Stoichiometry and Spectral Properties of the MoFe Cofactor and Noncofactor Redox Centers in the MoFe Protein of Nitrogenase from *Azotobacter vinelandii*†

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ABSTRACT: Reductive EPR and optical titrations of oxidized MoFe protein using reduced methyl viologen as reductant were used to quantitate the stoichiometry of the various spectroscopically and electrochemically distinct redox centers in the oxidized MoFe protein. Three centers were found to correlate with the EPR signal development (MoFe cofactor centers), and three centers were found to be independent of the EPR signal (P clusters) but to demonstrate distinct optical and kinetic properties. Oxidative EPR and optical titrations of reduced MoFe protein are reported which support the presence of three P-cluster centers. The optical titrations show a distinct change in kinetic behavior between the MoFe cofactor and P-cluster centers. Controlled potential coulometry demonstrates that incremental oxidation of reduced protein by methylene blue, thionine, or indigodisulfonate occurs specifically at three P-cluster sites. Subsequent oxidation by methylene blue and thionine (but not indigodisulfonate) causes the EPR signal to disappear. Three P-cluster sites, two EPR sites, and one presently uncharacterized site are suggested by the results of this study.

The MoFe protein is one of two component proteins required for biological reduction of dinitrogen to ammonia. The ATP-binding Fe protein comprises the other component of this system. Both proteins undergo redox reactions, but because of its larger size, complexity, and higher concentrations of redox active metal constituents, the MoFe protein has a greater potential for possessing diverse and perhaps even unusual redox centers that seem to be required for dinitrogen reduction. Indeed, present views (Hageman & Burris, 1978a,b; Zumft & Mortenson, 1975; Mortenson & Thorneley, 1979) of this protein endow it with the ability not only to stabilize "superreducing electrons" transferred to it from the Fe protein under ATP hydrolysis conditions but also to store a number of such electrons before discharging them into dinitrogen or another of its numerous alternate substrates.

Some of these diverse and unusual redox components have been isolated and are being studied, e.g., the MoFe cofactor (Shah & Brill, 1977; Rawlings et al., 1978; Burgess et al., 1980a). Others have been identified as being similar to, yet different from, known $Fe_4S_4(SR)_4^{2-}$ cluster centers (Zimmerman et al., 1978; Stephens et al., 1979; Kurtz et al., 1979), while still others, like the S center, have only been glimpsed (Smith & Lang, 1974; Zimmerman et al., 1978; Münck et al., 1975). The elucidation of the nature, function, and stoichiometric composition of these redox constituents in the protein are areas of intense interest and are essential for eventually understanding the mechanism of enzymatic nitrogen reduction. However, definitive quantitative study of these centers in the protein are hampered by the severe oxygen sensitivity as well as by the unknown and complex nature of these metal-based redox constituents. In studying this sensitive protein, it is essential to define its redox state both before and after performing physiochemical measurements in order to ascertain that the results of the measurement correspond to a well-defined change in the protein.

The controlled potential coulometry technique (Watt, 1979) provides a sensitive and convenient procedure for detecting redox changes, measuring their magnitude, and defining their position along the voltage axis. This technique along with EPR and optical spectroscopy has been applied to the MoFe protein to provide information regarding the nature and stoichiometry of the various redox centers in dye-oxidized and $S_2O_4^{2-}$-reduced MoFe protein.

Experimental Procedures

MoFe Protein. This protein isolated in the presence of excess $S_2O_4^{2-}$ with activities of 2200–2700 nmol of H_2 min^{-1} mg^{-1} was prepared by the method of Burgess et al. (1980b). The metal content ranged from 1.70 to 1.95 Mo atoms and 26 to 30 Fe atoms per 230 000 daltons. The six-electron oxidized form of this protein,[1] MoFe(3)(3), was prepared and characterized as previously described (Watt et al., 1980a,b). Reduced but $S_2O_4^{2-}$-free protein, MoFe(0)(0), was prepared by first reducing MoFe protein with excess $S_2O_4^{2-}$ and then removing the excess $S_2O_4^{2-}$ by chromatography on anaerobic acrylamide P-2 or Sephadex G-25 columns as described previously (Watt et al., 1980a,b). All chromatographic operations were conducted in specially designed Schlenk-type chromatography columns under a BASF (150 °C) purified argon atmosphere of 1.35 atm of pressure. Protein concentration was measured by biuret or Lowry colorimetric methods (Watt et al., 1980a) using 230 000 daltons for the molecular mass of the MoFe protein (Bulen, 1976). The absorbance at 400 nm for the oxidized (E_{400}^{ox} = 7.25 × 10^4 cm^{-1} M^{-1}) and reduced (E_{400}^{red} = 6.23 × 10^4 cm^{-1} M^{-1}) proteins was also used in some cases to determine protein concentration.

