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Redox and Spectroscopic Properties of Oxidized MoFe Protein from *Azotobacter vinelandii*†

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ABSTRACT: The MoFe protein from *Azotobacter vinelandii* undergoes a six-electron oxidation by various organic dye oxidants with full retention of initial activity. Reduction of the oxidized protein by $S_2O_4^{2-}$ and by controlled potential electrolysis indicates the presence of two reduction regions at −290 and −480 mV, each requiring three electrons for complete reaction. Control of the oxidation conditions provides a means for preparing two distinct MoFe protein species selectively oxidized by three electrons. Selective reduction of the redox region at −290 mV causes development of the EPR signal associated with fully reduced MoFe protein while reduction at −480 mV produces a change in the visible spectrum but has no effect on the EPR signal intensity. Kinetic differences for reduction of the two redox regions indicate that the cofactor region undergoes a more rapid reaction with reductant than the other metal redox sites.

The redox properties of the MoFe protein from different bacterial sources have been investigated by a number of workers [see Zumft & Mortenson (1975) and Mortenson & Thorneley (1979) for a review; Orme-Johnson et al., 1977; Watt & Bulen, 1976; O'Donnell & Smith, 1978; Watt et al., 1980]. Two groups of redox values for the MoFe protein have been reported in these studies, one occurring near −100 mV and the other near −450 mV vs. the normal hydrogen electrode (NHE).[1] In a recent comprehensive study by O'Donnell & Smith (1978), the redox potentials (in parentheses) for MoFe proteins from the organisms *Azotobacter vinelandii* (−42 mV), *Clostridium pasteurianum* (0 mV), *Bacillus polymyxa* (−95 mV), *Azotobacter croococcum* (−42 mV), and *Klebsiella pneumonia* (−180 mV) were measured by an EPR–potentiometric technique under similar conditions, thus giving a self-consistent set of data for these proteins. The redox reactions were all reported to be essentially reversible, except for the clostridial protein which could only be reduced at potentials −250 mV more negative than that required for oxidation. In all cases studied by O'Donnell and Smith and in all but one of the previously reported studies [Albrecht & Evans (1973) being the exception], a single redox region was observed by this EPR technique with $n = 1$.

It is important to note that the total number of electrons involved in the redox reaction(s) affecting the EPR signal was not measured in the EPR–potentiometric experiments. The $n = 1$ value was obtained from an analysis of the redox curve only, and the total number of electrons involved in the redox reaction was left unevaluated.

A spectrophotometric method was used to measure both a redox potential of −70 mV for the MoFe protein from *C. pasteurianum* (Walker & Mortenson, 1973) and the total number of electrons required for oxidation of the protein in this redox process. A single redox region was observed involving about four electrons during this optical titration performed at 460 nm.

These two spectroscopic techniques follow different properties of the protein and while both could report on the same redox events, it is conceivable that they report on quite different and even independent redox events. Evidence that this latter situation might be occurring comes from the oxidative EPR titrations reported by Orme-Johnson et al. (1977) and Zimmerman et al. (1978) and the electrochemical reductive titrations of Watt & Bulen (1976) and Watt et al. (1980). The oxidative EPR titrations show that three to four electrons are removed from $S_2O_4^{2-}$-free (but otherwise reduced) MoFe protein by thionine oxidation before the EPR signal intensity at $g = 3.65$ is affected. The next two to three electron equivalents of added thionine nearly abolish the EPR signal, producing a MoFe protein oxidized by approximately six electrons. The redox experiments of Watt et al. (1980) using polarographic and potentiostatic techniques clearly indicate two separate redox regions where dye-oxidized MoFe protein undergoes a methyl or benzyl viologen mediated reduction at various potentials at a carefully controlled platinum electrode.

The oxidative results of Orme-Johnson et al. (1977) and Zimmerman et al. (1978) and the reductive results of Watt et al. (1980) start from completely different oxidation states of the MoFe protein but are consistent in showing two separate redox regions, only one of which is responsible for the EPR signal. We report here details of our reduction measurements and include a more detailed description of the spectroscopic and electrochemical properties of the oxidized forms of the MoFe protein.

Experimental Section

MoFe Protein. This protein with activities ranging from 1800 to 2700 nmol of H_2 min^{-1} mg^{-1} was prepared by the method of Bulen & LeComte (1972) and Shah & Brill (1973) or a modification of this latter procedure by Burgess et al.

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[1] Abbreviations used: NHE, normal hydrogen electrode; SCE, saturated calomel electrode; EPR, electron paramagnetic resonance.

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(1980). The latter method gave higher specific activities than the other two and slightly more reproducible Fe and Mo analysis, although all MoFe protein preparations used in this study contained 22–27 Fe atoms and 1.75–2.0 Mo atoms per molecule of MoFe protein. The molecular weight value of 230 000 used in calculations involving the MoFe protein is the average value obtained from the extensive measurements of Bulen (1976 and unpublished results). Colorimetric biuret protein measurements based on dry weight protein values and the amino acid content were the primary analytical procedure used to calculate the molar concentration of the MoFe protein. Lowry protein determinations and absorbance values at 400 nm for the oxidized protein ($\epsilon_{400}^{ox} = 7.30 \times 10^4$ cm^{-1} M^{-1}) were sometimes used as secondary standardization procedures. Lowry and biuret determinations gave essentially the same value for the reduced protein, but the Lowry method underestimated the six-electron-oxidized MoFe protein concentration by 17%.

