Metal and sulfur composition of iron–molybdenum cofactor of nitrogenase

(Molybdenum–iron protein/dinitrogen reduction/iron–sulfur clusters)

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ABSTRACT The sulfur content of N-methylformamide solutions of cofactor from Clostridium pasteurianum nitrogenase has been determined to be 11.9 (±0.9) mol per mol of molybdenum. This number was determined radiochemically, using iron–molybdenum cofactor isolated from molybdenum–iron protein from bacteria grown on 35SO4. A total of 3.2 (±0.2) mol of sulfur per mol of molybdenum was found to be present in cysteine and methionine, probably arising from contaminating proteins not intrinsic to the cofactor. Combined with accumulated evidence that is discussed, these results lead to an updated stoichiometry of MoFe₆S₄, rather than MoFe₆S₅ as previously thought, for this cluster.

The molybdenum–iron protein of nitrogenase (MoFe protein) is thought to contain the catalytic site for the reduction of dinitrogen to ammonia (1). A cofactor containing both iron and molybdenum (FeMo cofactor) has been isolated from acid-denatured protein (2). This cofactor may be used to activate inactive, molybdenum-free, “MoFe” protein from a mutant strain of Azotobacter vinelandii, UW 45, suggesting an intrinsic role for the FeMo cofactor in the catalytic process. Physical studies of the FeMo cofactor and the MoFe protein have shown the following: (i) the characteristic EPR spectrum of the MoFe protein is elicited in a broadened form from the FeMo cofactor (3); (ii) the Mössbauer spectra of iron atoms associated with this $S = \frac{3}{2}$ center in the protein similarly are reproduced in studies of the cofactor (3); and (iii) the Mo x-ray absorption fine structure (EXAFS) analyses suggest that the structure of the molybdenum site in the cofactor is closely similar to that in the protein (4). This indicates that the structure of the iron and molybdenum cluster in the MoFe protein is relatively well represented in the isolated FeMo cofactor and that structural studies of the cofactor may help to solve the more difficult problem of the function of that site in the protein.

The structure of this species almost certainly is unique among biological metal–cluster clusters. Common iron–sulfur–clusters $[\text{Fe}_x \text{S}_y (\text{SR})_z]$ and Fe₆S₄(SR)₄] have not been detected as subspecies of the FeMo cofactor (3). Mössbauer and EPR spectroscopies of the MoFe protein (5, 6) show that each of the six iron atoms of the $S = \frac{3}{2}$ spin system is in a distinctive magnetic environment. Several clusters containing iron, molybdenum, and sulfur have been synthesized (7–9), but as yet none has duplicated the unusual physical and chemical properties of the FeMo cofactor.

Despite the absence of crystallographic information, some appreciation of the structure of this cluster might be gleaned from the body of spectroscopic data that is rapidly accumulating. However, a prerequisite of the design of models based on such information is an accurate knowledge of the stoichiometry of the FeMo cofactor. To date, the best information on that score arises from the rationalization of spectroscopic experiments, rather than from chemical analysis. Electron–nuclear double resonance (ENDOR) and EPR data suggest one molybdenum per spin system and two noninteracting spin systems per MoFe protein, implying the presence of one molybdenum in each cluster (10, 11). Mössbauer and ENDOR studies show at least six iron atoms coupled to the spin system in the protein (5, 6), and chemical analyses of solutions of isolated FeMo cofactor reveal six to eight iron atoms per molybdenum (2–4, 12, 13).

Isolation of the FeMo cofactor in the presence of dithionite makes quantitation of the amount of sulfur especially difficult. Initially, the FeMo cofactor was reported to contain six acid-labile sulfides per molybdenum (2), whereas more recent assays have suggested the number is closer to four (13). So small a number of sulfur atoms (less than the number of iron atoms) is incompatible with the composition of synthetic FeS clusters, as well as being contrary to recent Fe EXAFS spectroscopic studies of the FeMo cofactor (14). Furthermore, the only published study that suggests the presence of sulfur in any form but sulfide (15) has been disputed recently (13). Radiochemical analyses for sulfur (using 35S as a tracer) have the advantage of being insensitive to the presence of dithionite. They also are inherently more accurate and more precise than colorimetric techniques. A major problem is that radiochemical assays require some method to determine the specific activity of the 35S in the proteins as isolated. The source of the difficulty is the potential for the presence of unmeasured amounts of contaminating sulfate in the growth medium, sulfate being rather difficult to assay in the presence of the other components of the medium.

