Biochemistry **1989**, *28*, 9407–9412

A New Method for Extraction of Iron–Molybdenum Cofactor (FeMoco) from Nitrogenase Adsorbed to DEAE-cellulose. 2. Solubilization of FeMoco in a Wide Range of Organic Solvents[†]

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Received January 13, 1989; Revised Manuscript Received July 14, 1989

ABSTRACT: While the iron–molybdenum cofactor (FeMoco) of nitrogenase, a constituent of the active site for nitrogen reduction, can be extracted into *N*-methylformamide (NMF) and pyrrollidinone, the inability to solubilize it in any other organic solvents has hampered further understanding of its structure and chemical properties. A method to solubilize FeMoco, prepared in *N,N*-dimethylformamide (DMF) with Bu_4N^+ as counterion [McLean, P. A., Wink, D. A., Chapman, S. K., Hickman, A. B., McKillop, D. M., & Orme-Johnson, W. H. (1989) *Biochemistry* (preceding paper in this issue)], in acetonitrile, acetone, methylene chloride, tetrahydrofuran, and benzene is reported. FeMoco evaporated to dryness in vacuo dissolves readily in good yield (55–100%) and with no significant loss in specific activity. In addition, FeMoco can be extracted directly into these solvents from MoFe protein bound to a DEAE-Sepharose column if the protein is pretreated with DMF. Methods have also been developed to extract fully active FeMoco into acetone and acetonitrile in the absence of any amide solvents (NMF or DMF). Extraction of FeMoco into acetone (30% yield) involves only pretreatment of column-bound protein with methanol, while extraction into acetonitrile (22% yield) requires pretreatment with methanol followed by THF. We conclude that the presence of a suitable soluble cation confers solubility to the cofactor in many common organic solvents and that the solubility of FeMoco in a given solvent may be independent of the ability of that solvent to extract the cofactor from column-bound protein.

The MoFe protein component of nitrogenase contains an extractable cofactor, the iron–molybdenum cofactor (FeMoco) (Shah & Brill, 1977). FeMoco is clearly a constituent of the active site of nitrogenase (Hawkes et al., 1984), but its structure has remained obscure despite the long-recognized fact that a sulfur-bridged, heteropolynuclear cluster is present in cofactor preparations (Rawlings et al., 1978; Cramer et al., 1978; Antonio et al., 1982; Hoffman et al., 1982). The cluster is recognizable from its ability to reactivate the apoMoFe protein present in cofactorless mutants and from its distinctive EPR signature [for a review, see Orme-Johnson (1985)].

Shah and Brill (1977) demonstrated that FeMoco could be extracted from citric acid denatured MoFe protein into NMF. Smith (1980) showed that DMSO precipitation could replace the citric acid treatment in the Shah and Brill preparation, yielding a product of apparently lower molecular weight which Smith suggested might be due to a lack of citrate ligands in the DMSO-treated cofactor. Subsequently, Burgess et al. (1981) showed that the Shah and Brill cofactor preparation contains much citrate, phosphate, and chloride in addition to Mo, Fe, and S.

One of the major problems encountered in investigating the chemical, structural, and spectroscopic properties of FeMoco is the limited number of solvents in which the cofactor prepared by the citric acid method can be readily dissolved. Yang et al. (1982) examined in detail the procedure for extraction of FeMoco into NMF and formamide, and concluded that alkaline conditions were needed for extraction to occur. These studies clarified the complex nature of the NMF-extracted cofactor but did not suggest alternative solvents useful for further purification and characterization. Attempts to extract the cofactor into CH_3CN and DMF (Lough et al., 1986) gave very low recoveries. Walters et al. (1986) concluded from IR evidence that NMF was ligated to the cofactor via displacement of the secondary amide hydrogen. This suggested that a primary or secondary amide was essential and apparently rationalized the failure to extract the cofactor efficiently into other solvents.

We have recently shown that the cofactor *can* be extracted into DMF (a tertiary amide) in the presence of a number of organic salts, leading to the conclusion that the presence of an appropriate cation was essential for extraction (McLean et al., 1989). The cation requirements in this process have been overlooked in previous work. In basic NMF [e.g., as employed by Yang et al. (1982)], Na^+ or K^+ is present and effective, but salts of these cations are normally not readily soluble in less polar solvents. Since we have shown that proper selection of an *organic* cation results in extraction of FeMoco in DMF from denatured MoFe protein, it was of interest to see if this principle could be extended to other solvents. This paper details the successful extraction and solubilization of FeMoco in acetonitrile, acetone, THF, dichloromethane, and benzene, when the tetrabutylammonium cation is employed. The way is open to electrochemical, reactivity, and crystallization studies unimpeded by the less-useful properties of amide solvents.

