Electrochemical Characterization of the Iron-Molybdenum Cofactor from *Azotobacter vinelandii* Nitrogenase

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Abstract: The Fe-Mo-S-containing cofactor (FeMoco) isolated from the molybdenum-iron protein of *Azotobacter vinelandii* nitrogenase undergoes direct electrochemical reduction at a glassy carbon electrode in *N*-methylformamide (NMF). Two reductions are observed at -0.32 and -1.00 V vs. the normal hydrogen electrode by cyclic voltammetry; these appear to correspond to the [FeMoco(ox)] + $e^- \rightleftharpoons$ [FeMoco(s-r)] and [FeMoco(s-r)] + $e^- \rightleftharpoons$ [FeMoco(red)] redox processes of the cofactor bound within the intact MoFe protein. FeMoco(ox) to FeMoco(s-r) reduction in isolated cofactor is studied by chemical redox titrations with voltammetric, potentiometric, and EPR spectroscopic monitoring and is characterized as a chemically reversible, one-electron transfer involving an $S = 0$ to $S = {}^3/_2$ spin-state change. Review of electrochemical data reported for synthetic models of FeMoco in solvents other than NMF reveals that none of these compounds duplicate the redox properties of FeMoco exactly. Experimental examination reveals that several of these compounds also lack chemical stability in NMF; consequently, it appears that an exact chemical model for FeMoco has yet to be prepared.

Nitrogenase is the complex enzyme system that is responsible for biological fixation of dinitrogen to ammonia. The minimal composition of the catalytically competent enzyme system includes two separately purifiable proteins (MoFe and Fe proteins), MgATP, a reductant, protons, and substrate.[2] The larger MoFe protein (MW 230000) contains 2 Mo and ca. 32 Fe atoms distributed among at least six metal-containing prosthetic groups.[3-6] Two of these constitute apparently identical iron-molybdenum cofactors (FeMoco) that now have been identified[7] as the substrate-reducing sites in the enzyme and are considered[8] to have the approximate composition $MoFe_{(6-8)}S_{(4-9)}$. Since its first extrusion[8a] from the protein into *N*-methylformamide (NMF), FeMoco has elicited much interest both in its structure, reactivity and physical properties[8b,9-12] and as a synthetic challenge for bioinorganic chemists.[13]

Inasmuch as the principal function of FeMoco is to transfer electrons to substrate, we have undertaken experiments to characterize its electrochemical behavior. Our objectives are to determine the extent to which the redox properties of FeMoco correlate with those of the Mo-Fe-S centers in the intact protein and to establish correspondence between oxidation levels and spectroscopic properties of isolated FeMoco. The redox properties of FeMoco also have value as a guide to synthetic efforts[13] to prepare a chemical model for this Mo-Fe-S cluster. Finally, we also wish to know if FeMoco will exchange electrons with an electrode, as we envision that experiments using electrochemically mediated substrate reduction by FeMoco may aid in understanding nitrogenase function. We report here an initial characterization of the redox properties of isolated FeMoco using cyclic voltammetry (CV) and chemical redox titrations with voltammetric, potentiometric, and EPR spectroscopic monitoring.

Experimental Section

Materials and Methods. Molybdenum-iron protein from *A. vinelandii* was purified according to a previously published method.[14] FeMoco was isolated by the HCl/NaOH modification[8b,12] of the original extraction procedure,[8a] concentrated by either vacuum evaporation or ion-exchange chromatography as described below, and stored anaerobically under dry ice until used. FeMoco was assayed by reconstituting the FeMoco-deficient protein in crude extracts of *A. vinelandii* UW45 in the presence of sodium dithionite and determining the resultant acetylene-reducing activity.[12,14] Iron was determined with bathophenanthroline and molybdenum with toluene-3,4-dithiol.[15] *N*-Methylformamide (Aldrich) was stirred over solid sodium bicarbonate for 12-16 h, filtered, vacuum distilled, and frozen until used.[12b] This solvent was vacuum distilled again just prior to use in electrochemical experiments. Sodium dithionite (Eastman), tetraethylammonium bromide (Aldrich), potassium ferricyanide (J. T. Baker), and methyl viologen [MV^{2+}, Aldrich] were obtained commercially and used as a received. Sodium dithionite solutions were assayed spectrophotometrically with methylene blue[16] before use in redox titration experiments. Ferrocene (Aldrich) was sublimed before use. The following compounds were prepared by the indicated literature procedure: $[Bu_4N]_3[Mo(S_2C_2(CN)_2)_4]$,[17] $[Et_4N]_3[Fe(MoS_4)_2]$,[18]

