ISOLATION BY CRYSTALLIZATION OF THE Mo-Fe PROTEIN OF *AZOTOBACTER* NITROGENASE

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SUMMARY

Homogeneous Mo-Fe protein of *A. vinelandii* nitrogenase was isolated in high yields as white needle-like crystals by decreasing the ionic strength of a solution rich in the Mo-Fe protein and free of the Fe protein of nitrogenase. Molecular weight of the di-molybdo crystallized protein is 270,000-300,000; approximate ratios of Mo:Fe:CySH:labile S are 1:20:20:15. The crystallized protein in combination with the Fe protein was essential for nitrogenase reactions including reduction of N_2, C_2H_2, CN^-, acrylonitrile and azide, and ATP-dependent H_2 evolution; specific activity was 1488 nmoles H_2 evolved/minute/mg Mo-Fe protein.

Studies of the characteristics of nitrogenase (N_2ase) have been limited by the unavailability of homogeneous enzyme or of its components, the Mo-Fe protein and the Fe protein. Homogeneous Mo-Fe protein is now available by the crystallization procedure described in this report. The procedure is striking in its simplicity, requiring little time or specialized equipment. Exceptionally high recoveries permit the production of half-gram amounts of crystals by a single operator within several days of crude extract preparation.

Previous work in several laboratories has established that N_2ase preparations from sources including *A. vinelandii* (1), *Clostridium pasteurianum* (2), *Klebsiella pneumoniae* (3), *Bacillus polymyxa* (3), and soybean nodules (4) can be fractionated into two components, one rich in Mo-Fe protein and the other rich in

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Fe protein. Labile sulfide has been associated with both proteins. Enzymic activity requires the presence of both proteins.

METHODS

Azotobacter vinelandii strain O (ATCC 13705) was grown in 20 l. cultures in a Brunswick Fermentor at 30° on a modified N-free Burke's medium containing (in g/l) sucrose, 20; $MgCl_2$, 0.2; $CaCl_2$, 0.06; $FeCl_3 \cdot 6H_2O$, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.0025; KH_2PO_4, 0.2; K_2HPO_4, 0.8; pH was 7.0-7.2. Cells were harvested with a Sharples Supercentrifuge. The cell paste was frozen in liquid N_2 and stored at -80° for up to 14 months. Frozen cells were thawed, washed in cold 0.025 M potassium phosphate buffer (150 ml buffer per 100 g cell paste), and suspended in buffer to give a volume of 275 ml per 100 g washed pellet. Cells were broken in a French pressure cell and then centrifuged 16 hrs. in the GSA rotor of a Servall RC-2 B centrifuge at 10,000 rpm to yield a crude extract containing 45-55 mg protein per ml with a specific activity of 40-60 nmoles H_2 evolved/min/mg protein. Nucleic acids were removed with protamine sulfate (1). All subsequent operations were performed anaerobically. The extract was heated at 60° for 10 min (5) and N_2ase was precipitated with protamine sulfate and resolubilized by treatment with cellulose phosphate (1) to yield a preparation with a specific activity of 280-320 nmoles H_2 evolved/min/mg protein and containing 65-85% of the initial crude extract activity. The preparation was then treated with ca. 3 mg $Na_2S_2O_4$ per gram protein, stirred 10 min., and fractionated on a DEAE cellulose column (1) into the Mo-Fe protein of N_2ase in the proteins eluted with 0.25 M NaCl and the Fe protein of N_2ase in the proteins eluted with 0.35 M NaCl.

Molybdenum (6), iron (7), and labile sulfide (8) were determined by established methods. Protein was determined

by the Biuret method (9). Enzyme activity was determined as described elsewhere (1,5,10).

RESULTS

Crystallization of Mo-Fe Protein

A N_2ase preparation from 370 g of frozen cell paste purified through the cellulose phosphate resolubilization step (see Methods) and containing 3610 mg protein in 69 ml 0.01 M potassium phosphate, pH 7.0, was treated with *ca.* 10 mg $Na_2S_2O_4$, stirred 10 min., then fractionated on a DEAE cellulose column (4.7 cm x 11 cm) as described in Methods. The fraction eluted with 0.25 M NaCl in 0.015 M Tris-HCl, pH 7.2, was concentrated in an Amicon Corp. ultrafiltration cell to give a solution of 1712 mg protein in 38 ml 0.25 M NaCl in 0.015 M Tris-HCl. This was mixed with 350 ml 0.02 M Tris-HCl, pH 7.2, and considerable brown-gray material came out of solution. The brown material appeared amorphous and the lighter material crystalline. After stirring gently for 60 min. the preparation was centrifuged for 5 min. at 20,000 X g. The almost colorless supernatant liquid was discarded; the dark brown pellet was suspended in 50 ml 0.02 M Tris-HCl, pH 7.2, and centrifuged. The washed pellet was dissolved in 15 ml 0.25 M NaCl in 0.015 M Tris-HCl, pH 7.2, and centrifuged to remove a small amount of undissolved material which was discarded. The dark brown supernatant solution, containing 700 mg protein in 21 ml, was mixed with 180 ml 0.02 M Tris-HCl, pH 7.2. The brown color disappeared with the formation of a dense population of white crystalline needles measuring approximately 50μ long x 3μ at the widest point (Fig. 1). These were collected by centrifugation as before and dissolved in 0.25 M NaCl in 0.015 M Tris-HCl, pH 7.2, to give 591 mg of 2X crystallized protein in 16 ml. Centrifugation of this preparation yielded no pellet. Homogeneity of this