EXPERIMENTAL PROCEDURES

Manipulations of air-sensitive materials were carried out either in a Vacuum Atmospheres Model M040-1 glovebox with He atmosphere (<1 ppm O_2) or in glass vessels by Schlenck techniques (Beinert et al., 1978; Shriver, 1986). The oxygen content of the glovebox was continuously monitored. Preswollen DEAE-cellulose was purchased from Whatman, and

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[†] Supported by National Science Foundation Grant DMB85-2087.

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0006-2960/89/0428-9407$01.50/0

DEAE-Sepharose CL-6B and Sephadex LH-20 were purchased from Pharmacia. Bu_4NCl and Bu_4NBr were purchased from Aldrich and used as received. Disposable columns (0.7-, 1.0-, and 2.5-cm diameter) were obtained from Bio-Rad (Richmond, CA).

HPLC-grade DMF (Aldrich) was dried over Linde 4A molecular sieves and then vacuum distilled from K_2CO_3. Acetonitrile, acetone, benzene, and dichloromethane were all predried with 4A Linde molecular sieves and degassed via three freeze–pump–thaw cycles. THF was distilled from Na/benzophenone under an atmosphere of N_2. The dried THF was then degassed with three freeze–pump–thaw cycles. Methanol was dried over Linde 4A molecular sieves and vacuum distilled from sodium. All solvents contained $(Bu_4N)_2S_2O_4$ at 2–5 mM to remove O_2 unless otherwise stated. All aqueous solutions contained 25 mM Tris, pH 7.4, and 2 mM $Na_2S_2O_4$.

MoFe protein preparations, preparation of cofactor in DMF, cofactor assays, and metal determinations were all as described in the accompanying paper (McLean et al., 1989).

Solution of FeMoco in Various Organic Solvents from Dried Cofactor. MoFe protein (100 mg) in 0.25 M NaCl/Tris buffer was diluted 5-fold with degassed water and added to an anaerobic DEAE-cellulose column (2.5 × 3.5 cm). The column was then washed with 5 column volumes of Tris buffer to eliminate excess NaCl. DMF or a NMF/DMF (1:9 v/v) solution was passed through the column and the matrix stirred gently. The column was allowed to stand for 10 min, followed by washing with 3–5 column volumes of NMF/DMF (1:9 v/v). A 0.5 M Bu_4NBr– NMF/DMF (1:9 v/v) solution was passed through the column, eluting active FeMoco with a yield of 80–90% (based on activity) as previously reported. On the occasions when the initial extraction employed DMF alone, the column was treated with a solution of 1 M Bu_4NPF_6 and 50 mM 2,2′-bipyridine (bpy) in DMF to remove noncofactor iron, prior to elution of the cofactor with 0.5 M Bu_4NBr in DMF.

A second DEAE-cellulose column was poured in aqueous buffer and reequilibrated with DMF, and the FeMoco extracted above was diluted 5-fold with DMF and loaded on this column. The cofactor adsorbed in a dark band. The column was then washed with 3 column volumes of DMF, followed by addition of 1 column volume of 0.5 M Bu_4NPF_6/50 mM bpy in DMF. After 10 min, 3 column volumes of DMF was used to eliminate excess salt and bpy, and the cofactor was eluted with a solution of 0.1 M Bu_4NCl in DMF. The cofactor solution was then desalted by passage down an LH-20 column preequilibrated in neat DMF [this column was pretreated with an aliquot of $(Bu_4N)_2S_2O_4$ to remove oxidizing equivalents]. The desalted solution was collected and evaporated to dryness under vacuum at 25 °C with a liquid nitrogen cooled trap. The cofactor then was redissolved in various organic solvents such as acetonitrile, acetone, THF, dichloromethane, and benzene.

