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LARGE-SCALE PURIFICATION OF HIGH ACTIVITY *AZOTOBACTER VINELANDII* NITROGENASE

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Summary

A large scale, rapid, high-yield purification procedure for *Azotobacter vinelandii* nitrogenase proteins has been developed. Yields of approx. 600 mg of the FeMo protein (*Av*1) and approx. 550 mg of the Fe protein *Av*2 are routinely obtained using a procedure that requires only 28 h. The specific activities of *Av*1 and *Av*2, respectively, are 3000 and 2100 nmol H_2 evolved/min per mg. These activities are significantly higher than those commonly used in reactivity studies. Procedures for the isolation and concentration of large quantities of iron-molybdenum cofactor of nitrogenase are also reported. Techniques for anaerobic protein manipulation, generally applicable to the purification of oxygen sensitive proteins are also described.

Introduction

Nitrogenase has been isolated from a variety of prokaryotic organisms and its overall composition and requirements for substrate reduction have been extensively studied [1]. For catalytic activity, two component proteins are required, these being designated the FeMo protein and the Fe protein. The extreme similarity of the component proteins isolated from different sources is well documented [2] and a general picture has emerged concerning the composition and reactivity of nitrogenase. The FeMo protein has a molecular weight of 220 000—245 000 and an $\alpha_2\beta_2$ subunit pattern, and has been reported to contain two Mo, 24—32 Fe atoms, and an amount of acid-labile sulfide roughly

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Abbreviations: Tes, *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid; BASF, Badische Anilin und Soda Fabrik.

comparable to that of iron [3]. Some of the Fe and all of the Mo are located in an extractable iron-molybdenum cofactor, in a reported approx. 8 : 1 atomic ratio [4] with most and possibly all of the remaining Fe in the form of 4Fe-4S clusters [5]. The Fe protein has a molecular weight of 57 000—65 000, an α_2 subunit pattern and contains a single 4Fe-4S cluster [6,7]. In addition to both component proteins, ATP, Mg^{2+} and a reducing agent ($S_2O_4^{2-}$ in vitro) are required for nitrogenase turnover [8].

Past attempts to evaluate nitrogenase reactivity have utilized detailed stoichiometric [9—12], kinetic [13—17], electrochemical [16,18] and thermochemical [19] techniques to probe nitrogenase reactions. More recent studies have focused on rapid reaction studies between the Fe and FeMo proteins or between these and artifical electron donors or acceptors [20—22]. The specific activities and purity of the component proteins used in these studies vary considerably and often fall below the values commonly reported in the preparative papers. In our work on the FeMo and Fe proteins of *Azotobacter vinelandii* (designated *Av*1 and *Av*2, respectively) [23,24] important differences appear when high-purity, high-specific-activity proteins are used in place of low-activity impure preparations.

In addition to the above requirements for high-purity, high-specific-activity nitrogenase, the application of physical probes such as EPR [26], Mössbauer [26], and extended X-ray absorption fine structure (EXAFS) [27] to nitrogenase and its FeMo cofactor requires very large quantities of protein. The isolation of the FeMo cofactor from *Av*1 into *N*-methylformamide [4,25] and the demonstration [26,27] that the Mo site in this cofactor is extremely similar to the Mo site in the FeMo protein, represent a significant discovery. Large quantities of FeMo cofactor are now needed for efforts at crystallization and for the continuing EXAFS studies. Further, chemical studies on intact FeMo and Fe proteins, including extrusion studies [5], attempts at sequencing [29] and crystallization, also demand large amounts of the pure component proteins.

This paper reports the procedures and techniques that we have developed for the isolation of greater than 0.5 g quantities of *Av*1 and *Av*2 with specific activities significantly higher than those commonly used in reactivity studies [30—32]. Due to an increasing emphasis on the iron-molybdenum cofactor, we also include detailed procedures for its isolation and concentration in large quantities. The techniques for anaerobic protein manipulation presented here should prove generally applicable to the purification of other oxygen-sensitive proteins, e.g. hydrogenase and formate dehydrogenase.

Materials and Methods

Anaerobic technique

Atmosphere. A commercially available Ar/H_2 (90%/10%) (Airco) gas mixture is used in our anaerobic system. The Ar/H_2 is scrubbed to remove O_2 by passing it through a BASF catalyst column heated to 100°C. The presence of H_2 continually regenerates the catalyst and allows the use of an Engelhard Pt-based deoxo-cartridge for additional oxygen scrubbing. As flushing is not used

to deoxygenate solvents, large quantities of Ar/H_2 are not used and the use of the expensive argon is not prohibitive.

