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MOLECULAR WEIGHT DETERMINATION OF THE SUBUNITS OF MOLYBDOFERREDOXIN BY SEPHAROSE 6B IN THE PRESENCE OF 6 M GUANIDINE-HCl[1][2][3]

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Abstract

Molybdoferredoxin, a component of the nitrogenase of *Clostridium pasteurianum*, dissociates into two different subunits in the 6 M guanidine-HCl solution. The molecular weights of the subunits were found to be 62000 and 52500 based on Sepharose 6 B-6 M guanidine-HCl gel filtration.

Introduction

Molecular weight determination by SDS gel electrophoresis of the subunits of molybdoferredoxin (MoFd), a component of nitrogenase from *Clostridium pasteurianum*, has been reported by Nakos and Mortenson (1971). However, there are several reports which suggest that the charge properties of protein may affect the accuracy of molecular weight determination by SDS gel electrophoresis (Tung and Knight, 1971; Panyim and Chalkley, 1971).

When proteins are treated with 6 M guanidine–HCl in the presence of a reducing agent such as 2–mercaptoethanol, they behave like randomly coiled linear polymers (Tanford *et al.*, 1967). If the proteins have more than one polypeptide chain, all the subunits dissociate and unfold. Thus, regardless of what their native structure might have been, all proteins possess the same gross conformation in the reduced state in concentrated guanidine–HCl.

Porous cross-liked gels have been widely used in the field of biochemistry for the separation of biological macromolecules according to their size. Andrews (1965) has suggested that the elution position of a native protein from these gels can be correlated directly with its molecular weight; however, this

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treatment is adequate only when the unknown protein has the same shape or gross conformation as the proteins used to calibrate the column.

With knowledge of the protein structure in 6 M guanidine-HCl solution, Fish, *et al.* (1969) showed a correlation between molecular weight and elution volume for proteins chromatographed in 6 M guanidine-HCl. This technique has been successfully used by Bryce and Crichton (1971a, 1971b). In this study, the molecular weights of the subunits of MoFd, dissociated and calculated by Sepharose 6B in the presence of 6 M guanidine-HCl were determined in order to confirm the results from SDS gel electrophosis.

Materials and Methods

Carboxymethylation of MoFd and the markers

To a test tube, 4.3 g of guanidine-HCl, 15 mg of EDTA, and 0.1 ml of 2-mercaptoethanol were added and the tube then was sealed with a rubber serum stopper and degassed through a syringe barrel. The tube was filled with N_2 and the process repeated several times to remove all O_2. Tris buffer, pH 8.6 (5.23 g of Tris and 9 ml of 1.0 N HCl diluted to 30 ml with H_2O) also was degassed and added to the test tube through a syringe to give a final volume of 6 ml. The tube was unsealed in order to add 50 mg of protein in enough Tris buffer to give a final volume of 7.5 ml and resealed and degassed.

Protein solution prepared as described was incubated for 4 hours at room temperature with gentle shaking from time to time to ensure that all the protein dissolved and the tube was then wrapped with black electric tape to exclude light. A freshly prepared solution of 0.186 g of iodoacetic acid in 0.2 ml of 1.0 N NaOH was added to the protein solution such that the molar ratio of 2-mercaptoethanol to iodoacetic acid was 1.4.

The sulfhydryl groups of the cysteine residues react most rapidly. Since the excess iodoactic acid will react faster with 2-mercaptoethanol above pH 8.0 with thioether sulfer, alkylation of methionine can be kept to a minimum.

After one hour of incubation the reaction mixture was concentrated to 1 ml by ultrafiltration and then dialyzed against 6 M guanidine-HCl for 2 days. 0.2 ml (lysozyme only) to 0.3 ml of sample solution was applied to the column.

Preparation of sepharose 6B column in the presence of 6 M guanidine-HCl

Sepharose 6B with an agarose content of 6% (Pharmacia) was used for packing a gel column. Guanidine-HCl was added to give a final concentration of 6 M and allowed to deaerate without stirring at room temperature for 5 hours. The column (100×1.6 cm, Pharmacia) was then packed to give a gel bed of 87×1.6 cm. Extreme care was taken to prevent degradation of the agarose beads. The column was equilibrated with 6 M guanidine-HCl, pH 5.5,

for 3 days before the proteins were added. The flow of eluant was maintained at a rate of 1.4 ml per hour by use of a Marriot flask.

