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Purification and Some Properties of Molybdoferredoxin, a Component of Nitrogenase from *Clostridium pasteurianum**

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ABSTRACT: A method for the purification of molybdoferredoxin (Mo-Fe protein), a component of the nitrogenase complex from *Clostridium pasteurianum*, is presented. This method yields about 2 g of pure protein and can be prepared within 2 days from 1 kg of dried cells (170 g of crude protein). The molecular weight of the protein is 168,000 and has a partial specific volume based on sedimentation equilibrium studies of 0.72 ml/g. The protein contained the following metals and sulfur groups (moles/mole of protein): Mo, 1.0; Fe, 14.0; SH, 23; S^{2-}, 16. The protein showed a tendency to aggregate if oxidized or stored for several months in liquid nitrogen. Significant changes in both the visible and paramagnetic resonance spectra occurred as well as a 70% loss in acetylene reduction activity after 1-hr exposure to air. Less loss of activity resulted from short-term exposure to air even though the spectral change was optimal.

The nitrogenase complex from *Clostridium pasteurianum* has been resolved into two oxygen-sensitive protein components (Mortenson, 1965), named molybdoferredoxin (MoFd[1]) and azoferredoxin (AzoFd) (Mortenson *et al.*, 1967). AzoFd, a cold-sensitive protein, exists in solution as a dimer of molecular weight 55,000 and has been reported to contain four nonheme iron atoms and four acid-labile sulfide groups per dimer (Nakos and Mortenson, 1971a).

This paper describes the method currently used to purify MoFd and reports a revision in the metal content of the protein. A careful investigation of the molecular weight of MoFd was undertaken since values previously reported (Mortenson *et al.*, 1967; Dalton and Mortenson, 1970) were only estimations which ranged between 100,000 and 200,000. Several other properties of this protein are reported.

Experimental Section

Materials. Cacodylic acid, Tris (tris(hydroxymethyl)aminomethane), mersalyl (sodium *O*-[3-hydroxymercuri-2-methoxypropyl)carbamyl]phenoxyacetate), and ATP (sodium salt) were obtained from Sigma Chemical Co. Protamine sulfate was obtained from General Biochemical Co. Sephadex G-25, G-100, and G-200 were obtained from Pharmacia Fine Chemical Co. Other reagents were obtained from common commercial sources.

Methods. CELLS AND CELL-FREE EXTRACTS. *Clostridium pasteurianum* W5 cells were grown, harvested, and dried as described previously by Mortenson (1964). Batches of 2×10^3 to 3×10^3 g of dried cells were thoroughly mixed and distributed in 60-g portions, which were then sealed in bottles and stored under H_2 at $-20°$. This mixing minimized variation in the purification procedure from one preparation to another. Cell-free extracts were prepared by autolysis of the dried cells in anaerobic 0.05 M Tris-HCl buffer, pH 8.0, as described by Moustafa and Mortenson (1969).

ASSAYS. Protein concentration was estimated with the biuret reagent with bovine serum albumin (Sigma) as the standard. Acetylene reduction was used to measure enzymatic activity. The ethylene produced was analyzed quantitatively by gas chromatography as described by Bui and Mortenson (1968). Assays were carried out in 10-ml glass vials fitted with

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[1] Abbreviations used are: MoFd, molybdoferredoxin; AzoFd, azoferredoxin; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PSE, protamine sulfate extract; PSO$_4$, protamine sulfate.

serum stoppers. The reaction mixture contained in 2.0 ml: 10 μmoles of $MgCl_2$, 10 μmoles of ATP, 100 μmoles of lithium cacodylate buffer, pH 7.0, and 50 μmoles of acetyl phosphate or creatine phosphate. When acetyl phosphate was used, a cell-free extract of cells grown on NH_3 was included (10 mg of protein) as a source of ATP–acetate phosphotransferase. This extract also contained H_2–ferredoxin oxidoreductase and ferredoxin, which were essential when H_2 was used as the source of electrons. When the creatine phosphate ATP-generating system was used, the NH_3-cell extract was replaced with 0.5 mg of creatine phosphokinase (Worthington) and 10 μmoles of sodium dithionite ($Na_2S_2O_4$) were added as a source of electrons. The gas phase in all cases was H_2 with 300 μl of acetylene. H_2 did not affect acetylene reduction. The nitrogenase components were added as indicated below.

Molybdenum and iron were determined colorimetrically by the methods of Clark and Axley (1955) and Lovenberg *et al.* (1963), respectively, and by atomic absorption spectrophotometry. Calcium, zinc, magnesium, and cobalt were analyzed with a Perkin-Elmer 303 atomic absorption spectrophotometer, with a triple-slot Bolin burner head. Acid-labile sulfide was estimated by a modification of the method of Sheppard and Hudson (1930).

