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A New Method for Extraction of Iron-Molybdenum Cofactor (FeMoco) from Nitrogenase Adsorbed to DEAE-cellulose. 1. Effects of Anions, Cations, and Preextraction Treatments†

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ABSTRACT: A convenient and rapid method of obtaining the cofactor of nitrogenase (FeMoco) with a low and apparently limiting Fe/Mo ratio has been developed. FeMoco can be extracted from the MoFe protein bound to DEAE-cellulose. The cofactor is eluted in either *N*-methylformamide (NMF), *N*,*N*-dimethylformamide (DMF), or mixtures of these solvents by use of salts such as Et_4NBr, Bu_4NBr, Ph_4PCl, and Ph_4AsCl. The method is simple, is rapid (45 min), yields concentrated cofactor, and, unlike the original method [Shah, V. K., & Brill, W. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3249-3253] which requires anaerobic centrifugation, is easily scaled up. Furthermore, it gives yields of cofactor in excess of 70%. Its disadvantages are a high Fe:Mo ratio when DMF is the extracting solvent and a high salt concentration in the resultant FeMoco solution. These disadvantages are easily overcome by removing excess Fe by pretreating the cofactor with bipyridyl while still on the column. This gives Fe:Mo ratios of (6 ± 1):1 (11 trials) with specific activities ranging from 170 to 220 nmol of C_2H_4/[min·(nmol of Mo)]. Chromatography on Sephadex LH-20 removes ca. 99% of the excess salt. The adsorption of MoFe protein to DEAE-cellulose seems to facilitate denaturation by organic solvents so that pretreatment of the protein with acid, used in earlier methods, is unnecessary. There is an apparent dependence on the charge density of the anion employed for elution of FeMoco bound to DEAE-cellulose, such that $Cl^- > Br^- \gg I^-, PF_6^-$ is the order of effectiveness of the Bu_4N^+ salts of these anions. The extraction of cofactor in DMF with a comparable specific activity to that obtained in NMF proves that the two amides are equally good solvents for the cofactor and coordination of deprotonated solvent may not be *necessary* for extraction.

Nitrogenase consists of two proteins, the Fe protein and the MoFe protein [for a review, see Orme-Johnson (1985)]. The Fe protein is an $\alpha_2\beta_2$ tetramer with a metal content of Mo_2-Fe_{30-32} (Davis & Orme-Johnson, 1976). It contains an extractable MoFe-sulfur cluster (Shah & Brill, 1977) which is believed to be the active site for dinitrogen reduction. For example, the altered substrate reduction properties of the MoFe protein from *Klebsiella pneumoniae nifV* mutants are transferred to apoprotein via the cofactor extracted from the mutant protein (Hawkes et al., 1984). The product of the *nifV* gene appears to be required for the production of homocitrate, whose presence is needed for the synthesis of wild-type cofactor (Hoover et al., 1987). Mössbauer studies have shown that two distinct classes of iron centers (designated P and M centers) are present in MoFe protein (Münck et al., 1975; Zimmermann et al., 1978; McLean et al., 1987). The P clusters consist of four $[Fe_4S_4]$ centers (Zimmermann et al., 1978) while the two M centers each correspond to FeMoco (Huynh et al., 1980).

FeMoco can be extracted into NMF[1] from citric acid precipitated MoFe protein with an efficiency variously reported to be between 45 and 90% (Shah & Brill, 1977; Yang et al., 1982; Walters et al., 1986). The partial formula of the resultant FeMoco is $MoFe_{6-8}S_{8-9}$ (Nelson et al., 1983). The cluster extracted in NMF can reconstitute MoFe protein ac-

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[1] Abbreviations: bpy, 2,2′-bipyridine; DEAE-cellulose, (diethylaminoethyl)cellulose; DMF, *N*,*N*-dimethylformamide; EPR, electron paramagnetic resonance; NMF, *N*-methylformamide; TEAE-cellulose, (triethylaminoethyl)cellulose; Tris, tris(hydroxymethyl)aminomethane.

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tivity of mutants which lack FeMoco, e.g., *Azotobacter vinelandii* UW45 and *K. pneumoniae nifB* (Shah & Brill, 1977; Roberts et al., 1978; Smith, 1980). This provides a functional assay for the activity and purity of FeMoco preparations.

