

The Subunits of Human Hexosaminidase A

By ERNEST BEUTLER, AKIRA YOSHIDA, WANDA KUHL and JO ELLEN S. LEE
*Division of Medicine, City of Hope Medical Center, 1500 E. Duarte Road,
Duarte, CA 91010, U.S.A.*

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Previous studies of the subunit structure of hexosaminidase gave ambiguous results, but suggested that the enzyme was composed of six equally sized subunits. Dissociation of hexosaminidase A with *p*-chloromercuribenzoate produces an alkylated fragment with mol.wt. approx. 50000, which is converted into hexosaminidase S by treatment with dithiothreitol. Treatment of native hexosaminidase A with sodium dodecyl sulphate results in the formation of a large and a small fragment. However, although the native enzyme has a sedimentation coefficient of 5.8S, dissociation by *S*-carboxymethylation and maleic anhydride treatment results in subunits exhibiting a single schlieren boundary on analytical ultracentrifugation with a sedimentation coefficient of 2.18S. These results indicate that the enzyme is composed of four subunits, each with mol.wt. approx. 25000-27000. The mol.wt. of the native enzyme is calculated to be approx. 110000. Our data are consistent with the subunit structures of hexosaminidases A, B and S as being $\alpha_2\beta_2$, β_4 and α_4 respectively.

Two major forms of hexosaminidase (EC 3.2.1.30), hexosaminidase A and hexosaminidase B, are found in human tissues (Robinson & Stirling, 1968). A minor hexosaminidase, hexosaminidase S, is prominent in the tissues of patients with Sandhoff's disease, and is also found in normal tissues (Ikonne *et al.*, 1975; Beutler *et al.*, 1975a). We have previously (Beutler & Kuhl, 1975; Beutler *et al.*, 1975a) demonstrated that hexosaminidases B and S are homopolymers comprising β chains and α chains respectively, and that hexosaminidase A is a heteropolymer of α and β chains. We have assumed that α and β chains were approximately the same size. When hexosaminidase A was subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Srivastava *et al.*, 1974), however, more than one polypeptide band was observed. Although we have tentatively assumed that the presence of several bands represented incomplete dissociation of subunits by sodium dodecyl sulphate, a possibility that the α chain was several times the size of the β chain remained. We have therefore further investigated the subunit structure of the hexosaminidases, using a variety of analytical techniques.

Materials and Methods

Hexosaminidases A and B were purified to homogeneity from human placenta, as described in the preceding paper (Lee & Yoshida, 1976).

The protein was denatured, reduced and *S*-carboxymethylated, and the *S*-carboxymethylated protein treated with maleic anhydride as described by Cohen

& Rosemeyer (1969). The active enzyme and the *S*-carboxymethylated and maleic anhydride-treated enzyme were dialysed against 0.1M-KH₂PO₄/Na₂HPO₄, pH 7.0, overnight, and analytical ultracentrifugation was carried out in a Spinco model E ultracentrifuge at 56100 rev./min with a protein concentration of approx. 4 mg/ml (Lowry *et al.*, 1951).

Sedimentation-equilibrium centrifugation of the native and dissociated proteins was carried out with the use of Rayleigh interference optics in a Spinco model E ultracentrifuge (Yphantis, 1964). The buffer used was 0.1M-KH₂PO₄/Na₂HPO₄, pH 7.0, and the concentration of the protein was approx. 0.2 mg/ml. Centrifugation was carried out for 20 h at 27 690 rev./min for the native enzyme and 35 600 rev./min for the *S*-carboxymethylated and maleic anhydride-treated enzyme.

Polyacrylamide-gel electrophoresis was carried out in 7.5% acrylamide by using a Tris/glycine buffer system (pH 9.4) described by Davis (1964). For subunit analysis, the protein was dissociated with sodium dodecyl sulphate and 2-mercaptoethanol, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out in 5%, 7.5% and 10% acrylamide gels by the method described by Spiro (1966). Pancreatic ribonuclease (mol.wt. 13 700), aldolase (subunit mol.wt. 38 500) and bovine serum albumin (mol.wt. 68 000) were used as reference standards. Gels were stained with 0.025% (w/v) Coomassie Blue in 9% (v/v) acetic acid and 45% (v/v) methanol and were de-stained in 10% acetic acid. Radioactivity determinations were carried out by

slicing the stained gel into 1 mm slices, dissolving the slices in equal parts of 60% (w/v) HClO_4 and 30% (w/v) H_2O_2 at 70°C for 3h and counting in a scintillation counter after the addition of Scintisol (Isolab Inc., Akron, OH, U.S.A.).