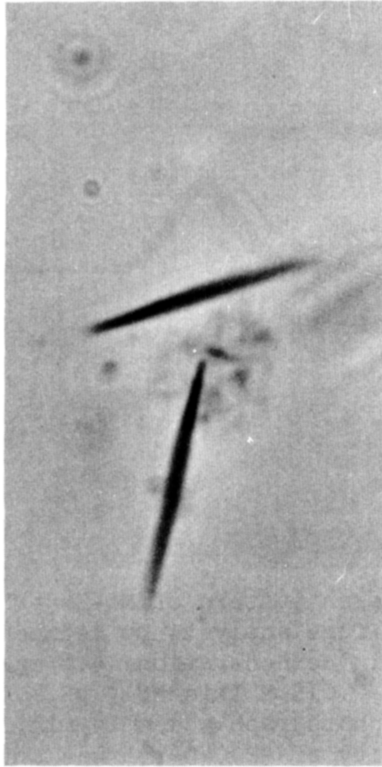

Figure 1. Phase contrast micrograph of crystals of Mo-Fe protein.

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preparation was indicated by ultracentrifuge analysis (Fig. 2) and by constancy of amino acid analyses and specific activity through three recrystallizations. All operations were performed under an atmosphere of N_2 and at room temperature, except that centrifugations were at 0-5°.

Characterization of Crystalline Mo-Fe Protein

Reactivity: The essentiality of the Mo-Fe protein for catalysis of N_2 reduction and C_2H_2 reduction is established by the data in Table I. ATP-dependent H_2 evolution and the reduction of other substrates of nitrogenase (11), including CN^-, acrylonitrile and azide

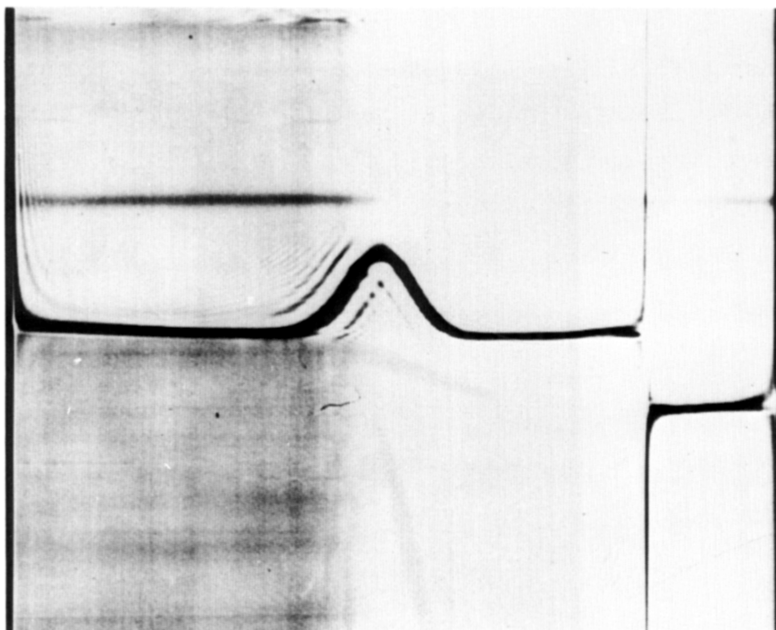

Figure 2. Schlieren pattern of Mo-Fe protein in ultracentrifuge analysis by sedimentation velocity. Protein concentration, 4.6 mg/ml in 0.25 M NaCl, 0.015 M Tris-HCl, pH 7.4; temperature, 20°; photograph taken 24 min. after full velocity of 42,040 rpm was attained; all operations performed anaerobically.