Only NMF and the related 2-pyrrolidinone have been reported to usefully extract substantial yields of FeMoco (Yang et al., 1982; Shah et al., 1984; Walters et al., 1986; Lough et al., 1986). Extraction of FeMoco into formamide gave fair yields (below 60%), but the solvent dissolved the denatured protein (Yang et al., 1982; Shah et al., 1984), complicating the separation of cofactor and protein. Many modifications to the original procedure of Shah and Brill (1977) have been proposed (Smith, 1980; Yang et al., 1982; Schultz et al., 1985; Lough et al., 1986), but the solvent of choice has remained NMF. The ability of NMF to readily extract FeMoco from denatured nitrogenase has been variously attributed to its high dielectric constant (Shah et al., 1984) or to the dissociability of its amide proton (Yang et al., 1982; Walters et al., 1986). Attempts to extract FeMoco in decent yield, separating cofactor from protein by treatment with other common organic solvents, have been unsuccessful. This has limited the range of experiments which could be done on the chemistry of the cofactor and has evidently impeded efforts to determine its structure. Recently, extraction into DMF/Et_4NOH has been reported; however, low specific activities and variable recoveries were observed (Lough et al., 1986).

FT-IR spectra of FeMoco preparations in NMF showed evidence for the coordination of deprotonated solvent (Walters et al., 1986). We concluded that NMF was ligated to FeMoco via amide nitrogen, with displacement of the amide proton by the cofactor. By analogy, we suggested that FeMoco was bound to the protein via deprotonated peptide N-groups. The apparent necessity for a dissociable amide proton for extraction of FeMoco from nitrogenase MoFe protein would by extension explain the failure of other organic solvents, such as DMF (a tertiary amide with lacks a N-bound proton), to extract FeMoco in an efficient manner.

We report here that FeMoco can be efficiently extracted from MoFe protein which is absorbed on a DEAE-cellulose column, using NMF or DMF as solvent, without prior acid denaturation of the protein.

Materials and Methods

All manipulations of air-sensitive substances (including chromatographic separations) were carried out either in a Vacuum Atmospheres Model M040-1 glovebox with a He atmosphere or in serum-stoppered glass vessels with Schlenck techniques (Shriver, 1986; Beinert et al., 1978). Tris-HCl buffers at pH 7.4 contained 2–5 mM $Na_2S_2O_4$ to ensure the absence of oxidizing equivalents from cofactor solutions, unless otherwise stated. The presence of dithionite was confirmed by use of strips of filter paper, soaked in 2 mM methylviologen dihydrochloride in 25 mM Tris-HCl, pH 7.4, and then dried. DMF and NMF were vacuum distilled from Linde 4A molecular sieves. These solvents were then degassed via three freeze–pump–thaw cycles. DMF contained 2 mM $(Bu_4N)_2S_2O_4$ and NMF contained 2 mM $Na_2S_2O_4$ in all procedures. Preswollen DEAE- and TEAE-celluloses were purchased from Whatman and Sigma, respectively. Both cellulose column materials were equilibrated with 0.25 M Tris. Sephadexes LH-20 and G-15 were purchased from Pharmacia and swollen in DMF. Bu_4NX (X = Br, Cl, I, PF_6), Ph_4AsCl, Ph_4PCl, and Et_4NBr were all purchased from Aldrich and used as received.

MoFe protein was purified from cell extracts of *Azotobacter vinelandii* (Av) according to Burgess et al. (1980) except the crystallization step was omitted. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoreses (Laemmli, 1970) indicated that the protein purity was greater than 90%, on the basis of the intensity of staining of minor bands. Stock solutions of protein were 25–100 mg/mL, sp act. = 1400–2300 nmol of C_2H_4·(min·mg)$^{-1}$ (most in the 1400–1600 range), in 0.25 M NaCl/25 mM Tris buffer.