The reduced but $S_2O_4^{2-}$-free MoFe protein is very sensitive to oxidation, and strict adherence to anaerobic techniques is required for its preparation and handling. The following procedure was adopted to assure that the reduced MoFe protein (free of $S_2O_4^{2-}$) was produced for the experiments to be described. After the protein was collected from the anaerobic desalting gel column, which removed excess $S_2O_4^{2-}$, 50–100-μL samples were transferred in specially designed

† From the C. F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received April 8, 1981; revised manuscript received August 4, 1981.* This work was supported by the U.S. Department of Agriculture Competitive Research Grants Office, Project No. 7800795.

[1] This nomenclature has been defined earlier (Watt et al., 1980a,b) and indicates the extent of oxidation (enclosed in parentheses) of each of the two redox regions known to be present in the protein. MoFe(3)(3) indicates both regions are oxidized by three electrons each while MoFe(0)(0) indicates both are fully reduced.

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syringes to a controlled potential electrolysis cell maintained at −590 mV vs. the normal hydrogen electrode (NHE) or to a polarogrphic cell containing $S_2O_4^{2-}$. If the protein had become oxidized during handling, the potentiostat controlling the cell at −590 mV would register a cathodic current flow (reduction of the protein would occur) and the polarograph would indicate a [$S_2O_4^{2-}$] decrease (reduction by $S_2O_4^{2-}$ would occur) upon protein addition. If the protein contained excess $S_2O_4^{2-}$, the potentiostat would indicate an anodic current (oxidation of $S_2O_4^{2-}$ would occur at this potential), and the polarograph would indicate an increase in $S_2O_4^{2-}$ (addition of $S_2O_4^{2-}$ to cell). No response by either the potentiostat or the polargraph upon sample addition was taken as an indication of fully reduced, $S_2O_4^{2-}$-free MoFe protein. This latter situation was not always encountered, as slight oxidation sometimes occurred. However, because protein concentrations were usually 4–8 mg/mL and since the potentiostat method is capable of measuring 0.5 nmol of reduction with about 7% uncertainty (Watt, 1979), we were able to quantitate the extent of oxidation of the reduced protein to the nearest 0.2 electron. The polarographic method is less sensitive and can only reliably estimate oxidation to the extent of about 0.5 electron. On the basis of these reduction methods, MoFe protein samples oxidized by less than 0.5 electron were used in the experiments to be described. The extent of oxidation was determined, and suitable corrections were applied in those experiments.

The transfer syringes were modified versions of gas-tight Hamilton 50- or 100-μL gas-flush fixed needle syringes. The modification enclosed the back of the syringe with a Kel-F enclosure and required the plunger to move through a vacuum tight "O" ring. This modification allows for evacuation and flushing of the entire syringe barrel with argon both behind and in front of the gas-tight plunger seal. This prevents drawing the reduced protein into the syringe barrel in which O_2 has contacted the glass behind the gas-tight seal and eliminates the possibility of protein oxidation resulting from the presence of residual O_2 adsorbed on the glass barrel.

Oxidants. Methylene blue and thionine (Eastman Chemicals) chromatographed on neutral alumina were used as oxidants. The concentrations were determined by controlled potential electrolysis and absorbance measurements at 660 (E = 70000 cm^{-1} M^{-1}) and 602 nm (E = 56300 cm^{-1} M^{-1}), respectively. Oxygen was used as air-saturated buffer solutions. Indigodisulfonate and dichlorophenolindophenol were recrystallized before use.

Reductants. Methyl viologen (Sigma Chemicals) and benzyl viologen (Schwarz/Mann) recrystallized from ethanol and dissolved in 0.05 tris(hydroxymethyl)aminomethane (Tris) and 0.25 NaCl, pH 8, were electrolyzed at −600 and −480 mV vs. NHE, respectively, to obtain the reduced form.

Spectroscopic Measurements. A Varian Model 4502 EPR spectrometer equipped with a liquid helium Dewar and transfer line was used for the low-temperature 7–15 K EPR measurements. Anaerobically prepared MoFe samples (0.2–0.3 mL) were frozen in 3-mm tubes under argon. Titrations of reduced protein with oxidants were carried out by either (1) adding small aliquots of 5–30 μL of oxidant to protein samples contained in anaerobic EPR tubes or (2) oxidizing ~1–2 mL of protein samples to various extents in a separate vessel and then transferring aliquots of the resulting solution to anaerobic EPR tubes. In either case, 20-min incubation periods of protein with oxidant (or reductant) were observed before the reactions were quenched by freezing in liquid nitrogen.