Oxidized MoFe Protein. MoFe protein in 0.05 M Tris and 0.25 M NaCl, pH 8.0, prepared by the methods indicated above contains excess $S_2O_4^{2-}$ to protect the protein from O_2 inactivation. The excess $S_2O_4^{2-}$ was removed, and the protein (0.5–1 mL of 20–50 mg/mL) was oxidized by careful addition of degassed oxidant solutions to the MoFe protein until a detectable excess of oxidant persisted for 10–30 min. The oxidants used were methylene blue, thionine, 2,6-dichlorophenolindophenol (DCPIP), and, in some cases, air. After oxidation by these reagents, the protein was placed on an anaerobic polyacrylamide P-2 or Sephadex G-25 column (2 × 5–8 cm) and eluted with anaerobic buffer. The excess oxidant and oxidation products remained near the top of the column, and the oxidized protein was collected anaerobically as it emerged from the column. Specific activities, total protein (biuret or Lowry), and Fe and Mo content were measured on this dye-oxidized gel-filtered MoFe protein.

Spectroscopic Measurements. A Varian Model 4500 EPR spectrometer equipped with a liquid helium Dewar and transfer line was used for the low-temperature 8–15 K EPR measurements. Anaerobically prepared MoFe protein samples (0.25 mL of 5–15 mg/mL) were frozen in 3-mm EPR tubes under an argon atmosphere. A Cary 118 spectrophotometer was used to record the visible–ultraviolet spectra of MoFe protein samples.

Reduction Measurements of MoFe Protein. The extent of oxidation of the dye-oxidized MoFe protein was determined by two methods. The first was a polarographic procedure (Watt et al., 1975) which measured the total number of electrons transferred to the MoFe protein by measuring the decrease in concentration of an amperometrically standardized $S_2O_4^{2-}$ solution after reaction with a known amount of oxidized MoFe protein.

For this measurement, 2 mL of degassed 0.05 M Tris, pH 8.0, containing 0.1 M NaCl was transferred by gas-tight syringe to an argon-flushed, two-compartmented polarographic H-cell. The H-cell contained the 2 mL of buffer in one compartment and a saturated calomel electrode in the other compartment. The dropping mercury electrode (DME) set at +0.30 V vs. SCE was placed in the 2 mL of buffer, and $Na_2S_2O_4$ (as small crystals or a solution) was added (with stirring) to the buffer until the desired concentration ($\sim 1 \times 10^{-4}$ M) of $S_2O_4^{2-}$ was attained. The concentration was determined, and then 0.1-mL samples of the oxidized MoFe protein were added to the $S_2O_4^{2-}$ solution in the cell. The solution was briefly stirred, and the $S_2O_4^{2-}$ concentration was remeasured. The change in $S_2O_4^{2-}$ concentration with addition of oxidized MoFe protein was used to calculate the electrons transferred from $S_2O_4^{2-}$ to MoFe protein, assuming the stoichiometry shown by reaction 1 at pH 8 and above which has been shown to be the preferred reaction for nitrogenase catalysis (Watt & Burns, 1975) and for other protein redox reactions involving $S_2O_4^{2-}$ (Mahew, 1978; Lambeth & Palmer, 1973).

$$S_2O_4^{2-} + 2H_2O \rightleftharpoons 2SO_3^{2-} + 4H^+ + 2e \quad (1)$$

The second technique of characterizing the extent of oxidation of the oxidized MoFe protein is a controlled potential electrolysis method (Watt & Bulen, 1976; Watt, 1979). This method is quite selective and measures the number of electrons transferred to the MoFe protein at precisely controlled potentials.

The controlled potential reduction experiments consist of bringing a 2-mL solution of buffer and mediator to the desired potential by using a three-electrode potentiostat (Harrar, 1975). When the desired potential is attained as evidenced by a small but constant current flow, a sample of oxidized MoFe protein is added. If electron transfer occurs from the electrode through the mediator into the MoFe protein, a current flow is observed as the protein undergoes reduction. Integration of this current flow over the time of reduction gives the total number of electrons transferred to the MoFe protein at the potential of the experiment. This procedure is capable of detecting 0.5 nmol of reduction to a precision of 5% (Watt, 1979). A number of such reduction measurements were made as a function of applied potential (Watt, 1979) to completely define the redox state of the protein.

Both the polarographic cell and the controlled potential reduction cell are equipped with gas lines which allow them to be flushed with various gases after passage through heated quartz tubes containing copper turnings to remove O_2 and run under any desired gaseous atmosphere. O_2 levels were usually <0.1 ppm. Both types of reduction measurements were done at 25.0 ± 0.3 °C.