Equipment. Schlenk techniques are very popular in anaerobic inorganic and organic chemical work [33,34] but have not been used extensively in biochemical manipulation. In our system a Schlenk manifold is used, where gas pressure maintenance and vacuum systems are independently constituted and only reach a common point at a three-way stopcock. This system allows a vessel connected to it (by vacuum tubing) to be either under pressure, under vacuum or isolated from the manifold. The gas delivery system starts with an Ar/H_2 tank regulated at 1.1 atm, followed by a mercury release valve (840 mmHg), the deoxo-cartridge, the O_2-scrubbing column and oil bubbler for observing gas flow. The gas manifold itself contains two 1000 ml round vessels which serve as ballast. The vaccum manifold has a Virtis solid CO_2/acetone macrotrap and a vacuum gauge to monitor the residual pressure in the system. The vessels employed include: (1) a serum-stopper capped Schlenk tube with side arm; (2) an anaerobic centrifuge tube with O-ring, Teflon adaptor and serum stopper; and (3) a three-necked round-bottom flask with adaptors for serum stoppers and connection to the manifold.

Operating principles and techniques. All centrifuge tubes, columns, buffer vessels and rubber tubing are maintained above atmospheric pressure (1.1 atm). Leaks into the atmosphere are indicated by the bubbler and are minimized by the use of new serum stoppers and rubber bands to hold glass joints in place. An inert atmosphere is produced in a given vessel by attaching it to a Schlenk manifold outlet through an adaptor or through a needle (for centrifuge tubes) and subsequently evacuating and filling several times with stirring if a solution is present. While evacuating and filling a given vessel other apparatus are isolated from the line. Anaerobic transfer of solutions between two vessels is accomplished by use of an 18—22-gauge stainless steel transfer line. With both vessels independently connected to the manifold, under positive pressure, the transfer line is inserted into the vessel of origin, through the septum but not (yet) into the liquid. The pressure differential between the vessel and the atmosphere causes a rapid flow of Ar/H_2 to sweep the air from the transfer line. Then, the other end of the line is inserted (while still blowing) into the receiving vessel. The transfer line in the vessel of origin is then brought below the surface of the liquid to be transferred. While maintaining this vessel on positive pressure, a small differential negative pressure is induced in the receiving vessel causing the solution to flow through the transfer line. The flow is stopped by returning the receiving vessel to full pressure. Volumes accurate to within 0.2 ml can be dispensed to or from a calibrated tube in this manner.

Anaerobic heat treatment of crude extracts uses a three-necked vessel set in a 70°C water-bath with stirring under Ar/H_2. Protein concentration is done using a BioRad hollow-fiber beaker concentrator with two ports for the fibers and two for the main chamber. After degassing and rinsing the beaker (0.025 M Tris-HCl (pH 7.4) + 1 mM $Na_2S_2O_4$) the dilute protein is added to the main chamber via a transfer line. The eluate is removed by pulling a slight vacuum (approx. 100 mmHg) on the fibers through a transfer line attached to a degassed collection vessel.

Glenco columns fitted with top plungers are used for column chromato-

graphy. Thick-walled saran tubing connects the columns to tygon pump tubing driven by Buchler peristaltic pumps. Columns are equilibrated with buffer containing 1 mM $Na_2S_2O_4$ until the $S_2O_4^{2-}$ can be detected coming off the column by its ability to reduce methyl viologen. Linear gradient elution is accomplished anaerobically by having equal volumes of maximal and minimal buffer in round-bottom flasks each attached to the Schlenk line. The maximal buffer is pumped into the minimal buffer with stirring as the minimal buffer is pumped onto the column. By setting the pump between the maximal and minimal buffers at one-half the column speed, a linear gradient is obtained.

Cell growth

Azotobacter vinelandii OP. *A. vinelandii* (American Type Culture Collection No. 13905 Rockville, MD), is grown in a modified Burk's nitrogen-free medium [35] containing 4.6 mM K_2HPO_4/1.5 mM KH_2PO_4/0.8 mM $MgSO_4 \cdot 7\ H_2O$/3.4 mM NaCl/0.3 mM $CaSO_4 \cdot 2\ H_2O$/0.01 mM $Na_2MoO_4 \cdot 2\ H_2O$/0.05 mM $FeSO_4 \cdot 7\ H_2O$ (dissolved in the same concentration of citric acid) and 2% sucrose. Large-scale cultures are grown in a 400 l fermentor aerated at 40 ft^3 per min. Growth is followed by measuring turbidity with a Klett-Summerson photoelectric colorimeter with a No. 64 filter. Cells in mid-logarithmic phase (Klett = 200) are harvested using a Sharples centrifuge and stored frozen at −80°C until needed. Yields are typically 1400 g wet weight per 350 l. *A. vinelandii* UW45 cells are grown as described above in a medium supplemented with 3.2 mM ammonium acetate and harvested 3 h after ammonia is exhausted as monitored qualitatively using Nessler's reagent.