Optical titrations and visible–ultraviolet spectra of both oxidized and reduced MoFe protein were recorded on a Cary 118 spectrophotometer. All quartz vacuum-tight cells fitted with two stopcocks at the top were evacuated and flushed with argon at 1.35 atm. With one stopcock open to the scrubbed argon source, the other stopcock was opened to allow reduced or oxidized protein to be added with a gas-tight transfer syringe under a flow of argon. After the protein was added, the stopcocks were closed, and the spectrum was recorded. The cell was then reconnected to the argon source, and samples (50–100 μL) of protein were removed to verify the redox state of the protein by controlled potential coulometry. Aliquots of titrant (either methylene blue for oxidation or reduced methyl viologen for reduction) were then added to the protein, and the absorbance was monitored at 25 °C until a constant absorbance reading was obtained. Optical titrations were carried out at 425 and 700 nm by using the 0.1 A range. The absorbance was offset 0.3–1.2 A units for protein solutions containing 2–6 mg/mL. The diluting buffers, oxidant solutions, and reductant solutions were all passed through a fine Millipore filter to remove any light-scattering particles prior to recording spectra or performing optical titrations.

Electrochemical Methods. A variation of the coulometric reduction technique (Watt, 1979) was used to evaluate which of the two redox centers known to be present in the MoFe protein (Watt et al., 1980b) undergoes reaction when limiting oxidant reacts with reduced MoFe protein or when limiting reductant reacts with oxidized MoFe protein. This variation required the use of two controlled potential electrolysis cells to characterize the MoFe protein redox state. One was controlled at −400 mV which is on the plateau separating the two redox centers, and the other was controlled at −600 mV which is on the plateau of the most negative redox center. If a sample of the MoFe protein of unknown oxidation state is added to each cell, the degree of oxidation of the most positive redox center is obtained from the cell controlled at −400 mV. Reduction at −600 mV gives a measure of the total reduction of the protein so that the reduction at −600 mV minus that at −400 mV gives the extent of oxidation of just the most negative redox center. In this manner, the total extent of oxidation of the protein as well as the degree of oxidation of each of the two separate redox regions is easily determined.

Results

Reduced, $S_2O_4^{2-}$-Free MoFe Protein. Anaerobic chromatography of $S_2O_4^{2-}$-containing MoFe protein on acrylamide P-2 or Sephadex G-25 provides a means for preparing reduced MoFe protein free of $S_2O_4^{2-}$. Controlled potential electrolysis of reduced MoFe protein using methyl viologen as mediator at −590 mV showed no significant reduction, indicating that the protein is fully reduced and free of $S_2O_4^{2-}$. This protein was used for all oxidative titrations discussed below.

Controlled Potential Coulometry. The sensitivity and selectivity of this method provides a means for determining whether certain oxidants react preferentially with either of the two distinct redox centers present in reduced MoFe protein. Figure 1a shows a sequential oxidation of reduced MoFe protein with methylene blue followed by reduction of the protein at both −400 and −600 mV. The figure shows that no reduction occurs in the protein at −400 mV when up to three electrons have been removed by the oxidant. However, all added oxidation equivalents are accounted for in the protein when reduction is carried out at −600 mV. This result demonstrates that only the most negative redox region was oxidized by the added methylene blue. Only with the addition of more than 3 equiv of methylene blue does reduction at −400 mV occur in the protein, indicating that the more positive redox centers have undergone oxidation. Thionine produces the same

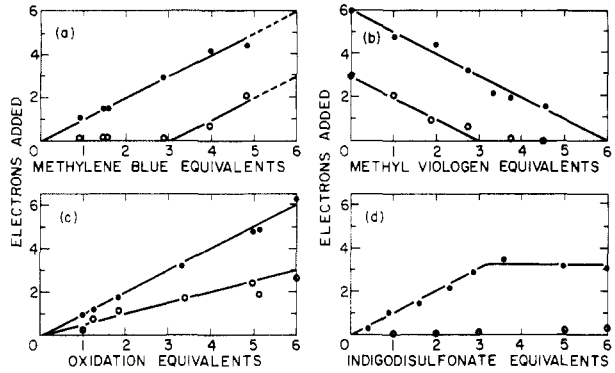

FIGURE 1: Curves resulting from (a) the addition of methylene blue to reduced protein followed by controlled potential reduction at −600 (filled circles) and −400 mV (open circles); (b) the addition of the indicated amounts of electrochemically reduced methyl viologen to oxidized MoFe followed by controlled potential reduction at −600 (filled circles) and −400 mV (open circles); (c) same as (a) except air-saturated buffer was used as oxidant; (d) same as (a) except indigodisulfonate was used as oxidant. In all figures, the vertical axis is the coulometrically measured electron uptake by the protein that was previously reacted with the equivalents of reagents indicated along the horizontal axis.

result as that for methylene blue. Figure 1d shows that the milder oxidant indigodisulfonate only oxidizes the more negative redox centers. No reduction was observed at −400 mV even with MoFe protein which had been reacted with a 10-fold excess of this oxidant. These oxidants clearly discriminate between the two redox centers of the MoFe protein, preferring first to react completely with three noncofactor centers and then with the EPR centers. Oxidation of the protein with air-saturated buffer gave the results in Figure 1c. No preference for either redox center of the protein occurs with this oxidant.