In order to facilitate understanding of the structure of the FeMo cofactor, we have reexamined the MoFe:S stoichiometry of this cluster, using atomic absorption spectroscopy to assay for Fe and Mo, and radiochemical analyses for S. To obtain the specific activity of the sulfur in the isolated proteins and cofactor we have used Clostridium pasteurianum as the source of the MoFe protein, and we have simultaneously purified to homogeneity the well-characterized eight-iron ferredoxin from that organism (16). This ferredoxin (16 mol of S/8 mol of Fe/mol of protein) serves as an excellent internal standard for the specific activity of 35S.

MATERIALS AND METHODS

Carrier-free H₂ 35SO₄ was obtained from New England Nuclear. [14C]Toluene was the product of Amersham-Searle. Reagent grade nitric acid was distilled twice prior to use. Oxygen was

Abbreviations: MoFe protein, molybdenum–iron protein of nitrogenase; FeMo cofactor, iron–molybdenum cofactor of the MoFe protein; ENDOR, electron–nuclear double resonance; EXAFS, x-ray absorption fine structure.

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removed from carbon monoxide by equilibration of the gas with a solution of 5% pyrogallic acid in 20% NaOH, and from other gases by passage through Oxyisorp (Messer-Griesheim) and Deoxo (Engelhardt) cartridges. All chemicals used were of reagent grade. C. pasteurianum strain W6 was the gift of Leonard Mortenson. Glassware used during analysis for Mo and Fe was triply acid-washed (17); glassware thus treated and the plastics used introduced essentially no Fe or Mo contamination according to atomic absorption analysis. Activity assays with the MoFe and Fe proteins were performed as published (12). Protein concentrations were determined by biuret assay (18).

Growth of $^{35}$S-Enriched C. pasteurianum. The minimal sulfate requirement for these bacteria was estimated by growth of 100-ml cultures on various amounts of sulfate. The medium used for the growth of labeled $^{35}$S-labeled C. pasteurianum was as previously described (16), except that it contained 0.2 mM sulfate (19). To 14 liters of medium containing 10 mM (1 Ci = 3.7 x 10$^{10}$ becquerels) of $^{35}$SO$_4$ was added a 1-liter inoculum of bacteria grown on natural-abundance materials. After growth of the bacteria (OD$_{550} = 1.7$) they were harvested in a Sharples centrifuge, yielding 43.6 g of cell paste. This was stored overnight at 156 K.

Isolation of $^{35}$S-Enriched Proteins. The isolation of the nitorgenase proteins was performed anaerobically. All steps were carried out under 1.1 atmosphere of 5% H$_2$ in Ar. All solutions were degassed and contained 2 mM Na$_2$S$_4$O$_6$ unless it is stated otherwise. Columns were made anaerobic by rinsing with buffer containing dithionite until the effluent was reducing to methyl viologen, and they were loaded by cannulation. Other manipulations were essentially as described (12).

Cells were lysed anaerobically under an atmosphere of 20% CO in H$_2$/Ar by addition of 40 mg of lysozyme and 5 mg of DNase I to a suspension in 450 ml 50 mM Tris-HCl, pH 8.0. After 1.5 h at 37°C the debris was pelleted by centrifugation, and the supernatant was loaded onto a DEAE-cellulose DE-32 column (Whatman; 3 x 20 cm) equilibrated with 50 mM Tris-HCl, pH 7.1 (at 30°C) and cooled to 4°C. Elution by a gradient of 300 ml each of 0.15 M NaCl/25 mM Tris-HCl, pH 7.1, and 0.60 M NaCl/25 mM Tris-HCl, pH 7.1 (both equilibrated under 20% CO in 5% H$_2$ in Ar), yielded successively MoFe protein, Fe protein, and the ferredoxin.

MoFe protein was purified further, essentially as published (19). After concentration by ultrafiltration (Amicon XM-100A membrane) the protein was purified by chromatography on Sepharose 6B (Pharmacia; 2.5 x 70 cm) with 50 mM Tris-HCl, pH 8.0, as elution buffer. The product was loaded onto a DEAE-cellulose DE-32 column (3 x 20 cm) equilibrated with 50 mM Tris-HCl, pH 8.0, and eluted with a gradient of 500 ml each of 50 mM Tris-HCl, pH 8.0, and 0.3 M NaCl/50 mM Tris-HCl, pH 8.0. After concentration to approximately 35 mg/ml by ultrafiltration, this protein was frozen and stored under liquid nitrogen.