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$[Et_4N]_3[Fe(WS_4)_2]$,[19] $[Bu_4N]_3[Mo_2Fe_6S_8(SPh)_9]$,[20] and $[Bu_4N]_2[Fe_4S_4(SPh)_4]$.[21]

FeMoco. FeMoco samples were concentrated by one of two procedures before use. In the vacuum concentration method,[14] samples of freshly isolated FeMoco were maintained under working vacuum at 30 °C until the original solution volume of ca. 125 mL was reduced to ca. 2–4 mL (ca. 8–10 h). The chromatographic procedure was based on our observation[22] that FeMoco bears a negative charge and can be adsorbed on an anion-exchange resin. Typically, a 5 × 1 cm column of DE-52 DEAE-cellulose (Whatman) in its acetate form was prepared anaerobically within a glass-wool-plugged 10-mL graduated pipet from a slurry of the resin with degassed NMF. The column was rinsed with a solution of 1 mM $Na_2S_2O_4$ in NMF until a positive test on the eluate for dithionite, using methyl viologen, was observed and then with degassed NMF until a similar test was negative. Dilute FeMoco samples were then transferred to the column, rinsed with NMF, and eluted as a single, intense, green-brown band with 0.1 M $[Et_4N]Br$/NMF. All manipulations of FeMoco by chromatography and in the EPR and electrochemical experiments described below were carried out under an atmosphere of rigorously purified argon in a Vacuum Atmospheres glovebox.

Altogether three samples concentrated by the vacuum evaporation technique and eight by the chromatographic procedure were prepared. Some of the chromatographed samples had been vacuum concentrated previously. The samples ranged from 0.4 to 1.7 mM Mo concentration, had an Fe:Mo ratio of 6.3 (±0.5):1, contained less than 15% extraneous Fe by the *o*-phenanthroline assay,[12b] and exhibited activities ranging from 150 to 300 nmol C_2H_2 reduced $(min)^{-1}$ $(ng\text{-}atom\ Mo)^{-1}$ in the UW45 reconstitution assay. There was no discernible effect of concentration procedure on these parameters. However, it was our observation that the chromatographic procedure provided a superior product for electrochemical analysis. The principal reduction wave of FeMoco(ox) at −0.4 V vs. the normal hydrogen electrode (NHE) was very prominent in these samples, whereas a −0.6-V reduction wave often accompanied the −0.4-V wave in the vacuum concentrated samples. Chromatography also served to remove proteinaceous material from the FeMoco samples, and the 0.1 M $[Et_4N]Br$/NMF eluant conveniently became the supporting electrolyte in electrochemical experiments. Sodium chloride resulting from the protein denaturation step served as supporting electrolyte in the vacuum-concentrated samples; addition of 0.1 M $[Et_4N]Br$ to these samples was without effect on their behavior.

All 11 FeMoco samples prepared for this study were examined by cyclic voltammetry and found to exhibit the electrochemical features described in this report. However, the precise quantitative data reported here were drawn from two of these samples only, one purified by chromatography (sample a in Table I) and the other by vacuum concentration (sample b). This limitation in samples resulted from the relatively large volume (>2 mL) of concentrated (0.5–1.0 mM in Mo) FeMoco solution required to conduct experiments from which electrochemical, EPR, and redox titration data could be obtained concomitantly. Not all samples were obtained in such quantity.