Figure 1b is a similar type of experiment to those just described, except it is carried out in the opposite redox direction. The MoFe protein is first oxidized by six electrons and then sequentially reduced with methyl viologen. The decrease in the extent of reduction at −400 mV with added methyl viologen, up to 3 equiv, indicates that the more positive redox centers (EPR centers) are first selectively reduced by this reductant. Subsequent equivalents then reduce the more negative centers.

Oxidative Titrations of *Reduced MoFe Protein.* Figure 2 shows an optical titration of reduced MoFe protein monitored at both 425 and 700 nm using methylene blue ($E_{1/2}$ = −25 mV at pH 8.0) as oxidant. Similar results were obtained with thionine ($E_{1/2}$ = +20 mV at pH 8.0) as oxidant. The absorbance at each wavelength rapidly increases with addition of methylene blue up to 3 equiv. After 3 equiv, subsequent additions cause a much slower absorbance increase at 425 nm, but no further change occurs at 700 nm. The absorbance change at 425 nm is nearly constant per added equivalent for the first 3 equiv of methylene blue and is only slightly larger per equivalent for the next 2–3 equiv. After approximately 5 equiv has been added, the presence of unreacted methylene blue is observed in the spectrum. The spectrum of the protein remains unchanged with time (~20 min), as does the concentration of methylene blue, indicating a stable state exists in which partially reduced protein (~1 electron remaining) and oxidized methylene blue are both present. Further addition of methylene blue causes an incrementally smaller change to occur in the protein spectrum at 425 nm and a continued increase in the concentration of oxidized methylene blue. An apparent equilibrium condition thus exists between partially reduced MoFe protein and oxidized methylene blue.

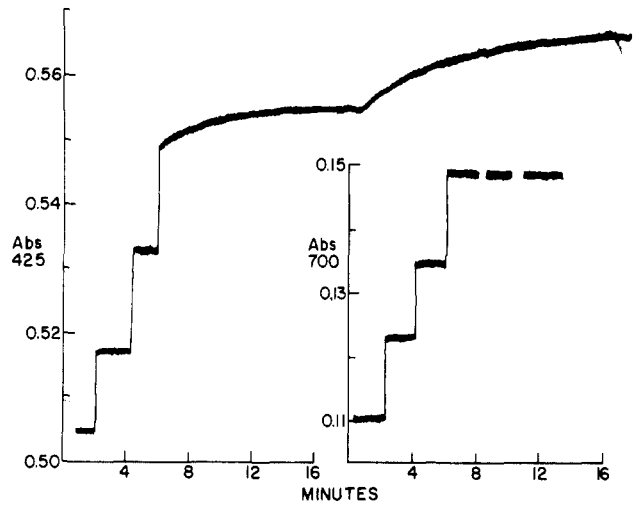

FIGURE 2: Methylene blue titration of reduced MoFe protein (2.52 mg/mL) followed at 425 nm (left figure) and at 700 nm (right figure). The constant absorbance of the original protein at each wavelength is shown at the lower left of each figure. The absorbance increase at each wavelength was recorded for the sequential addition of four aliquots (1.07 equiv each) of methylene blue. The slower kinetic component appears after the addition of the third aliquot (3.21 equiv) as a slow change in the protein absorbance at 425 nm. The rapid kinetic component is eliminated after 3.0 equiv of methylene blue.

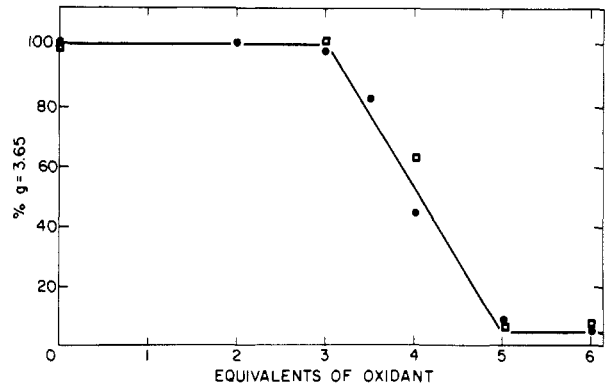

FIGURE 3: EPR titrations of reduced MoFe protein with methylene blue (●) and thionine (□). The vertical axis is in percent reduction, as measured at the g = 3.65 component of the EPR signal. In both titrations, the protein was verified to be completely reduced by controlled potential coulometry before oxidant was added.