Extraction of FeMoco into Various Organic Solvents from a DEAE-Sepharose Column. (*a*) *Pretreatment with DMF.* MoFe protein (15 mg) was bound to DEAE-Sepharose CL-6B (0.7 × 1.0–2.0 cm) and reequilibrated in DMF as described above. DEAE-Sepharose was used to replace DEAE-cellulose due to its superior flow properties in the organic solvents used. This was followed by addition of a second organic solvent (acetonitrile, acetone, THF, CH_2Cl_2, or benzene). The column was stirred, allowed to stand for 10 min, and then washed with 2 column volumes of the organic solvent. A 0.1 M Bu_4NCl solution in either acetonitrile, acetone, or dichloromethane was added to elute the FeMoco. When benzene or THF was the solvent, a saturating solution ([salt] $<$ 0.05 M) of Bu_4NBr was used for elution of cofactor, since the chloride salts are far less soluble.

(*b*) *No Pretreatment with DMF.* This was done as above except that the DMF wash was omitted. MoFe protein was loaded on a DEAE-Sepharose column and washed with Tris buffer. This was followed by addition of 3 column volumes of either acetonitrile, acetone, THF, or CH_2Cl_2. The column was stirred and allowed to stand for 20 min. In the case of dichloromethane, careful stirring was necessary to prevent the column material from floating. Two additional column volumes of the solvent were then passed through the column. In the cases where little or no FeMoco was eluted with tetrabutylammonium salts in the less polar solvents, the columns were then rewashed with DMF, and cofactor was extracted in DMF/Bu_4NCl solution as described above.

(*c*) *Extraction of FeMoco into CH_3CN with Pretreatment with THF.* MoFe protein was bound to a DEAE-Sepharose column and washed with Tris buffer. The column was washed with methanol to remove water as FeMoco is not directly extracted into methanol. This was then followed by addition of THF. The column was stirred and allowed to stand for 20 min, followed by passage of 5 column volumes of CH_3CN. Addition of 0.1 M Bu_4NCl in acetonitrile resulted in the extraction and elution of FeMoco.

Extraction of FeMoco after Exposure of the Solvent-Denatured Protein to an Aqueous Solution. Equal amounts of MoFe protein (15 mg) were loaded onto six DEAE-Sepharose columns (0.7 × 10 cm) and washed with Tris buffer. The columns were then washed with 3 column volumes of the appropriate solvent: DMF, MeOH, CH_3CN, acetone, CH_2Cl_2, and THF. The columns were stirred and allowed to stand for 20 min, followed by further washing with the organic solvent. Several column volumes of aqueous Tris buffer were then passed through the column, after which the column was stirred and allowed to stand for 1 h. Subsequent addition of an aqueous 1 M NaCl/25 mM Tris solution to each column was used to elute MoFe protein. Acetylene reduction and protein assays were done on samples of eluate according to standard techniques. After addition of NaCl, the columns were washed with buffer and reequilibrated in DMF. A 0.1 M Bu_4NCl/DMF solution was passed down each column to elute the FeMoco.

A control column, not given the intermediate aqueous buffer treatment between organic solvent treatment, was run concurrently in these experiments. After the MoFe protein was added to a DEAE-Sepharose column, the control column was equilibrated in DMF. The column was stirred and allowed to stand for 1 h. This was followed by addition of 0.1 M Bu_4NCl/DMF solution, resulting in the extraction of cofactor.

RESULTS

Perhaps the simplest method for preparing a large amount of purified FeMoco is to use one of the column methods described in McLean et al. (1989). For the present study, FeMoco was prepared in NMF/DMF (1:9 v/v), extraneous Fe was removed with bipyridine on a second DEAE-cellulose column run in DMF, and then the cofactor was eluted with DMF containing Bu_4NCl. After desalting on an LH-20 column, 0.5-mL aliquots of the resultant cofactor solution containing 25 nmol of Mo were then evaporated to dryness in vacuo to form a tacky brown-green solid. The solid was then dissolved in 0.10 mL of the following solvents: acetonitrile, acetone, methylene chloride, tetrahydrofuran, and benzene. In these initial solubility studies the cofactor preparation had an activity of 100 nmol of C_2H_4 produced/

Table I: Solubility of FeMoco[a] in Various Solvents after Evaporation to Dryness

solvent	dielectric constant	% recovery of FeMoco[b]
CH_3CN	38	95
acetone	28	75
CH_2Cl_2	9	55
THF	1.9	85
benzene	1.2	99

[a] Extracted and desalted as described under Experimental Procedures. The specific activity of FeMoco was 100 nmol of C_2H_4/[min·(nmol of Mo)], and the Mo:Fe ratio was 1:(9 ± 1). [b] Analyses as described in text.