PURIFICATION OF MOLYBDOFERREDOXIN. MoFd was purified by protamine sulfate fractionation and gel filtration on Sephadex G-100. The purification procedure varied from one preparation to another so that it was not possible to follow a constant pattern. Instead, molybdenum content was monitored by atomic absorption spectrophotometry (a rapid method) and the purification was carried to a constant Mo: protein ratio. The buffer used in all purification procedures was 0.05 M Tris-HCl, pH 8.0. All operations, except centrifugation steps, were carried out under an atmosphere of H_2. For methods of anaerobic preparation, see Mortenson (1971). Protamine sulfate was prepared as an anaerobic solution (50 mg/ml for the first step and 15 mg/ml for subsequent steps). In the first step protamine sulfate was added dropwise from a separatory funnel, and in subsequent steps was added by syringe through a serum stopper. Protamine sulfate percentages were calculated on the basis of the weight of protein in the starting solution in each step.

Nucleic acids and their degradation products were precipitated from the crude extract with 2.0% protamine sulfate and discarded. AzoFd precipitated in the 2.0–5.0% fraction and was resolubilized and further purified as described by Moustafa and Mortenson (1969). Approximately 75% of the MoFd precipitated between 5 and 12% protamine sulfate. The precipitate was centrifuged (1500*g*, 15 min) and resolubilized by mixing with a sevenfold excess (based on the weight of protamine sulfate added) of phosphocellulose (Whatman P-11) adjusted to a pH value of 8.0 with 8 N NaOH. The cellulose–protamine sulfate complex was removed by centrifugation and the supernatant sulution (PSE I) was stored at −20°, to be pooled with other similar extracts.

The pooled PSE I preparations were diluted with deaerated buffer to adjust the protein concentration to approximately 20 mg/ml. After each 0.5% increment of protamine sulfate was added, a 1.0-ml sample was withdrawn and centrifuged and the supernatant solution was analyzed for protein and molybdenum. Addition of about 3.5–4% protamine sulfate removed contaminating protein but no molybdenum. This was removed by centrifugation. Protamine sulfate was then added until approximately 90% of the molybdenum had been removed from solution (usually about 6% protamine sulfate is required). The precipitated protein was centrifuged and resolubilized as in the preparation of PSE I. The protein concentration of the supernatant solution (PSE II) was again adjusted to approximately 20 mg/ml.

The PSE II was subjected to another protamine sulfate fractionation and MoFd was again monitored by molybdenum analysis. The 0–2.5% fraction was again centrifuged and discarded. The 2.5–5% precipitate was centrifuged (1500*g*, 15 min) and the MoFd exchanged from the protamine sulfate with phosphocellulose as described with PSE I.

The resultant supernatant solution (PSE III) was introduced onto an anaerobic 5.0 × 50 cm Sephadex G-100 column. Two components, the first Fd and the second MoFd, were separated and the MoFd band was collected under H_2, concentrated, and frozen by dropwise addition from a syringe into liquid nitrogen (Yankulov *et al.*, 1964). Storage in liquid nitrogen preserved nearly full activity for up to 1 year.

SPECTRA. Spectra were recorded with a Cary 14 recording spectrophotometer with 1-cm light-path Thunberg-type cuvets fitted with serum stoppers.

ULTRACENTRIFUGATION. Ultracentrifuge studies were carried out in a Spinco Model E ultracentrifuge equipped with a Beckman Type RS dynograph for recording the concentration distribution of solute molecules in the analytical cell. Data for the ln c *vs.* r^2 plots were derived through the use of a Digital Linc 8 computer connected directly to the ultracentrifuge recorder (Filmer, 1971). The molybdoferredoxin concentrations ranged between 0.17 and 0.42 mg per ml. Samples were prepared anaerobically with nitrogen as the gas phase unless otherwise stated.

ELECTRON PARAMAGNETIC RESONANCE SPECTRA. Electron paramagentic resonance spectra were recorded in a Varian V-4502 electron paramagnetic resonance spectrometer, with accessories for low-temperature measurement.

Results

Purity of Molybdoferredoxin. The results of a representative purification are shown in Table I.