Preparation of Tetrabutylammonium Dithionite. A DEAE-cellulose column, 1.5 × 3.0 cm, equilibrated in 25 mM Tris was loaded with 10 mL of 1 M $Na_2S_2O_4$/25 mM Tris solution. The column was then equilibrated with DMF and allowed to stand for 30 min during which time the column turned yellow. The column was scrubbed with a further 5 mL of DMF. Addition of 0.25 M Bu_4NBr/DMF resulted in the elution of a yellow solution which was reducing to benzylviologen. The solution was evaporated to dryness, resulting in a yellow solid which became white on prolonged evacuation. Benzylviologen ($E°$ = −0.321 V) in DMF was then spectrophotometrically titrated with a tetrabutylammonium dithionite solution in DMF: the tetrabutylammonium dithionite solid was >70% pure, on the basis of the reducing power of a weighed aliquot of this solid (data not shown).

Analysis of the MoFe Cofactor. The activity of FeMoco was assayed by activation of crude extracts of derepressed *Klebseilla pneumoniae* UN106 *nifB*$^-$ cells (St. John et al., 1975; Roberts et al., 1978; Smith, 1980), broken in a French pressure cell after being diluted with 2 volumes of 0.1 M Tris, pH 7.4, containing 5 mM sodium dithionite. The activation reaction contained 25 mM Tris-HCl, pH 7.4, 5 mM $Na_2S_2O_4$ (0.3 mL), and UN106 extract (0.1 mL) in an Ar-filled serum vial. FeMoco (2–20 μL) prepared as described below was added and incubated at 22–30 °C for 5–30 min. A 50-μL aliquot was then removed for assay (Burgess et al., 1980). The specific activity of the FeMoco is reported as nmol of C_2H_4 produced/[min·(nmol of Mo)]. It was determined that NMF and DMF at concentrations up to 5% did not affect either activation of apoprotein or nitrogenase activity. Activity was linear with FeMoco addition in the range used for assays (Shah & Brill, 1977). The recovery was based on the total activity of the MoFe protein used in the cofactor extraction.

Atomic absorption analyses were obtained with a Perkin-Elmer Model 2380 spectrometer, using a HGA-400 graphite furnace. Mo was measured by atomic absorption; Fe was determined colorometrically with ferrozine (Carter, 1971). Arsenic was detected spectrophotometrically by the reduction of arsenomolybdate by hydrazine sulfate (Kingsley & Schaffert, 1951; Onishi & Sandell, 1953). Samples containing tetraphenylarsonium were dried and subsequently digested at 100 °C for 1 h in 0.1 mL of H_2SO_4 containing 10 μL of 30% H_2O_2. The samples were diluted with 5 mL of 10^{-5} M $FeSO_4$ and heated for an additional hour to decompose excess peroxide. Upon subsequent addition of 2 g of powdered zinc, arsine was evolved through a bent glass tube into 2.5 mL of 0.001 M I_2/KI. After 30 min, 0.25 mL of 1% ammonium molybdate in 5 N H_2SO_4 and 0.1 mL of 0.15% hydrazine sulfate were added, and the solutions were heated (80–90 °C) for 10 min. The resulting absorption due to molybdenum blue was determined at 840 nm. No interference from exogenous Mo(VI) (as ammonium molybdate), from added Fe^{3+} (as Fe_2O_3), or from $Na_2S_2O_4$ was observed. The detection limit was approximately 10 nmol of As.

FeMoco Extraction from MoFe Protein Bound to DEAE-cellulose. A DEAE-cellulose column (Econocolumns, 0.5-, 0.7-, 1.5-, or 2.5-cm diameter, Bio-Rad, Richmond, CA) was poured from a suspension of DEAE-cellulose in 0.25 M

Tris-HCl, pH 7.4. The column bed was then washed with 25 mM Tris buffer (containing 2–5 mM $Na_2S_2O_4$) until the eluent turned methylviologen papers blue. Stock MoFe protein was diluted with 3–5 volumes of 25 mM Tris buffer, and diluted protein solution was loaded on the column to form a brown band. The column was then washed with 2–4 column volumes of 25 mM Tris. The column was subsequently washed with several column volumes of one of the following: NMF, DMF, or DMF/NMF (9:1 v/v). After the first column volume, the matrix was usually stirred to avoid channeling and to ensure complete removal of water. The column was then allowed to stand for 10–20 min. The FeMoco was eluted with the original solvent containing 0.2–0.5 M organic salt: (for NMF) Et_4NBr or Et_4NCl; (for DMF or DMF/NMF) Bu_4NX (X = Cl, Br), Ph_4AsCl, or Ph_4PCl.