[min·(nmol of Mo)], and the Mo:Fe ratio was 1:9. In each case we recovered 55% to nearly 100% of the activity (Table I), and the specific activity was unchanged. This demonstrated that the Bu_4N^+ salt of FeMoco was soluble in these solvents up to 30–60 μM. It should be noted that the volume of cofactor solution recovered differed from 100 μL in some cases due to evaporation and adsorption of the solvent into the septa. We have obtained FeMoco in these solvents in subsequent experiments at higher concentrations: CH_3CN ~ 10 mM; DMF ~ 10 mM; acetone ~ 1 mM; CH_2Cl_2 > 100 μM; THF 50–100 μM (data not shown).

We next attempted to extract the cofactor directly into these solvents from MoFe protein bound to a DEAE column. The aqueous buffer was displaced with DMF, and the column was equilibrated with one of the following solvents: acetonitrile, acetone, dichloromethane, tetrahydrofuran, or benzene. Following this, the addition of solvent containing dissolved organic salt resulted in elution of the cofactor with varying degrees of efficiency. Addition of a 0.1 M Bu_4NCl solution in these solvents resulted in the extraction of FeMoco with 80–100% yields in acetonitrile [Mo:Fe of 1:(15 ± 3)], 55–90% in acetone [Mo:Fe of 1:(16 ± 2)], and 20–40% in dichloromethane [Mo:Fe of 1:(12 ± 2)]. In these solvents, the specific activity was between 160 and 240 units/nmol of Mo, indicating that the reconstitution of *nifB⁻* extracts was not inhibited by these solvents in the amounts used. The Mo:Fe ratios were between 1:12 and 1:20, indicating that most the P-cluster Fe ended up in the cofactor preparation. It should be noted that difficulties were experienced in determining [Mo] in CH_2Cl_2 by atomic absorption, and the [Mo] values may be underestimated. When THF or benzene was the solvent, the bromide salt was used since the chloride salts do not dissolve appreciably in THF or benzene. FeMoco was eluted with 15% and 4% efficiency, respectively. Since a large concentration of anion is required to displace FeMoco from the DEAE column, the low recoveries are probably due to low solubility of the salt in these solvents.

If the DMF wash in the above method was omitted, different results were obtained (Table II). MoFe protein was bound to the column, and then methanol was added to remove the aqueous phase and facilitate the transition to the second organic solvent. This was not particularly important in the case of acetonitrile and acetone but was essential for dichloromethane, which is not miscible with water. Methanol does not extract cofactor from MoFe protein on DEAE columns (data not shown) and was used here to remove water. The column was equilibrated in the second organic solvent, and solvent containing salt was added. In the case of acetone (salt = Bu_4NCl), extraction gave a 30% yield of cofactor with a specific activity of 200 ± 20 nmol of C_2H_4/[min·(nmol of Mo)] with Mo:Fe = 1:17. In the case of acetonitrile less than 10% was extracted, and for dichloromethane or methanol no detectable cofactor was eluted. In these cases the column was subsequently washed with solvent to eliminate excess salt followed by addition of DMF. In both cases, subsequent addition of DMF and Bu_4NCl *did* result in the elution of FeMoco with the expected specific activities but in low yield.

Table II: Effect of Pretreatments on Ability To Extract FeMoco into Non-Amide Solvents

pretreatment solvent(s)[a]	eluting solvent[a]	% recovery	sp act.	act. recovered with DMF[a] %	act. recovered with DMF[a] sp act.
MeOH[c]	acetone	30	200	0	nd
MeOH	CH_3CN	10	nd	15	120
MeOH	CH_2Cl_2	0	nd	23	240
MeOH → DMF	CH_3CN	90	200	nd	nd
MeOH → THF	CH_3CN	22	210	nd	nd

[a] Pretreatment, elution, and subsequent recovery of activity with DMF as described under Experimental Procedures. [b] Specific activity is expressed as nmol of C_2H_4 produced/[min·(nmol of Mo)]. [c] No activity was eluted by MeOH.