Results

Human γ -globulin, repurified by isoelectric focusing in 1% Ampholine, pH range 8–10 (LKB model 8100; LKB, Stockholm, Sweden), was added to homogeneous hexosaminidase A as a stabilizer, and the mixture was treated with *p*-chloromercuri[^{14}C]-benzoate (Schwarz/Mann, Orangeburg, NY, U.S.A.), by using the technique described previously with merthiolate (Beutler *et al.*, 1975b). Dissociation of the enzyme in a manner identical with that which we have reported with merthiolate (Beutler *et al.*, 1975b) was observed (Plate 1a). A more slowly moving catalytically active protein, hexosaminidase B, and catalytically inactive highly radioactive fragment, designated ' α ' unit, were formed. Control studies with γ -globulin alone failed to reveal the formation of any of these proteins, and no detectable ^{14}C radioactivity moved into the gel. The ' α ' fragment was removed from the gel by electrophoresis and its molecular weight determined by Sephadex G-150 chromatography and also by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after treatment with maleic anhydride, giving values of 46000 and 44000 respectively.

Since it was apparent that the ' α ' unit represented alkylated polypeptide chain(s), an attempt was made to determine whether this material could be converted into hexosaminidase S, which we have previously shown to be an α -chain homopolymer (Beutler & Kuhl, 1975). Indeed, addition of 30 μM -dithiothreitol to the *p*-chloromercuribenzoate (10 μM)-treated mixture resulted in removal of the radioactivity (*p*-chloromercuribenzoate) from the polypeptides and the appearance of a catalytically active band in the position of hexosaminidase S (Plate 1b).

Polyacrylamide-gel electrophoresis of purified hexosaminidase A in the presence of sodium dodecyl sulphate resulted in detection of two protein components, one with mol.wt. approx. 55000, and another with mol.wt. approx. 25000 (Plate 2), as we have reported previously (Srivastava *et al.*, 1974). Since the mol.wt. of native enzyme is about 110000, the result of *p*-chloromercuribenzoate treatment and of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis might be interpreted as indicating that the enzyme consists of an α subunit with mol.wt. approx. 45000–55000 and two β subunits with mol.wt. approx. 25000–27000. However, it is also possible that incomplete dissociation of the α subunits artificially might produce the larger subunit.

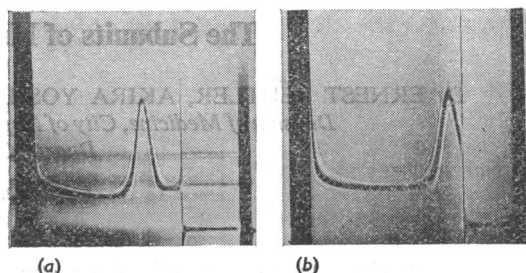


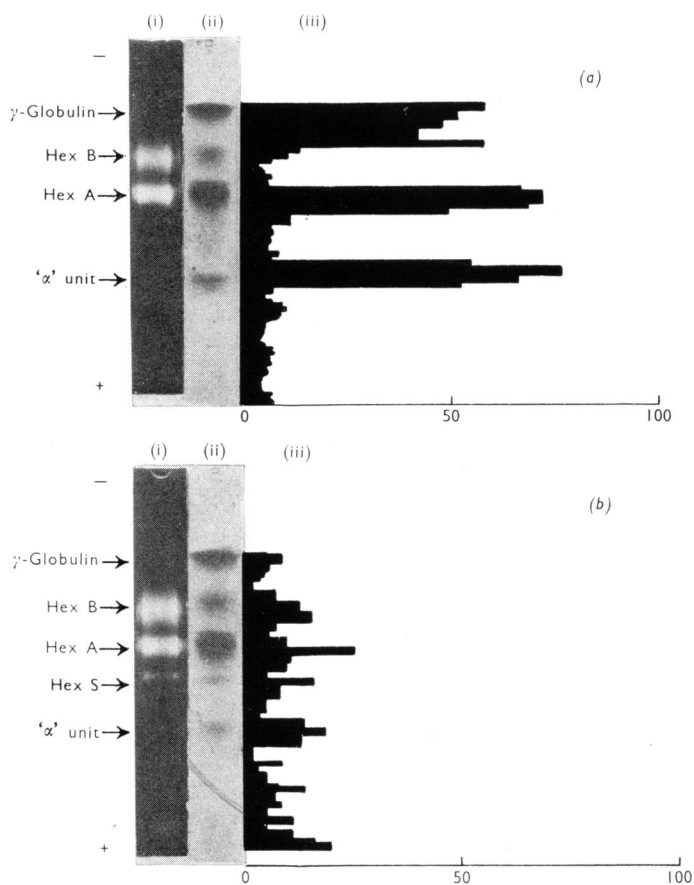
Fig. 1. Ultracentrifugation patterns of human hexosaminidase A

Centrifugation was carried out in 0.1M-phosphate buffer, pH7.0, at 56100rev./min at 20°C. (a) Native enzyme, 32min after reaching top speed; (b) *S*-carboxymethylated maleic anhydride-treated enzyme, 32min after reaching top speed.