Disc electrophoresis on polyacrylamide gel at pH 8.0 by the method of Peacock *et al.* (1965) indicated that MoFd was pure (Figure 1b). When run in the presence of sodium dodecyl sulfate, two major subunits bands of MoFd were seen (Figure 1c)

Molecular Weight. The molecular weight of MoFd has been estimated by gel filtration on Sephadex G-200 and gave a value around 170,000 (Nakos and Mortenson, 1971b). However, this study was hampered by the lack of standard proteins of molecular weight greater than MoFd. Treatment of the protein with 1% sodium dodecyl sulfate followed by electrophoresis in gels containing 0.1% sodium dodecyl sulfate gave rise to subunits of molecular weight 50,700 and 59,500 as determined by analytical sodium dodecyl sulfate disc gel electrophoresis (Nakos and Mortenson, 1971b). This suggests that, on the basis of a 170,000 molecular weight for MoFd, there are 2 subunits of 59,500 and one of 50,700.

Estimation of the molecular weight by the sedimentation equilibrium technique with several different MoFd preparations gave values between 140,000 and 330,000 depending upon length of storage of the preparations. It was necessary first to know the partial specific volume of the protein before the molecular weight could be calculated. The concentration distribution of MoFd in D_2O and H_2O by the method of Edelstein and Schachman (1967) results in an average value of 0.72 ml/g for the partial specific volume (Figure 2).

A variety of MoFd preparations have been used for the

TABLE I: Purification of Molybdoferredoxin.

	Protein (mg/ml)	Vol (ml)	Mo Protein[b]	Fe Protein[b]	S^{2-} Protein[c]	Specific Activity[c]	% Recovery of Units
Crude extract	24.1	3600	0.41	6.6	5.9	48	100
2.0% PSO_4 super.[e]	20.0	3500	0.40	6.6	5.8	58	98
PSE I	21.2	900	0.70	12.5	6.0	192	77
2.0% PSO_4 super.	18.0	840	0.80	12.5		200	74
PSE II	46.0	200	0.82	13	10.7	337	75
2.5% PSO_4 super.	42	195	0.90	12.8			
PSE III	24	150	1.08	13.9	11.3	750	65
Sephadex G-100[a]	31.2	72	1.09	14.2	12.6	910	50

[a] Effluent concentrated prior to analysis. [b] Based on mol wt 1.70×10^5. The values are reported as if all the protein fractions were pure (*i.e.*, the protein complex has a molecular weight of 170,000). [c] Units are: nmoles of ethylene/min per mg of protein in MoFd fraction. Creatine phosphate–creatine phosphokinase was used as ATP generator; $Na_2S_2O_4$ was used as source of electrons in highly purified fractions; H_2 was used otherwise: MoFd fraction, approx 0.3 mg; AzoFd, approx 1.5 mg. [d] Results as high as 1200 have been obtained but this is dependent on the AzoFd preparation used. [e] Super. = supernatant.

molecular weight determinations. In each case at least three protein concentrations are run so that extrapolation of the molecular weight to zero protein concentration can be made. Freshly prepared samples of MoFd give a concentration distribution within the analytical cell which, when plotted as log concentration (*i.e.*, log absorbance at 280 nm) against r^2 (r = the distance from the axis of rotation), is linear (Figure 3). Samples which have been stored in liquid nitrogen for at least 1 month do not give linear plots (Figure 4).

The freshly prepared samples give a value for the molecular weight of about 168,000 when extrapolated back to zero concentration (Figure 5). Two slopes, one of which corresponds to a molecular weight of approx 255,000, and the other to approx 140,000 are obtained when the older samples are used (Figure 4). The 140,000 and 255,000 molecular weight figures were obtained at a single protein concentration and the data were not extrapolated to zero concentration. If it were, a higher molecular weight would have been obtained (see Figure 5). Molecular weight values up to 1.3×10^6 have been found. The high slope corresponding to the higher molecular weight with the older samples probably results from aggregation in solution of the protein units of 168,000. The lower molecular weight slope corresponds to the monomer. This aggregation on storage has little if any effect on the activity of MoFd since the activity of older preparations are not markedly decreased.

Metal Content. Atomic absorption spectrophotometry is routinely used to estimate the metal content of MoFd during purification. Tris-HCl buffer at pH 8.0 reduces the absorption of standard metal solutions to approximately one-half that obtained with standard solutions prepared in distilled water. Sodium dithionite increases the absorption of iron and

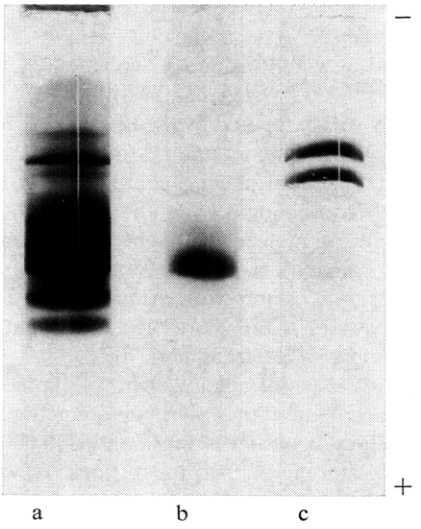

FIGURE 1: Disc electrophoresis pattern of MoFd. Run at pH 8.3 in borate–EDTA–Tris buffer, 2.5 mA per column; (a) sample from PSE I, (b) purified MoFd, (c) purified MoFd treated with 1% sodium dodecyl sulfate and the run in the presence of 0.1% sodium dodecyl sulfate.