Table III: Effect of Organic Solvents on MoFe Protein Absorbed to DEAE[a]

pretreatment solvent	properties of MoFe protein eluted with 1 M NaCl[a]: act. %	% (mg) protein	sp act.[c]	FeMoco[b] extracted with final DMF/Bu_4NCl: % yield	sp act.
DMF	0	2	0	30	170
DMF (control)	nd	nd	nd	28	228
MeOH	0	0	0	30	211
CH_3CN	14	44	353	20	155
acetone	14	36	443	18	180
THF	7	11	716	18	210
CH_2Cl_2	31	61	572	0	0

[a] MoFe protein was absorbed to DEAE, pretreated with organic solvent, and then reequilibrated in aqueous buffer. Subsequent addition of 1 M NaCl resulted in the elution of MoFe protein. The initial MoFe protein sp act. was 1105 nmol of C_2H_4/(min·mg). FeMoco was extracted from protein that remained column bound as described under Experimental Procedures. [b] Analyses as described in text. [c] Sp act. in nmol of C_2H_4/(min·mg).

It appears that acetonitrile by itself is not capable of efficiently extracting the cofactor. However, the pretreatment of the column-bound MoFe protein with DMF *does* result in efficient extraction of cofactor into this solvent. This prompted us to carry out a further set of experiments, in which water was displaced from the column with methanol, followed by addition of a second solvent, and the cofactor was removed in acetonitrile with salt (Table II). Pretreatment of the column with THF was followed by extraction in CH_3CN. It should be mentioned that pretreatment with methanol followed by elution in acetonitrile and salt resulted in 0 and 5% extraction of FeMoco, respectively, suggesting that neither pretreatment effectively facilitates release of the cofactor.

To examine the extent to which each solvent denatured the MoFe protein, an experiment was done in which MoFe protein bound to a DEAE-Sepharose column was equilibrated in an organic solvent (Experimental Procedures) followed by reequilibration with aqueous buffer. After a 1-h incubation, buffer containing 1 M NaCl as used to elute MoFe protein. In the case of DMF and methanol no active MoFe protein or protein material was eluted, and the color due to the metal–sulfur clusters remained on the column (Table III). In the case of acetonitrile, acetone, THF, and CH_2Cl_2 partially active MoFe protein was eluted. This suggests that DMF and methanol very effectively denature the protein such that it is immobilized and can no longer be eluted from the column. In the cases of acetonitrile, acetone, or THF, some protein could be eluted but with reduced specific activity indicating at least partial denaturation. In the case of CH_2Cl_2, 30% of the activity was recovered suggesting that MoFe protein is denatured to a much lesser extent. After the above treatment the columns were reequilibrated in DMF followed by extraction of cofactor in every case except dichloromethane. The specific activity was between 160 and 210 nmol of C_2H_4/[min·(nmol of Mo)] with yields between 20 and 30% (Table III). This suggested that although there was significant loss of activity of the protein with exposure of the treated column to aqueous buffer, there was not substantial loss of extractable FeMoco activity compared to the control. We infer that the solvents denature the protein to varying degrees under the conditions used here, causing it to remain bound to the column under aqueous conditions that would normally cause it to elute from the column.

In the above experiment, methanol-treated protein retained significant FeMoco activity when exposed to water. We next investigated whether removal of the P-clusters by bipyridine/methanol would decrease the yield of FeMoco as a result of exposure to water. A DEAE column with bound MoFe protein was treated with MeOH and bipyridine, resulting in the extraction of the Fe associated with the P clusters. The column was reequilibrated in aqueous solution and allowed to stand for 1 h. This was followed by addition of DMF and extraction of cofactor with Bu_4NCl. The specific activity of the cofactor was 185 nmol of C_2H_4/[min·(nmol of Mo)] indicating that there was only a small loss of specific activity upon exposure to water. This is in contrast to the case when purified FeMoco is bound to a column and exposed to water, resulting in a 70% loss of specific activity. The latter was consistent with the lability of cofactor in water, observed by Shah and Brill (1977). We suggest that FeMoco in the extraction experiment still remains in a protein pocket protected from water even after the removal of the P-clusters with methanol. Further experiments on the stabilizing role of the P-clusters are in progress.

