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DETERMINATION OF THE STOKES RADIUS OF NATIVE AND SUCCINYLATED GLUTAMATE DEHYDROGENASE ON SEPHAROSE 4B

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SUMMARY

The Stokes radius of native and of succinylated beef liver glutamate dehydrogenase was measured by gel filtration on Sepharose 4B. Methods for the determination of the Stokes radius of a protein on a gel were compared. The succinylated enzyme was found to lose all glutamate dehydrogenase activity and has a very elongated conformation.

INTRODUCTION

It was observed by OLSON AND ANFINSEN[1] that beef liver glutamate dehydrogenase (GDH) associates reversibly at high enzyme concentrations. As reported in literature, the molecular weight of the dilute enzyme varies between 2.5 and 4.0×10^5 (refs. 2–5). Recently EISENBERG AND TOMKINS[6] and DESSEN AND PANTALONI[7] found 313,000 and 310,000, respectively, for the molecular weight of the protomers. The reported molecular weight of the oligomer varies between 1×10^6 and 2.2×10^6 (refs. 1, 2, 6, 8–10) and recent investigations point to a linear indefinite type of subunit association for this enzyme[7,11,12].

The size of the oligomer cannot be measured on Sephadex since it is completely excluded from those gels. Recently, agaropectin-free agarose was prepared in bead form by HJERTÉN[13]. This product is now available from Pharmacia under the trade name Sepharose and it can be used for the gel filtration of large macromolecules. Under these conditions, the Stokes radius of these molecules can be calculated from the elution volume, V_e, of the macromolecule and from the column constants using the basic equation of ACKERS[14]. The elution volume, V_e, is a function of the effective hydrodynamic volume of the molecule and a chemical modification of a protein may change the effective volume of the molecule by altering its shape.

In the present work, succinylated glutamate dehydrogenase was prepared and

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the Stokes radii of the native and the succinylated enzymes were determined and compared in order to find out if succinylation of the enzyme had caused a noticeable change in the shape of the molecule.

MATERIALS AND METHODS

Beef liver glutamate dehydrogenase (GDH) or L-glutamate NAD (P)-oxidoreductase (deaminating) EC 1.4.1.3, bovine thyroglobulin and catalase were obtained from Sigma Chemicals. Sepharose 4B was purchased from Pharmacia Canada Ltd.

Succinylation of glutamate dehydrogenase

The enzyme was succinylated according to the method of HABEEB[15]. The enzyme (110 mg, 10 mg/ml) was dialyzed for 20 h at 2° against 2 l of 0.05 M potassium phosphate at pH 7.6. The volume of the solution after dialysis was 25 ml. Three milliliters of dioxane containing 164 μmoles of succinic anhydride were added dropwise with stirring over a period of 30 min. This quantity of reagent represents a 24 times excess over the number of succinylable amino groups[5,16]. The pH was maintained at 7.8 with 1 N NaOH. The enzyme solution was then concentrated to a volume of 15 ml by the addition of dry Bio-Gel P-4 (ref. 17).

Column preparation

Sepharose 4B was obtained as a suspension which was diluted by the addition of 5 volumes of 0.05 M phosphate buffer at pH 7.6. The suspension was left at 10° for a few hours and then poured into a Pharmacia column (2.5 × 45 cm) up to a height of 40 cm. The column was equipped with an upflow adapter and washed with two volumes of eluent. The bed volume, V_t, was determined at the end of the experiment by filling the column to the same height with water[18].

Column standardization

The void volume, V_0, of the Sepharose 4B column was measured with 2 ml of a suspension of lyophilized *E. coli* cells (1 mg/ml, *E. coli* strain 9001 NCTC, Batch No. 6, Seravac Laboratories Ltd., Great Britain). The volume of eluent was measured directly in a graduate cylinder at the outlet of a LKB Uvicord monitor set at 254 mμ. Bovine thyroglobulin (10 mg, 1 ml), bovine liver catalase (40 mg, 2 ml), glutamate dehydrogenase (20 mg, 2 ml) and succinylated glutamate dehydrogenase (8.3 mg, 2 ml) were applied separately and in duplicate on top of the column. The column was run under a pressure of 25 cm of water giving a flow rate of 2.8 ml/cm^2/h.

Ultracentrifugation

Native and succinylated glutamate dehydrogenase were dialyzed for 24 h at room temperature against 4 l of 0.15 M phosphate buffer at pH 8.0. The concentration of enzyme was measured at 279 mμ (ref. 1). The sedimentation velocity was measured at 20° and at a concentration of 4.14 mg/ml in a Beckman ultracentrifuge Model E at 59,780 r.p.m.

Electrophoresis

Cellulose acetate strips (1 in. × 6¾ in. Sepraphore III, Gellman Co.) were used.