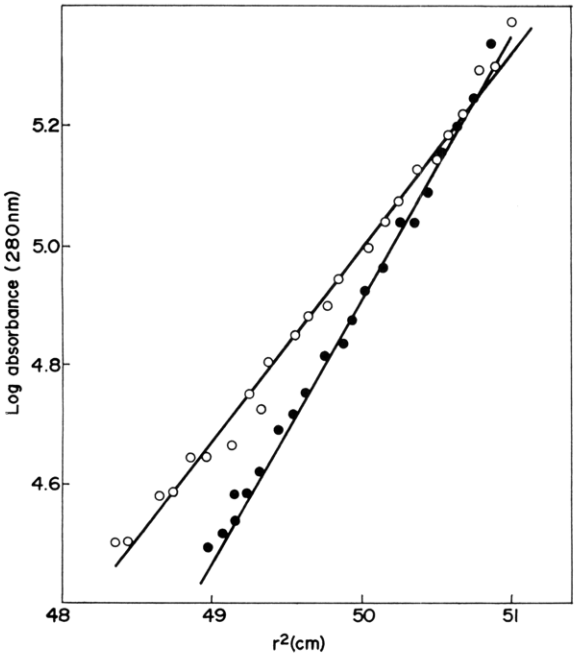

FIGURE 2: Equilibrium sedimentation in 90% D_2O–10% H_2O and in H_2O. Scans were taken after 24 hr at 10° and 6000 rpm. The loading concentration of protein was 0.3 mg/ml for the D_2O–H_2O sample and 0.28 mg/ml for the H_2O sample: D_2O–H_2O (○—○); H_2O (●—●).

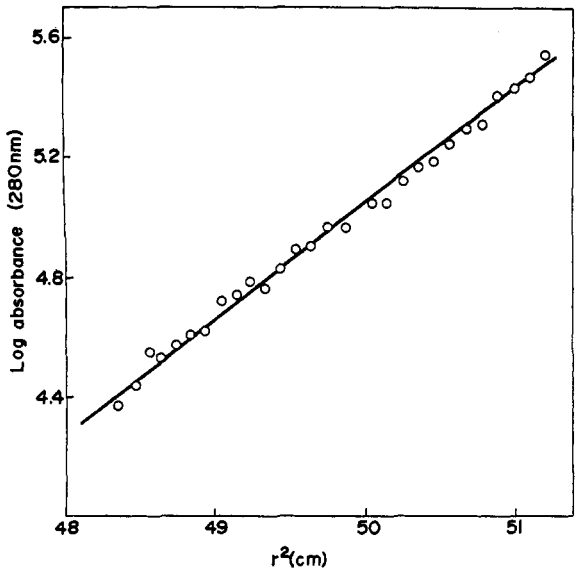

FIGURE 3: Equilibrium sedimentation of freshly prepared molybdoferredoxin in 0.05 M Tris-HCl buffer, pH 8.0. The scan was taken as described under Figure 2. The loading concentration of protein was 0.26 mg/ml.

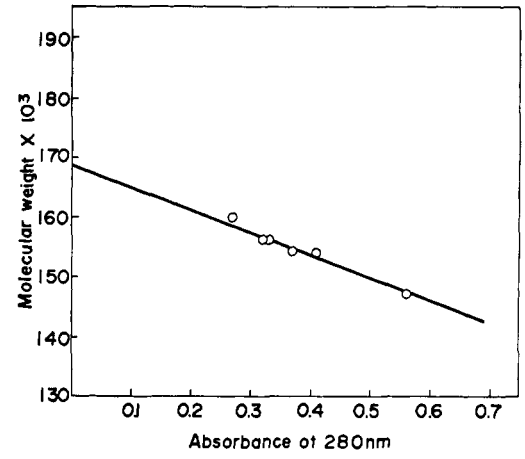

FIGURE 5: Determination of molecular weight of freshly prepared molybdoferredoxin by equilibrium sedimentation. The molecular weights at six specific protein concentrations were calculated from the equation: $M_w = (2RT/(1) - \bar{v}\rho)\omega^2)$ (d ln c/dr^2), where (d ln c/dr^2) is the slope of the line obtained from the ln c *vs.* r^2 plot for each protein concentration (see Figure 3), R = the gas constant (ergs/degree mole), T = degrees Kelvin, $\bar{v}$ = partial specific volume (ml/g), ρ = solvent density (g/ml), ω = angular velocity (rad/sec), and r = radius (cm). The one point not on the line represents a sample shaken 5 min in air prior to the 24-hr centrifugation. The gas phase in the cell for this sample was air.

molybdenum standards approximately twofold. Care must be taken to avoid its presence in samples for metal analysis.