Enzymatic assay

Nitrogenase. C_2H_2 reduction and H_2 evolution assays are performed at 30°C in 9.5 ml calibrated vials fitted with rubber serum stoppers and metal caps (Pierce), containing 0.1 atm C_2H_2 and 0.9 atm Ar for C_2H_2 reduction and 1.0 atm Ar for H_2 evolution. The reaction mixture used contains, in 1 ml, 38 mM Tes-KOH (pH 7.4)/2.5 mM ATP/5.0 mM $MgCl_2$/30 mM creatine phosphate/20 mM neutralized $Na_2S_2O_4$/0.125 mg creatine phosphokinase. The reaction vessels are automatically evacuated and flushed with the appropriate gas using an automatic instrument [42]. Then $Na_2S_2O_4$ is injected, as well as the desired amount of *Av*2 and, after preincubation (30°C for 5 min), the reaction is started by adding *Av*1 to give the appropriate molar ratio of the two components for a total of 1 mg protein per reaction.

After shaking at 30°C for 8 min, the reaction is terminated by adding 0.25 ml of 2.5 M H_2SO_4. Separate experiments reveal the reaction to be linear for at least 10 min. Gas samples, 200 μl at bottle pressure, are taken with a pressure-lock syringe (Precision Sampling) and are analyzed using a Hewlett-Packard gas chromatograph with a Poropak N column (He) for C_2H_2 reduction and a home-built gas chromatograph with a thermal-conductivity detector and a Molecular Sieve 5A column (Ar), for H_2 evolution. Calibration gases are obtained from Applied Science Laboratories (State College, PA). The sensitivity to H_2 or C_2H_4 is averaged from several determinations and the number of nmol H_2 or C_2H_4 in a given injection volume, at bottle pressure, is converted to a total number of nmol in the bottle using the calibrated volume for each

bottle minus the liquid volume (1.25 ml). 1 unit of *Av*2 or *Av*1 corresponds to an amount of protein that, together with the optimum level of the other protein, produces 1 μmol of H_2 per min under standard assay conditions.

FeMo cofactor. Isolated FeMo cofactor is assayed by a modification of the previously described procedure [4]. FeMo cofactor (300 nmol Mo per ml) is diluted with 10 vols 0.025 M phosphate buffer, pH 7.5, immediately prior to the addition of a 1—15 μl aliquot to 0.3 ml *A. vinelandii* UW45 crude extract. After incubation for 30 min at room temperature, 0.1 ml *A. vinelandii* UW45/FeMo cofactor is added to the C_2H_2 reduction assay mixture as described above without the addition of Fe and FeMo proteins. *A. vinelandii* UW45 crude extracts are prepared as described below.

When acid-treated protein is used instead of isolated cofactor the protein is diluted anaerobically to 2 mg/ml with H_2O, taken to pH 2.0 with 0.2 N HCl for 3 min, neutralized with an equal volume of 0.2 N NaOH and made 1.2 mM with respect to $Na_2S_2O_4$ prior to its addition to *A. vinelandii* UW45 crude extracts [43].

Molybdenum cofactor. Protein is acid-treated anaerobically [44] and assayed for NO_2^- [55] as described elsewhere. It has been reported that the activity of Mo cofactor as isolated from xanthine oxidase, increased with storage [46]. However, this finding can be attributed to a diminution of nitrite reductase activity upon storage of the *Neurospora crassa* nit-1 crude extracts. Sulfite, a potent inhibitor of nitrite reductase [45], is added to the Mo cofactor assay system to avoid this problem.

Analytical methods

Protein was determined by Biuret [36] and/or Lowry [37] methods calibrated with bovine serum albumin. Iron [38] and molybdenum [39] analysis and SDS-gel electrophoresis [40] were performed using published procedures. Salt concentration was determined using a Radio-meter (Copenhagen) conductivity meter calibrated with NaCl in 0.025 M Tris-HCl, pH 7.4, containing 1 mM $Na_2S_2O_4$. An ISCO Monitor (UA-5) was used to follow absorbance of column fractions at 405 nm.