The electrophoresis was run at a potential difference of 100 V for 2 h in cold 0.05 M phosphate buffer at pH 7.6. The strips were stained with Ponceau S (0.01% in 5% trichloroacetic acid), washed with acetic acid and ethanol and cleared with 10% acetic acid in methanol[10].

Ackers' equation[14]

This equation was used to calculate the Stokes radii.

$$K_D = \frac{V_e - V_0}{V_t} = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104\left(\frac{a}{r}\right) + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right]$$

In this equation, K_D is the distribution coefficient of the protein on the gel, V_e, the elution volume, V_0, the void volume of the column, V_t, the effective imbibed volume of the gel, a, the Stokes radius of the molecule, and, r, the effective pore radius of the gel.

RESULTS

Succinylated glutamate dehydrogenase

Preliminary reports on the succinylation of glutamate dehydrogenase have been presented by FRIEDEN[20]. Succinylation renders the enzyme very soluble in 0.05 M Tris-HCl at pH 8 and the succinylated enzyme may be left at room temperature for at least one week without any increase in the turbidity of the solution. The extinction coefficient $E^{1\%}_{279\,\mathrm{m}\mu}$ was found to be 9.48, which is close to the value found by OLSON AND ANFINSEN[1] for the native enzyme. This indicates that the tyrosine residues were not modified by succinylation in these conditions. A similar observation was reported for the acetylation of this enzyme[5] and for the succinylation of globular proteins[21]. The modified enzyme is inactive at concentrations 1.7 and 2.4 times the concentration normally used to determine the glutamate dehydrogenase activity. COLMAN AND FRIEDEN[5] have shown that an excess of acetic anhydride inactivates the enzyme.

Sedimentation velocities

Table I shows the sedimentation velocity of native and succinylated glutamate dehydrogenase. A single and symmetrical peak was obtained for both of these proteins at a concentration of 4.14 mg/ml. OLSON AND ANFINSEN[1] found a value $S_{20,w}$ of 25 S for the native enzyme at a concentration of 4 mg/ml. The sedimentation velocity of the succinylated enzyme was not standardized by the method of SCHACHMAN[22] since the partial specific volume of the modified enzyme is not known.

TABLE I

SEDIMENTATION VELOCITY OF NATIVE AND SUCCINYLATED GLUTAMATE DEHYDROGENASE

Protein	*Sedimentation velocity (S)*	*Standardized sedimentation velocity (S)*
Native glutamate dehydrogenase	22.2	24.8
Succinylated glutamate dehydrogenase	21.0	—

TABLE II

GEL FILTRATION OF PROTEINS ON SEPHAROSE 4B

Protein	V_e *(cc)*	V_e/V_0
Thyroglobulin	137	2.34
	137	2.34
Catalase	160	2.74
	158	2.71
Native GDH	130	2.22
	131	2.24
Succinylated GDH	71.3	1.22
	61.4	1.05

Electrophoresis

Native glutamate dehydrogenase gave a single band at 5.3 cm from the origin in the direction of the anode. Under the same conditions, the succinylated enzyme gave a single band which stayed at the origin.

Gel filtration

Two pure proteins of known Stokes radius were run on Sepharose 4B in addition to the native and succinylated enzyme. The results of the gel filtration are given in Table II. ACKERS' equation was used to calculate the effective pore radius, r, of the gel using K_D and the Stokes radius of thyroglobulin or catalase. The Stokes radius of catalase was calculated using ACKERS' equation and the r value obtained with thyroglobulin; the Stokes radius of thyroglobulin was then calculated in the same way using the r value obtained with catalase. The calculated Stokes radii of these two proteins are given in Table III. These are within $\pm 8\%$ of the values in literature. This gives a measure of the accuracy of ACKERS' equation when applied to filtration on Sepharose 4B in these conditions. Using the average value of r calculated previously, the Stokes radii of the native and succinylated enzyme were calculated (Table IV).

The logarithms of the Stokes radius of catalase and of thyroglobulin were plotted against their reduced elution volume V_e/V_0 (Fig. 1). From that curve, the

TABLE III

EFFECTIVE PORE RADIUS OF SEPHAROSE 4B[a]

Protein	K_D	a/r	r *(mμ)*	a *(mμ)*	
				Calculated value	*Accepted value*
Catalase	0.722	0.0750	69.8	4.82	5.23 (ref. 25)
Thyroglobulin	0.564	0.1262	64.2	8.81	8.10 (ref. 26)

[a] $K_D = (V_e - V_0)/V_t$, where V_e is the elution volume of the molecule, V_0, the void volume of the column and, V_t, the effective imbibed volume of the gel, a, the Stokes radius of the protein and r, the effective pore radius of the gel.