Mediators, Buffers, and Reference Electrodes. Saturated calomel electrodes (SCE) were used as reference electrodes both for the polarographic measurements of total $S_2O_4^{2-}$ consumption by oxidized MoFe protein and for the controlled potential reduction measurements. These voltage values at 25 °C were converted into voltage values with reference to the more familiar normal hydrogen electrode (NHE) by

$$V_{NHE} = V_{SCE} + 0.2415 \text{ V} \quad (2)$$

Tes, Tris, and phosphate buffers (0.05–0.1 M at pH 7–8) containing 0.1–0.25 M NaCl were used in all of the measurements reported here. The mediators with their indicated redox potentials were methyl viologen (−453 mV), benzyl viologen (−350 mV), ethyl viologen (−480 mV), isopropyl viologen (−495 mV), phenosafranine (−252 mV), and methylene blue (+11 mV) at concentrations ranging from 1×10^{-5} to 1×10^{-4} M in the buffers mentioned, and they were used to mediate electrons from the platinum electrode to the oxidized MoFe protein. The mediators were used separately in the potential range where they were effective and never as mixtures.

Results

MoFe Protein Oxidation Conditions. The excess $S_2O_4^{2-}$ was removed and the protein oxidized by adding a controlled excess of anaerobic oxidant to MoFe protein samples. A number of variables were examined to determine their effect on the outcome of the oxidation. These were pH variation (6.5–8.5), temperature (20–40 °C), NaCl concentration (0.1–0.5 M),

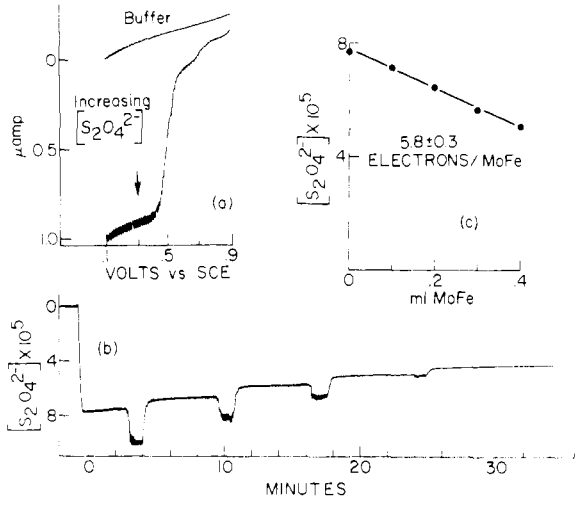

FIGURE 1: (a) A dropping mercury polarogram of $Na_2S_2O_4$ in 0.05 M Tris and 0.25 M NaCl, pH 8 (lower curve), in the voltage range 0.1–0.9 V vs. SCE. The upper curve is a polarogram of the buffer only. The current in microamperes is proportional to the $S_2O_4^{2-}$ concentrations. Precise calibration was accomplished by adding aliquots of spectrophotometrically (λ = 420 nm, ϵ = 1020 M^{-1} cm^{-1}) standardized, anaerobic $Fe(CN)_6^{3-}$ to 2.00 mL of $S_2O_4^{2-}$ solution contained in the polarographic cell. Limiting $Fe(CN)_6^{3-}$ reacts according to $Fe(CN)_6^{3-} + {}^1/_2S_2O_4^{2-} + H_2O \rightleftharpoons Fe(CN)_6^{4-} + SO_3^{2-} + 2H^+$ at pH 8 or greater. (b) Addition of four separate 0.10-mL samples of oxidized MoFe protein to standardized $S_2O_4^{2-}$ monitored at 0.3 V as in (a). After each addition of protein to the $S_2O_4^{2-}$ solution, the mixture was thoroughly stirred for ~30 s and the change in $[S_2O_4^{2-}]$ was determined. The protein concentration was 10.4 mg/mL or 4.52 nmol/addition. (c) The change in $[S_2O_4^{2-}]$ (corrected for dilution) from (b) plotted against milliliters of added protein. The solid curve is a least-squares fit of the experimental points, the slope of which was used to calculate 5.8 ± 0.3 e/mol of MoFe protein.

redox potential of the oxidant, and length of exposure of the protein to excess oxidant. Following oxidation, during which one or more of the variables were changed, the excess oxidant and other small molecular products of $S_2O_4^{2-}$ oxidation were removed by anaerobic chromatography on acrylamide P-2 or Sephadex G-25 columns. The protein emerging from these columns had specific activities and Mo and Fe contents identical with those of the starting protein, showing that oxidation under the variable conditions used had no detrimental effects on the MoFe protein activity. At NaCl concentrations of 0.1 M or below, the oxidized MoFe protein began to crystallize on the gel columns, making chromatography difficult and precluding further evaluation of NaCl effects on oxidation below 0.1 M NaCl. Temperature had no significant effect on determining the outcome of MoFe protein oxidation, but the other variables did, as will be indicated next.

Reduction of Oxidized MoFe Protein. The oxidation of the MoFe protein with a slight excess of oxidized methylene blue [the excess oxidized methylene blue concentration was estimated to be (1–5) × 10^{-5} M] in 0.25 M NaCl, pH 8.0, for 15–30 min produced an active MoFe protein which was EPR silent and had an increased absorbance (per milligram of protein) in the 350–800-nm range over the $S_2O_4^{2-}$-reduced protein. No absorbance peaks in the 600-nm range were evident for anaerobic or aerobic protein samples, indicating the absence of oxidized and reduced methylene blue. Reduction of seven separate oxidized protein samples by $S_2O_4^{2-}$ in the polarographic cell required 6.0 ± 0.4 electrons (3 $S_2O_4^{2-}$ ions) per mol of MoFe protein. A typical reduction experiment is shown in Figure 1.

