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The Molecular Weight of, and Evidence for Two Types of Subunits in, the Molybdenum–Iron Protein of *Azotobacter vinelandii* Nitrogenase

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The weight-average molecular weight of the Mo–Fe protein isolated from *Azotobacter vinelandii* has been determined by sedimentation-equilibrium techniques. In buffer, the value is 245000±5000; in 8M-urea, the value is 61000±1000. The protein was separated into two components by chromatography on CM-cellulose in 7M-urea, pH4.5. These components have similar molecular weights but were shown to differ in charge, amino acid content and arginine-containing peptides. It is proposed that the tetramer has the subunit composition ($n^{\alpha}{}_2n^{\beta}{}_2$).

The Mo–Fe protein‡ is a component of the nitrogenase of *Azotobacter vinelandii*. Since the separation of nitrogenase components (Bulen & LeComte, 1966) several methods have been used to determine their physical properties and to study their multimeric structure. A substantial disagreement about the value of the molecular weight of each component led us to apply sedimentation-equilibrium techniques to this problem. Some results have already been published (Swisher *et al.*, 1975). The present paper provides further supporting data and evidence that two types of structural subunits are present in the Mo–Fe protein.

Materials and Methods

Materials

Dithiothreitol, creatine kinase, Tris, Tes and ATP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; phosphocreatine was from Calbiochem, La Jolla, CA, U.S.A.; ^2H_2O was from Bio-Rad, Richmond, CA, U.S.A.; trypsin and carboxypeptidase A were from Worthington, Freehold, NJ, U.S.A. DEAE- and CM-cellulose were from Reeve Angel, Clifton, NJ, U.S.A.; sodium dodecyl sulphate was from Matheson, Coleman and Bell, Cincinnati, OH, U.S.A.; sodium dithionite was from Vine Chemical Co., Widnes, Cheshire, U.K.; and high-purity nitrogen and acetylene from Airco, Portland, OR, U.S.A. Traces of oxygen present in the nitrogen were removed by passing it through R3-11 catalyst from BASF, Paramus, NJ, U.S.A.

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‡ Abbreviations: Mo–Fe protein, molybdenum–iron protein; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulphonic acid.

Nitrogenase assay

The activity of the Mo–Fe protein was assayed in the presence of an excess amount of Fe protein by measuring the reduction of acetylene to ethylene. The assay system of Bulen & LeComte (1972) was used. Protein concentration was measured by the biuret method (Gornall *et al.*, 1949), and samples containing dithionite were exposed to air for 1h to eliminate interference (Shah & Brill, 1973).

Purification procedure

A. vinelandii strain OP (A.T.C.C. no. 13705) was grown on nitrogen-free medium (Burns *et al.*, 1970) in a 25-litre fermenter. Bacteria were harvested in the exponential phase 16–20h after inoculation.

Mo–Fe protein was prepared by a modification of the method of Shah & Brill (1973). The bacteria were broken with a French press and the heat step was omitted. Protein from the first DEAE-cellulose column was diluted with O_2-free 25mM-Tris/HCl buffer, pH7.5, and applied directly to a second anaerobic DEAE-cellulose column. All other details did not differ from the procedure of Shah & Brill (1973). For critical experiments the Mo–Fe protein was crystallized twice.

Ultracentrifugation

All experiments used a Spinco model E instrument equipped with temperature control, electronic speed control and both interference and schlieren optics. An An-H rotor fitted with 12mm cells containing sapphire windows and aluminium- or carbon-filled Epon centrepieces was used. Interference patterns were measured with a Nikon Shadowgraph and the data were processed by computer programs written in this laboratory for a Varian 620/i computer. All sedimentation-velocity runs were made at room temperature (20°C) and 56000rev./min.

The Chervenka (1970) modification of the meniscus-depletion method of sedimentation equilibrium was used. Protein samples were ultrafiltered with an Amicon (Lexington, MA, U.S.A.) 8MC micro-ultrafiltration unit. After 5 or more vol. of buffer had equilibrated with the protein, the ultrafiltrate was suitable for use as the reference 'diffusate'. Samples were introduced into anaerobic centrifuge cells and all operations were carried out in a purified nitrogen atmosphere. Densities of solutions were determined with a DMA 02C Digital Precision Densitometer.

The apparent partial specific volume $\bar{v}$ $(\partial\rho/\partial c)$ of the Mo–Fe protein was evaluated with a magnetic densitometer (Goodrich *et al.*, 1969). For this purpose the protein solution was prepared in 20mM-potassium phosphate buffer, pH7.0. The values obtained were confirmed by the method of Edelstein & Schachman (1967). The protein solution was prepared in ^2H_2O buffered with 25mM-potassium phosphate, pH7.4, containing 0.25M-NaCl and 0.1mg of sodium dithionite/ml.