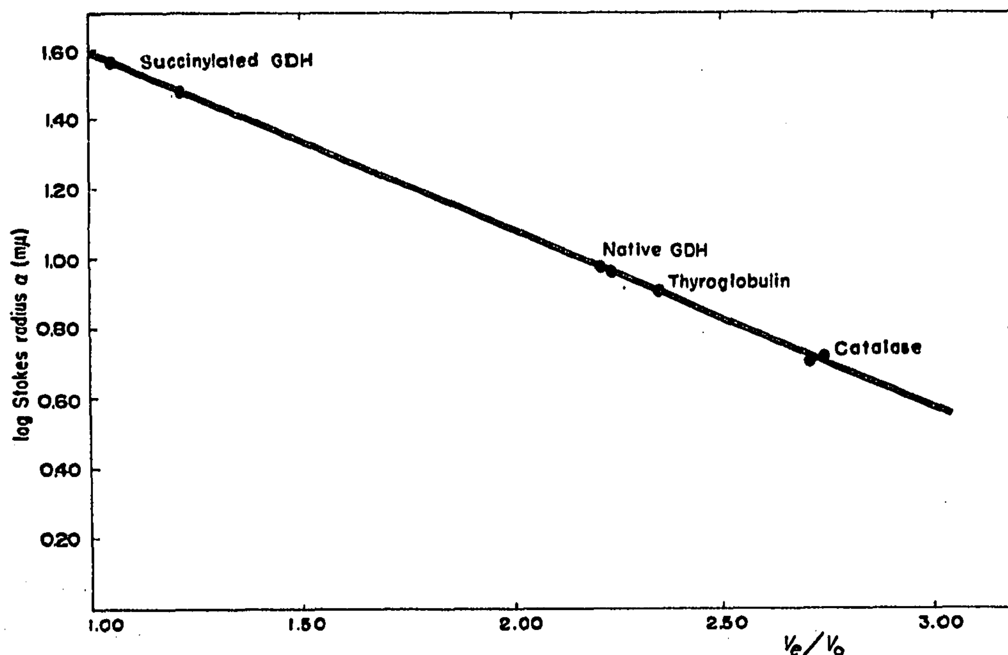

Fig. 1. Determination of the Stokes radius of native and succinylated glutamate dehydrogenase on Sepharose 4B.

Stokes radii of native and succinylated glutamate dehydrogenase were found to be 9.37 and 33.1 mμ, respectively. These values are comparable to the calculated values using ACKERS' equation (Table IV).

DISCUSSION

At a concentration greater than 0.8 mg/ml, glutamate dehydrogenase exists as an oligomer[1,4,6]. At this concentration, an average value of 9.5 mμ was obtained for the Stokes radius of the native enzyme on Sepharose 4B. Using the Stokes–Einstein equation[23], $D = kT/6\pi\eta a$ when D is the diffusion coefficient, k, the Boltzmann constant, T, the absolute temperature, η, the viscosity coefficient and a, the Stokes radius of the protein, the diffusion coefficient of the oligomer was found to be 2.15×10^{-7} cm^2/sec. OLSON AND ANFINSEN[1] have reported a value of 2.38×10^{-7} cm^2/sec for the diffusion coefficient of glutamate dehydrogenase at a concentration of 4 mg/ml. Gel filtration on Sepharose 4B provides a rapid method to determine the Stokes radius of large molecules. The ACKERS' equation or the use of a standard curve (log Stokes radius against V_e/V_0) gave comparable results.

TABLE IV

STOKES RADIUS OF NATIVE AND SUCCINYLATED GDH

Protein	K_D	a/r	a (mμ)
Native GDH	0.514	0.1445	9.7 ± 0.5
	0.521	0.1419	9.5 ± 0.5
Succinylated GDH	0.058	0.4961	33.3 ± 5.6

The observations made on the succinylated enzyme indicate that the succinylated molecule is considerably elongated. We observed also that succinylated glutamate dehydrogenase did not move on electrophoresis. The electrophoretic mobility of a molecule being proportional to its charge and inversely proportional to the square of its radius and to its degree of asymmetry[24], a large increase in the Stokes radius of the succinylated enzyme may explain its electrophoretic behavior.

The fast migration of the succinylated enzyme on Sepharose 4B is not due to an increase in molecular weight by agglomeration or polymerization since a decrease in the sedimentation velocity was observed (Table I). An increase in the Stokes radius of a molecule accompanied by a decrease in the sedimentation velocity indicates a decrease in the diffusion coefficient due to an increase in the frictional coefficient produced by an increase in the degree of asymmetry of the molecule. Succinylation of this enzyme does not seem to alter its state of aggregation, but causes a large increase in the Stokes radius of this molecule indicating an important change in shape.

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