The protein itself also influences the determination of molybdenum by atomic absorption spectrophotometry. Addition of MoFd or bovine serum albumin to known amounts of molybdenum standard gives values 1.5 to 1.6 times higher than expected. Some component of the protein, not peculiar to MoFd, causes a stimulation of the molybdenum response in the flame. No such stimulation is observed when the colorimetric analysis is used (Clark and Axley, 1955). The latter method is always used when accurate results are desired, whereas the former method is employed when accuracy is less important than speed.

Based on a molecular weight of 168,000 and using the colorimetric assay, MoFd contains one atom of molybdenum. Previous estimations of 2 Mo per protein of molecular weight about 200,000 were in error because atomic adsorption was used and the values were not corrected for the protein effect (Dalton and Mortenson, 1970).

The protein decreases the sensitivity of iron determinations by atomic absorption spectrophotometry whereas no affect is observed on the colorimetric determination (Lovenberg *et al.*, 1963). Based on these analyses, MoFd contains 14 iron atoms per molecule.

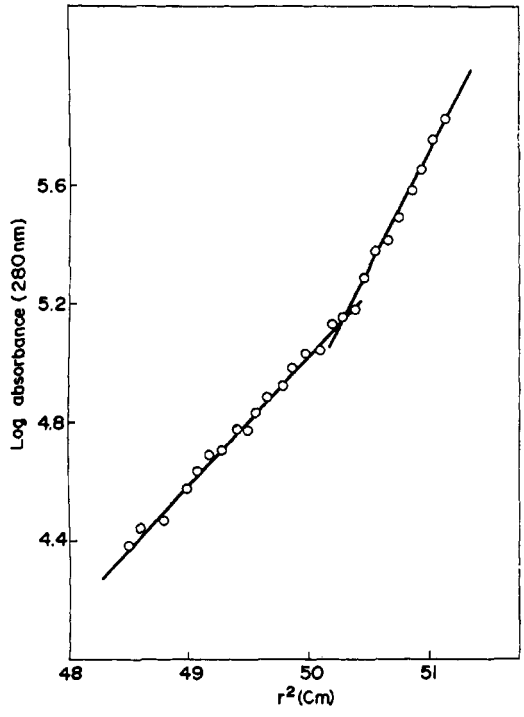

FIGURE 4: Equilibrium sedimentation of molybdoferredoxin in 0.05 M Tris-HCl buffer, pH 8.0, stored 3 months in liquid nitrogen. The scan was taken as described under Figure 2 except that the centrifugation speed was 7200 rpm. The loading concentration of protein was 0.41 mg/ml.

TABLE II: Composition of Molybdoferredoxin (Mol Wt, 168,000).

Mo	1[a]
Fe	14[a]
Ca	1.7[a]
Mg	0.5[a]
Zn	0
Co	<0.01[a]
SH	23[a]
S^{2-}	16[a]
[S]	85[a]
C	49.9[b]
H	6.7[b]
N	15.3[b]

[a] Moles/mole of protein. [b] Per cent of protein.

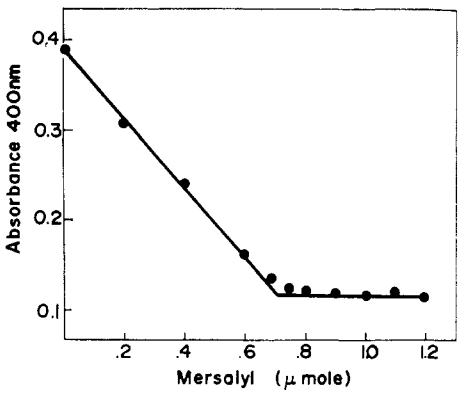

FIGURE 6: Titration of MoFd with sodium mersalyl; 2.1 mg (12.7 nmoles) of molybdoferredoxin in 50 μl was added to a cuvet fitted with a rubber serum stopper containing an anaerobic solution of 2.9 ml of 0.05 M Tris buffer, pH 8.0. An anaerobic solution of sodium mersalyl (10 mM) was added in 10-μl increments, *via* syringe, with a 30-min interval between additions. The experimental cuvet was read at 400 nm against a blank containing Tris buffer.