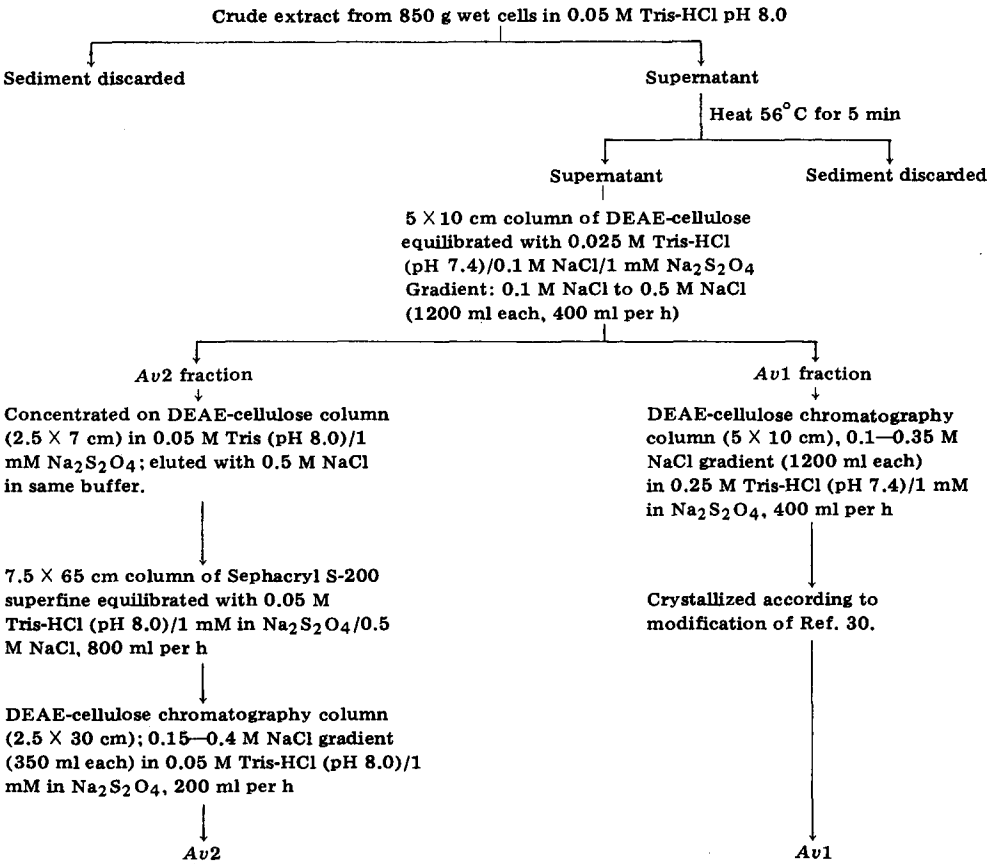
Chemicals

All chemicals employed, unless stated otherwise, were of the purest grade available. ATP, FAD, NADPH, Trizma base, Tes and creatine phosphokinase were obtained from Sigma Chemical Company (St. Louis, MO). Creatine phosphate was synthesized using published procedures [41]. Ar/H_2, Ar and C_2H_2/Ar were high purity gases obtained from Airco. Sephacryl S-200 superfine was obtained from Pharmacia Fine Chemicals (Piscataway, NJ), DE52 from Whatman (U.K.) and $Na_2S_2O_4$ from British Drug House Ltd. (Poole, U.K.). *N*-Methylformamide and *N,N*-dimethylformamide were obtained from Aldrich and were vacuum distilled prior to use. All other chemicals were Baker Analyzed Reagents.

Nitrogenase purification

Preparation of crude extracts. Cells (850 g) are washed with 1.4 vols. of 0.05 M Tris-HCl, pH 8.0, centrifuged at 10 000 × *g* for 10 min, resuspended in 1.4 vols. of the same buffer and ruptured in a Manton-Gaulin homogenizer at

12000 lb/inch^2. The crude extract is obtained by spinning the lysate at 14000 $\times g$ for 20 min at 4°C in a Sorval-RC2 centrifuge. Scheme I is a flow



Scheme I. Flow diagram of the purification of the two nitrogenase component proteins.

diagram of the purification procedure starting with this crude extract. All steps that follow are carried out in the absence of oxygen using the Schlenk system described above and all gels and buffers are 1 mM with respect to $Na_2S_2O_4$. The crude extract is incubated for 1 h at room temperature with 10 μg/ml deoxyribonuclease and ribonuclease and then heated with stirring to 56°C for 5 min. The precipitate is removed by centrifugation at 70000 $\times g$ for 45 min in a Beckman preparative ultracentrifuge (L2-53) at 4°C. Heat treatment at this stage of the purification has the advantages of eliminating 60% of the total protein with only minor loss of the total units of Fe and FeMo, of greatly reducing the amount of DEAE-cellulose and buffer needed to separate *Av*1 and *Av*2 [30] and of eliminating *A. vinelandii* flavodoxin, a major contaminating protein in the *Av*2 purification.