In order to resolve this problem, the native enzyme and the enzyme dissociated by *S*-carboxymethylation and maleic anhydride treatment were subjected to analysis by analytical ultracentrifugation and sedimentation-equilibrium centrifugation. Schlieren patterns of both samples showed single sedimentation boundaries (Fig. 1). The sedimentation coefficient ($s_{20,w}$) of the native enzyme was calculated to be 5.8S, and that of the dissociated enzyme was 2.18S. Since the molecular weight of protein is roughly proportional to $(s)^4$, the results suggest that the structure of the enzyme is, in fact, tetrameric. Under these conditions there was no evidence for the existence of a large subunit with mol.wt. about 55000. The tetrameric structure of the enzyme was confirmed by the molecular weight of the native and dissociated protein estimated by the sedimentation-equilibrium method (Yphantis, 1964). The mol.wt. of the native enzyme was estimated at about 110000, assuming a partial specific volume of 0.730ml/g, which was roughly calculated from the amino acid composition of the protein (Lee & Yoshida, 1976). The subunit mol.wt. of the dissociated protein was estimated as about 30000 after correction for the increase of molecular weight due to association with maleate, but disregarding the possible slight decrease of partial specific volume due to *S*-carboxymethylation and maleic anhydride treatment (Hass, 1964).

Discussion

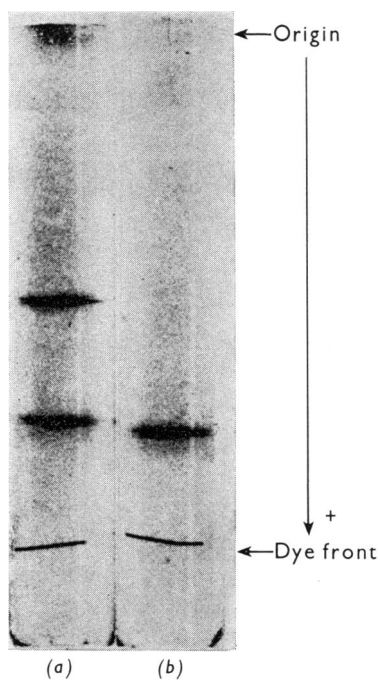
The difficulties in determining the molecular weight of glycoproteins and of their subunits is well known. In earlier studies, we estimated that the mol.wt. of the smallest subunit of the human hexosaminidases was approx. 18000. This led us to conclude that the enzyme was a hexamer. However, data obtained by



EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of human hexosaminidase A (a) after treatment with p-chloromercuri[¹⁴C]benzoate and (b) after treatment with dithiothreitol after the p-chloromercuri[¹⁴C]benzoate treatment

For both parts: (i) enzymic stain for hexosaminidase (Hex); (ii) Coomassie Blue stain for protein; (iii) radioactivity (c.p.m.) in gel slices. Highly purified γ -globulin was added to the homogeneous enzyme as a stabilizer. All studies were performed on the same gel. Methods are described in the text.



EXPLANATION OF PLATE 2

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis

Electrophoresis was carried out in 7.5% acrylamide gel (Spiro, 1966). The protein was stained with Coomassie Blue and the dye front was marked by insertion of a piece of wire. (a) Hexosaminidase A; (b) hexosaminidase B.

sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and by dissociation by *p*-chloromercuribenzoate also left open the possibility that a large α subunit and smaller β subunits were present in the enzyme. Now, determining the sedimentation constant of the native and chemically dissociated enzyme, we find that the enzyme is a tetramer. Only a single band appears on centrifugation of the dissociated enzyme, ruling out the possible existence of a larger subunit. The subunit size was estimated as 25000–27000 daltons on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and the slightly larger apparent size (30000 daltons) of the *S*-carboxymethylated maleic anhydride-treated enzyme estimated by the sedimentation-equilibrium method is probably due to the change in partial specific volume produced by chemical modification of the subunits.

Thus it appears that hexosaminidase A is composed of a combined total of four α and β subunits, each with a mol.wt. of about 27000. The α subunits appear to have a strong tendency to aggregate and are not dissociated either in sodium dodecyl sulphate/2-mercaptoethanol or by thiol reagents, such as *p*-chloromercuribenzoate. However, *S*-carboxymethylation and treatment with maleic anhydride does dissociate the subunits. We therefore propose that the correct structures of hexosaminidase S and hexosaminidase B are α_4 and β_4 respectively. Hexosaminidase A contains both α and β chains, and, although a symmetrical structure of $\alpha_2\beta_2$ is most attractive, the possibility that hexosaminidase A contains unequal numbers of α and β chains also deserves consideration. However, an excess of α chains appears to be ruled out by the high yield of hexosaminidase B after merthiolate treatment of hexosaminidase A (Beutler *et al.*, 1975b); the possi-

bility that hexosaminidase A is composed of three β chains and only a single α chain is ruled out by the finding that the hexose content of hexosaminidase A is only one-half of that of hexosaminidase B (Lee & Yoshida, 1976). This indicates that most of the carbohydrate is on the β chain: if hexosaminidase A were $\alpha\beta_3$ it would have to contain at least three-quarters as much hexose as does hexosaminidase B (β_4).

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