Similar results are observed with the closely related oxidant thionine, although nearly complete oxidation of the protein occurs with 6 equiv of this slightly stronger oxidant. Indigodisulfonate oxidation causes optical changes to occur at both 700 and 425 nm identical with those shown in Figure 2 for methylene blue. However, no further change occurs at either wavelength when more than 3 equiv of this oxidant are added. The optical titrations are consistent with the results from the previous section in showing that indigodisulfonate specifically oxidizes three noncofactor centers and leaves the EPR centers fully developed. No reaction is observed when a 10-fold excess of oxidized methyl viologen ($E_{1/2}$ = −450 mV at pH 8.0) or benzyl viologen ($E_{1/2}$ = −365 mV at pH 8.0) is reacted with reduced MoFe protein, even though thermodynamic considerations (Watt et al., 1980a,b) would predict some reaction.

EPR titrations of reduced MoFe protein with methylene blue as oxidant are shown in Figure 3. The EPR signal is essentially unchanged after three electrons have been removed from the protein. However, subsequent removal of electrons causes the signal intensity to decrease sharply to near zero after a total of 5 equiv of methylene blue or thionine has been added. Titration of the protein with indigodisulfonate up to 10 equiv

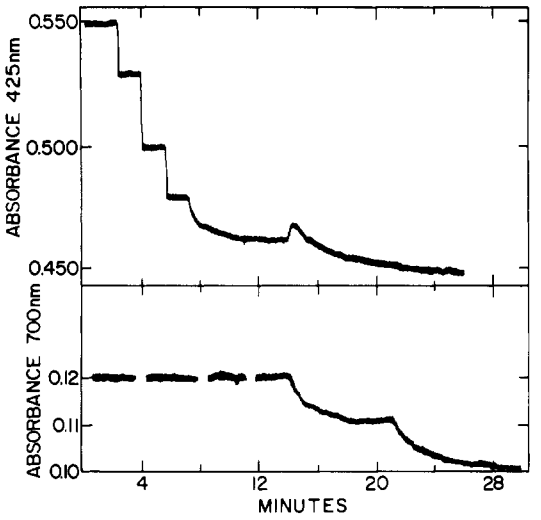

FIGURE 4: Reduced methyl viologen titration of oxidized MoFe protein (2.03 mg/mL) monitored at 425 nm (upper figure) and at 700 nm (lower figure). The initial absorbance of the oxidized protein MoFe(3)(3) is at the upper left of each figure. The absorbance changes for a series of 1-equiv additions of reduced methyl viologen are recorded. The change in kinetic behavior occurs at both wavelengths after the addition of 3 equiv of reduced methyl viologen.

showed no decrease in the EPR signal intensity.

With either methylene blue or thionine, incubation periods of at least 20 min must be observed before the reactions are quenched by freezing, because the rate of oxidation of the MoFe cofactor center (displaying the EPR signal) by these oxidants is not very rapid and incomplete reaction is easily observed with shorter contact times. The results in Figure 2 also demonstrate this kinetic effect by showing that 15–20 min are required before the absorbance change of the protein at 425 nm becomes stable when more than 3 equiv of methylene blue are added. The slow rate of oxidation of the MoFe cofactor center of the protein is thus clearly demonstrated by both the optical measurements and the EPR results.

Reductive Titrations of Oxidized MoFe Protein. An optical titration at both 425 and 700 nm of six-electron oxidized MoFe protein with electrochemically reduced methyl viologen as tritrant is shown in Figure 4. The first 3 equiv of methyl viologen cause a rapid decrease in the absorbance at 425 nm, but no change occurs in the 700-nm region. The fourth and fifth equivalents cause a noticeably slower decrease in absorbance at 425 nm, accompanied by a similar slow decrease in absorbance at 700 nm. After more than about 5 equiv of methyl viologen, smaller changes at 425 and 700 nm occur per added equivalent, and a definite appearance of reduced methyl viologen is seen in the spectrum, as evidenced by a broad band at 600 nm and a sharp band at 397 nm. At this point in the titration, both reduced methyl viologen and partly oxidized protein coexist in a stable, apparent equilibrium state.

An EPR titration of oxidized MoFe protein with reduced methyl viologen is shown in Figure 5. Oxidized MoFe protein does not display an EPR signal; however, the typical EPR signal of reduced MoFe protein develops upon addition of methyl viologen and reaches a maximum at 3 equiv. Further development of signal intensity does not occur upon subsequent methyl viologen additions. Benzyl viologen produces similar effects to those of methyl viologen, but because it is a weaker reductant, reduction by more than the three electrons required to develop the EPR signal does not readily occur.