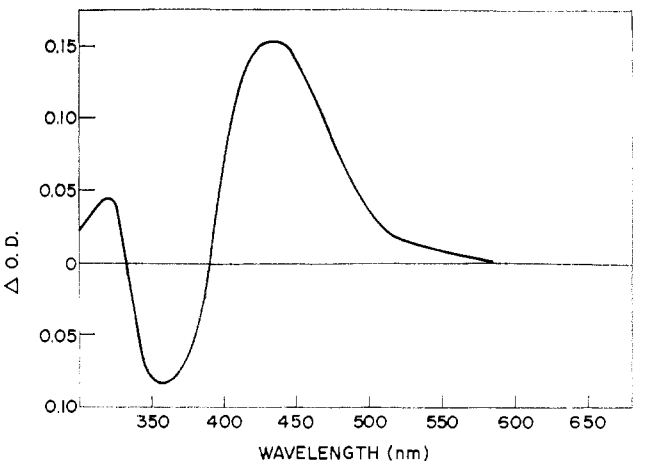

FIGURE 8: Difference spectrum of reduced minus oxidized MoFd. Conditions for the native (reduced) protein were the same as for Figure 5 except that the protein concentration was 2.0 mg/ml. Oxidized MoFd was prepared by exposing similar sample to air for 1 min. The cuvet containing native protein was placed in the reference cell.

Calcium and magnesium (and a trace of cobalt) were also found, but zinc was absent (Table II).

Sulfur-Containing Groups. Acid-labile sulfide (S^{2-}) and sulfhydryl groups (SH) are of special interest in MoFd. MoFd contains 16 acid-labile sulfide groups (Table II). By titration with sodium mersalyl 55 titratable SH equivalents are found (Figure 6) and since the 16 sulfide groups account for 32 SH equivalents, the remaining 23 equivalents are assumed to be sulfhydryl groups.

Spectrum. The spectrum of native MoFd is shown in Figure 7. The protein exhibits a broad absorption between 300 and 600 nm, but no distinctive peaks or shoulders are observed.

Enzymatic Activity. MoFd has no known enzymatic activity alone. All the activities of nitrogenase are catalyzed only when MoFd is added to AzoFd.

Effect of Oxidation. The effect of oxidation on MoFd was examined spectrophotometrically and by electron paramagnetic resonance spectroscopy and enzymatic assay. Exposure of MoFd to air for 1 hr results in the loss of only 70% of the enzymatic activity. MoFd turns reddish brown (as opposed to very dark brown for the native protein) within a minute after exposure to air, a phenomenon which can be recorded spectrophotometrically. The difference spectrum in Figure 8 is obtained by exposing MoFd in one cuvet to air for 20 sec and measuring the spectrum against a reference cuvet containing an equivalent amount of native MoFd. An attempt was made to follow the course of oxidation by measuring the increase in absorbance at 435 nm, but the maximum change occurred within 30 sec, about the time required to expose the protein to air and place it in the instrument. Obviously, the spectra

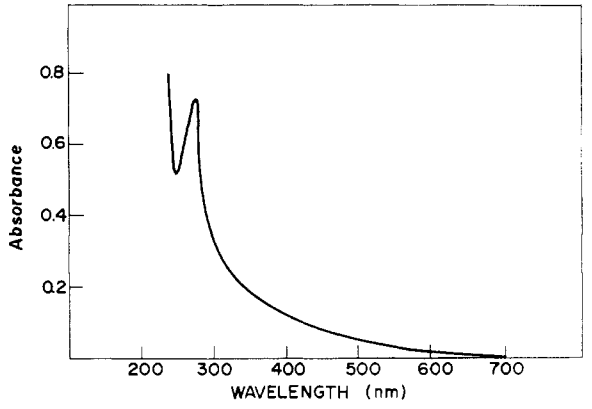

FIGURE 7: Spectrum of native MoFd. MoFd was added anaerobically to a cuvet containing an anaerobic solution of 0.1 M TES buffer, pH 7.0, to give a final protein concentration of 0.5 mg/ml and read against a blank containing no protein in a Cary 14 recording spectrophotometer.