Elimination of Excess Fe from a DMF Solution of FeMoco. Extraneous iron could be removed with 2,2′-bipyridine (bpy) by a modification of the procedure of Schultz et al. (1985). This was done prior to initial elution of the FeMoco from the DEAE column, on a second DEAE column, or by a combination of both. (a) MoFe protein bound to DEAE-cellulose that had been exchanged into DMF was equilibrated with 10 mM bpy in DMF. After being stirred, the column was left for 10–20 min. Several column volumes of the bpy solution were then passed until no further red color [indicative of the $Fe(bpy)_3^{2+}$ complex] eluted. The column was washed with 5 column volumes of DMF to remove the bpy and then FeMoco eluted with 0.5 M Bu_4NBr in DMF. (b) The extraneous iron could also be removed on a second DEAE column. The cofactor was diluted 5-fold with DMF and loaded onto a 0.7 × 1.0 cm DEAE column equilibrated with DMF. This was followed by addition of 1 column volume of 10 mM bpy/0.5 M Bu_4NPF_6 in DMF. Bu_4NPF_6 aided the extraction of the iron. The column was allowed to stand in this solution for 10 min. The column was then washed with DMF, resulting in a pink eluate. After washing with 5 mL of DMF to ensure there was no bpy left on the column, FeMoco was eluted as a concentrated solution with 0.5 M Bu_4NBr/DMF.

Removal of Excess Salt from FeMoco. A 1.5 × 30 cm LH-20 column preswollen in DMF was poured and prewashed with 2 mL of 0.1 M $(Bu_4N)_2S_2O_4$ in DMF followed by DMF eluant. The eluant containing $(Bu_4N)_2S_2O_4$ was reducing to benzylviologen, indicating an excess of reducing equivalents. Concentrated FeMoco (0.5 mL) solution was loaded to this column; the brown band moved down the column and eluted when 16 mL of the solvent had passed through [the synthetic Fe–sulfur cluster, $Fe_4S_4(SPh)_4(Ph_4As)_2$ (MW = 1554) (Averill et al., 1973), eluted after 17 mL].

Extraction of FeMoco from Denatured MoFe Protein Pellets. Duplicate MoFe protein samples (0.7 mL containing 7 mg of protein) were placed in Ar-filled glass centrifuge tubes, and FeMoco was prepared by the method of Shah and Brill (1977) with the following modification: after the DMF wash to remove water from the precipitated protein, the FeMoco was extracted into a solution of 0.2 M Bu_4NBr in DMF instead of into NMF. A parallel control was extracted with NMF. After 30 min at 4 °C, samples were centrifuged to remove protein. This extraction was repeated a second time.

In another experiment, the citric acid denaturation step was omitted, and the MoFe protein was denatured by addition of DMF at neutrality. MoFe protein (10 mg, 0.3 mL) was placed into each of four Ar-filled 15-mL conical test tubes capped with serum stoppers. Four volumes of DMF (1.2 mL) containing 0.5 mM $(Bu_4N)_2S_2O_4$ was added by syringe. The solution was vortexed for 15 s and centrifuged in an IEC Centra-7R desktop centrifuge for 10 min at 2200 rpm. The solution was removed from the pellet and 2 mL of DMF added, vortexed, and allowed to stand at 0 °C for 35 min. After centrifugation as above, the DMF layer was removed and the pellet resuspended in 2.0 mL of 0.2 M Bu_4NBr in DMF, additionally containing 10 mM bpy in one of the two tubes, and allowed to stand 30 min at 0 °C and a further 15 min at room temperature. The solutions were then centrifuged as before and the supernatants recovered.

Table I: Extraction of FeMoco from MoFe Protein of *A. vinelandii* When the Protein Is Bound to DEAE-cellulose

eluant			range of		
solvent	salt	[salt] (mol/L)	activity yield (%)	sp act. of FeMoco[d]	Fe:Mo ratio[e]
NMF	Et_4NCl	0.2–0.5	50–80	225 ± 20	(6.7–7.9):1
DMF	Bu_4NBr	0.2–0.5	50–90	220 ± 20	(14–18):1
NMF/DMF[a]	Bu_4NBr	0.2–0.5	55–78	200 ± 30	(8–10):1
bpy/DMF[b]	Bu_4NBr	0.2–0.5	50–85	200 ± 20	(5.2–7.5):1
DMF[c]	Bu_4NPF_6	0.5			
DMF[c]	Bu_4NBr	0.02–0.1			

[a] NMF:DMF ratio is 1:9. [b] [bpy] = 0.02–0.05 M. [c] No FeMoco was extracted, but some Fe was detected in the eluate. [d] nmol of C_2H_2 reduced (nmol of cofactor present)$^{-1}$ min^{-1}. [e] Fe and Mo determined as in the text.