Both optical and EPR titrations of oxidized MoFe protein with electrochemically reduced methylene blue have been attempted. In neither case did any observable reaction occur.

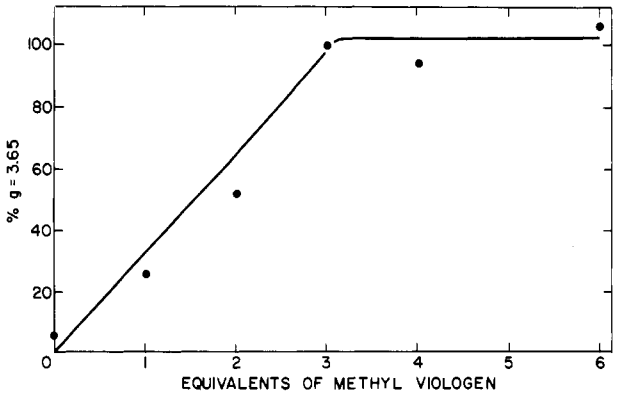

FIGURE 5: EPR titration of six-electron oxidized MoFe protein with electrochemically reduced methyl viologen. The vertical axis represents the percent development of the $g = 3.65$ component of the EPR signal. The 100% signal development was obtained from protein samples reduced either with excess methyl viologen or with solid $Na_2S_2O_4$. The oxidized protein was verified to be oxidized by 5.90 ± 0.25 electrons by coulometric and polarographic methods before the titration was initiated.

This same negative result was obtained by the controlled potential coulometry technique which possesses greater sensitivity for detecting reaction than either the optical or EPR experiments. Reduced methylene blue is therefore completely incapable of reducing either redox region of oxidized MoFe protein.

Discussion

Oxidized MoFe Protein—MoFe(3)(3). Oxidized MoFe protein undergoes reduction at two distinct redox potentials (Watt et al., 1980a,b). Reduction at the most positive midpoint potential of −290 mV vs. NHE corresponds to reduction of the MoFe cofactor center while reduction at the most negative midpoint potential of −480 mV corresponds to reduction of noncofactor centers.[2] The reduction stoichiometry was reported to be three electrons for each redox region (Watt et al., 1980a,b), which has been verified by results presented here. Figure 5 confirms a three-electron requirement for the full development of the EPR spectrum from the EPR silent, six-electron oxidized MoFe protein. Figure 4 also confirms this result by showing a rapid decrease in absorbance of oxidized MoFe protein at 425 nm with addition of aliquots of reduced methyl viologen up to a total of 3 equiv. After three added equivalents of reduced methyl viologen, a distinct change in behavior is observed in which a much slower decline in absorbance occurs. These EPR and optical titrations combined with the results from Figure 1b and other results (Watt et al., 1980a,b) all show that reduced methyl viologen selectively reduces the most positive of the two redox centers of oxidized MoFe protein which has been previously correlated with the EPR signal and consequently identifed as the MoFe cofactor center (Watt et al., 1980a,b). Reduced methyl viologen thus selectively reacts with the oxidized MoFe cofactor center of the MoFe protein.

Figure 4 shows no change in protein absorbance in the 650–800-nm range as the three electron requiring MoFe cofactor reduction occurs. However, when more than three electrons are added, the absorbance in this spectral region decreases. This absorbance decrease results from reduction occurring in the more negative redox region of the "P-cluster"

[2] These noncofactor centers will be referred to in the following discussion as "P clusters" following the designation of Zimmerman et al. (1978).

centers. This decrease in absorbance at wavelengths greater than 650 nm during reduction of oxidized protein is specific to these "P clusters" and affords a method not only to determine their stoichiometry in the protein (three are found to be present) but also to assess their redox status by optical spectroscopy at room temperature. These clusters also undergo a broad decrease in absorbance centered near 425 nm, but this is not unique because the MoFe cofactor also undergoes a similar broad decrease in absorbance in this spectral region.