Electrochemistry and Chemical Redox Titrations. Cyclic voltammetry and chemical redox titrations were conducted in a small-volume cell that consisted of a Teflon collar of ca. 100 μL internal volume threaded onto the shaft of an inverted glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN; 0.071 cm^2 area), which served as both the working electrode and cell bottom. A Pt wire auxiliary electrode was inserted through the Teflon collar. The reference electrode was a miniature Ag/AgCl (1 M KCl) electrode (MI-401, Microelectrodes, Inc., Londonderry, NH), which was immersed directly in the sample solution at the time of measurement. The glassy carbon electrode was polished with diamond paste or 1-μm alumina on a Buehler microcloth before each experiment. The Teflon cell was evacuated for 12–24 h in the antechamber of the glovebox before use. About 50 to 75 μL of FeMoco solution was transferred to the cell for electrochemical measurements. In chemical titration experiments, appropriate quantities of aqueous 0.02 M $Na_2S_2O_4$ or 0.02 M $K_3[Fe(CN)_6]$ were transferred to vials containing 350-μL aliquots of FeMoco solution by microsyringe, and the solutions were mixed by stirring manually with a sealed melting point capillary; 60 μL was then transferred to the electrochemical cell and the remainder was transferred to a matched quartz EPR tube and frozen as described below. The equilibrium (i.e., the rest or zero-current) potential of these sample solutions was determined by measuring the potential difference between the glassy carbon and Ag/AgCl electrodes with an Orion 701 meter. Cyclic voltammograms were generated with a Bioanalytical Systems CV-1B potentiostat and recorded with an IBM 7424-MT recorder. The Orion meter, potentiostat, and recorder were connected to the cell by shielded cable passing through an air-tight seal in the glovebox.

The potential of the Ag/AgCl (1 M KCl) reference electrode was calculated to be +0.231 V vs. NHE.[23] Potentials measured against this electrode were corrected to the NHE by use of the relationship $E(NHE) = E(Ag/AgCl) + 0.231$ V and rounded to the nearest 0.01 V. Except as noted, all potentials are given in V vs. NHE. As a further means of calibrating electrode potential measurements,[24] we determined the formal potential of the ferrocene/ferrocenium ion couple (measured as the average of cathodic and anodic peak potentials by CV and employing the correction stated above) to be +0.666 V vs. NHE in 0.1 M $[Et_4N]Br$/NMF. We also measured formal potentials of −0.358 and −0.215 V vs. NHE, respectively, in 0.1 M $[Et_4N]Br$/NMF for the following half-reactions:

$$[Mo(S_2C_2(CN)_2)_4]^{3-} + e^- \rightleftharpoons [Mo(S_2C_2(CN)_2)_4]^{4-} \quad \text{(ref 17)}$$

$$MV^{2+} + e^- \rightleftharpoons MV^{+} \quad \text{(ref 25)}$$

The uncertainty in the formal potentials reported for the FeMoco redox processes is estimated to be ±0.02 V, and that in FeMoco current parameters to be ±15%. For standard compounds, these uncertainties are on the order of ±0.01 V and ±5%.

EPR Measurements. EPR spectra were recorded at 5 mW power on a Varian 4502 spectrometer at a constant temperature of 10 K maintained by liquid helium boil-off. The spectrometer was interfaced to an Apple II+ microcomputer for data acquisition and integration of spectra. Spin concentrations were estimated by double integration of spectra and comparisons with results for 0.1 and 1 mM aqueous solutions of Cu(II)–EDTA. Spin concentrations were calculated by the method of Aasa and Vanngard.[26] As described in ref 27, 10 K is above the temperature for optimal signal development in FeMoco, and measured spin concentrations (spins per Mo) are ca. 0.80 times the full value. To ensure sample consistency for EPR spectral monitoring, all EPR samples were frozen in liquid N_2 exactly 10 min after addition of reagents.

Results and Discussion

Previous studies[4-6] have shown that iron–molybdenum cofactor within the MoFe protein (designated "FeMoco") exists in three oxidation states:

$$\text{"FeMoco(ox)"}\ (S = 0;\ \text{EPR-silent}) \underset{-0.17\ \text{V}}{\overset{\text{(a)}}{\rightleftharpoons}} \text{"FeMoco(s-r)"}\ (S = {}^3/_2;\ \text{EPR-active}) \underset{-0.465\ \text{V}}{\overset{\text{(b)}}{\rightleftharpoons}} \text{"FeMoco(red)"}\ (S = \text{integer};\ \text{EPR-silent})$$

"FeMoco(s-r)" is the EPR-active, semi-reduced form that is produced and maintained in the presence of excess $Na_2S_2O_4$. "FeMoco(ox)" is prepared by dye oxidation of "FeMoco(s-r)". "FeMoco(red)" is the substrate-reducing state that has been achieved to date only in the full nitrogenase system. The enzyme itself does not exchange electrons with an electrode; redox mediators (e.g., viologens) are required to effect electron transfer.[6,28,29] Even so, redox couple a is not fully reversible. Its average potential of −0.17 V vs, NHE is derived from midpoint potentials of −0.29 and −0.05 V measured in the reducing and oxidizing directions, respectively, for this couple in *A. vinelandii* MoFe protein.[6,28] The substrate turnover potential of nitrogenase, −0.465 V vs. NHE,[6,30,31]