Controlled potential electrolysis (Watt, 1979) of the oxidized protein at −600 mV (NHE) confirms a six-electron reduction

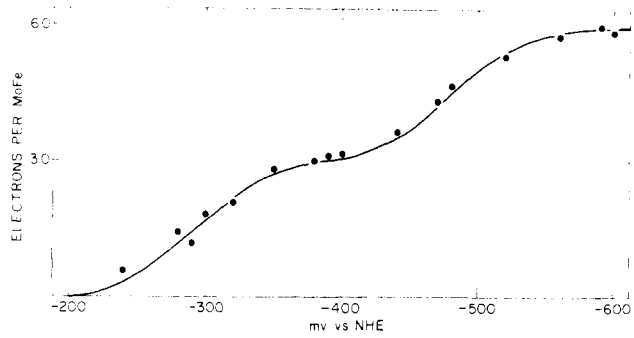

FIGURE 2: The number of electrons transferred to oxidized MoFe protein as a function of applied potential. The solid curve is drawn for two reversible n = 1 processes with midpoint potentials of −290 and −480 mV, respectively, vs. the normal hydrogen electrode. The filled circles are experimentally determined. The redox region at −290 mV is referred to in the text as region I while that at −480 mV is referred to as region II.

(6.0 ± 0.3) stoichiometry. In addition, this technique shows in Figure 2 that two redox regions separated by a narrow plateau are present, each requiring three electrons for reduction. The $S_2O_4^{2-}$ reduction shown in Figure 1 and the controlled potential electrolysis experiment at −600 mV (NHE) fully reduce the two separate, three-electron-requiring redox states shown in Figure 2 and account for a total of six electrons.

The presence of two separate redox states and the ability to prepare each one separately as discussed below require a convenient designation for indicating which redox region, I or II, of the MoFe protein shown in Figure 2 is oxidized. The extent of *oxidation* of the MoFe protein will be indicated by two sets of numbers enclosed in parentheses. The first number indicates the extent of oxidation which occurs in the voltage region I, and the second number indicates the extent of oxidation which occurs in the voltage region II shown in Figure 2. Thus, MoFe(3)(3) indicates a redox state of the protein formed by removal of three electrons each from I and II of Figure 2; MoFe(0)(0) indicates that neither I or II is oxidized (this state corresponds to the isolated, $S_2O_4^{2-}$-reduced MoFe protein); MoFe(3)(0) and MoFe(0)(3) are two distinct oxidation states in which only I and II, respectively, are oxidized by three electrons.

The presence of the plateau at 400 mV shown in Figure 2 indicates that electrolysis at this plateau voltage should selectively reduce the more positive redox state I by three electrons and form MoFe(0)(3) from MoFe(3)(3). This selective reduction has been carried out and verified by transferring samples of the MoFe protein from the reduction cell controlled at the plateau voltage of −400 mV to a second electrolysis cell controlled at −600 mV (where complete reduction occurs) or to the polarographic cell containing $S_2O_4^{2-}$. In both cases three electrons were required to reduce the preelectrolyzed MoFe protein as shown by eq 3. Activity measurements performed at each step of the experiment represented by eq 3 demonstrated retention of full activity for the various redox states of the MoFe protein shown.

$$\text{MoFe(3)(3)} \xrightarrow[-400\ \text{mV (NHE)}]{\text{redn}} \text{MoFe(0)(3)} \begin{cases} \xrightarrow{S_2O_4^{2-}} \text{MoFe(0)(0)} \\ \xrightarrow[-600\ \text{mV}]{\text{redn}} \text{MoFe(0)(0)} \end{cases} \quad (3)$$

The presence of the two separate redox states shown in Figure 2 suggests that oxidation conditions might be found such that each redox state could be separately formed [i.e., MoFe(0)(3) and MoFe(3)(0)]. The variables discussed above can be manipulated to accomplish this reproducibly for

MoFe(0)(3), but success rates for cleanly and reproducibly preparing MoFe(3)(0) are somewhat variable. For example, oxidation of the MoFe protein at pH 8 in 0.05 M Tris and 0.1 M NaCl with a slight excess of methylene blue for 5 min produces MoFe(0)(3). The reduction stoichiometry of this oxidation state was determined by controlled potential electrolysis at −600 mV and by polarographic $S_2O_4^{2-}$ consumption, each measuring a three-electron reduction. In addition, controlled potential electrolysis of this protein in the voltage region −250 to −600 mV produced only a single redox curve identical with that of II in Figure 2 with a reduction potential of −470 mV and an $n = 1$ value (Watt et al., 1980).

Oxidation at pH 6.5–7.0 in 0.05 M Tes and 0.5 M NaCl with a slight excess of methylene blue for 5–10 min produced MoFe(3)(0). This was demonstrated by controlled potential reduction of the protein in the voltage region −250 to −600 mV, where a single redox reaction was observed with a reduction potential of −300 mV and $n = 1$, requiring a total of three electrons for reduction (Watt et al., 1980). Polarographic measurements of $S_2O_4^{2-}$ consumption for reduction of this form of the oxidized MoFe protein also showed a three-electron reduction requirement. Thus, selective oxidation of the MoFe protein under the conditions described produces the two electrochemically distinct three-electron-oxidized MoFe protein species, MoFe(0)(3) and MoFe(3)(0).