Assay of fractions

The fractions from the column were collected with an automatic fraction collector with 30 minutes allowed for each fraction. The absorbance at 280 nm was measured within a 1 ml cuvette. For MoFd, in addition to U. V. absorbance, Folin-phenol reagent was used to determinate protein concentration after the protein containing fractions were dialyzed against H_2O for 20 hours. Blue dextran was measured at 630 nm. and DNP–alanine at 360 nm.

Markers

The following proteins with known molecular weight were used as markers:

BSA	68,000
Catalase	60,000
Ovalbumin	43,000
Pepsin	35,000
Trypsin	23,000
Lysozyme	14,000

1% blue dextran 2,000 (pharmacia) and 0.1% DNP-alanine (Sigma) in 6 M guanidine–HCl solution were used for measuring the column void volume (Vo) and the total volume accessible to solvent (Vi) respectively.

Treatment of data

The position at which a substance elutes from the gel filtration column is still expressed in term of elution volume, Ve, but weight was used instead of volume as suggested by Fish *et al.* (1969). The other terms, Vo and Vi, were also determined by using weight instead of volume.

As shown in Figure 5, the plot of the distribution coefficient, Kd, versus the logarithm of molecular weight markers was used for the standard curve. The distribution coefficient, Kd, is defined as:

$$Kd = \frac{Ve - Vo}{Vi - Vo}$$

Results and Discussion

The elution pattern of carboxymethylated MoFd is shown in Fig. 1. The patterns based on U. V. absorbance and Folin-phenol reagent are different. The pattern based on Folin-phenol reagent is not symmetrical and suggests that more than one kind of protein may be present.

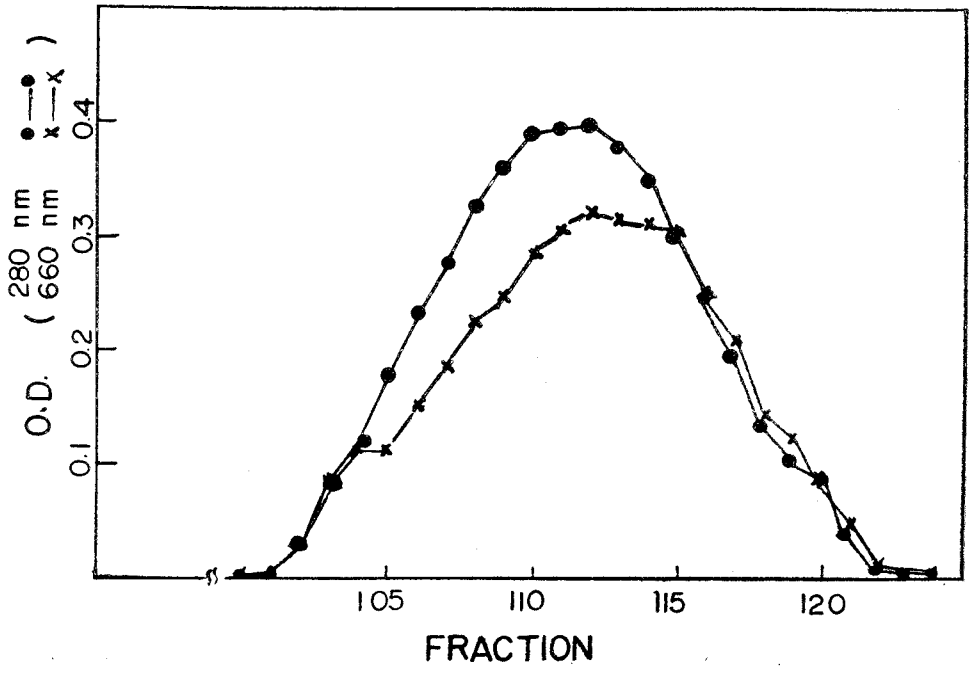

Fig. 1. Gel filtration on Sepharose 6 B in 6 M guanidine-HCl of carboxymethylated MoFd.

The elution positions of carboxymethylated MoFd and some markers run under the same conditions are shown in Fig. 2 in order to indicate their relative elution positions. It can be seen that corboxymethylated MoFd has an elution position very close to carboxymethylated catalase which has a known molecular weight of 60,000. It suggests that the carboxymethylated MoFd has molecular weight around 60,000, a value much lower than the native MoFd (Nakos and Mortenson, 1971) but similar to the value for the heavier subunit obtained from SDS gel electrophoresis. Figures 1 and 2 also suggest that MoFd dissociates into subunits in 6 M guanidine–HCl after carboxymethylation, but that the subunits are not well separated by the Sepharose 6B gel filtration technique.