TABLE I

ESSENTIALITY OF Mo-Fe PROTEIN FOR N_2 REDUCTION AND C_2H_2 REDUCTION

Expt.	Mo-Fe Protein (2X crystallized)	Fe Protein Fraction	µmoles N_2 reduced	µmoles C_2H_2 reduced
	0	0.54	0.14	
1	0.36	0	0.00	
	0.12	0.54	0.72	
	0	0.48		1.13
2	0.51	0		0.00
	0.17	0.48		3.88

Reaction vessels contained, in 1.0 ml at pH 7.0, 5 µmoles Mg Cl_2, 5 µmoles ATP, 30 µmoles creatine phosphate, 10 units creatine kinase, 20 µmoles $Na_2S_2O_4$, and mg protein as indicated. Atmospheres: Expt. 1, 1.0 atm. N_2; Expt. 2, 0.8 atm Argon and 0.2 atm C_2H_2. Incubation time, 15 min. Temperature, 30°.

also specifically required the Mo-Fe protein. Titration of the Fe protein with Mo-Fe protein provided the activity curve of Figure 3; calculated from the initial portion of the curve, specific activity is 1488 nmoles H_2 evolved/min/ mg Mo-Fe protein.

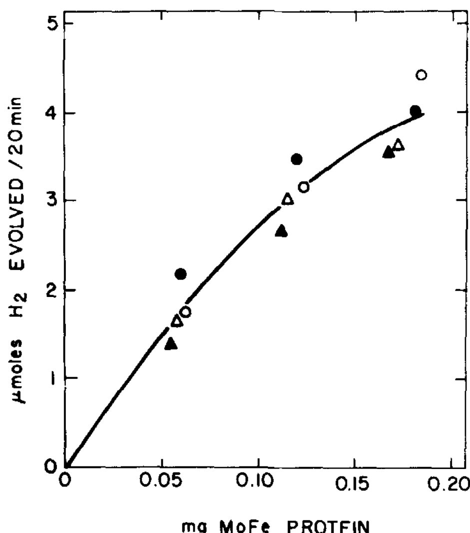

Figure 3. Activity titration of Fe Protein Fraction with Mo-Fe Protein. Reaction mixtures as in Table I except atmosphere was Argon; vessels contained 0.54 mg Fe Protein fraction and indicated amounts of 1X (O), 2X (●), 3X (Δ) and 4X (▲) crystallized Mo-Fe protein.

Molecular weight, metal, and labile sulfide analyses: The Fe, Mo and labile sulfide ($S^=$) content of three preparations is given in Table II. An emission spectrogram showed only slight traces of metals other than Mo and Fe. The calculated minimum molecular weight based on one atom of Mo per molecule is ca. 150,000. Molecular weight determined by Archibald approach to equilibrium is ca. 270,000, which suggests either a dimer or two Mo per molecule. The iron and labile sulfide contents are 34-38 Fe and 26-28 $S^=$ per molecular weight of 270,000. Amino acid analyses, which

TABLE II

METAL AND SULFIDE ANALYSES

	Preparation	μg Atoms per gram Protein Mo	Fe	$S^=$
1	Crystallized 2X	8.05	116	98
2	Crystallized 2X	7.41	131	105
2a	Crystallized from 2	7.90	122	103
3	Crystallized 2X	8.28	121	-
3a	Crystallized from 3	6.64	116	-
3b	3, after 16 hrs. dialysis	6.50	126	-

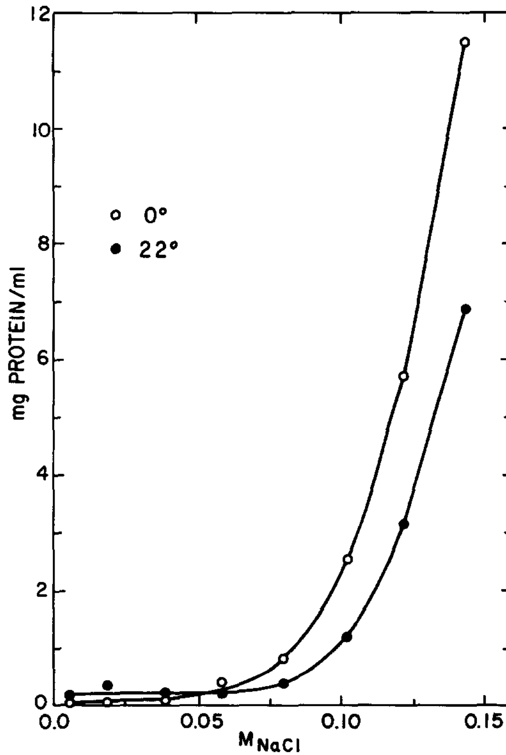

Figure 4. Solubility of Mo-Fe protein as a function of M_{NaCl} at 0° and 22°. Recrystallized Mo-Fe protein was added to the indicated NaCl solutions in 0.015 M Tris-HCl, pH 7.4, at each temperature, and stirred for 10 min.; undissolved protein was removed by centrifugation and protein concentration of the supernatant solution was determined; all operations performed anaerobically.

will be reported elsewhere, indicate an approximate equivalence of cysteine and metal. The protein can be dissociated into subunits; preliminary evidence indicates the presence of more than one type of subunit (12).