Oxidation with thionine gave results identical with those described for methylene blue. However, the stronger oxidant DCPIP seems to react more rapidly with the MoFe protein than methylene blue does, and only the MoFe(3)(3) has been produced. Oxidation beyond this six-electron state to form MoFe(>3)(>3) also occurs with DCPIP as reported by Watt & Bulen (1976) and Watt et al. (1980) but will not be further discussed here. Oxygen is quite uncontrollable in its reaction with the MoFe protein, but with care reasonably active MoFe protein can be prepared in which six electrons have been removed, MoFe(3)(3).

Spectroscopic Properties of Oxidized MoFe Protein. Oxidation of the MoFe protein by six electrons to form MoFe(3)(3) produces an EPR-silent protein from the original EPR-active MoFe(0)(0) protein. There is also an increase in absorbance of the MoFe protein when oxidation occurs. The ability to selectively reduce MoFe(3)(3) by well-defined increments at voltages indicated in Figure 2 and then record the spectral properties of this partially reduced protein affords a method for correlating spectral properties with the extent of reduction. In this manner, the redox region in Figure 2 giving rise to the EPR signal or to the change in the visible–UV spectrum of MoFe protein can be clearly delineated.

Figure 3 is a series of EPR spectra resulting from selective reduction of Mo(3)(3) at the indicated potentials. The EPR signal begins to develop during reduction at voltages corresponding to I of Figure 2, reaches a maximum at the plateau voltage, and remains unchanged during reduction of region II (Figure 2). The EPR signal is therefore only associated with redox state I in Figure 2. In other words, MoFe(0)(3) is EPR active while MoFe(3)(3) is not.

The visible–UV spectral change upon reduction of MoFe(3)(3) is more difficult to quantitate as a function of the degree of reduction as was done with the EPR signal because the optical change is quite small. Therefore, the visible–UV spectrum was recorded for three distinct states of the MoFe protein. These were MoFe(3)(3), MoFe(3)(3) selectively reduced at the plateau voltage of Figure 2 to form MoFe(0)(3), and fully reduced protein MoFe(0)(0). These three measurements are sufficient to show that a visible–UV spectral change occurs when II undergoes reduction [MoFe(3)(3) → MoFe(3)(0)] and a lesser absorbance change occurs upon reduction of I [MoFe(3)(3) → MoFe((0)(3)]. Further details of the visible–UV spectral properties of the MoFe protein will be reported later.

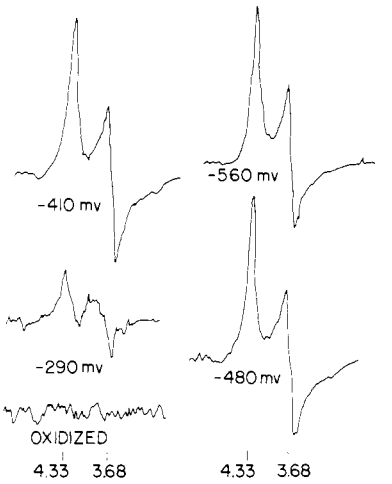

FIGURE 3: EPR spectra of oxidized MoFe protein reduced at the indicated potentials. Benzyl viologen (5×10^{-5} M) was used as mediator in the voltage region −250 to −420 mV and methyl viologen (5×10^{-5} M) in the range −400 to −600 mV. The protein concentration was 5.01 mg/mL. The spectra were taken with the same instrument settings: signal level 2500, modulation amplitude 9 G, power 6.5 mW, all at 10 K.

The two chemically oxidized forms of the MoFe protein, MoFe(0)(3) and MoFe(3)(0), obtained by controlling the variables during methylene blue oxidation as discussed above, substantiate the relationship between spectral properties and redox state just discussed. When MoFe(0)(0) is oxidized to produce Mo(0)(3), an increase in optical absorbance occurs but the EPR signal is not diminished. However, when MoFe(3)(0) is formed by oxidation of MoFe(0)(0), the EPR signal disappears but little change in the visible–UV absorbance occurs. Thus, MoFe(3)(0) and MoFe(0)(3) are both electrochemically and spectroscopically distinct oxidation states of the MoFe protein.

Electrochemical Reduction of MoFe(3)(3). Controlled potential electrolysis of MoFe(3)(3) at the plateau voltage of −400 mV with methyl viologen as mediator to form MoFe(0)(3) proceeds at a faster rate than total reduction at −600 mV to form MoFe(0)(0). At −400 mV the concentration of reduced methyl viologen (the electron transfer form of the mediator) is only 10% of its concentration at −600 mV. This observation suggests that the reduction of region II of Figure 2 by methyl viologen is a slower process than reduction of region I. Analysis of Figure 4, which is a reduction curve of MoFe(3)(3) at −600 mV, demonstrates this biphasic reduction behavior. In general, electrolysis curves result from first-order mass-controlled reactions and produce straight lines when the natural logarithm, ln, of the current is plotted against time (Harrar, 1975). The insert in Figure 4 is such a plot of the curve and clearly indicates a composite curve formed by the blending of two limiting straight lines. This is the expected behavior for two first-order reduction reactions of MoFe(3)(3) occurring at different rates. From the slopes of the straight line sections of the inset in Figure 4, the electrochemical rate constants for the two rate processes were estimated and used to resolve the composite curve into its component reactions. This analysis revealed the ratio of the fast to the slow reaction to be about 3 and further showed that each reaction accounts for approximately three electrons. The fast reaction is