Reduced MoFe Protein—MoFe(0)(0). This protein has been reported (Orme-Johnson et al., 1977; Zimmerman et al., 1978) to be oxidized by thionine in two distinct phases. In the first phase, oxidation by four electrons was reported during which no change was observed in the EPR signal. However, upon further oxidation by two electrons, the EPR signal disappears. The results in Figure 3 for thionine and methylene blue oxidation are consistent with a two-phase oxidation but differ significantly in numberical detail. Figure 3 shows that oxidation by three electrons leaves the EPR signal intensity unchanged but oxidation beyond three electrons attenuates the signal linearly until complete quenching occurs at 5 equiv. The EPR samples used to obtain Figure 3 were incubated at least 20 min at room temperature with the oxidant prior to freezing in liquid N_2. This time interval is seen to be necessary from Figure 2 which shows that the optical change associated with oxidation of the protein (or consumption of methylene blue, not shown) is not complete in shorter contact times. Orme-Johnson et al. (1977) and Zimmerman et al. (1978) waited only 5 min (a period of time too short for complete oxidation) before quenching the reaction in isopentane at −140 °C. This difference in sample treatment may account for the discrepancy between their EPR titration results and those in Figure 3 and also for the fact that approximately 25% of the EPR signal remained after their titration was completed.

The optical titration in Figure 2 is consistent with the presence of two types of redox centers in the protein, each requiring three electrons, because after 3 equiv of methylene blue has been added, a noticeably slower reaction occurs upon addition of the next 3 equiv of methylene blue. As discussed above, this slower reaction corresponds to oxidation of the cofactor centers, because after the fourth equivalent of methylene blue is added and allowed to react completely, the EPR signal has been reduced by half (and upon reaction with the fifth, the signal is eliminated). Also, the absorbance in the 650–800-nm region increases with the first 3 equiv of oxidant (demonstrated above to exclusively occur upon "P-cluster" oxidation) but then remains constant with further additions.

The results of Figures 1–3 clearly indicate that oxidation of reduced MoFe protein with methylene blue first occurs at the most negative redox centers and requires three electrons. As the EPR signal is still present (the MoFe cofactor remains reduced), we conclude that the three-electron oxidation occurs at noncofactor or "P-cluster" sites. The increase in absorbance in the 650–800-nm region accompanied by no dimunition of the EPR signal intensity demonstrates the exclusive oxidation of the "P-cluster" centers.

Other Redox Reactions. Thionine is a closely related thiazine analogue of methylene blue. Its redox potential is about 50 mV more positive than that of methylene blue, but this difference does not seem sufficient to alter interaction with the reduced MoFe protein, and results with this oxidant are similar to those in Figures 1–3 obtained with methylene blue. Dichlorophenolindophenol ($E_{1/2}$ = +250 mV, pH 8.0) is a much stronger oxidant than either thionine or methylene blue, and its interaction with reduced MoFe protein is rapid and complete to form the six-electron oxidized protein, MoFe(3)(3), with no indication of any intermediate oxidation state species forming. However, the weaker oxidant indigodisulfonate ($E_{1/2}$ = −165 mV, pH 8.0) is not capable of oxidizing the MoFe cofactor EPR centers, and only a three-electron oxidized form of the protein results from reaction with this oxidant. These facts plus the equilibrium-like behavior displayed by methylene blue by incompletely oxidizing the MoFe protein to MoFe(3)(3) at molar ratios of 3:1 (methylene blue is a two-electron oxidant) indicate that the redox potential of methylene blue and that of the reduced cofactor centers of the MoFe protein are similar. Indeed, from both the measurement of unreacted oxidized methylene blue present ($E^{0\prime}$ = −25 mV at pH 8) when 6 equiv is reacted with reduced MoFe and the result that the MoFe protein can be oxidized by six electrons (Watt et al., 1980a,b) with excess methylene blue, an oxidation potential for the MoFe cofactor centers in the protein can be estimated to be −20 to −80 mV. This value is in the range of previously reported results, in parentheses, of O'Donnell & Smith (1978), Walker & Mortenson (1974), and Zumft et al. (1974) for the EPR centers of MoFe protein from *Clostridium* (0 to −50 mV), *Klebsciella* (−180 mV), and *Azotobacter* (−50 mV). This calculated oxidation potential and those previously measured values differ significantly from our reported *reduction* potential of −290 mV for the oxidized MoFe cofactor centers and −480 mV for the "P-cluster" centers in the protein. We have previously suggested (Watt et al., 1980a) that this behavior is explainable in terms of electrochemical hysteresis. This explanation simply expresses the fact that an apparent reversible oxidation reaction of a redox center occurs at a potential quite different from the apparent reversible reduction of this same center. The difference in potential between oxidation and reduction represents an activation energy or overpotential which often results from such molecular events as protein conformational changes, changes in orientation or structure of the redox clusters themselves, or differences in the number or type of ligand atoms of the cluster occurring upon redox reaction.