Results and Discussion

The recovery of activity as FeMoco from MoFe protein bound to a DEAE-cellulose column was generally between 50 and 90% for these amide solvent systems [NMF, DMF, NMF/DMF (1:9 v/v)]. It was found that stirring the column thoroughly after the first column volume of solvent had passed prevented channeling and gave the better yields (70–90%). The specific activity of the extracted cofactor in all solvents was 200 ± 30 nmol of C_2H_4/[min·(nmol of Mo)]. The range of reported specific activities for Kpl is 1600–2200 nmol of C_2H_4/(min·mg) (Smith, 1980), which along with the mass of the protein/mol of Mo (1.2×10^5 g) gives a range of expected specific activities for cofactor of 180–240 nmol of C_2H_4/[min·(nmol of Mo)] (Smith, 1980; Hawkes et al., 1984). The high specific activities of the FeMoco suggest that >85% of the Mo present is active FeMoco. We find no significant difference between the specific activities (based on Mo content) of FeMoco extracted in NMF and DMF (Table I). Cofactor solutions eluted from 100 mg of MoFe protein bound to 2.5 × 2.5 cm DEAE columns were typically 0.1–0.2 mM Mo. Desalting resulted in approximately 2-fold dilution of these cofactor solutions.

The iron content of the extracted FeMoco (expressed as a function of molybdenum content) did vary according to the solvent system used for the extraction (Table I). *N*-Methylformamide gave the lowest Fe:Mo ratios [(6.7–7.9):1], indistinguishable from previously published results using the method of Shah and Brill (1977), suggesting relatively little contamination with noncofactor Fe. Considerable color remained on the column after elution of the FeMoco in NMF, suggesting that this treatment did not extract all the Fe–S centers from this protein. On the other hand, FeMoco prepared in DMF eluted from the column as an intense dark brown solution with a Fe:Mo ratio of (14–18):1, which accounts for essentially all the Fe in the protein (Davis & Orme-Johnson, 1976). Very little, if any, color remained on the column. Thus, it appears that all the Fe associated with the MoFe protein was extracted in pure DMF whereas the protein remains bound to the column.

It was observed that the Fe:Mo ratio could be lowered considerably if 10% NMF was present in the DMF solution [Fe:Mo = (8–10):1] and that a dark color remained on the

column after this treatment (Table I). NMF and DMF thus differ strikingly in their selectivity for elution of non-FeMoco iron.

Removal of Extraneous Iron. The greater volatility of DMF makes it easier to work with than NMF and so efforts were directed at decreasing the Fe:Mo ratio in the DMF-based process while retaining high yields and specific activities of product. Two alternative approaches were used with comparable success, and typically they were combined.

The first method was to treat the protein bound to the column with a DMF solution of bpy prior to elution of the FeMoco (Materials and Methods). This allowed active FeMoco with lower Fe:Mo ratios to be obtained in a single step. Treatment of MoFe protein bound to a DEAE column in DMF with bpy caused the non-cofactor Fe^{2+} to form the red $Fe(bpy)_3^{2+}$ cation. The lower iron to molybdenum ratios were obtained when the amount of bpy added was in 20-fold excess of the noncofactor[2] Fe on the column. It should be noted that the treatment with bpy does not have any demonstrable effect on the subsequent activation of Kpl activity in UN106 $NifB^-$ extracts (Schultz et al., 1985). This is consistent with the demonstration by Rawlings et al. (1978) that added bpy did not inactivate cofactor nor complex Fe unless the cluster structure was destroyed by the addition of mercurial. Sixty percent of the iron was removed by this treatment, which suggests that *all* the non-cofactor Fe might be scrubbed from the column prior to FeMoco extraction.

