the MgATP reactions.

$(RS)_4]^{3-}$ and $[Fe_4S_4(RS)_4]^{2-}$ redox states. Figure 2 shows that the protein-bound cluster undergoes a redox reaction with a midpoint potential of −300 mV, a result in agreement with the redox behavior of other proteins containing only a single $[Fe_4S_4]$ cluster. Our results, therefore, do not support the presence of a two electron redox process (Thorneley et al., 1976) or the presence of more than one $[Fe_4S_4]$ cluster per Av_2 as reported (Braaksma et al., 1983).

The redox behavior of the $[Fe_4S_4]$ cluster in Av_2 is very responsive to the presence of nucleotides, as shown in Figure 2. In all cases studied (except MgAMP, where no change is seen), the midpoint potential is shifted in a negative direction compared to that of Av_2 in the absence of nucleotides. The shift of −130 mV for MgATP, −190 mV for MgADP, and −85 mV for the CH_2 analogue of ATP is a result of the differential in binding of the various nucleotides to Av_2(ox) relative to Av_2(red). In an attempt to substantiate this conclusion, direct binding studies of MgATP and MgADP to Av_2(ox) and Av_2(red) were carried out. However, measured nucleotide binding constants to the Av_2(red) in the presence of $S_2O_4^{2-}$ gave poorly reproducible values that seemed to be technique dependent. For example, flow dialysis gave a dissociation constant of $K_{diss} \sim 2 \times 10^{-8}$ M^2, a values more than a factor of 10 different from that obtained by using EPR changes ($K_{diss} = 2.5 \times 10^{-7}$ M^2) for the same set of conditions. While these measurements were in progress, Lindahl et al. (1985), Hagen et al. (1985), Watt et al. (1985), and Howard et al. (1985) presented EPR and chemical reactivity evidence showing that the Av_2(red) system in the presence of $S_2O_4^{2-}$ is heterogeneous, consisting of at least two distinct species, making direct binding measurements quite meaningless.

Because of this heterogeneous nature of Av_2 in the presence of $S_2O_4^{2-}$ and because of the technical difficulties of handling reduced but $S_2O_4^{2-}$-free Av_2 solutions, we have developed an indirect method for measuring MgATP binding to Av_2(red). This method uses the well-defined reactions summarized by reactions 1–3. When these reactions are appropriately combined, they yield reaction 4, which predicts that the reduction

$$Av_2(ox) + e^- \rightleftharpoons Av_2(red)$$
$$E_{1/2} = -300 \text{ mV} \quad \Delta G = +6.92 \text{ kcal/mol}$$

$$Av_2(ox)(MgATP)_2 \rightleftharpoons Av_2(ox) + 2MgATP$$
$$K_{diss} = 1.72 \times 10^{-8} \text{ M}^2 \quad \Delta G = +10.7 \text{ kcal/mol}$$

sum: $Av_2(ox)(MgATP)_2 + e^- \rightleftharpoons Av_2(red) + 2MgATP$
$E_{1/2} = -764$ mV $\quad \Delta G = +17.6$ kcal/mol (4)

of $Av_2(ox)(MgATP)_2$ to form dissociated Av_2(red) and free MgATP should occur at −764 mV. Binding of MgATP to Av_2(red) will result in a positive deviation in this $E_{1/2}$, in direct proportion to the binding strength of MgATP to Av_2(red). The direct reduction of $Av_2(ox)(MgATP)_2$ occurs at $E_{1/2} = -430$ mV (Figure 2), which, when combined with (4), yields a K_{diss} of 2.2×10^{-6} M^2 for (6). This K_{diss} value differs by a factor

$$Av_2(ox)(MgATP)_2 + e^- \rightleftharpoons Av_2(red) + 2MgATP$$
$$E_{1/2} = -764 \text{ mV} \quad \Delta G = +17.6 \text{ kcal/mol} \quad (4)$$

$$Av_2(red)(MgATP)_2 \rightleftharpoons Av_2(ox)(MgATP) + e^-$$
$$E_{1/2} = +430 \text{ mV} \quad \Delta G = -9.92 \text{ kcal/mol} \quad (5)$$

sum: $Av_2(red)(MgATP)_2 \rightleftharpoons Av_2(red) + 2MgATP$
$K_{diss} = 2.2 \times 10^{-6}$ M^2 $\quad \Delta G = +7.68$ kcal/mol (6)

of 130 from the corresponding value of 1.72×10^{-8} M^2 obtained for Av_2(ox) according to reaction 3. These results clearly show a much stronger affinity of MgATP for Av_2(ox) than for Av_2(red). A similar quantitative treatment for MgADP binding to Av_2(ox) and Av_2(red) cannot be carried out because the binding constant for MgADP binding to Av_2(ox) is too large to be determined by the methods employed in this study. However, from the −190-mV shift in reduction potential of Av_2(ox) in the presence of MgADP, a much stronger binding to both Av_2(ox) and Av_2(red) compared to MgATP is expected.

Ljones (1973) reported that as the $S_2O_4^{2-}$ concentration becomes exausted under normal assay conditions, the observed increase in absorbance at 430 nm arises from Av_2 oxidation as it transfers its electron to the MoFe protein. Similar conclusions had been drawn earlier from EPR experiments (Orme-Johnson et al., 1972) on the nitrogenase assay system. Figure 7 confirms these results and clearly demonstrates that Av_2 can serve as the sole reductant in the Av_2-catalyzed, MgATP-dependent H_2-evolution reaction. This figure also shows very low but significant Av_2 oxidation actively occurring with MgADP, which, however, could be a result of contamination of the MgADP with low levels of MgATP. The reaction shown in Figure 7 is a very sensitive method for determining if Av_2 solutions are free of contaminating MoFe protein.

The self-oxidation of Av_2 in the presence of $S_2O_4^{2-}$ was first clearly described by Stephens et al. (1983) and later verified by Cordewener et al. (1983) and Burns et al. (1985). The phenomenon is not well understood, but our results indicate that the presence of $S_2O_4^{2-}$ (or a byproduct of decomposition) is a contributing factor because in its absence Av_2(red) does not spontaneously oxidize. We have attempted to demonstrate that one of the mixed spin-state species present in $S_2O_4^{2-}$ solutions of Av_2 is the catalytic form that is responsible for the spontaneous oxidation phenomenon but have no concrete evidence for this hypothesis. What our results have shown is that the spontaneous oxidation reaction produces active, single electron oxidized Av_2(ox) that is spectroscopically identical (vis–UV, CD) with dye-oxidized Av_2(ox) but electrochemically distinct. Reaction 1 and Figure 1 show that dye-oxidized

Av_2(ox) undergoes a single electron reduction reaction at −300 mV while spontaneously oxidized Av_2(ox) is similarly reduced at −380 mV. Both oxidized forms undergo reduction at −430 mV in the presence of MgATP, indicating that MgATP drives both into a common thermodynamic state. Spontaneously oxidized Av_2 thus appears to be in an intermediate thermodynamic state between dye-oxidized Av_2(ox) and Av_2(ox)-$(MgATP)_2$, but the nature of this intermediate state is presently unknown.

Registry No. ATP, 56-65-5; MgATP, 1476-84-2; MgADP, 7384-99-8; nitrogenase, 9013-04-1.

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