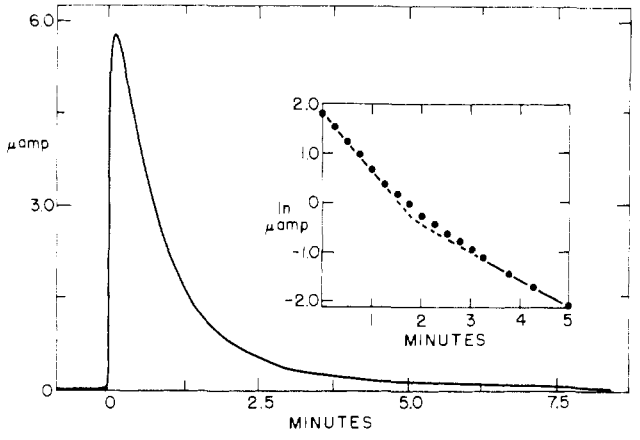

FIGURE 4: The curve resulting from the controlled potential electrolysis of 50 μL of oxidized MoFe protein (6.21 mg/mL) at −590 mV. The inset is a plot of the natural logarithm of the current against time.

MoFe(3)(3) being reduced to MoFe(0)(3), and the slower reaction is MoFe(0)(3) being reduced to MoFe(0)(0). This latter result was confirmed by preparing MoFe(0)(3) separately and comparing its rate of reduction with the slow reduction reaction of the insert in Figure 4.

Reduction of MoFe(3)(3) in the Presence of Gaseous Substrates. The reduction of MoFe(3)(3) by $S_2O_4^{2-}$ as monitored polarographically under atmospheres of N_2, Ar, 10% CO in argon, or 10% acetylene in argon required six electrons for reduction. No indication of interaction of these gases with the MoFe protein during reduction was observed. Controlled potential reduction of MoFe(3)(3) under these same gaseous atmospheres in the voltage range given in Figure 2 produced curves identical with those shown, with the same redox potentials and reducing equivalents being required. Curves identical with those in Figure 2 were observed under argon at pH 7.0, 7.5, 8.0, and 8.5.

Discussion

The close similarity of the EPR signal from the isolated MoFe cofactor and that for the MoFe protein indicates that the EPR signal displayed by the protein arises from the protein-bound cofactor center (Rawlings et al., 1978). We have now demonstrated that the EPR signal and hence the MoFe cofactor center are associated with a distinct redox region (region I of Figure 2) of the MoFe protein involving three electrons centered at a potential of −290 mV vs. NHE. A second redox region also involving three electrons is centered at −480 mV but seems to be associated with redox centers on the protein independent of the MoFe cofactor. A similar conclusion (though numerical details differ) has also been reached by studies of EPR and Mössbauer spectra of incrementally thionine-oxidized MoFe protein (Orme-Johnson et al., 1977; Zimmerman et al., 1978). In these latter studies, the Fe atoms associated with the MoFe cofactor were distinguishable from Fe atoms bound elsewhere in the protein, indicating that two separate redox regions were responding to oxidation. Our results and those of Orme-Johnson et al. (1977) and Zimmerman et al. (1978) are in agreement in establishing the presence of two redox regions involving a total of six electrons that are electrochemically and spectroscopically distinct. One redox region involves the MoFe cofactor center, only, and the other involves Fe clusters elsewhere in the protein.

Although there is overall agreement between the two sets of results just discussed, there appears to be a quantitative discrepancy in the redox stoichiometry of the two separate redox regions. The separately prepared MoFe(0)(3) and MoFe(3)(0) species (Watt et al., 1980), the controlled potential electrolysis measurements in Figure 2, the polarographic monitoring of $S_2O_4^{2-}$ consumption in Figure 1, and the results represented in reaction 3 all indicate that each redox region accepts three electrons whereas the thionine oxidation experiments (and quantitation of both the EPR and Mössbauer spectra) of Orme-Johnson et al. (1977) and Zimmerman et al. (1978) indicate that two electrons are present, one each in presumably two separate cofactor centers (M centers), and a total of four electrons are present, one each in four separate clusters (P clusters) bound to the protein. In other words, the total number of electrons involved is quantitatively the same but the two sets of experiments allocate the six electrons differently between the two redox regions. In the terminology we have been using, our results indicate that MoFe(3)(3) describes the oxidized protein while the results of Orme-Johnson et al. (1977) and Zimmerman et al. (1978) suggest MoFe(2)(4).