DISCUSSION

That the nitrogenase iron-molybdenum cofactor is anionic is suggested by its migration upon electrophoresis and by its adsorption to anion exchangers (Yang et al., 1982). In the original procedure for the preparation of FeMoco developed by Shah and Brill (1977), MoFe protein in aqueous solution was precipitated by citric acid treatment, followed by neutralization, treatment of the protein pellet with DMF to remove water, and extraction of the cofactor with NMF. FeMoco prepared in NMF by this procedure does not readily dissolve in other organic solvents (Lough et al., 1986). *N*-Methylformamide and more recently pyrollidinone were the only solvents that would extract the cofactor from such a protein pellet (Walters et al., 1986). With hindsight this is easily explained, since the only cations available as counterions for the anionic FeMoco were Na^+ and K^+. Salts of these cations do not readily dissolve in nonpolar solvents. If the Bu_4N^+ counterion was supplied, we showed that FeMoco could be extracted from such a protein pellet with DMF (McLean et al., 1989). We concluded that the presence of the soluble cation was responsible for the solubility of FeMoco in DMF. We have exploited the anionic property of FeMoco in this and the preceding work (McLean et al., 1989) to develop a procedure in which FeMoco can be prepared in DMF from MoFe protein bound to an ion exchange column. The advantages of this procedure are detailed in the preceding article. The experiments presented here were done in order to examine the generality of the above observation using other organic solvents, in particular some of the more common solvents used for NMR, electrochemistry, and crystallography.

As shown here, the cofactor prepared with the Bu_4N^+ counterion in DMF and dried down was readily soluble in acetonitrile, acetone, THF, dichloromethane, and even benzene (Table I). The retention of specific activity and high yields demonstrated that neither the solvent nor the evaporation to dryness affected the activity of the cofactor as determined by its ability to reconstitute the MoFe protein activity in extracts of a *nifB*⁻ mutant. These solvents differ widely in dielectric constant and metal basicity. Efficient extraction of FeMoco had been thought to require the presence of a secondary amide which became N-ligated to the cofactor with loss of the amide proton under basic conditions (Yang et al., 1982; Walters et al., 1986). While a basic solution of cofactor in NMF may contain coordinated, deprotonated solvent (Walters et al., 1986), the present work shows this is not a prerequisite for solubility. We note, however, that since the FeMoco dried to a tacky residue, it is possible that traces of DMF remained. This will be checked in future experiments by NMR. We have not investigated the long-term stability of FeMoco in these solvents in any detail.

Direct extraction of FeMoco into these solvents was then attempted, with the column method mentioned above for preparation of FeMoco. Pretreatment of the column-bound protein with DMF resulted in facile subsequent extraction of the cofactor in these organic solvents. The purpose of the DMF treatment was to remove water from the column, analogous to the DMF wash in the Shah and Brill (1977) procedure, and also to facilitate the use of water-immiscible solvents such as THF and CH_2Cl_2. However, since it was conceivable that the FeMoco prepared in this way could contain ligated DMF, further experiments were done to determine if soluble FeMoco could be prepared in the absence of an amide. In these cases, the DMF wash was replaced by a methanol wash. When this was done, low yields of FeMoco were obtained with all solvents tested (CH_3CN, acetone, CH_2Cl_2) (Table II). Only acetone gave appreciable extraction of FeMoco (30%). When CH_3CN, methanol, and CH_2Cl_2 were used, extraction of cofactor did not occur. In the case of the water-miscible solvents, omission of the methanol wash did not change the result (data not shown), indicating that the low recoveries were not due to the use of methanol to remove water from the column. These observations indicate that the solubility of FeMoco in a particular solvent may be independent of the ability of that solvent to extract the cofactor from column-bound protein, the latter no doubt including *both* denaturation and solvation.

It was thus of interest to determine to what extent the protein is denatured on the column by other solvents which by themselves (Table I) are efficient solvents for cofactor. Column-bound MoFe protein was treated with the above solvents for 20 min for denaturation, followed by exposure to aqueous solution and elution of the MoFe protein with aqueous buffer containing a high NaCl concentration. The predicted results can be divided into four classes depending on the degree of denaturation: (a) If the protein was not denatured, it would be recovered in high yield at or near its original specific activity. (b) Partially denatured protein which was not rendered insoluble (i.e. not irreversibly bound to the column matrix) would elute at a lower specific activity and yield. (c) Denatured protein which became insoluble and could not be eluted with an aqueous high salt solution but which did not expose its cofactor to inactivation by water would subsequently yield active FeMoco. (d) The last class is denatured protein which stays on the column and which is denatured to such an extent that the FeMoco is exposed to the aqueous environment. This would cause loss of FeMoco activity and poor yields on subsequent treatment of the column with DMF.