Fe protein also was purified further, essentially as published (19). After dilution with an equal volume of 50 mM Tris-HCl, pH 8.0, it was loaded onto a DEAE-cellulose DE-32 column (2 x 8 cm) equilibrated with that buffer. After elution in a small volume of 0.5 M NaCl/50 mM Tris-HCl, pH 8.0, the protein was purified by chromatography on a Sephacryl S-200 column (Pharmacia; 2.5 x 70 cm) eluted with 0.5 M NaCl/50 mM Tris-HCl, pH 8.0. After concentration by ultrafiltration (Amicon PM-30 membrane), the protein was diluted with an equal volume of 50 mM Tris-HCl, pH 8.0, and loaded onto a DEAE-cellulose DE-32 column (3 x 20 cm) equilibrated with the same buffer. Elution was accomplished by a gradient of 350 ml each of 0.15 M NaCl/50 mM Tris-HCl, pH 8.0, and 0.4 M NaCl/50 mM Tris-HCl, pH 8.0. The product was concentrated by ultrafiltration, frozen, and stored under liquid nitrogen.

The ferredoxin was concentrated by ultrafiltration (Amicon UM-2 membrane) and desalted by chromatography on Bio-Gel P-2 (Bio-Rad) with 50 mM Tris-HCl, pH 8.0 (no dithionite), in an inert atmosphere box. The dithionite-free protein was oxidized by exposure to air. Further manipulations of this protein were performed aerobically in the absence of dithionite, as described by Rabinowitiz (16). The protein was loaded onto a DEAE-cellulose DE-32 column (3 x 20 cm) equilibrated with 50 mM Tris-HCl, pH 8.0. After a rinse of 500 ml of 0.15 M NaCl/50 mM Tris-HCl, pH 8.0, and 0.35 M NaCl/50 mM Tris-HCl, pH 8.0. Fractions with $A_{350}/A_{290} \geq 0.75$ were combined, concentrated by ultrafiltration (Amicon UM-2 membrane), and further purified by chromatography on a Sephadex G-75 column (Pharmacia; 2.5 x 90 cm) eluted with 50 mM Tris-HCl, pH 8.0. Samples with $A_{350}/A_{290} \geq 0.77$ were combined, concentrated by ultrafiltration, and purified by ammonium sulfate fractionation. The initial cut between 65% and 85% saturated ammonium sulfate was purified further by repeated crystallization in 80% saturated ammonium sulfate.

Isolation of FeMo Cofactor from MoFe Protein. FeMo cofactor was isolated by a procedure similar to that of Shah and Brill (2). All solutions contained 5 mM sodium dithionite added as a 1 M solution in 1 M Tris-HCl, pH 8.0. A 0.5-ml aliquot of $^{35}$S-labeled MoFe protein (29 mg/ml) was diluted first with 0.5 ml of 0.25 M NaCl/0.025 M Tris-HCl, pH 7.4, and then with 2 ml of water. Addition of 100 $\mu$l of 1 M citric acid was followed after 150 s by 400 $\mu$l of 0.5 M Na$_2$HPO$_4$. At this point the protein precipitated. The suspension was allowed to stand at ice temperature for 1 h, after which the protein was separated by centrifugation at 120 x g for 10 min. The supernatant was removed and replaced with 1 ml of dimethylformamide (Aldrich, vacuum distilled in the absence of grease), which was layered onto the protein. The sample was resuspended by brief (5-s) agitation on a Vortex mixer, the protein was separated by centrifugation at 520 x g for 5 min, and the supernatant was removed. This dimethylformamide wash was repeated. No color was detected in these washes. After the second dimethylformamide wash, 0.5 ml of N-methylformamide (Aldrich, vacuum distilled in the absence of grease) containing 5 mM Na$_2$HPO$_4$ (added as a 0.5 M solution in water) was added. The sample was agitated for 5 min on a Vortex mixer and allowed to stand on ice approximately 10 min. The protein was separated by centrifugation at 1,450 x g for 10 min, and the dark-green clear cofactor solution was removed and stored in a double-septum vial. A second wash with N-methylformamide/phosphate yielded a light-green solution, and a third was colorless. The first two washes were combined and concentrated in vacuo to approximately 0.2 ml. After filtration, samples were taken for analysis.