Solubility: The Mo-Fe protein is almost insoluble at NaCl concentrations less than 0.08 M, as indicated by the crystallization procedure and as shown in detail in Figure 4. Solubility increases sharply as the salt concentration is increased and protein concentrations in excess of 70 mg/ml are obtainable at 0.25 M NaCl. From the data of Fig. 4 the heat of solution for the protein is calculated to be -5.4 Kcal/mole and is independent of NaCl concentration. Qualitatively similar solubility behavior is observed when NaCl is replaced by KCl, KNO_3, Na_2SO_4, $MgCl_2$, NH_4Cl, Na-formate, Na-acetate, Na-phosphate, ammonium formate or Tris-HCl, indicating a general ionic strength effect.

Spectra: The visible and UV spectrum (Fig. 5) of the Mo-Fe protein shows a shoulder at 410-420 mμ, but otherwise lacks distinguishing features. On treatment with $Na_2S_2O_4$ the absorption at 410-420 mμ diminishes but a peak at 420 becomes more pronounced and slight absorption at 525 mμ and 557 mμ develops. Based on a molecular weight of 270,000, the millimolar extinction coefficients are 470 at 280 mμ and 85 at 412 mμ. Other properties including Mössbauer and magnetic susceptibility determinations, which will be reported elsewhere, suggest a predominance of high-spin Fe^{+3} and a small amount of high spin Fe^{+2} in the native protein; EPR measurements show resonances at g values of 4.30, 3.67, 2.01 and 1.94 in the reduced protein at 4°K.

In view of the known similarities of nitrogenases from different sources with respect to fractionation by DEAE cellulose chromatography (1-4) and cross-reactivities of fractions (3), extension of this crystallization procedure to Mo-Fe

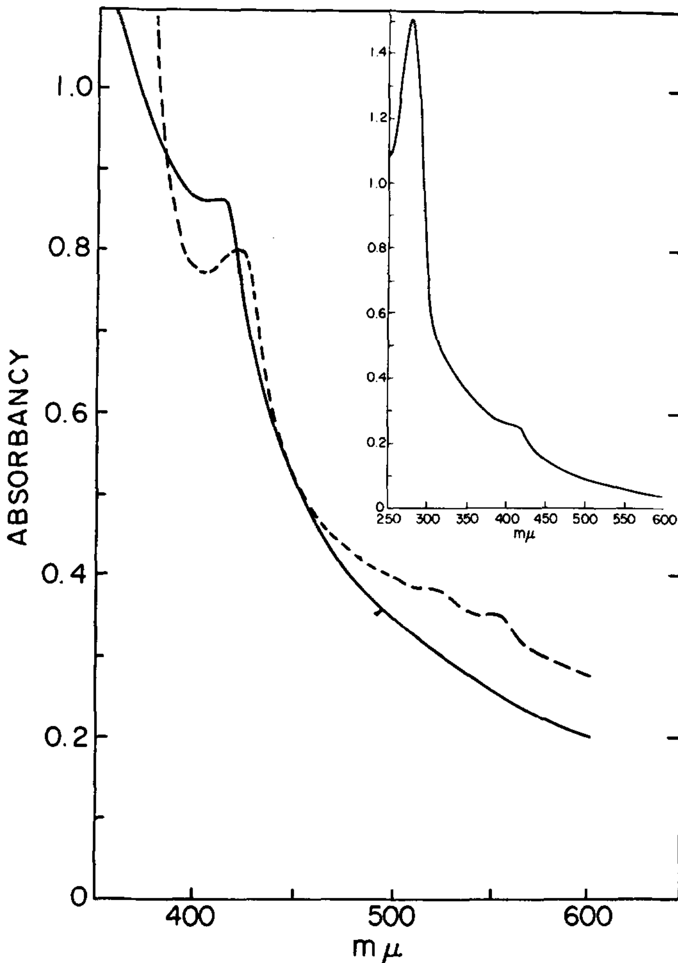

Figure 5. Visible-UV spectra of Recrystallized Mo-Fe Protein. Cuvettes contained 2.73 mg or (inset) 0.87 mg protein per ml 0.25 M NaCl, 0.01 M Tris-HCl, pH 7.4, under N_2. Native protein (—); plus $Na_2S_2O_4$(---).

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proteins from sources other than Azotobacter can be expected.

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