Polyacrylamide-disc-gel electrophoresis

An apparatus similar to that described by Davis (1964) was used. The method of Weber & Osborn (1969) was used when the gels contained sodium dodecyl sulphate. The buffer used was 25mM-Tris/HCl, pH7.5. Electrophoresis was also carried out at pH2.9 (100mM-acetic acid) or pH9.2 (50mM-Tris/acetate) in the presence of 8M-urea. Gels containing urea were polymerized by the method of Peterson (1972). Gels were stained with Coomassie Blue R-250 in 12.5% (w/v) trichloroacetic acid for 12h and destained in 5% (v/v) acetic acid.

Protein was prepared for electrophoresis in urea by dissolving in 8M-urea/1% 2-mercaptoethanol/1% EDTA sodium salt. Thiol groups were blocked with iodoacetamide by the method of Crestfield *et al.* (1963). The carboxymethylated Mo–Fe protein was dialysed against water and freeze-dried.

Peptide 'mapping'

Protein solutions were prepared as for electrophoresis in urea. In some cases the thiol groups were oxidized by performic acid (Hirs, 1967).

Samples of the dry *S*-(carboxyamidomethyl)-protein (2–5mg) were suspended in 0.2M-*N*-ethylmorpholine acetate, pH8.5, or 0.1M-NH_4HCO_3, pH7.4. Trypsin was added as a 1mg/ml aqueous solution and incubated at room temperature with effective stirring. Trypsin was added at 5h intervals in such quantity that it never exceeded 2% of the weight of Mo–Fe protein.

Peptide 'maps' were obtained by spotting 3–10μl of hydrolysate (containing about 0.05mg) as a 0.5cm-diam. spot on cellulose thin-layer plates (Eastman Kodak, Rochester, NY, U.S.A.). Plates were developed in the first dimension by chromatography with 3-methylbutan-1-ol/pyridine/water (7:7:6, by vol.). Development in the second dimension involved electrophoresis at pH2.0 (2% formic acid, 8% acetic acid, v/v) or pH3.5 (0.5% pyridine, 5% acetic acid, v/v). The developed plates were sprayed with an arginine-specific reagent (Yamada & Itano, 1966). After 30min the plates were illuminated with u.v. light and the fluorescent spots counted.

Results and Discussion

Purity

When assayed in the presence of a 50-fold excess of Fe protein the activity of twice-crystallized protein was 1500nmol of ethylene/min per mg of Mo–Fe protein. Shah & Brill (1973) reported 1640, and Burns & Hardy (1972) reported 1440nmol/min per mg.

Freshly prepared protein examined by sedimentation-velocity ultracentrifugation formed a single symmetrical boundary. Solutions of protein which had been frozen and thawed, or kept at 5°C for 24h, formed an additional boundary, indicating a lower-molecular-weight contaminant. At temperatures near 0°C, no such boundary was visible up to 24h. Protein solutions left at room temperature for 24h developed both high- and low-molecular-weight artifacts. All samples when subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gave only one boundary.

Molecular weight of the Mo–Fe protein

Since the protein tended to become polydisperse with time, the Chervenka (1970) modification of sedimentation-equilibrium ultracentrifugation was used. Equilibrium was reached in 12–15h. Twice-crystallized protein in 50mM-Tris/HCl buffer, pH7.8, containing 0.25M-NaCl and 10mM-sodium dithionite was used. A 25mM-potassium phosphate buffer, pH7.0, could also replace Tris/HCl. Centrifugation was at 9000–10000rev./min at 0–5°C. The linearity of the data is typified by a previously published Figure (Fig. 1 of Swisher *et al.*, 1975). A plot of molecular weight against concentration revealed that there was no variation throughout. The average molecular weight from several experiments was 245000 ± 5000.

The data plots showed no indication of dissociation even at the lowest protein concentration, 0.12mg/ml. However, when protein only once crystallized was used, or protein solutions were subjected to freezing and thawing, downward curvature of plots (molecular weight against concentration) indicated the presence of dissociation artifacts. Analysis of the data from

these ultracentrifuge experiments by the method of Roark & Yphantis (1969) provided evidence that the heterogeneity was due to two components of mol.wt. 125000 and 250000. These values point to a dissociation of the tetramer to dimer. Further analysis of the data by the relationships derived by Adams & Fujita (1963) led to the conclusion that the tetramer and dimer were not in chemical equilibrium, and hence irreversible denaturation was being observed.