Assay of FeMo Cofactor Activity. These were performed in a manner similar to that previously described (12). Crude extract of A. vinelandii UW45 was provided by Paul Lindahl and stored frozen in liquid nitrogen. The concentrated FeMo cofactor solution was diluted 1:10 into 0.025 M sodium phosphate, pH 7.5, containing 20 mM sodium dithionite. Aliquots of this solution (2-20 $\mu$l) were added to 300-$\mu$l samples of the crude extract, incubated at 30°C for 90 min, and then placed on ice. Of these samples, 100 $\mu$l was taken with a 20-fold excess of the iron protein from A. vinelandii for assay of nitrogenase activity. Specific activity was taken from the slope of the line fit to a plot of activity vs. molybdenum concentration.

Assay of $^{35}$S. The amount of $^{35}$S in samples was determined by liquid scintillation counting. A solution of 0.8% 1,4-bis-[2-(5-phenylloxazolyl)]benzene, 0.65% 2,5-diphenyloxazole, 33% Triton X-100 in toluene was used as scintillation fluid. A small amount of the sample was added to 70 $\mu$l of 0.3 M KOH in a
scintillation vial and was followed by the scintillation fluid.
Methanol (300 µl), dioxane, or both were used to clear turbid solutions. Efficiency was assessed by adding a known amount of [14C]toluene and recounting. Disintegrations per minute were corrected for decay (t1/2 = 87.2 days) and all were standardized to the same date.

Assay of Fe and Mo. These elements were assayed by atomic absorption spectroscopy with a Perkin-Elmer model 2380 spectrometer, equipped with a graphite furnace, at 248.3 nm (Fe) and 313 nm (Mo). Samples were digested by incubation overnight at 100°C in 0.5 M HNO3 and were evaporated to dryness under a flow of N2. Samples were dissolved in 1.00 ml of either 0.5 or 0.1 M HNO3. For assay of Mo, enough NH4Cl was added to yield a 2% solution in order to suppress interference from Fe. The amount of Fe and Mo in these samples was determined by the method of standard additions. Lines were fit to the data by least-squares analysis, and standard deviations were assigned to the slope and intercept. Standard deviations were propagated throughout the analysis by standard techniques (20).

Analysis of Amino-Acid-Bound 35S. Aliquots of both the labeled FeMo cofactor and the ferredoxin were treated with performic acid (21), lyophilized, and hydrolyzed by heating for 24 hr with 6 M HCl at 120°C in sealed containers. The solutions were lyophilized and the residues were redissolved in 100 µl of 0.01 M HCl. Aliquots (10 µl) of each was subjected to thin-layer chromatography on silica gel (1B-F, Baker) using 1-propanol/concentrated ammonium hydroxide, 70:30 (vol/vol), as eluting solvent. Authentic cysteic acid and methionine sulfone were used as standards. The radioactivity was quantified by two methods. One procedure involved scraping off the ninhydrin spots corresponding to the standards and directly measuring the amount of 35S in each spot by scintillation counting. For the second method, the radioactivity was first visualized by fluorography (22). The intensities of the spots were quantitated by comparison of the optical density of the darkened portions of the film with spots produced by known quantities of [35S]sulfate and [35S]methionine (New England Nuclear).

RESULTS

The ferredoxin used to determine the specific activity of the 35S in the proteins exhibited A280/A390 = 0.838 and A280/A390 = 1.17, which may be compared to the reported values of 0.83 and 1.2, respectively, for the purified protein (16). This ferredoxin solution was analyzed by atomic absorption spectroscopy and found to have a concentration of 4.72 ± 0.42 mM iron, corresponding to 9.44 ± 0.84 mg of Fe. The same solution contained 26.3 ± 0.7 Ci/ml of 35S, leading to a specific activity of 2.79 ± 0.26 mCi/mmol in the bacterial proteins. Utilizing the concentration of the protein as determined by atomic absorption spectroscopy, we estimate ε390 = 31,700 M⁻¹ cm⁻¹ for this eight-iron ferredoxin, in excellent agreement with the value of 30,600 M⁻¹ cm⁻¹ estimated for the analogous protein from Clostridium acetii urici (16).