The reason for the discrepancy in allocation is not clear at present but may be related to the direction of the redox reactions. The results reported here and those of Orme-Johnson et al. (1977) and Zimmerman et al. (1978) were obtained by examining redox states of the MoFe protein produced by different procedures. Our results were obtained by first oxidizing the MoFe protein by six electrons and then selectively reducing the oxidized protein, while the results of the other studies were obtained by selectively oxidizing reduced protein. If oxidation and reduction were completely reversible, it would not matter which direction the redox reactions of the MoFe protein proceeded. However, there are clear indications that complete redox reversibility does not occur with the MoFe proteins. O'Donnell & Smith (1978) report for *Clostridium* MoFe protein that oxidation occurs at a potential 150 mV more positive than does reduction. We have found (G. D. Watt, unpublished results) considerable hysteresis with *Azotobacter* MoFe protein in which oxidation occurs at values 100–200 mV more positive than the reduction results shown in Figure 2. This lack of reversibility suggests that certain redox sites might be "locked" into metastable states depending on the redox direction used to form them. Complete oxidation followed by reduction might favor the electron distribution shown in Figure 2 whereas selective oxidation of reduced protein might favor the distribution of MoFe(2)(4) observed by Orme-Johnson et al. (1977) and Zimmerman et al. (1978). Spectral observation of these apparently different redox distributions over longer time intervals than previously used or under conditions (higher temperature or protein denaturing conditions, etc.) that would promote metastable to stable conversions might demonstrate the occurrence of metastable states, if present.

Although this hysteresis hypothesis may partially explain the apparently different redox states MoFe(2)(4) and MoFe(3)(3), the presence of the separately prepared MoFe(0)(3) and MoFe(3)(0) redox states (Watt et al., 1980) that are electrochemically and spectroscopically distinct makes this explanation less direct. These latter redox states are the two comprising Figure 2, and since they have been prepared only by selective oxidation of reduced MoFe protein (as distinct from complete oxidations followed by partial reduction), they should not be effected by the "hysteresis effect". The presence of the EPR signal in MoFe(0)(3) but not in MoFe(3)(0) and the clear reduction stoichiometry of 3.0 ± 0.4 (polarographic reduction) and 3.0 ± 0.2 (controlled potential electrolysis) of these species support the formulation MoFe(3)(3) for the six-electron-oxidized state of the MoFe protein. Furthermore,

the development of the EPR signal associated with the MoFe cofactor as a function of voltage shown in Figure 3 demonstrates that the EPR signal (and therefore the MoFe cofactor reduction) reaches a maximum when three electrons are added to MoFe(3)(3). The oxidative EPR titrations and associated EPR and Mössbauer spectra reported by Orme-Johnson et al. (1977) and Zimmerman et al. (1978) showing the presence of MoFe(2)(4) differ from the results reported here. The resolution of this discrepancy will have an important bearing on the MoFe cofactor composition in the MoFe protein because the proposed MoFe(2)(4) state has been interpreted in terms of 2 cofactor units/MoFe protein whereas the formulation MoFe(3)(3) tends to suggest only 1.

Comparison of Redox Potential. The redox potential for the EPR-active redox center in *Azotobacter* MoFe protein determined by O'Donnell & Smith (1978) following the EPR signal height as a function of applied potential is at least 200 mV more positive than that determined in Figure 2 for this redox center. Both methods used mediators to establish equilibrium between the electrode and protein, but the method of Smith and O'Donnell used chemical redox reagents [$S_2O_4^{2-}$ or $Fe(CN)_6^{3-}$] to set the potential of the protein–mediator mixture, whereas the method used in the present study varied the potential of this mixture by electrochemical means and required no additional reagents. The redox potentials reported here were also measured at a well-defined temperature of 25.0 °C, but due to the requirement that the EPR signal of the MoFe protein is only observable at temperatures of <30 K, the temperature of the frozen EPR samples used by O'Donnell and Smith is not thermodynamically defined and lies somewhere between that set at room temperature and the temperature at which the EPR signal was recorded. The two sets of measurements are therefore not directly comparable because of this temperature difference. A possible consequence of this can be deduced from ΔH values (Watt & Burns, 1975) for the redox reactions of mediators and reagents commonly used in redox measurements from which the variation of their potential with temperature, dE/dT values, was calculated. The redox potentials in some cases are strongly temperature dependent and may cause shifts in the set potential as the temperature is lowered during freezing. This would cause a redistribution of electrons among the mediators and protein during the freezing process which could alter the EPR spin population and give an apparent redox potential different from that set at room temperature. This effect in part might explain the difference in our results and those of O'Donnell & Smith (1978).

An earlier attempt by O'Donnell & Smith (1978) to explain the discrepancy between their redox potential of −42 mV for the EPR center in *A. vinelandii* MoFe protein and our previously reported (Watt & Bulen, 1976) result of −320 mV for oxidized MoFe protein suggested that the mediators used to determine this latter value were outside their useful range. This was not the case in our previous study (where both benzyl and methyl viologens were used) nor is it in the present study where care was taken to match the mediator with the applied potential. A further comment by O'Donnell and Smith concerning our previous study was that no protein parameter was monitored during reduction. It is true that no spectroscopic parameter was monitored in our previous study; however, the capacity of the protein to accept electrons as measured by the electrolysis technique used here and in our previous study must be considered to be as fundamental and as sensitive a property of protein reduction as any spectroscopic response. Nevertheless, in the present study we have measured both the number of electrons accepted by the oxidized protein and the EPR and vis–UV spectral response to this reduction. Figures 2 and 3 show that the measured EPR signal closely correlates with the reduction of the protein as measured by controlled potential electrolysis.