Determination of apparent partial specific volume

Since a reliable molecular weight can be calculated from sedimentation-equilibrium data only if the value of $\bar{v}$ is known, three methods were used to evaluate it.

By using the values for amino acid composition reported by Kleiner & Chen (1974) we obtained the value of $\bar{v} = 0.73$ ml/g.

Data obtained by the method of Edelstein & Schachman (1967) were satisfactory at protein concentrations above 0.5mg/ml. The molecular weight, corrected for ^2H exchange, was 243000±6000 and confirmed the $\bar{v}$ value above.

An attempt was made to determine the $\bar{v}$ value directly by magnetic densimetry. Experimental difficulties prevented us from obtaining results of the desired precision, but the average of the values obtained was 0.73.

Molar extinction coefficient

By using accurate dry-weight values (Goodrich & Reithel, 1970) for protein solutions whose absorbance had been measured carefully, we were able to calculate a reliable extinction coefficient. Assuming a mol.wt. of 245000, the absorption coefficient at 280nm (25mM-potassium phosphate, pH7.0) was 420000±2000 litre·mol^{-1}·cm^{-1}. When the protein concentration was measured by the method of Lowry *et al.* (1951) the calculated value of the coefficient agreed within a 5% experimental error.

Evaluation of the monomer molecular weight

The results of gel electrophoresis of protein treated with sodium dodecyl sulphate suggested a monomer molecular weight of 56000±3000. To confirm and establish this value more reliably, sedimentation-equilibrium ultracentrifugation experiments were done. Fig. 1 is a plot of molecular weight against concentration for a typical experiment. The average mol.wt. obtained was 61000±1000, assuming $\bar{v}$ = 0.73ml/g.

Both techniques gave no indication that more than one kind of polypeptide was present in the tetramer. This result differs from the observations on Mo–Fe protein from other bacterial species (Huang *et al.*, 1973; Eady *et al.*, 1972). Since it seemed possible that there might be two types of polypeptide subunit present having the same molecular weight, but differing in charge, electrophoresis on gels containing 8M-urea was used.

Electrophoresis of carboxyamidomethylated Mo–Fe protein in 100mM-acetic acid (pH2.9)/8M-urea on polyacrylamide gels resulted in two boundaries. Fig. 2 shows the patterns obtained from an optical

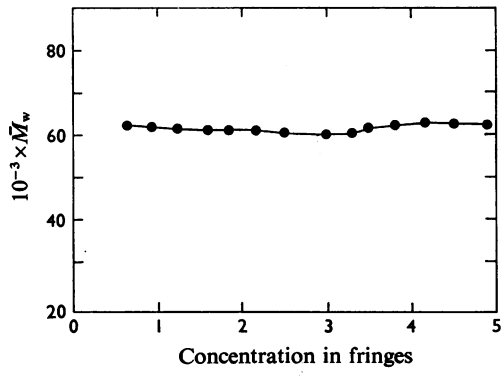

Fig. 1. *Monomer molecular weight of Mo–Fe protein in 8M-urea*
Mo–Fe protein was dialysed for 24h against 60mM-potassium phosphate buffer (pH7.0)/125mM-2-mercaptoethanol/2.5mM-EDTA (sodium salt)/8M-urea. The sample was ultracentrifuged (Chervenka, 1970) at 22000rev./min and 24°C.

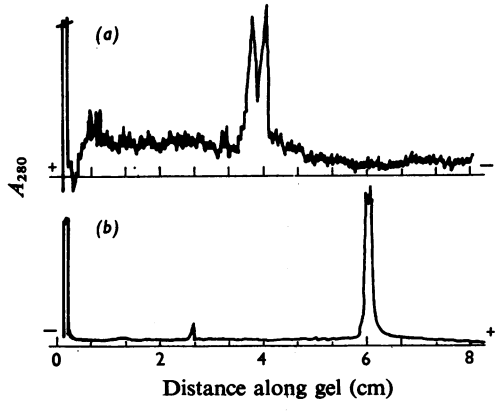

Fig. 2. *Electrophoresis of carboxyamidomethylated Mo–Fe protein in 8M-urea*
Electrophoresis of carboxyamidomethylated protein was carried out (*a*) in 8M-urea/100mM-acetic acid, pH2.9, or (*b*) at pH9.2 (50mM-Tris/acetate). The point of sample application is at the left of the optical scanning pattern.

scanning of the stained disc gels. Identical results were obtained when 100mM-citric acid, pH2.2, was used instead of acetic acid. Moreover, protein carboxymethylated with iodoacetic acid and native protein yielded similar results. In each case the densities of the two bands were equal.