The nitrogenase iron protein was purified to a specific activity of 2,050 nmol of ethylene produced per min per mg of protein. This protein contained 4.0 ± 0.4 Fe atoms per protein molecule, on the basis of a molecular weight of 64,000. The iron protein is reported to contain 32 methionines, 12 cysteines, and 4 acid-labile sulfides, for a total 38 atoms of sulfur per protein molecule (23). Thus the ratio of sulfur to iron in this protein is 9.5:1. Our result of 9.7:1 is in excellent agreement with the known ratio, and supports the validity of this approach in determining such stoichiometries in this type of system.

The nitrogenase MoFe protein was purified to a specific activity of 1,950 nmol of ethylene produced per min per mg of protein. This protein contained 16.1 ± 0.6 mol of iron per mol of molybdenum. From this material we isolated three different preparations of FeMo cofactor in N-methylformamidine. These preparations yielded a specific activity of 175 ± 25 nmol of ethylene produced per min per nmol of Mo. The results of assaying each of these preparations for molybdenum, iron, and sulfur are shown in Table 1. Each sample was assayed individually, and the mean and standard deviation of those assays is reported. We find an average of 8.2 ± 0.4 mol of Fe per mol of molybdenum in these FeMo cofactor solutions. Utilizing the same procedure for isolation of the FeMo cofactor, other workers have found seven or eight iron per molybdenum (2, 12, 13). Procedures that include an iron chelation step yield active FeMo cofactor with six iron per molybdenum (13).

In these N-methylformamidine solutions, we found 11.9 ± 0.9 mol of sulfur per mol of molybdenum. We estimated the amount of sulfur contributed by protein-bound amino acids as follows. Samples of the FeMo cofactor solutions were oxidized with performic acid, hydrolyzed, and chromatographed. Fluorography of these chromatograms revealed only three radioactive spots: one at the origin (Rf = 0.00), one that migrated with cysteic acid (Rf = 0.19), and one that migrated with methionine sulfone (Rf = 0.33). The relative amounts of 35S associated with each spot as determined by fluorography were 72.7 ± 2.1, 11.0 ± 1.3, and 16.3 ± 0.9%, respectively. By direct scintillation counting of the spots scraped from the plates, the relative amounts of radioactivity were found to be 73.5 ± 1.3, 9.5 ± 0.8, and 17.0 ± 0.7%, respectively. The ratio of cysteic acid to methionine sulfone found in the FeMo cofactor solution closely corresponds to that found in the MoFe protein (2:3) (24), thereby suggesting that these amino acids represent apoprotein isolated with the FeMo cofactor. After chromatography of samples of the ferredoxin, only two spots were visualized by fluorography, one at the origin and one migrating with cysteic acid. The relative amounts of label in each spot were 46.3 ± 3.7 and 53.8 ± 3.9%, respectively. The ferredoxin is known to contain equimolar amounts of sulfide and cysteine, and no methionine (16).

From this, a reasonable hypothesis is that adventitiously co-purified apoprotein contributes 26.9 ± 1.7% of the sulfur in these solutions of FeMo cofactor, equal to 3.2 ± 0.2 mol of sulfur per mol of molybdenum. On the basis of a total of 100 cysteine plus methionine residues per 220,000 molecular weight (24),

Table 1. Molybdenum, iron, and sulfur content of the FeMo cofactor and Fe protein from C. pasteurianum

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Mo, mM</th>
<th>Fe, mM</th>
<th>S, mM</th>
<th>Fe/Mo</th>
<th>S/Mo</th>
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<tr>
<td>FeMo cofactor I</td>
<td>5</td>
<td>0.121 ± 0.009</td>
<td>1.05 ± 0.08</td>
<td>1.49 ± 0.14</td>
<td>8.7 ± 0.9</td>
<td>12.3 ± 1.5</td>
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<tr>
<td>FeMo cofactor II</td>
<td>3</td>
<td>0.155 ± 0.010</td>
<td>1.29 ± 0.09</td>
<td>1.79 ± 0.17</td>
<td>8.2 ± 0.3</td>
<td>11.5 ± 1.3</td>
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<tr>
<td>FeMo cofactor III</td>
<td>3</td>
<td>0.152 ± 0.011</td>
<td>1.14 ± 0.03</td>
<td>1.80 ± 0.17</td>
<td>7.8 ± 0.2</td>
<td>11.8 ± 1.4</td>
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<tr>
<td>FeMo cofactor average</td>
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<td>8.2 ± 0.4</td>
<td>11.9 ± 0.9</td>
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<td>Amino acid sulfur</td>
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<td>3.2 ± 0.2</td>
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<td>FeMo cofactor less amino acids</td>
<td></td>
<td>8.7 ± 1.0</td>
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<td>Fe protein</td>
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<td>This work</td>
<td></td>
<td>0.74 ± 0.06</td>
<td>7.2 ± 0.7</td>
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<td>S/Fe = 9.7 ± 1.2</td>
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<tr>
<td>From sequence (22)</td>
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<td></td>
<td>S/Fe = 9.5</td>
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</table>
this leads to a ratio of 0.03 mol of apoprotein per mol of cofactor in these solutions. Other workers (13) have reported less-than-stoichiometric amounts of amino acids in several-fold more concentrated solutions of the FeMo cofactor. Assuming that such amino acids arise from saturating amounts of apoprotein in the N-methylformamide solutions, this would lend support to our hypothesis. Taking such an impurity into account leads to the conclusion that the FeMo cofactor has 8.7 ± 1.0 mol of non-amino-acid sulfur associated with each mol of molybdenum in solution.