SDS gel electrophoresis was used to examine the protein containing fractions after guanidine–HCl was removed by dialysis. Fig. 3 shows the patterns of four different fractions. From these patterns, it is clear that the earlier fractions have a higher percentage of 60,000 subunit, and the later fractions a higher percentage of 50,000 subunit. The percentage of 60,000 and 50,000 subunit in each fraction based on the color intensity after staining with coomassie blue are indicated in table 1. The O. D. at 660 nm (Folin phenol reagent) for the two subunits in each fraction was evaluated from their percentage of color intensity. Profiles of the two subunits based on the O. D. at 660 nm, compared with the total O. D. at 660 nm (see Fig. 1) are shown in Fig. 4.

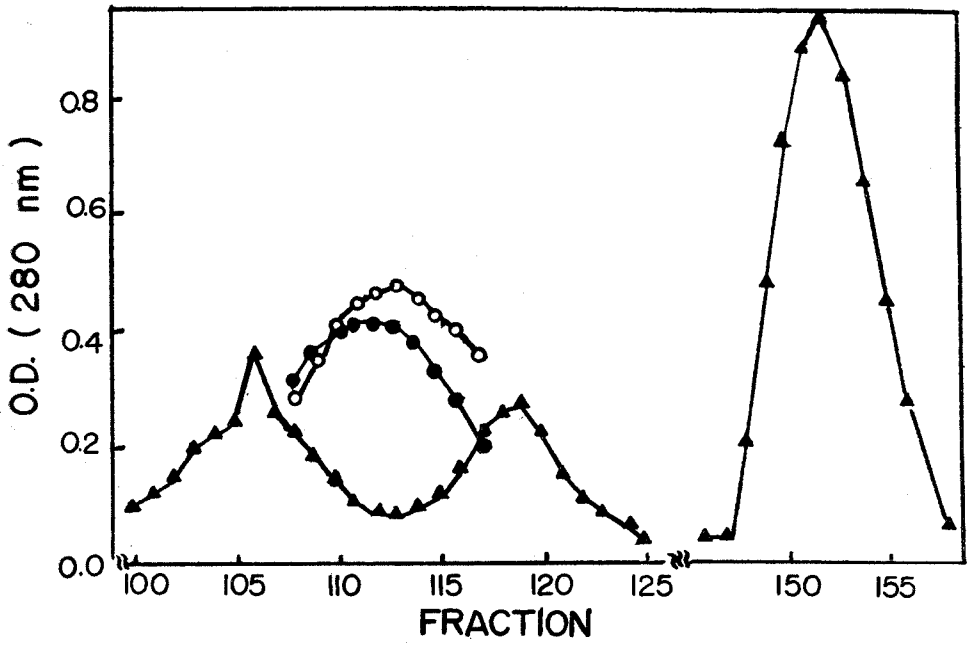

Fig. 2. Chromatography on Sepharose 6 B in 6 M guanidine HCl of carboxymethylated MoFd and protein markers. (a) ●—●—●: carboxymethylated MoFd; (b) ○—○—○: carboxymethylated catalase; (c) ▲—▲—▲: carboxymethylated BSA, ovalbunin and lysozyme. (a), (b) and (c) were run separately.