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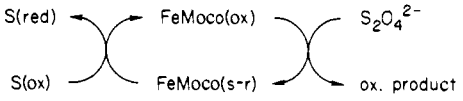
Table I. Cyclic Voltammetric Data for FeMoco.[a]

sample	[Mo] (mM)	$(E_{pc})_1$ (V)	$(E_{pa})_1$ (V)	$(E_0')_1$ (V)	$(\Delta E_p)_1$ (mV)	$(I_{pc})_1$	$(I_{pa})_1$	$(E_0')_2$	$(\Delta E_p)_2$	$(I_{pc})_2$
a	0.86	−0.45 (−0.63)	−0.21	−0.33	240	120 (20)	219	−0.96	240	235
b	1.35	−0.39 (−0.60)	−0.26	−0.32	130	150 (180)	224	−1.05	155	280

[a] Samples a and b refer to chromatographically purified and vacuum concentrated samples, respectively; detailed experimental conditions are given in the Experimental Section. Subscripts 1 and 2 refer to the electrode reactions in eq 1 and 2. Data for the −0.6-V wave associated with the first reduction process are listed in parentheses under $(E_{pc})_1$ and $(I_{pc})_1$. $(E_0')_1$ and $(E_0')_2$ are formal potentials in V vs. NHE measured as the average of cathodic (E_{pc}) and anodic (E_{pa}) peak potentials by CV. $\Delta E_p = E_{pa} - E_{pc}$ in mV. Measurements at 0.1 V s^{-1} for sample a and 0.2 V s^{-1} for sample b. $I_p = i_p/\nu^{1/2}AC$ in units of $\mu A\ s^{1/2}\ V^{-1/2}\ cm^{-2}\ mM^{-1}$ measured at ν = 0.05–0.02 V s^{-1}. All I_p's are corrected for background current at the glassy carbon electrode in 0.1 M $[Et_4N]Br$/NMF at the potential of measurement as well as the current resulting from all preceding electrochemical processes.

is assigned to couple b, although this estimate may be too positive because it neglects any contribution from the energy-yielding ATP hydrolysis that is mandatory for catalysis.[32] Based on data for ferredoxins and their synthetic analogues,[33] we estimate[34] that, as a relatively small, mainly inorganic anion,[22] FeMoco should experience shifts of ca. −0.4 V in its redox potentials upon transfer from an aqueous protein matrix into NMF. Thus, couples a and b are predicted to have potentials of ca. −0.57 and −0.87 V vs. NHE, respectively, in isolated FeMoco.

Oxidation State of Isolated FeMoco. Inasmuch as FeMoco is prepared and stored in the presence of excess dithionite, we expected to find this material in its semi-reduced form when examined electrochemically and spectrally. However, we find that FeMoco in NMF "self-oxidizes" to its EPR-silent state, rather quickly under anaerobic, ambient conditions and slowly, yet completely, when stored under dry ice. A possible mechanism for this "self-oxidation" is shown below, where S(ox) is a material,



possibly protic in nature, in the solvent or FeMoco preparation which is capable of oxidizing FeMoco(s-r) to FeMoco(ox).[35] The cycles shown are sustained by the presence of excess dithionite, but when this reductant is consumed, FeMoco is left in its oxidized state. Consistent with this observation, we find that samples of FeMoco(ox) contain no detectable $S_2O_4^{2-}$.[36] Despite oxidation, these samples retain full activity in the UW45 reconstitution assay and develop an intense EPR signal upon addition of $Na_2S_2O_4$. Experimental results in this paper are reported for "self-oxidized" samples exclusively. On two occasions, we noticed that, when FeMoco was examined relatively promptly (2–3 days) after preparation and storage under dry ice, it was present either partially or totally as FeMoco(s-r) based on the $S = {}^3/_2$ signal and the electrochemical response of the sample.

Cyclic Voltammetry of FeMoco. Cyclic voltammetry of "self-oxidized", EPR-silent FeMoco shows two reduction processes, one beginning at ca. −0.4 V vs. NHE and a second at ca. −1.1 V (Figure 1a, Table I). The related oxidation waves occur at −0.21 and −0.85 V, respectively, on the reverse potential scan.[37] Figure 1b shows that only a single anodic wave at ca. −0.2 V is observed when the potential scan is reversed after the first reduction process, even when this consists of waves at both −0.4 and −0.6 V. These results suggest that oxidized cofactor, FeMoco(ox), is interconverted with its semi-reduced form, FeMoco(s-r), by reduction at ca. −0.4 V and oxidation at ca. −0.2 V.