**DISCUSSION**

Using a combination of radiochemical techniques and atomic absorption spectroscopy, we have shown that the FeMo cofactor from *C. pasteurianum* contains 8.7 ± 1.0 mol of non-amino-acid sulfur per mol of molybdenum. Thus we conclude that there are eight or nine sulfur atoms per molybdenum in this cluster. That synthetic MoFeS clusters prepared as models for the FeMo cofactor generally have contained even numbers of sulfur atoms per molybdenum might favor the lower number. On the other hand, none of these models has been successful in mimicking the distinctive properties of the cofactor.

In these experiments we have found 8.2 ± 0.4 mol of iron per mol of molybdenum in the FeMo cofactor. Other workers have found seven or eight iron atoms per molybdenum in similar preparations (2, 12). When samples of FeMo cofactor are prepared in this fashion and treated with iron-chelating reagents, an active species with only six iron atoms per molybdenum is obtained (13). This is in accord with both Mössbauer and ENDOR experiments (5, 6), which support the concept that there are six iron atoms spin-coupled together in this cluster, within the holoprotein. Of course, it may be that the FeMo cofactor contains six tightly bound, spin-coupled iron atoms as well as one or two uncoupled iron atoms that may be removed and restored easily. In that case the FeMo cofactor preparations with six iron per molybdenum might well scavenge iron from the *A. vinelandii* UW45 crude extract solution during reconstitution, but it seems quite reasonable to assume instead that the ratio of iron to molybdenum in the FeMo cofactor is indeed six, and that any extra iron results from adventitious copurification (13).

The importance of these experiments lies in the discovery that the FeMo cofactor contains eight or nine mol of non-amino-acid sulfur per mol of molybdenum—about twice the amount detected by previous workers. This has two important consequences. First, recent EXAFS investigations into the local structure about the iron atoms in the FeMo cofactor have found an average of three to four sulfur atoms in the vicinity of the average iron atom (14). It is difficult to envision a structure that would account for that observation and yet would contain less than one sulfur per iron atom. The presence of S and Fe in a ratio of about 1.5:1 is much more compatible with the Fe EXAFS results. Second, even in the absence of the structural evidence, a Mo:Fe:S ratio of 1:6:4 would be much more difficult to rationalize in terms of "conventional" metal clusters than is a ratio of 1:6:9. For example, the Fe:S ratio in the iron-sulfur clusters in the ferredoxin we used as an internal standard is 1:1. From these points of view, this revision of the ratio of sulfur to molybdenum is intuitively satisfying.

There are several possible reasons why previous workers have obtained such low values for the amount of sulfur in the FeMo cofactor. It should be noted that the technique used here determines the total sulfur present, not merely acid-labile sulfur. Both Mo EXAFS spectroscopy (4) and chemical assays of acid-denatured MoFe protein (22) have suggested the presence of a MoS₂ core in the FeMo cofactor. The usual assay for acid-labile sulfur has been shown to yield low values for the sulfide content in some MoS₂-containing clusters of known stoichiometry (26). Failure to detect molybdenum-bound sulfur in earlier determinations might account for the difference between our results and those previously reported. Similarly, organic sulfur compounds would not be included in an acid-labile sulfur assay but would be detected radiochemically. As yet we have no compelling evidence for such organic sulfur ligands, and we tentatively ascribe the three sulfur amino acid residues we find in this cofactor preparation to a small (3 mol %) amount of residual apoprotein.

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