Alternatively, extraneous iron was removed by treating FeMoco with bpy on a second DEAE column (see Materials and Methods). The specific activity of FeMoco preparations treated in this way was 210 ± 20, and the recovery of activity was ca. 70%. The Fe:Mo ratio was improved from 16:1 to (5–7):1. Schultz et al. (1985) reported a Fe:Mo ratio of (5.8–6.8):1 using this method, with *NMF* as the solvent. We found that, if the bpy solution contained 0.5 M Bu_4NPF_6, a somewhat lower Fe:Mo was obtained [(5.2):1 versus 7:1] without affecting either the recovery of activity or the specific activity. These differences seem marginally significant considering the potential errors in both calculations.

Examination of Role of Cations on FeMoco Solubility. Extraction of FeMoco from the DEAE-cellulose column typically required a 0.2–0.5 M solution of salt in the amide solvent. Tetraethylammonium bromide (Et_4NBr) worked well in NMF or NMF/DMF (1:9) but not in DMF, most probably due to its low solubility in the latter solvent. Similarly, Bu_4NBr, Ph_4AsCl, and Ph_4PCl worked well in DMF or NMF/DMF but not in NMF, again because of low solubility. The effect of salt on extraction of FeMoco into DMF from citric acid denatured protein was tested given the fact that DMF alone is not sufficient to extract FeMoco (Shah & Brill, 1977). MoFe protein was denatured according to the Shah and Brill (1977) method, and the pellet was washed with DMF. Subsequent treatment with DMF containing 0.2 M Bu_4NBr extracted 49% of the FeMoco activity compared to 58% in an NMF control sample. In a separate experiment, the protein was denatured by addition of 4 volumes of DMF, omitting treatment with citric acid. The resultant pellets were extracted with either DMF + Bu_4NBr, DMF + Bu_4NBr + bpy, DMF + Bu_4NPF_6, or DMF + Bu_4NPF_6 + bpy. Following centrifugation, protein pellets were observed, and in the absence of bpy, colorless supernatants were obtained, which indicated that FeMoco had not been extracted. In the cases in which bpy was present, a small amount of pink color [$Fe(bpy)_3^{2+}$] was observed in the supernatant solution. The pellets were then resuspended, allowed to stand for 48 h at room temperature in an anaerobic glovebox, and then centrifuged, resulting in a faint green solution indicative of FeMoco. There was only 10% recovery of the activity in Bu_4NBr/DMF. In the presence of 0.2 M Bu_4NPF_6, a green solution was observed indicative of FeMoco. The recovery of activity as FeMoco was less than 5%. These experiments indicate that even although DMF alone denatures the protein in aqueous solution, it does not render the metal centers susceptible to extraction. The observation that DMF alone is effective when used on a column-bound MoFe protein indicates that the DEAE-cellulose matrix may play a role in the denaturation process in the new method described, in which citric acid is not used. The high charge density in DEAE-cellulose may profoundly affect the conformation of a strongly negatively charged molecule like the MoFe protein and thus affect the course of the cofactor extraction process.

Effect of Anion on Elution of FeMoco from DEAE-cellulose in DMF. Elution of FeMoco from DEAE columns in DMF was achieved by washing the column with a solution of a salt of an organic cation. The concentration of salt at which FeMoco was eluted in DMF also showed a strong dependence on the nature of the anion. This was further studied with FeMoco bound to a DEAE column (no protein present) and various salts of Bu_4NX (X = Cl, Br, I, PF_6).

FeMoco was prepared in DMF containing Bu_4NBr on a DEAE column. It was then diluted 3-fold with DMF [containing 0.5 mM $(Bu_4N)_2S_2O_4$], and equal aliquots were loaded on to small DEAE columns (0.5 cm diameter × 1 cm) equilibrated in DMF. The columns were then washed with DMF to remove excess salt and then treated with increasing concentrations of Bu_4NX (X = Cl, Br, I, PF_6) in DMF to elute the FeMoco. Solutions of 0.02, 0.10, 0.20, and 0.50 M salt were used. FeMoco eluted as a dilute solution in 0.02 M Bu_4NCl. When the anion was bromide, a fraction of the FeMoco leached from the column at 0.1 M salt; however, the major part of the FeMoco eluted easily at 0.2 M. No FeMoco activity eluted when Bu_4NI (0.5 M) or Bu_4NPF_6 (1.0 M) was used, but the FeMoco could subsequently be eluted if either the chloride or bromide salt was applied. We also found that the extraction of FeMoco from *protein* bound to DEAE was not obtained with 0.5 M Bu_4NPF_6 and that the presence of Bu_4NPF_6 in DMF solutions of Bu_4NBr did not noticeably change the concentration of the latter required to elute the cofactor.