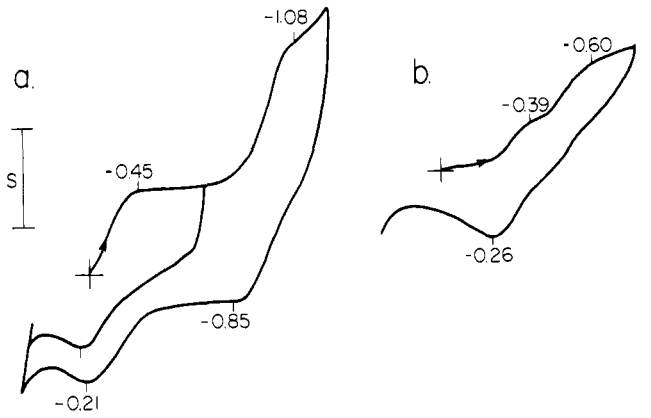


Figure 1. Cyclic voltammograms of FeMoco in NMF at a glassy carbon electrode. Samples a and b were prepared by chromatography and vacuum concentration, respectively (see Experimental Section). The Mo concentrations (mM), sweep rates (V s^{-1}), and current sensitivities (S in μA) are: (a) 0.86, 0.1, 4; and (b) 1.35; 0.2, 16. Peak potentials in V vs. NHE are marked on each curve. In (b), the horizontal axis was expanded by 1.25 times, and the CV trace was limited to the first redox process.

$$[\text{FeMoco(ox)}] + e^- \rightleftharpoons [\text{FeMoco(s-r)}] \qquad (E_0')_1 \qquad (1)$$

A formal potential, $(E_0')_1$, of −0.32 V is calculated for this couple from the CV data in Table I.

Figure 1a and Table I show that, as the voltammetric scan is extended to more negative potentials, a second quasi-reversible reduction with formal potential $(E_0')_2$ = −1.00 V is observed. If direct correspondence is assumed between redox processes in protein-bound "FeMoco" and isolated cofactor, then this wave should represent reduction of FeMoco(s-r) to the substrate-reducing state (eq 2). The formal potentials of reactions 1 and 2 agree reasonably well with the values predicted above from the potentials of couples a and b in the intact MoFe protein.

$$[\text{FeMoco(s-r)}] + e^- \rightleftharpoons [\text{FeMoco(red)}] \qquad (E_0')_2 \qquad (2)$$

The extent to which reactions 1 and 2 correspond to reversible one-electron transfers was examined through studies of the cyclic voltammetric behavior of two reversible one-electron systems in 0.1 M $[Et_4N]Br$/NMF, namely, $[Mo(S_2C_2(CN)_2)_4]^{3-/4-}$,[17] and $MV^{2+/+}$.[25] These couples exhibit a peak potential separation (ΔE_p)

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(34) A reasonable estimate of E_0' in aqueous solution for the 2+/1+ couple of 4Fe–4S ferredoxins is −0.40 ± 0.10 V vs. NHE (Table I, ref 33a), which compares with an E_0' of −0.80 ± 0.10 V in DMF or $(CH_3)_2SO$ for the same couple in synthetic $[Fe_4S_4]$ clusters (Table II, ref 33a).

(35) A referee has suggested that small amounts of dioxygen entering the sample during dry ice storage could be responsible for this conversion. Although we cannot definitively dismiss this possibility, we note that dioxygen oxidation of FeMoco at room temperature degrades its UW45 reconstitution activity and no such degradation was observed for the "self-oxidized" samples.

(36) This "self-oxidation" mechanism may also explain the observation[9] that the EPR signal of FeMoco(s-r) in NMF is quenched by anaerobic dilution into DMF because $Na_2S_2O_4$ is only sparingly soluble in the latter solvent.

(37) The voltammetric scan is relatively flat in the region between the two redox waves. This is due in part to the presence of material that is reduced in the vicinity of −0.6 V (see Figure 1b), but it may also reflect the involvement of FeMoco(s-r) in a catalytic homogeneous reaction similar to its "self-oxidation" reaction.