*Separation of Av*1 *and Av*2. *Av*1 and *Av*2 are separated on a DEAE-cellulose column (5 X 10 cm) previously equilibrated with 0.025 M Tris-HCl (pH 7.4)/0.1 M in NaCl and eluted with a linear gradient from 0.1—0.5 M NaCl (2400 ml at 400 ml per h) in the same buffer. The proteins are monitored by their

absorbance at 405 nm. The molar extinction coefficient at 400 nm for dithionite-reduced *Av*1 is $6 \cdot 10^4$ and for *Av*2 is $6.6 \cdot 10^3$ l · mol^{-1} · cm^{-1}, Fig. 1 is an elution profile of the first DEAE-cellulose column showing the separation of *Av*1 and *Av*2 and the salt gradient. *Av*1 typically elutes at approx. 0.12 M NaCl and *Av*II elutes at approx. 0.22 M NaCl. A previous procedure for *A. vinelandii* nitrogenase purification used batch elution of the two components with 0.25 M NaCl for *Av*1 and 0.5 M NaCl for *Av*2 [30]. When this procedure was scaled up 30-fold in our laboratory, more than 75% of the *Av*2 was eluted with the *Av*1 in 0.25 M NaCl.

*Further purification of Av*1. Following the DEAE-cellulose gradient, the *Av*1 fraction is diluted with an equal volume of 0.025 M Tris-HCl (pH 7.4), loaded on a 5 X 10 cm DEAE-cellulose column preequilibrated with the same buffer, and eluted with an 0.1—0.3 M NaCl linear gradient (2400 ml) at 400 ml per h. The peak fraction as monitored at 405 nm is collected, concentrated 18-fold in a hollow-fiber beaker (molecular weight cutoff = 80 000), transferred to two ultrafiltration cells (Amicon, Lexington, MA) with XM100A membranes and concentrated to 10 ml. The salt concentration of the protein is determined and it is diluted to 0.028 M NaCl with 0.025 M Tris-HCl, pH 7.4, and crystallized according to a modification of previous methods [30]. The *Av*1 solution is transferred to two 30 ml centrifuge tubes, and heated to 38°C in a water bath for 1 h. (The ultrafiltration cell is washed with 0.025 M Tris-HCl (pH 7.4)/ 0.25 M NaCl (5 ml) and this membrane wash is frozen for later recrystallization.) *Av*1 crystals are centrifuged at 20 000 X *g* for 20 min at 38°C and the supernatant is discarded. The crystals are washed with 0.025 M Tris-HCl (pH 7.4)/0.028 M NaCl, centrifuged at 20 000 X *g* (20 min at 38°C) and the supernatant is saved for later recrystallization. The crystals are gently resuspended in 6 ml per tube 0.025 M Tris-HCl (pH 7.4)/0.25 M NaCl (10 ml per tube) and centrifuged at 20 000 X *g* for 20 min at 4°C. Purified *Av*1 is formed into pellets by allowing drops from a 24-gauge needle to fall directly into liquid N_2. The pellets are stored in perforated liquid scintillation vials in liquid N_2. The typical concentration of this *Av*1 is 35 mg per ml.

*Further purification of Av*2. Following the first DEAE-cellulose separation,

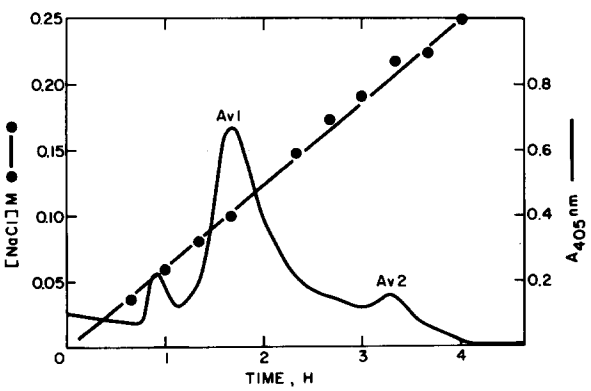


Fig. 1. Separation of *Av*1 and *Av*2 fractions on DEAE-cellulose. The column was developed as described in Results.

*Av*2 is diluted with 0.05 M Tris-HCl, pH 8.0, and adsorbed onto a DEAE-cellulose column (2.5 × 7 cm) equilibrated with the same buffer. The protein is eluted by running the column in reverse with 0.5 M NaCl in the same buffer. Using this technique, the concentration of 350 ml crude *Av*2 to 25 ml is accomplished in 2 h.

The concentrated *Av*2 is then loaded onto a Sephacryl S-200 superfine column (7.5 × 65 cm) preequilibrated with 0.05 M Tris-HCl (pH 8.0)/0.5 M NaCl. Without the addition of NaCl, *Av*2 does not elute from the Sephacryl column. *Av*2 is eluted at 800 ml per h and the peak fraction as monitored at 405 nm is collected, diluted with 2 vols. 0.05 M Tris-HCl, pH 8.0, and loaded onto a second DEAE-cellulose column (2.5 × 30 cm) previously equilibrated with 0.05 M Tris-HCl, pH 8.0. *Av*2 is eluted with a 0.15—0.4 M NaCl linear gradient (700 ml) at 100 ml per h, diluted and concentrated on DEAE-cellulose as described above. *Av*2 is stored as pellets in liquid N_2 for further use. The typical concentration of this *Av*2 is 30 mg/ml.