The results reported here readily demonstrate this hysteresis effect in both redox directions. The apparent reversible oxidation potential of ~ −50 mV for the MoFe cofactor center suggests that an excess of reduced methylene blue should cause reduction of the oxidized MoFe protein. However, no reaction is observed as monitored by the EPR spectrum, by the optical spectrum at 660 nm (oxidized methylene blue should form with E_{660} = 70 000 M^{-1}), or with controlled potential coulometry of methylene blue reduced at −200 mV vs. NHE. All three methods are very sensitive, and the measured lack of reduction reliably demonstrates the nonreversibility of the reaction. In a similar way, the reported (Watt et al., 1980a) reduction potentials in oxidized MoFe protein of −290 (the MoFe cofactor centers) and −480 mV (the "P clusters") to form the reduced protein suggest these centers should undergo oxidation by an excess of oxidized methyl or benzyl viologen. However, the three methods mentioned above again fail to detect any reaction demonstrating the lack of reversibility of this reaction.

Interpretation of the Titration Results. Oxidation of reduced MoFe protein by the first 3 equiv of methylene blue occurs rapidly and exclusively at the "P-cluster" (noncofactor) sites and indicates that three such centers are present in the protein. The results reported by Watt et al. (1980a,b), the electrochemical results, the EPR and optical titrations in Figures 1–3, and the separate preparation and characterization of MoFe(0)(3) (Watt et al., 1980a,b) all support this inter-

pretation. However, this conclusion is at variance with earlier EPR and Mössbauer studies of Orme-Johnson et al. (1977) and Zimmerman et al. (1978) for *Azotobacter* MoFe protein and also with their more recent results with Clostridial MoFe protein (Huynh et al., 1980), indicating four such centers to be present. We are unable to completely rationalize these apparently contradictory results from our experiments which were done under similar if not identical conditions to theirs. However, the methods used to remove excess $S_2O_4^{2-}$ from the reduced protein may offer an explanation. In our work, $S_2O_4^{2-}$ was removed chromatographically and its absence verified by sensitive electrochemical methods. In the other studies, $S_2O_4^{2-}$ was removed by an in situ oxidation at −325 mV. If residual $S_2O_4^{2-}$ remained or if a reductant not oxidizable at −325 mV but capable of reducing thionine was still present, this could account for an apparent four-electron oxidation instead of three.

Our result of only three "P cluster" being present in the protein is also significantly less than the 3.4–4.0 extrudable $Fe_4S_4(SR)_4^{2-}$ clusters measured by Kurtz et al. (1979) which are thought to originate from the "P clusters" of the *Azotobacter* MoFe protein. This elegant ^{19}F NMR based extrusion method accounts for nearly 100% of the noncofactor iron present in the protein of 245 000 daltons containing 30 ± 2 iron atoms. For the *Azotobacter* protein, however, a molecular weight of 230 000 (Bulen, 1976; W. A. Bulen, unpublished results) seems more reasonable than that of 245 000 used by Kurtz et al. (1979), and on this basis, the iron analysis of the protein actually used becomes 28 ± 2 instead of the 30 ± 2 used in the extrusion calculations. Taking these two minor refinements into account, we calculate that the reported number of extrudable $Fe_4S_4(SR)_4^{2-}$ clusters decreases from 3.4–4.0 to 2.9–3.5, in reasonable agreement with our result of three.

The methylene blue and thionine titrations of reduced MoFe protein in Figure 3 show that oxidation of the protein by two electrons (between the third and fifth equivalent of oxidant) completely eliminates the EPR signal. Further oxidation by one more electron forming MoFe(3)(3) (Watt et al., 1980a,b) occurs, as evidenced by controlled potential coulometry, by $S_2O_4^{2-}$ consumption followed polarographically, and by optical changes occurring in the 425-nm spectral region. It is clear then that a previously unsuspected EPR silent redox center is present in the reduced protein which undergoes oxidation by the sixth equivalent of oxidant. Some properties of this center can be inferred from previously reported data and that presented here. The controlled potential reduction of oxidized MoFe(3)(3) shown in Figure 1b and previously reported results (Watt et al., 1980a) show that three electrons are required to reduce the MoFe cofactor centers and to develop fully the EPR signal. This same result is seen in the EPR and optical titrations of oxidized MoFe(3)(3) shown in Figures 4 and 5. However, EPR integration has established that only two EPR centers are present in the protein (Münck et al., 1975; B. Hoffman and G. D. Watt, unpublished results). This EPR silent center thus accounts for the third redox component being reduced in the experiments just described, and therefore it must possess kinetic properties and a redox potential nearly identical with those of the two EPR centers of the protein. The rapid, three electron requiring kinetic component of the biphasic reduction reaction of MoFe(3)(3) and a redox potential of −290 mV for the three-electron reduction step previously reported (Watt et al., 1980a) support this conclusion.

Acknowledgments

We thank Drs. J. W. McDonald, W. E. Newton, E. I. Stiefel and B. K. Burgess for helpful discussion and assistance.

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