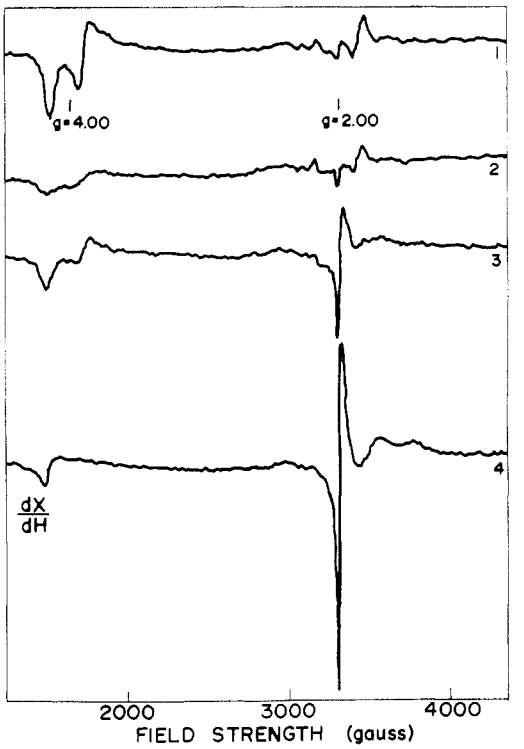

FIGURE 9: Electron paramagnetic resonance spectra of MoFd. Field modulation, 1000 kcps; gain setting, 200; chart speed, 1000 G/min; microwave frequency, 9.234. (1) Native MoFd, −238°; (2) MoFd + 5 mM $Na_2S_2O_4$, − 239°; (3) MoFd aerated at 25° for 1 min, −239°; (4) MoFd aerated at 25° for 5 min, −239°. All tubes contained 5.25 mg of MoFd and 2.5 μmoles of Tris-HCl, pH 8.0.

changes are not entirely indicative of a loss of enzymatic activity since inactivation requires over 60-min exposure to air.

Oxidation of MoFd can also be observed by change in its electron paramagnetic resonance spectrum. The first spectrum (Figure 9) is that of native MoFd. Small signals are evident in the regions of *ca.* $g = 4.3$ and $g = 2.01$. The effect of oxidation is shown in the third and fourth spectra (Figure 9). Oxidation is effected by shaking samples of MoFd in air for 1 and 5 min. The oxidized protein is syringed into electron paramagnetic resonance tubes, which are then immersed in liquid nitrogen.

After 1-min exposure to air, the signal at $g = 4.3$ (3, Figure 9) begins to disappear and a large, sharp signal appears at $g = 2.01$. After 5-min exposure to air, the $g = 4.3$ signal is almost completely gone and the $g = 2.01$ signal increases in size. The second spectrum in Figure 9 is that of native MoFd plus sodium dithionite. Reduction by dithionite (second spectrum) eliminates the signal at $g = 4.3$. Reduction of the oxidized samples with dithionite fails to diminish the $g = 2.01$ signal or to restore the $g = 4.3$ signal.

TABLE III: Metal Composition of MoFe Protein from Various Organisms.[a]

Organism	Fe	Mo	Reference
Cl. pasteurianum	89	6.9	This paper
	94	6.1	Vandecasteele and Burris (1970)
A. vinelandi	63	5.1	Bulen and LeComte (1966)
	126–141	7.4	Burns *et al.* (1970)
A. chroococcum	78.5	6.5	Kelly (1969)

[a] Values expressed as nmoles of metal/mg of protein calculated from data presented in the papers.

Discussion

The purification procedure described in this paper yields a product which is pure when examined by disc electrophoresis either in the presence or absence of sodium dodecyl sulfate (Figure 1b, 1c). Only the two subunits are seen in the latter. The procedure described here was representative of three different preparations. Variations do occur, since some preparations required only two protamine sulfate fractionations instead of the three described here. Such variation made it essential to monitor either the activity or some specific component of MoFd. The molybdenum content was the most convenient measure of the degree of purification if atomic absorption analysis was used.

Molybdoferredoxin can be purified within 2 days by the method reported. Although there are more steps involved in the purification than the method employed by Vandecasteele and Burris (1970), up to 2 g of purified protein was obtained compared to about 60 mg by the latter authors. We have found that purification of MoFd by DEAE-cellulose column chromatography caused a loss in activity (total units). If MoFd more than 70% pure was introduced to the column, we also noted a loss of sulfide, iron, and molybdenum from the protein.

The molecular weight (168,000) reported here is close to a value that was previously reported (Morris *et al.*, 1969). The value, 270,000, found by Burns *et al.* (1970), for the FeMo protein from *Azotobacter vinelandii* may represent a dimer of the protein since their Mo content was one per about 150,000.

The iron, sulfhydryl, and sulfide contents of the clostridial protein are much lower than that for the azotobacter protein, 14, 23, and 16, respectively, compared to 40, 40, and 30. If one compares the iron and molybdenum results obtained here with the values obtained from organisms other than *Azotobacter*, a close similarity is obvious (Table III).