Results and Discussion

The purification of the two component proteins from cell rupture to storage of homogeneous *Av*1 and *Av*2 in liquid N_2 requires a total of 28 h with one full-time operator and a part-time assistant and can easily be divided into 3 days. If large quantities of *Av*1 are needed for work with FeMo cofactor, two preparations for *Av*1 alone can be accomplished in 40 h with one full-time operator and a part-time assistant and can easily be divided into 5 days.

*Properties of Av*1 *and Av*2

Yield. The quantitation of the purification of *Av*1 and *Av*2 is shown in Table I. An average of five preparations of *Av*1 gave yields of 601 ± 120 mg

TABLE I

The specific activity is nmol e^- pairs per min per mg protein as measured by C_2H_2 reduction for crude extracts and H_2 evolution for all other samples. H_2 evolution measurements in crude extracts are precluded by the presence of an active uptake hydrogenase.

Fraction	Volume (ml)	Activity units ($\times 10^{-6}$)	Total protein (mg)	Spec. act.	Activity (% units recovered)
*Av*1					
Crude extract	1630	4.53	76 300	59.4	100
Crude extract heated	1265	3.77	28 600	131.8	83
1st DEAE-cellulose	490	4.44	5 600	793	98
2nd DEAE-cellulose	435	3.13	3 050	1026	69
Crystals	16.5	1.71	588	2907	38
[Side fraction for recrystallization	18	0.62	611	1014	14]
*Av*2					
Crude extract	1630	1.93	76 300	25.3	100
Crude extract heated	1265	1.81	28 600	63.4	94
1st DEAE-cellulose	340	1.50	2 720	55.3	78
Sephacryl	210	1.25	1 350	927	65
2nd DEAE-cellulose	17.5	1.16	580	2017	61

FeMo. Yields as high as 900 mg have been obtained with cells stored at −80°C for less than 1 month. Cells stored 1 year at −80°C or longer give greatly reduced yields although no difference was found in the specific activity of the final proteins. Cells stored at −20°C instead of −80°C give much lower activities for *Av*2, while *Av*1 is unaffected.

Side fractions from the crystallization of *Av*1 can be combined and purified starting with the second DEAE-cellulose column step. Recovery of *Av*1 from this procedure is 60%, thus bringing the overall recovery of activity to approx. 50%.

An average of four preparations of *Av*2 gave yields of 560 ± 60 mg protein.

Activity. Specific activities are determined by titrating one component with the other as described in Materials and Methods. Table II shows the ratio used to determine the specific activity of each component. A detailed study of the effect of component ratio on total electron flow and distribution of electrons to products can be found elsewhere [23].

An average of five preparations of *Av*1 gave specific activities of 3070 ± 330 nmol of H_2 per min per mg *Av*1. An average of four preparations of *Av*2 gave specific activities of 2100 ± 170 nmol H_2 per min per mg *Av*2. Specific activity as measured by C_2H_2 reduction of *Av*1 or *Av*2 under 0.1 atm C_2H_2 gave lower numbers. For example, one preparation gave *Av*1 activity of 2907 nmol H_2 per min per mg. vs. 2106 nmol C_2H_4 per min per mg. However, analysis of the C_2H_2 reduction assay vials for H_2 gave 721 nmol H_2 per min per mg, which accounts for 90% of the missing electrons. Thus, with homogeneous preparation of *Av*1 and *Av*2, 0.1 atm C_2H_2 is not saturating as it does not completely inhibit H_2 evolution.

Metal analysis. An average of five preparations of *Av*1 gives 114 ± 10 nmol Fe, per mg and 7.34 ± 0.6 nmol Mo per mg *Av*1. The molar ratio of Fe to Mo in these preparations is 15.5 ± 0.3. Based on two atoms Mo per *Av*1, our preparations would have 31—32 Fe atoms per *Av*1 protein which agrees well with the most recently reported analyses and with inferences drawn from Mössbauer spectroscopic (MCD) studies [6].

An average of four preparations of *Av*2 gives 58 ± 6 nmol Fe per mg *Av*2. Based on an assumed M_r of 64 000, this value corresponds to 3.7 Fe atoms per *Av*2, in agreement with the identification of a single [4Fe-4S] center in *Av*1 by extrusion and MCD techniques [7,53].

Table II

ROUTINE ASSAY

Molar ratio *Av*2 to *Av*1 based on M_r 64 000 for *Av*2 and 240 000 for *Av*1 [24]. Specific activity in nmol H_2 per min per mg *Av*2 or *Av*1.

*Av*2		*Av*1	
Ratio	Specific activity	Ratio	Specific activity
0.7	607	24	2451
1.0	1772	30	3634
1.3	2017	40	3613
1.6	1472		

Tests for homogeneity. Both components are homogeneous by the criterion of gel electrophoresis. Neither component protein shows any activity in the absence of the other and the proteins have no uptake hydrogenase activity [24]. In addition to these standard tests for homogeneity, a criterion based on the presence of molybdenum-containing cofactors can be applied.