In the case of DMF and methanol, no protein, Mo, or Fe was eluted from the column (Table III). This indicated that the protein has been irreversibly bound to the column, suggesting significant denaturation had taken place. In the case of acetonitrile, acetone, and THF, some active protein was eluted, suggesting these solvents did not denature the protein to the same degree as methanol or DMF. [Treatment of the protein with CH_3CN/bpy solution did not result in the extraction of the P-center Fe, suggesting that acetonitrile does not open the protein enough to facilitate either P- or M-center extraction (data not shown).] In the case of methylene chloride most of the protein was eluted but at lower specific activity suggesting that, while the protein was not irreversibly adsorbed to the column, some denaturation with inactivation occurred. This indicates that with the exceptions of DMF and MeOH the above solvents are not good denaturants for column-bound MoFe protein. We conclude that the pretreatment of the protein with DMF denatures the protein to an extent that provides access of other organic solvents to FeMoco. However, such denaturation with either DMF or methanol does not expose the FeMoco to the aqueous milieu, since the yield of FeMoco from the denatured protein that had been exposed to water was the same as that of the untreated control (Table III). Thus pretreatment with DMF or MeOH does not render the FeMoco susceptible to aqueous destruction.

It is clear that there is a difference between the effects of methanol and DMF, since the methanol-treated protein, though denatured, does not release cofactor when subsequently treated with other solvents under conditions for FeMoco elution (Table II). This raises the question of whether it is necessary to remove the P-clusters to facilitate extraction. The difference between DMF and MeOH may be that pretreatment with DMF may displace the P-clusters to a column-bound form (the clusters are anionic) permitting access of other solvents to FeMoco whereas MeOH does not. In other experiments, the column-bound protein was treated with DMF/bpy or MeOH/bpy to denature the protein *and* remove P-clusters. Both treatments gave active FeMoco after exposure of the protein to aqueous buffer. This indicated that even removal of the P-clusters does not necessarily expose FeMoco to water. In the case of acetonitrile a small increase in cofactor yield was observed (10–15%) for the MeOH/bpy-treated column (data not shown). By contrast, acetonitrile easily eluted FeMoco from the column pretreated with either DMF or THF (Table II). This suggests that while extraction of the P-cluster iron may be necessary, it may *not* be a sufficient precondition for the extraction of cofactor.

If DMF and methanol denature the protein (adsorbed to DEAE) to similar degrees, why does DMF extract cofactor but methanol does not, even *after* removal of the P centers? Since methanol is a weaker coordinating ligand than DMF in most inorganic complexes, one possible reason for cofactor extraction is that the DMF may displace one or more amino acid residues bound to the cofactor, whereas methanol cannot. We have consistently observed a color change of the column-bound protein upon exchange from aqueous buffer to both DMF and NMF but not methanol or the other solvents. This may indicate changes in the chromophore on ligation of the solvent, but this remains to be investigated.

We suggest that DMF may penetrate to the FeMoco binding pocket and displace its ligands, without opening it up enough to allow access to aqueous buffer but allowing the FeMoco to be eluted if other organic solvents are subsequently used. McLean et al. (1989) showed that simple precipitation of the MoFe protein with DMF in a test tube did *not* facilitate the extraction of either the P-center Fe with bipyridine or the FeMoco with a 0.5 M Bu_4NBr solution. It is clear that, in this liquid system and in the absence of other denaturants, DMF cannot denature the protein to the extent that either of the metal centers can be extracted. In contrast, column-bound MoFe protein was easily denatured by DMF, and the P-clusters were readily removed with DMF/bpy (see preceding paper). Therefore, the requirements for denaturation and P-cluster removal differ depending on whether the protein is bound or in solution. This also illustrates that it is possible to denature the protein (i.e., render insoluble) without exposing any of its clusters for extraction.