To check the possibility of interconversion, the gels were sliced to separate physically the two presumptive components. The gel slices were minced and the protein fractions eluted by soaking in 8M-urea/0.1M-acetic acid for 12h at 37°C. The eluted proteins were again subjected to electrophoresis on disc gels as above, and their mobilities were unchanged. Only one boundary was now present, indicating that no ready interconversion could occur.

C-Terminal amino acid analysis

The action of carboxypeptidase A on Mo–Fe protein is evident from the results given in Table 1. Increasing the hydrolysis time beyond the normal 5h did not change the amino acid yield. In a typical experiment, 54nmol of Mo–Fe protein was exposed to 1.0mg (or 28nmol) of carboxypeptidase. Addition of more hydrolase increased the amino acid yield only slightly. All yields were corrected for transfer losses on the basis of norleucine recovery.

Table 1. *Amino acids hydrolysed from Mo–Fe protein by carboxypeptidase A*

For this analysis, by the method of Ambler (1972), the protein was prepared by dissolving in 8M-urea/1% 2-mercaptoethanol and keeping at 43°C for 4h. After dialysis against water and freeze-drying, the dry protein was weighed and dissolved in 0.2M-*N*-ethylmorpholine acetate, pH8.5, containing 1% sodium dodecyl sulphate. The final concentration of about 3mg/ml was known from precise measurements of weight and volume. To samples (1.00ml) were added 0.1–2.0mg of carboxypeptidase A and the mixture was incubated at 37°C for 1–20h. Liberated amino acids were absorbed by dry beads of Dowex 50W (X8; H^+ form) added until the pH fell to 3 or less. After washing and elution with 5M-NH_3, the amino acid samples were analysed with a Technicon amino acid analyser. In the top set of values a 30% transfer loss of hydrolysed amino acids was assumed on the basis of standard norleucine recovery. For the bottom set of values two additions of enzyme were made 10h apart.

Carboxypeptidase (mg added)	Yield (nmol/nmol of subunit)		
	Alanine	Serine	Valine
0.1	0.27	0.08	0.05
1.0	0.85	0.41	0.35
2.0	0.86	0.44	0.42
1.0+1.0	0.81	0.40	0.35

We interpret our results to show that alanine is the *C*-terminal residue of all polypeptide chains in the Mo–Fe protein. Moreover, since serine and valine were released in about one-half the yield of alanine, it seemed likely that polypeptide sequences of two kinds are present.

Peptide 'mapping'

When carboxyamidomethylated Mo–Fe protein was exposed to trypsin, the solution remained turbid despite repeated additions of trypsin over a 48h period. Analysis of the hydrolysates on cellulose thin-layer plates showed much unhydrolysed material at the origin. Despite this, 30–45 spots were visible after spraying. Mo–Fe protein oxidized with performic acid yielded similar results. Complete solubilization was achieved by treating carboxyamidomethylated protein with trypsin in 0.2M-*N*-ethylmorpholine buffer, pH8.5, containing 10mM-$CaCl_2$ and 5M-urea. Urea was removed from the peptide mixture with a small column of Sephadex G-10.

The number of arginine peptides expected on the basis of the amino acid composition (Kleiner & Chen, 1974) was 26 if the subunits were identical, or 52 for two subunits with unique polypeptide sequences. In all cases, even when complete hydrolysis seemed uncertain, more than 30 spots were observed after spraying. Again, no more than 45 spots were ever seen, suggesting some homology between the two polypeptides.

Chromatography on CM-cellulose in 7M-urea

Mo–Fe protein in sodium acetate buffer containing 7M-urea was fractionated on CM-cellulose columns and eluted with gradients of sodium acetate containing 7M-urea. Experiments were done with solutions of pH4.0, 4.6 and 5.1. The appropriate ionic strength required for elution was determined in each case. From the A_{280} of the fractions it appeared that the protein was eluted as a broad peak at either pH4.0 or 5.1. At pH4.5, sufficient resolution was obtained so that these protein fractions could be compared by electrophoresis in gels containing urea.

Fig. 3 shows the elution profile for one such experiment. Various fractions were examined by electrophoresis in gels containing 8M-urea. Leading fractions from the first peak contained the component having the highest mobility in urea gels (n^β), with only a trace of the component having the lower mobility (n^α). The second peak contained component n^α with only a trace of component n^β.