This allowed the anions to be ranked in the order $Cl^- > Br^- \gg I^-, PF_6^-$, i.e., in order of increasing size and softness. [Tetrabutylammonium chloride at 0.02 M would not elute the cofactor as a concentrated solution, and therefore, higher concentrations (0.2 M) were used in routine preparations, especially when FeMoco was being extracted from MoFe protein bound to columns.] Qualitatively similar results were obtained in NMF/DMF (1:9). This phenomenon was not studied in NMF.

It seems most likely that the dominant role of the halide is simply one of ion exchange, i.e., to displace FeMoco which is strongly ion paired to the DEAE column or to the protein. Although we cannot experimentally separate extraction and elution when the protein is present, we note that the same concentrations of salts elute FeMoco from DEAE-cellulose whether or not the protein is present. In DMF, it has been shown that ionic salts are strongly ion paired. Since FeMoco is an anion and the column material is a polycation, the co-

[2] The term noncofactor iron refers to iron atoms not needed to be added to extracts containing apoprotein for formation of MoFe protein to occur.

factor is presumably strongly ion paired to the cationic column in such a manner that only a small anion such as Br^- or Cl^- with a relatively large charge density could displace it. The anions I^- and PF_6^- do not elute FeMoco probably because, at comparable concentrations, I^- and PF_6^- have much lower affinities for the column. This suggests that FeMoco has a high charge density which requires an anion with similar charge density to displace it from the column.

The possibility that the FeMoco could be covalently bound to the DEAE column via a bond to an amine group on the column was effectively eliminated by the use of a TEAE-cellulose column, which contains a quaternary amine unable to enter into covalent interactions. The *same* anion specificity was observed for the elution of FeMoco from TEAE-cellulose as from DEAE-cellulose except that higher concentrations were needed for elution.

Removal of Excess Salt. FeMoco solutions containing high salt were desalted via a 1.5 cm × 30 cm LH-20 column.[3] The column was poured in DMF and washed with 0.1 M $(Bu_4N)_2S_2O_4$ in DMF solution to remove O_2 or other oxidants prior to addition of FeMoco. Failure to do so resulted in the loss of 75% of the FeMoco activity. The brown FeMoco band eluted after 16 mL. Recovery of FeMoco activity was 88%. The efficiency of removal of excess salt was determined with FeMoco prepared with $AsPh_4^+$ cation. Arsenic assays before and after desalting show that >99% of the salt was removed after two passages down this desalting column (data not shown). Using this method, we intend to determine the limiting Mo:As ratio, and thus the magnitude of the negative charge of the cofactor.

We conclude that the presence of a soluble cation determines the solubility of FeMoco in DMF, and we have shown (Wink et al., 1989) that this is true for many other organic solvents. We believe that the previous failures to extract FeMoco into DMF or other organic solvents were primarily caused by the absence of a suitable cationic counterion. Indeed, the original protocol for the preparation of FeMoco involved washing of the protein pellet with DMF, in order to remove water. Under these conditions, FeMoco was not extracted into DMF (Shah & Brill, 1977). The latter observation has been substantiated by many other workers (Smith, 1980; Nelson et al., 1983; Schultz et al., 1985; Lough et al., 1986). Our hypothesis about the primacy of the hydrophobic cation was supported by our experiments in which we successfully extracted FeMoco into DMF containing 0.2 M Bu_4NBr, using the batch methods of Shah and Brill (1977). Displacement of FeMoco either from the protein or from DEAE requires an *anion* of high charge density. Work is currently in progress to extend these observations and to use these results to advance our knowledge of the chemistry and structure of FeMoco.

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Registry No. FeMoco, 72994-52-6; DMF, 68-12-2; NMF, 123-39-7.

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[3] Similar experiments were previously reported by Smith (1980) and Yang et al. (1982), who used NMF and hydrophilic gel exclusion matrices. We found the hydrophobic matrix LH-20 to give equally effective desalting columns.