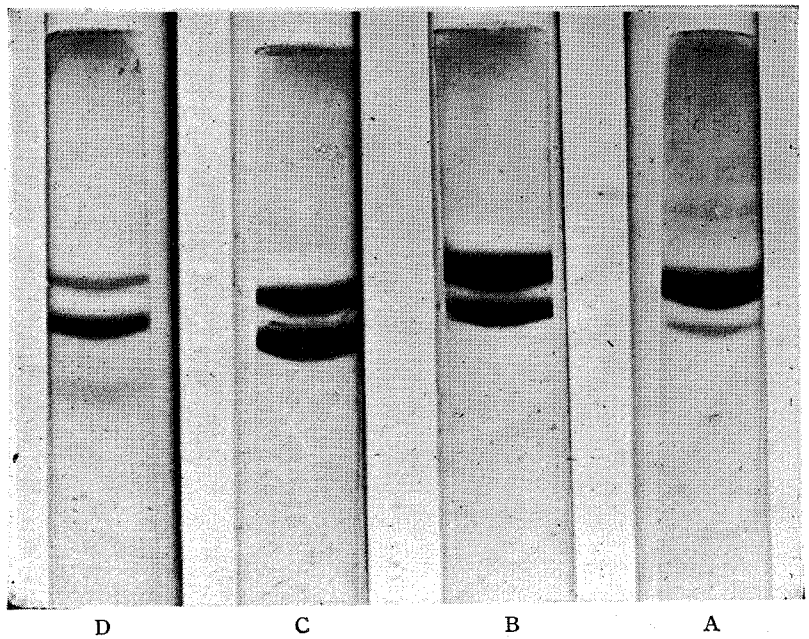

Fig. 3. SDS gel electrophoresis pattern of carboxymethylated MoFd eluted from Sepharose 6 B. A: fraction 107; B: fraction 111; C: fraction 114; D: fraction 116.

Table 1. *The ratio of 60,000 and 50,000 subunits in each fraction based on microdensitometer trace of the SDS gel for each fraction.*

Fraction number	* Total O.D. (660 nm) (60,000+50,000)	% of area (by microdensitometer trace)		O.D. (660 nm) for each subunit	
		60,000	50,000	60,000	50,000
107	0.187	90	10	0.1683	0.0187
108	0.225	80	20	0.1800	0.0450
109	0.245	74	26	0.1813	0.0637
110	0.285	65	35	0.1850	0.1000
111	0.307	57	43	0.1650	0.1320
112	0.324	51	49	0.1652	0.1588
113	0.315	47	53	0.1480	0.1670
114	0.310	43	57	0.1333	0.1767
115	0.305	36	64	0.1098	0.1952
116	0.245	30	70	0.0735	0.1715
117	0.208	24	76	0.0499	0.1581
118	0.143	20	80	0.0286	0,1144

* See Fig. 7 for the O.D. at 660 nm (Folin reagent)

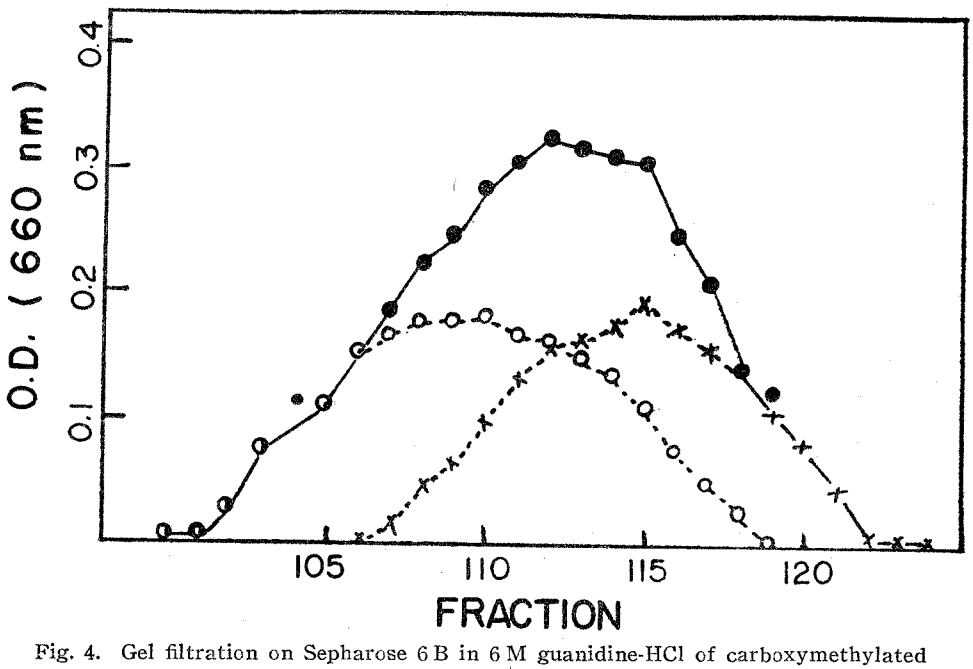

Fig. 4. Gel filtration on Sepharose 6B in 6 M guanidine-HCl of carboxymethylated MoFd. ×——×——×: the lower band calculated from SDS gel electrophoresis; ○——○——○: the upper band calculated from SDS gel electrophoresis; ●——●——●: O.D. was obtained from the whole fraction by Folin reagent.