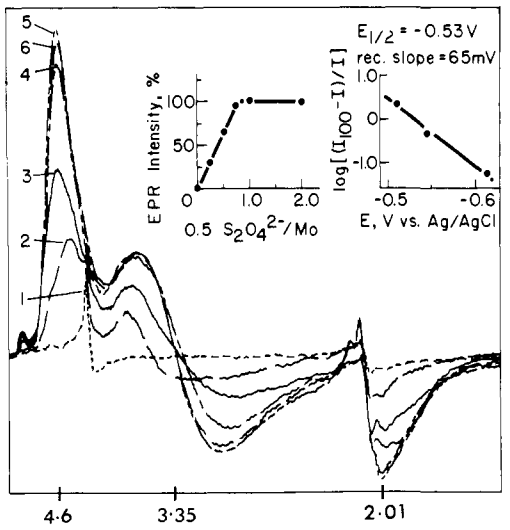

Figure 2. EPR spectra of 0.86 mM FeMoco(ox) incrementally reduced with aqueous $Na_2S_2O_4$. Experimental details are reported in footnote *a* of Table II. Spectra were recorded at 10 K under identical spectrometer settings at 5 mW power. (1) is the spectrum of EPR-silent FeMoco(ox). (2)–(6) are spectra recorded after addition of 0.25, 0.50, 0.75, 1.0, and 2.0 electron equiv of $S_2O_4^{2-}$, respectively, per g-atom of Mo. The weak signal at $g = 4.3$ in (1) arises from an unidentified non-FeMoco Fe^{3+} species in low concentration. The first inset plots the doubly integrated EPR signal intensity in relative units against electron equivalents $S_2O_4^{2-}$ added per g-atom of Mo. The second inset is a Nernst plot of these data according to eq 3. The potentials shown are V vs. Ag/AgCl (1 M KCl) and may be corrected to V vs. NHE by adding +0.23 V.

of 90 mV at 0.1 V s^{-1} sweep rate and peak current parameters $i_p/\nu^{1/2}AC$ = 366 and 390 μA $s^{1/2}$ $V^{-1/2}$ cm^{-2} mM^{-1}, respectively, over the sweep rate range ν = 0.05–0.2 V s^{-1}. A value of ΔE_p > 57 mV (the theoretical value for a Nernstian one-electron transfer[38]) for these standard compounds probably reflects the uncompensated solution resistance in our electrochemical cell. Because ΔE_p for reactions 1 and 2 is greater than 90 mV (range = 120–240 mV at ν = 0.1 V s^{-1}), some charge-transfer irreversibility accompanying the reductions of FeMoco(ox) and FeMoco(s-r) is indicated. This result is not surprising when the hysteresis associated with redox couple (a) of protein-bound "FeMoco" is recalled.[6,28]

Cyclic voltammetric peak current parameters ($I_p = i_p/\nu^{1/2}AC$), calculated for the FeMoco redox processes to determine if these quantities approximated those of a one-electron transfer, are presented in Table I. Given that FeMoco is a somewhat larger molecule[39] and undergoes electron transfer less reversibly than the standard compounds, it might be expected to exhibit smaller values of I_p. However, current parameters for the first reduction process are generally still too small to be accounted for by these effects alone. Only in the case of the vacuum-concentrated sample b, where the relatively well-resolved –0.4 and –0.6-V waves occur, does the total value of $(\bar{I}_{pc})_1$ approximate that for a one-electron transfer in NMF (Table I). Difficulty in measuring peak currents for overlapping CV waves may account for some of the discrepancy in FeMoco I_p values, but we cannot exclude the possibility that a fraction of FeMoco(ox), which is active in UW45 assays, exists in an electrochemically inactive form under some conditions. In Table I, the mean values of $(I_{pc})_2$, the current parameter for reduction of FeMoco(s-r) following electrochemical reduction of FeMoco(ox), and $(I_{pa})_1$, the current parameter for oxidation of FeMoco(s-r) produced by the one-electron chemical reduction of FeMoco(ox), are 258 and 222 μA $s^{1/2}$ $V^{-1/2}$ cm^{-2} mM^{-1}, respectively. These figures increase to 331 and 284 μA $s^{1/2}$ $V^{-1/2}$ cm^{-2} mM^{-1}, when the molybdenum concentration is corrected for the 78% EPR activity exhibited in Figure 2 by sample a (vide infra).