In 1970, it was reported that acid-treated preparations of *Av*1 and *Clostridium pasteurianum* molybdenum-iron protein (*Cp*1) activated the inactive nitrate reductase in crude extracts of the *N. crassa* mutant nit-1 [47]. This result led to the erroneous conclusion that nitrogenase and nitrate reductase share a common Mo-containing cofactor. In 1976, it was first shown that while acid-treated crude preparations of *Cp*1 could activate the mutant neurospora enzyme, homogeneous *Cp*1 could not [48]. This result was later confirmed for the *A. vinelandii* system [46].

The data in Fig. 2 indicate the original result was due to a contaminant in the *Cp*1 and *Av*1 preparations available in 1970. The Mo profile in Fig. 2 shows three major peaks corresponding to the three peaks identified for the *C. pasteurianum* system as: I, Mo storage protein; II, FeMo; and III, MoO_4^{2-} [49]. All *Av*1 activity was associated with peak II. When all fractions were acid-treated and added to the *A. vinelandii* UW45 assay for FeMo cofactor activity, only those fractions that contained *Av*1 had FeMo cofactor activity. When all fractions were acid-treated and added to the *N. crassa* nit-1 assay for Mo cofactor activity, only fractions 10—15 exhibited this activity. This Mo cofactor activity did not coincide with any of the three Mo peaks, but did overlap both peaks I and II. Thus, if the *Av*1 peak fractions were pooled, they would exhibit Mo cofactor activity. Preliminary results indicate the contaminant to be *A. vinelandii* nitrate reductase. Table III demonstrates, in agreement with other work [46,48], that the contaminating Mo cofactor activity can be completely eliminated by further purification of *Av*1.

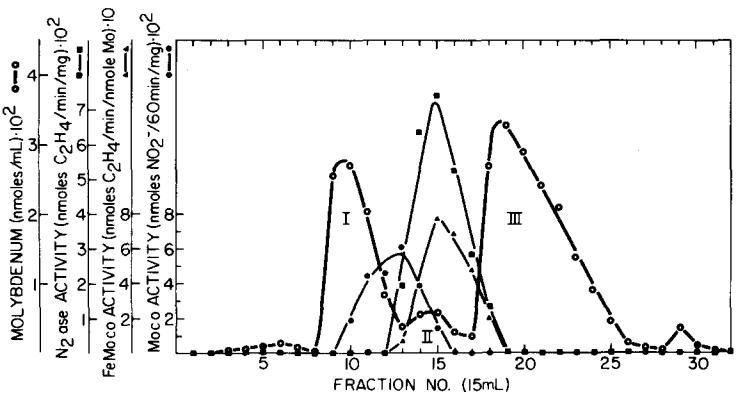


Fig. 2. Separation of Mo, *Av*1, FeMo cofactor activity (FeMoco) and Mo cofactor activity (Moco) on DEAE-cellulose. Crude extract from 100 g *Av* was not heat treated. Column was 2.5 × 30 cm, equilibrated with 0.025 M Tris-HCl, pH 7.4. Linear gradient was run from 0—1 M NaCl (350 ml each), in the same buffer at 100 ml per h. Fractions were collected anaerobically and assayed as described in Materials and Methods.

TABLE III

MOLYBDENUM COFACTORS ACTIVITY

A. vinelandii (*Av*) UW45 FeMo cofactor activity in nmol C_2H_4 formed per min per mg acid-treated protein. Nit-1 Mo factor activity in nmol NO_2^- formed per h per mg acid-treated protein.

Fraction	*Av* UW45 FeMo cofactor	Nit-1 Mo cofactor
*Av*1 1st DEAE-cellulose	405	255
*Av*1 2nd DEAE-cellulose	525	94
*Av*1 crystallized homogenous	1488	0
Side fraction (ultrafiltration before crystallization)	0	572

FeMo cofactor

General. The original procedure for FeMo cofactor isolation was outlined in 1977 [4]. Since that time, much effort has been expended to understand the FeMo cofactor [26,27]. Below, we describe a modification of the published procedure [4] and include details that are essential to successful isolation of FeMo cofactor using this method.

Isolation of FeMo cofactor. All operations are carried out in 12-ml clinical centrifuge tubes fitted with rubber serum stoppers under an Ar/H_2 atmosphere (1.1 atm) at 4°C. All reagents are kept on ice except for the 0.2 M Na_2HPO_4, which crystallizes from solution at 4°C. All reagents contain 1.2 mM $Na_2S_2O_4$ added as a 0.1 M aqueous, neutralized (to pH 7.4 with NaOH) solution, just prior to use. If $Na_2S_2O_4$ is added to the citric acid earlier, it will precipitate and result in lower yields of FeMo cofactor. $Na_2S_2O_4$ has low solubility in *N,N*-dimethylformamide.