The two reduction potentials in Figure 2 of −290 and −480 mV measured for oxidized MoFe(3)(3) are in the redox range measured for other iron–sulfur proteins (Stombaugh et al., 1976; Yoch & Carithers, 1979) and are also close to (−300 mV) and identical with (−450 mV) the redox potential that is required for nitrogenase catalysis (Watt & Bulen, 1976; Evans & Albrecht, 1974). These thermodynamic results and also preliminary kinetic information obtained during the redox measurements indicate that both redox regions of the MoFe protein are kinetically and thermodynamically competent to transfer electrons within the turnover time of nitrogenase catalysis and thus could be involved in the catalytic process. However, no direct evidence has yet been obtained indicating this to be the case.

Internal Electron Transfer in MoFe Protein. The ability to make MoFe(3)(0) by selective oxidation of MoFe(0)(0) shows that the two sets of redox centers in the MoFe protein do not communicate internally. The redox state MoFe(3)(0) is thermodynamically unstable with respect to MoFe(0)(3) as shown in Figure 2 and should spontaneously undergo internal electron transfer if a pathway were available to the more stable MoFe(0)(3) state. MoFe(3)(0) is stable for periods of hours with no detectable change occurring, and thus we conclude that the two sets of redox centers are kinetically isolated in the oxidized MoFe protein. This may not be the case in the complete fixing system where the MgATP–Fe protein might remove kinetic barriers, making internal electron transfer possible. We have evidence that the Fe protein in the nitrogenase complex but in the absence of MgATP shifts redox region I of the MoFe protein to a more negative value, making it nearly coincident with redox region II.

The Meaning of $n = 1$. The solid line in Figure 2 is that predicted for two reversible $n = 1$ Nernstian redox processes with midpoint potentials of −290 and −480 mV vs. NHE. The actual experimental points conform closely to this calculated behavior and suggest the presence of two reversible redox regions in the oxidized MoFe protein. The value of $n = 1$ for a redox process that requires a total of three electrons poses an interesting question about the actual number of redox centers participating in the redox steps of I or II in Figure 2. One possibility is that there are three *independent* redox centers, each accepting one electron at the same potential. A similar situation for the two-electron-requiring clostridial ferredoxin has been analyzed by Eisenstein & Wang (1969). Their analysis would predict the result that is observed in Figure 2. However, another possibility might be that a redox center (or group of centers) exists that can accept a total of three electrons but is only accessible through yet another rate-determining center (an electron "gate") that only transfers electrons one at a time. The overall effect is a total reduction of three electrons with an apparent $n = 1$ value. Kinetic experiments for reduction of oxidized MoFe protein that determine the reaction order of both the protein and reductant may be useful in distinguishing these possibilities and are presently underway in our laboratory.

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Resonance Raman Spectra of Flavin Derivatives Containing Chemical Modifications in Positions 7 and 8 of the Isoalloxazine Ring†

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ABSTRACT: The resonance Raman spectra of riboflavin, 7,8-dichlororiboflavin, 8-chlororiboflavin, 8-bromoriboflavin, 8-(methylmercapto)riboflavin, 7-chlorolumiflavin, 8-norlumiflavin, 7,8-norlumiflavin, and 3-CH_2COOH-lumiflavin were measured in complex with riboflavin binding protein, which was used as a fluorescence quenching agent. Shifts in the positions of Raman bands in the vicinity of 1250, 1405, 1550, and 1585 cm^{-1} were observed in the spectra of many of these flavin derivatives. Comparable shifts were found in the IR spectra (solid KBr) of the uncomplexed flavins. The perturbed bands have been previously assigned to reasonably localized stretching modes in the isoalloxazine system, which are well removed from the 7 and 8 positions. Thus, a direct effect on these bands due to modification of the substituents at positions 7 and 8 is precluded. These observations have led us to conclude that these Raman bands are associated with highly delocalized aromatic framework vibrations.

For some time, evidence has been building which suggests that the different chemical reactivities of the various classes of flavoproteins are due, at least in part, to differences in the flavin–protein interactions for each class (Massey et al., 1969; Massey & Hemmerich, 1980). Direct evaluation of this suggestion via X-ray crystallography is not yet a viable option. Secondary approaches for evaluating flavin–protein interactions, e.g., circular dichroism, protein modification, dye probes, and solvent perturbation, have been used on some flavoenzymes (Blankenhorn, 1978; Mayhew, 1971; Edmondson & Tollin, 1971; Bright & Porter, 1975; Williams, 1975). However, these studies provide information primarily on the protein moieties involved. Information on the nature of the flavin involvement in these flavin–protein interactions is potentially available via ^{13}C NMR using enriched flavins (Yagi et al., 1976; Grande et al., 1977). However, this technique has not been exploited to any appreciable extent. The most extensive information on the role of the flavin in flavin–protein interactions to date has come from reconstitution studies employing flavin analogues and a series of apoflavoenzymes (Massey & Hemmerich, 1980).

Recent resonance Raman studies on flavins and flavoproteins (Dutta et al., 1977; Benecky et al., 1979; Nishina et al., 1978; Kitagawa et al., 1979) have established the feasibility of using resonance Raman spectroscopy to study the flavin and its interactions with proteins. The extensive vibrational information available through Raman spectroscopy and also the

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