Fig. 4 clearly suggests that MoFd has two subunits differing in molecular weight. Although the subunits are not well separated, the elution peak of each is clearly shown.

Fig. 5 shows the plot of the distribution coefficient, Kd, versus the logarithm of molecular weight of markers in this study. By least square analysis a linear relationship can be expressed in the form:

$$\log (\text{molecular weight}) = (10.023) - (23.171)\ \text{Kd}.$$

The estimated distribution coefficients, Kd, of the subunits of carboxymethylated MoFd were 0.165 and 0.206. These corresponds to a value of 62,000 and 52,500 respectively, a result very similar to the SDS gel electrophoresis.

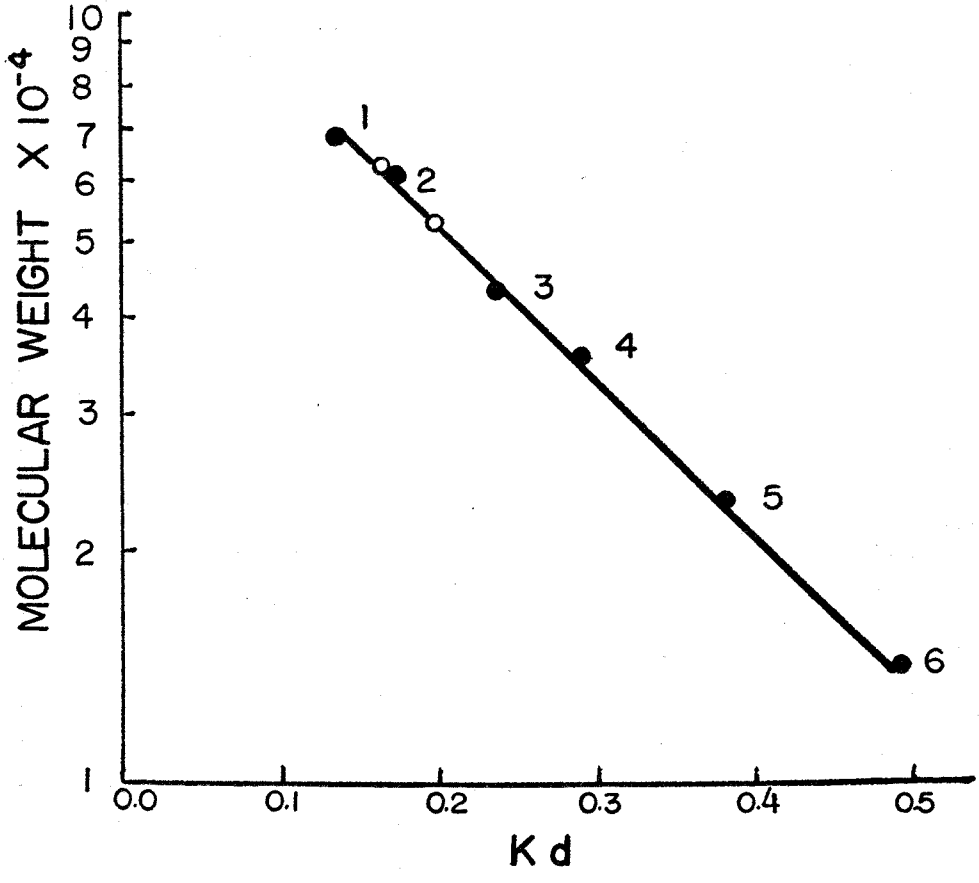

Fig. 5. Molecular weight determination by gel filtration on Sepharose 6B in the presence of 6 M guanidine-HCl. The extrapolated value of the subunits of MoFd are 52,500 and 62,000. The marker proteins are: 1: bovine serum albumin; 2: catalase; 3: ovalbumin; 4: pepsin; 5: trypsin; 6: lysozyme.

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以 Sepharose 6B-6 M guanidine-HCl 膠質過濾分析法對 molybdoferredoxin 的次單位分子量的測定

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Molybdoferredoxin 在 6 M guanidine-HCl 溶液中分離成兩種不同的次單位，它們的分子量根據 Sepharose 6B-6 M guanidine-HCl 膠質過濾分析的結果分別是 62,000 和 52,500。