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Table II. Electrochemical and EPR Data for Titration of FeMoco with Sodium Dithionite[a]

$S_2O_4^{2-}$ added (e$^-$ equiv/ g-atom Mo)	rest potential (V vs. NHE)	i_{pc}[b] (μA)	i_{pa}[b] (μA)	I[c]
0	–0.230	3.5	0.0	0.03
0.25	–0.279	2.5	*d*	0.22
0.50	–0.334	2.0	2.4	0.49
0.75	–0.382	1.2	4.1	0.69
1.00	–0.447	0.4	5.8	0.73
2.00	–0.469	0.0	*e*	0.72

[a] Experiment was conducted by adding the indicated quantity of dithionite as an aqueous 0.02 M $Na_2S_2O_4$ solution to each of six 350-μL aliquots of chromatographically concentrated 0.86 mM FeMoco in 0.1 M $[Et_4N]Br$/NMF; 60 μL was transferred to the electrochemical cell for potentiometric and voltammetric measurements and the remainder used for subsequent EPR measurements as described in the Experimental Section. [b] Voltammetric peak currents determined in separate experiments by sweeping in the appropriate direction from the rest potential of the solution. Currents were measured at 0.2 V s^{-1} and corrected for background current at the glassy carbon electrode in 0.1 M $[Et_4N]Br$/NMF at the same potential. [c] Double integral of EPR signal intensity reported as spins per Mo using aqueous Cu(II)–EDTA as calibrant. Data were corrected for the reading at zero added dithionite and for dilution before plotting in Figure 2. [d] Not measured. [e] Not measurable owing to the presence of excess dithionite.

The last two values are not unreasonable for a one-electron transfer in NMF and support the electron stoichiometry assigned in eq 1 and 2.

Chemical Redox Titrations of FeMoco. To better establish the stoichiometry of eq 1, sample a of Table I, which contained the –0.4-V reduction wave as the principal wave of FeMoco(ox), was examined by titration with sodium dithionite while monitoring the CV, potentiometric, and EPR spectroscopic responses. Results are presented in Table II and Figure 2. The sample is initially EPR-silent and has a rest potential of –0.23 V. As dithionite is added, the following changes occur: (i) the solution rest potential shifts from values more positive than –0.3 V to values more negative than –0.3 V; (ii) CV traces recorded from the rest potential show a decrease in primary reduction current and an increase in primary oxidation current; and (iii) an $S = {}^3/_2$ EPR signal develops that reaches constant intensity after addition of slightly less than one electron equivalent of dithionite per g-atom of Mo. Addition of a second electron equivalent of dithionite does not increase the magnitude of the signal (trace 6, Figure 2). The first inset in Figure 2 shows a plot of the doubly integrated EPR signal intensity vs. electron equivalents of dithionite (0.5 $S_2O_4^{2-}$) added per g-atom of Mo. It indicates full development of the EPR signal after addition of 0.78 electron per molybdenum. In the second inset, a Nernst plot is constructed from these data according to the relationship below, where I_{100} is the intenstiy of the fully

$$E = (E_0')_1 + (2.3RT/nF) \log [(I_{100} - I)/I] \qquad (3)$$

developed signal and I is the intensity at potential E. The plot has a potential at half-intensity $[(E_0')_1]$ of –0.30 V and a reciprocal slope of 65 mV (n = 0.91 at 25 °C).

In a complementary experiment, a sample of FeMoco(ox), which had been quantitatively reduced to FeMoco(s-r) with $Na_2S_2O_4$, was sequentially reoxidized by adding aliquots of aqueous $K_3[Fe(CN)_6]$ solution. The EPR signal intensity of the FeMoco(s-r) solution was 0.79 spin/Mo. The partially reoxidized sample exhibited a rest potential of –0.32 V and an EPR signal intensity of 0.33 spin/Mo. Substitution of these values into eq 3 gives $(E_0')_1$ = –0.33 V, in good agreement with the dithionite titration.