Our procedure uses 240 mg *Av*1 as starting material, 15 mg per ml in 0.025 M Tris-HCl (pH 7.4)/0.25 M NaCl, distributed 30 mg per tube. The protein is diluted to 5.0 mg per ml with water (final volume equals 6 ml). Dilution with water prior to acid treatment is critical. The protein is acid treated by adding 1 ml of a 0.1 M citric acid solution. After 165 s, the protein is neutralized by adding 1 ml of a 0.2 M Na_2HPO_4 solution. After standing for approx. 25 min, the mixture is centrifuged at 210 $\times g$ for 10 min in a clinical centrifuge. If this centrifugation is too severe, a dark gray waxy pellet will result which cannot be extracted with *N*-methylformamide. The colorless supernatant is discarded and the pellet is washed twice with 4 ml of *N,N*-dimethylformamide followed by centrifugation at 660 $\times g$ for 10 min. Immediately after the addition of *N,N*-dimethylformamide, the tube is placed on a vortex mixer for 5 s and centrifuged for 10 min. Longer exposure to *N,N*-dimethylformamide results in a light yellow color in the supernatant and a dark brown pellet which cannot be extracted with *N*-methylformamide. The colorless supernatant is discarded and the pellet is extracted twice by vortexing vigorously for 5 min with 2 ml of *N*-methylformamide containing 2.4 mM Na_2HPO_4 added as an 0.2 M aqueous solution. The pellet is removed by spinning at 1100 $\times g$ for 10 min. The dark brown supernatant contains FeMo cofactor. This procedure can be easily performed by one person in 3 h. The first *N*-methylformamide extraction yields approx. 80 nmol Mo per ml and the second extraction yields

approx. 20 nmol Mo per ml. The recovery of Mo is approx. 90%. The FeMo cofactor solution is dark brown. If a pink cast develops, the FeMo cofactor has been inactivated and the solution should be discarded.

Concentration of FeMo cofactor. A major problem in working with the FeMo cofactor is that the final *N*-methylformamide solution contains only approx. 50 nmol Mo per ml. Since FeMo cofactor is in *N*-methylformamide, it cannot be concentrated using usual biochemical techniques. Vacuum distillation is complicated by the low vapor pressure and high boiling point of *N*-methylformamide, but can be used to concentrate 32 ml of FeMo cofactor to 2 ml in 1 h.

In a vacuum atmosphere dry box under Ar, FeMo cofactor is placed in a 200 ml round-bottom flask with a stir bar and is connected to a 500 ml round-bottom solid CO_2/acetone trap using a short glass U-tube with 24/40 joints. The trap is connected to a high vacuum line (10^{-7} atm) through a glass stopcock adaptor and a flexible steel tube. During concentration, the flask containing FeMo cofactor is placed in a room-temperature water-bath.

The most important features of this system are the large surface area of the trap, the short distance and lack of constrictions between the *N*-methylformamide solution of FeMo cofactor and the trap and the room temperature water bath.

Purity of FeMo cofactor. Following concentration, a large amount of white precipitate is formed which can be removed by filtration through a fritted glass filter using standard Schlenk techniques [50]. This step removes residual *Av*1, $Na_2S_2O_4$, Na_2HPO_4 and possibly other salts. A detailed analysis of this FeMo cofactor appears elsewhere [50,51].

An average of six FeMo cofactor preparations gives specific activities of 254 ± 30 nmol C_2H_2 reduced per min per nmol Mo. This activity would correspond to approx. 2000 nmol C_2H_2 reduced per min per mg FeMo protein as measured in crude extracts. These numbers have also been obtained for *Klebsiella pneumonia* FeMo cofactor [52] and FeMo cofactor from other organisms [4]. Higher numbers have been reported for *A. vinelandii* FeMo cofactor [4] corresponding to approx. 3500 nmol C_2H_2 reduced per min per mg *Av*1 using starting material with activities of only 1800 nmol C_2H_2 reduced per min per mg *Av*1 [30]. We have been unable to reproduce these results.

An average of six FeMo cofactor preparations as isolated gives ratios of 6.92 + 0.14 Fe per Mo. A detailed discussion of our lower values in metal analyses and the EPR properties of this FeMo cofactor appear elsewhere [50, 51].

Concluding remarks

The procedures presented here lead to *Av*1, *Av*2 and FeMo cofactor in high percent yields and large quantities. The two proteins have specific activities which are significantly higher than those commonly used in reactivity studies. The techniques used involve the melding of standard biochemical and Schlenk vessel manipulations and are generally applicable to the anaerobic purification of oxygen-sensitive proteins.

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