The two eluted peaks were never equal in size, despite the fact that our results with gel electrophoresis indicated two components in equal amounts. The difference could be ascribed to differences in

tryptophan and tyrosine content, but the data of Table 2 suggest that the absorbance of component n^{α} should be 83% of that of component n^{β}. This cannot account entirely for the difference in peak areas, since component n^{α} appears to be only 50% of component n^{β} on the basis of A_{280} of the eluted fractions.

However, other data showed that 30–40% of the protein applied to the column was not being eluted, even when 2M-sodium acetate was used for elution. It is quite possible that component n^{α} is much more strongly adsorbed and that this accounts for the disparity in yield.

The amino acid contents of components n^{α} and n^{β} are given in Table 2. Very noticeable differences exist in the content of threonine, serine, glycine, isoleucine, leucine and phenylalanine. These differences were much larger than the 1–3% deviations among duplicate determinations of the same sample.

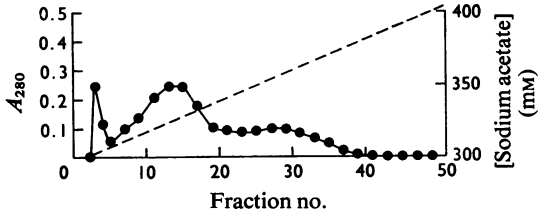

Fig. 3. *CM-cellulose chromatography of Mo–Fe protein in* 7M-*urea*

Whatman CM-32 cellulose was equilibrated with 250mM-sodium acetate (pH4.6)/7M-urea. A (0.5cm × 15cm) column was formed with a flow rate of 12ml/h. Carboxymethylated Mo–Fe protein (10–15mg) was dissolved in the buffer described and applied to the column. A linear gradient of 300–400mM-sodium acetate/7M-urea was begun, and fractions collected at 9min intervals. ●, A_{280} (protein); ----, sodium acetate gradient.

Discussion

Our experiments have shown that, to obtain linear data in the ultracentrifuge determination of the molecular weight, it was necessary to use (at least) twice-crystallized Mo–Fe protein, to maintain solution temperatures near 0°C and to decrease the time to a minimum. With a reliable molecular weight for the intact protein it was possible to establish that it was a tetramer consisting of subunits identical in weight.

Separation of two subunit fractions of differing electrophoretic mobility and amino acid composition led to the conclusion that two types of polypeptide chains were present as in the Mo–Fe protein from *C. pasteurianum* (Dalton *et al.*, 1971) and from *K. pneumoniae* (Eady *et al.*, 1972). This, together with

Table 2. *Amino acid analysis of the two structural subunits, n^{α} and n^{β}, of Mo–Fe protein*

Fractions eluted from the CM-cellulose column were pooled, dialysed against water and freeze-dried. These samples were hydrolysed in constant-boiling HCl for 24h at 110°C in evacuated hydrolysis tubes. The number of residues/61 000 daltons is based on the average of two duplicate samples. The deviation between the duplicate runs is indicated.

	Content (residues/61 000 daltons)				
				Mo–Fe protein	
Amino acid	n^{α}	n^{β}	$(n^{\alpha}+n^{\beta})/2$	Kleiner & Chen (1974)	Burns & Hardy (1972)
Lys	39.6±0.3	37.5±0.5	38.6	36.7	40.1
His	15.5±0.1	11.5±0.2	13.5	14.1	12.4
Arg	22.2±0.3	28.0±0.2	25.1	24.3	24.3
Cys	8.7±0.1	10.0±0.1	9.4	7.3	7.6
Asp	63.9±0.5	56.8±0.1	60.4	56.2	56.2
Thr	32.4±0.3	24.1±0.3	28.2	26.3	26.0
Ser	30.3±0.8	37.8±0.7	34.0	26.0	26.0
Glu	61.3±0.4	62.0±0.7	61.6	59.6	56.5
Pro	28.8±0.9	25.6±0.6	27.2	24.8	22.9
Gly	45.3±0	53.0±0.9	49.1	46.0	46.6
Ala	36.1±0.5	41.8±0.7	43.5	33.9	38.1
Val	26.7±0.7	24.7±0.2	25.7	36.4	30.0
Met	20.5±0.3	18.2±0.1	19.3	17.2	19.5
Ile	18.6±0.1	25.1±0.3	21.9	30.5	30.2
Leu	45.3±0.2	35.3±0.5	40.3	40.4	42.9
Tyr	17.9±0.2	22.5±0.3	20.2	19.8	17.8
Phe	30.2±0.7	21.3±0.3	25.7	28.0	23.1
Trp	9.0	10.7	9.85	7.1	11.3

the molecular-weight data, strongly suggests that the tetrameric Mo–Fe protein from *A. vinelandii* is an $\alpha_2\beta_2$ type.

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