The foregoing results demonstrate that FeMoco is converted from its oxidized to semi-reduced state by a chemically reversible one-electron transfer. By analogy with the intact protein, this redox process involves an $S = 0$ to $S = {}^3/_2$ spin-state change:

$$\underset{\substack{\text{(EPR-silent;}\\ S = 0)}}{[\text{FeMoco(ox)}]} + e^- \rightleftharpoons \underset{\substack{\text{(EPR-active;}\\ S = {}^3/_2)}}{[\text{FeMoco(s-r)}]} \quad (E_0')_1 \qquad (4)$$

Based on the similarity in potentials and our ability to monitor oxidized and reduced species during the course of these titrations, we believe this chemical process to be the same as that observed by direct CV of FeMoco(ox) (eq 1). From the combined CV and EPR titration data, $(E_0')_1 = -0.32 \pm 0.02$ V vs. NHE.

Comparison of Redox Properties of Model Compounds with FeMoco. Electrochemical data constitute valuable functional criteria to complement structural[9,10] and other[11,12] criteria for judging the appropriateness of synthetic models[13] for FeMoco. To illustrate this point, we attempted to determine the electrochemical behavior of the following compounds in 0.1 M $[Et_4N]Br/NMF$: $[Fe(MoS_4)_2]^{3-}$,[18,40] $[Fe(WS_4)_2]^{3-}$,[19,41] and $[Mo_2Fe_6S_8(SPh)_9]^{3-}$.[20,42] Each of these molecules either decomposes in NMF or exhibits behavior so different from its reported electrochemistry in other solvents that we assume a significant chemical change has taken place. The Fe_4S_4 cluster,[33] $[Fe_4S_4(SPh)_4]^{2-}$, also decomposes in NMF. Chemical reactivity of this nature, under conditions where the cofactor itself is stable, is prima facie evidence that these compounds are not closely correlated models for FeMoco. However, we reviewed the electrochemical response of selected model compounds in other solvents to judge the extent to which these species match the redox properties of FeMoco.

Based on the results in this paper, two quasi-reversible redox couples separated by 0.7 V at ca. −0.3 and −1.0 V vs. NHE in a polar aprotic solvent or at ca. +0.1 and −0.6 V vs. NHE in aqueous solution could indicate close correlation with FeMoco. Perusal of electrochemical data reported for Mo–Fe–S–O cluster complexes, however, shows that none fully satisfy this criterion. For example, the irreversible oxidation that eliminates the $S = {}^3/_2$ EPR signal from $[Fe(MoS_4)_2]^{3-}$ and the quasi-reversible 3- to 4- reduction of this compound occur at +0.16 and −1.5 V vs. NHE, respectively, in MeCN.[18,19,43] The $MoFe_3S_4$ monocubanes[44] exhibit two reversible redox couples, $[MoFe_3S_4]^{4+/3+}$ and $[MoFe_3S_4]^{3+/2+}$. These occur at ca. 0.0 and −0.9V NHE, respectively, in MeCN, thus exhibiting a larger separation (0.94 ± 0.12 V) than the redox couples of FeMoco. The weakly interacting $MoFe_3S_4$ centers in $[Mo_2Fe_6S_8(\mu_2\text{-}OMe)_3(S\text{-}t\text{-}Bu)_6]^{3-}$ and related dicubanes[13a,45] span the same oxidation levels, but exhibit two pairs of closely spaced one-electron transfers separated by ca. 1.3 V in DMF. The cyclic voltammograms of the $Fe(SR)_6$-bridged dicubanes[13a,46] resemble those of FeMoco, but their redox potentials [ca. −0.6 V for bridge Fe(III)/Fe(II) and ca. −1.2 V for $[MoFe_3S_4]^{3+/2+}$ with EtS− substituents in DMF and MeCN] are too negative. It must be reemphasized that none of these model data were collected in NMF, a solvent "managed" only with great difficulty,[12] even though only small differences in redox potentials are expected among NMF, DMF, and MeCN in the absence of specific solvent interactions. None of the model compound data reported to date appear to duplicate the redox properties of FeMoco exactly. Thus, a reasonable objective for future FeMoco model studies would be the preparation of compounds that exhibit both chemical stability and appropriate electrochemical response in *N*-methylformamide.

Conclusions

We conclude from this study that: (i) FeMoco exchanges electrons directly with an electrode; (ii) both redox processes associated with protein-bound "FeMoco" appear to be observed in isolated FeMoco and have formal potentials of −0.32 and −1.00 V vs. NHE in NMF; (iii) the FeMoco(ox) to FeMoco(s-r) reduction at −0.32 V is characterized as a quasi-reversible one-electron transfer involving an $S = 0$ to $S = {}^3/_2$ spin-state change; and (iv) no synthetic compound has yet duplicated these electrochemical properties exactly.

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