Total sulfur analysis of clostridial MoFd revealed the presence of 85 sulfur atoms per protein. If there are 23 present as sulfhydryl and 16 as labile sulfide, then about 46 are present in methionine and cystine. Since there are approximately 45 methionine and 27–29 $^1/_2$-cystine residues, this would suggest that there is one disulfide linkage. Further work on amino acid determination and sequence is in progress.

Electron paramagnetic resonance signals at $g = 4.28$ have been attributed to high-spin ferric iron in pyrocatechase (Nakazawa *et al.*, 1969). The signals near $g = 4.3$, however, are unlike the usual high-spin ferric signals and must await further investigation. The signals near $g = 2.0$ are also difficult to interpret. Oxidized ferredoxin exhibits a signal at $g = 2.01$ which has been attributed to high-spin ferric iron in a weak crystal field (Palmer *et al.*, 1966). Reduced ferredoxin shows a complex signal similar to that observed for the so called "1.94 protein" (Brintzinger *et al.*, 1966) and had been attributed to low-spin ferric iron in tetrahedral symmetry. Bray and Meriwether (1966) have found a $g = 1.978$ signal in xanthine oxidase which they attributed to a Mo^{5+}–thiol complex. In MoFd, if both ferric iron and Mo^{5+} are present, these two signals could interfere with one another. The appearance of the large signal at $g = 2.01$ with oxidation could be a result of the oxidation of complexed Fe^{2+} to Fe^{3+}. The lack of a Mo^{5+} signal suggests that the Mo is in some oxidation state other than Mo^{5+}, a state that would not exhibit an electron paramagnetic resonance signal.

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Purification and Properties of a *c*-Type Cytochrome from *Micrococcus denitrificans**

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ABSTRACT: A *c*-type cytochrome can be removed from intact cells of *Micrococcus denitrificans* by washing with weak buffer containing KCl, then with weak buffer. Under proper conditions the yield of the cytochrome can be 40% of the pigment obtainable from broken-cell extracts. Under these conditions only small amounts of contaminating protein and no other cytochromes are removed by the washings. Purification of the cytochrome *c* from the washings is a simple procedure. The cytochrome has an absorption spectrum similar to that of mammalian *c*-type cytochromes, but the ratio of dicarboxylic to diamino acids is twice as great in the bacterial pigment. Only one histidine is present. The molecular weight of *M. denitrificans* cytochrome *c* is slightly larger than that of the mammalian cytochrome (around 135 amino acids as compared to 104) and considerably larger than the *c*-type cytochrome which purified from *Pseudomonas aeruginosa*.

Although purified soluble cytochrome *c* and membrane-bound or purified cytochrome *c* oxidase of the respiratory chain system from different eukaryotic species will interact rapidly (plants, yeasts, molds, insects, vertebrates of all classes), the oxidases of most bacteria oxidize the eukaryotic cytochromes *c* very slowly, if at all. Conversely, most of the isolated bacterial cytochromes of the *c* type are not rapidly oxidized by mammalian oxidases (Smith, 1968). Some of the soluble *c*-type cytochromes from the bacterial respiratory chain systems have absorption spectra and redox potentials similar to those of the mammalian-type pigment, but the isoelectric points are often different (Smith, 1968). This is of interest in relation to the postulated electrostatic interaction involved in the reaction of oxidase preparations from mammalian sources with eukaryotic cytochromes *c* (Davies *et al.*, 1964; Smith and Minnaert, 1965). The complete amino acid sequence of only one respiratory chain bacterial *c*-type cytochrome is known, that from *Pseudomonas aeruginosa* (Ambler, 1963a,b). It resembles eukaryotic cytochromes *c* in several respects: the attachment of the heme group is the same; there are no free sulfhydryl groups in either; and the charge distribution between the heme and the amino terminus is rather similar in the two types.

A soluble *c*-type cytochrome was isolated from *Micrococcus denitrificans* by Kamen and Vernon (1955) and found to have an isoelectric point on the acid side of neutrality, in contrast to the mammalian pigment with an isoelectric point above pH 10 (Margoliash and Schejter, 1966; Flatmark and Vesterberg, 1966). In spite of this difference, the oxidase of *M. denitrificans* can oxidize both the bacterial and mammalian cytochromes at very rapid rates (Kamen and Vernon, 1955; Smith *et al.*, 1966) and the mammalian oxidase can oxidize the bacterial *c*-type cytochrome at a rather low rate (Kamen and Vernon, 1955; Smith *et al.*, 1966). Thus these activities of the *c*-type cytochrome and the oxidase differ from those of most other bacterial species. In order to make a further study of these reactions and gain more knowledge of the bacterial *c*-type cytochrome, a simpler method for isolating and purifying this pigment was devised. The method is described here, together with some of the properties and the amino acid composition of the cytochrome.

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