From these experiments one may conclude that there is no absolute requirement for an amide solvent to extract FeMoco and that under carefully selected conditions the cofactor demonstrates typical solubility properties expected for any organometallic or inorganic salt. The nature of the binding of cofactor to the MoFe protein remains unknown. Recent site-directed mutagenesis (Brigle et al., 1987) and spin-echo studies (Thomann et al., 1987) of MoFe protein suggest the possible covalent ligation of cofactor to the protein via residues which are not cysteine residues as commonly found with other Fe–S clusters [for review, see Averill and Orme-Johnson (1978)]. DMF, acetone, and THF may be able to displace such a protein ligand and thus facilitate extraction. Solvents such as CH_2Cl_2, acetonitrile, and methanol do not efficiently extract FeMoco, suggesting that these solvents are not capable of displacing such a ligand. It is conceivable that the cofactor is bound to the protein through a combination of covalent and electrostatic interactions. Although the solvent and salt may act primarily by disrupting the electrostatic/hydrogen-bonding interactions between the cofactor and protein, covalent ligation by the protein may require the active participation of the solvent in displacement of cofactor. This would restrict the number of solvents capable of *displacing* cofactor from the protein and might explain the difference between DMF and MeOH. It is clear that once FeMoco is removed from the protein it is readily soluble in all these solvents, and similarly, cofactor is easily eluted from DEAE-Sepharose by all these solvents. We note that it is conceivable that there is solvent or halide ligation to the cofactor under the extraction conditions reported here and that the net negative charge of *extracted* cofactor may be a consequence of the ligation of such anionic ligands. Whether the solvent is bound to the cofactor will be reexamined in subsequent NMR, EPR, and IR experiments. We have done preliminary EPR experiments of FeMoco in DMF and CH_3CN and do not observe the expected signal. Instead, a signal at $g = 2$ appears. The cofactor is active, and we have no explanation for the observed results. This is under current investigation. Despite these theoretical uncertainties, we have arrived at a convenient and efficient method for working with FeMoco not only in *N*-methylformamide but in other organic solvents of diverse types, a method which we have described here in detail.

ACKNOWLEDGMENTS

We thank Marc Walters for his comments on the manuscript.

Registry No. FeMoco, 72994-52-6; DMF, 68-12-2; THF, 109-99-9; CH_2Cl_2, 75-09-2; CH_3CN, 75-05-8; acetone, 67-64-1; benzene, 71-43-2.

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Effect of Chloride Ion on the Thermal Decay Process of the Batho Intermediate of Iodopsin at Low Temperature[†]

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Received May 2, 1989; Revised Manuscript Received July 20, 1989

ABSTRACT: The photochemical and the subsequent thermal behaviors of iodopsin (Cl^--bound form) and *N*-iodopsin (iodopsin whose Cl^- was replaced by NO_3^-) in CHAPS–phosphatidylcholine (PC) were studied by low-temperature spectrophotometry. Irradiation of the iodopsin preparation at −185 °C produced a photo-steady-state mixture composed of iodopsin, bathoiodopsin, and isoiodopsin. Bathoiodopsin was thermally reverted to the original iodopsin. These results were almost the same as those reported previously [Yoshizawa, T., & Wald, G. (1967) *Nature 214*, 566–571] in which iodopsin was extracted with 2% digitonin. Therefore, photochemical and subsequent thermal behaviors of iodopsin were independent of the detergent to solubilize iodopsin. Irradiation of *N*-iodopsin at −185 °C produced the similar photo-steady-state mixture. However, *N*-bathoiodopsin was thermally converted to the next intermediate, presumably *N*-lumiiodopsin. These results suggest that the batho–lumi transition of iodopsin at low temperature is likely to be inhibited by the Cl^- bound to the protein moiety of iodopsin, while at room temperature the Cl^- bound to iodopsin could be released on the conversion process of batho- to lumiiodopsin.

Since the discovery of iodopsin (Wald et al., 1937), differences in the biochemical and spectroscopic nature between the rod pigment rhodopsin and the cone pigment iodopsin have been investigated with great interest. Although the difficulty of purification of cone pigments prevented their precise characterization, the following unique natures of iodopsin have been elucidated.

Like rhodopsin, iodopsin has an 11-*cis*-retinal as its chromophore, but there are distinct differences owing to difference in the protein moiety between them (Wald et al., 1955; Matsumoto et al., 1975). One of the most prominent differences in the primary photochemical process is that bathoiodopsin produced by irradiation of iodopsin at liquid nitrogen temperature is thermally reverted to the original iodopsin, while bathorhodopsin bleaches to *all-trans*-retinal and opsin through

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[†] This work was supported in part by Grants-in-Aid for Specially Promoted Research (63065002) and for Scientific Research on Priority Areas of the Molecular Mechanism of Photoreception from the Japanese Ministry of Education, Culture and Science, and in part by a Special Coordination Fund of the Science and Technology Agency of the Japanese government.

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0006-2960/89/0428-9412$01.50/0