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FURTHER CHARACTERIZATION OF TWO FORMS OF *N*-ACETYL-α-GALACTOSAMINIDASE FROM HUMAN LIVER

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Summary

1. 2 forms of *N*-acetyl-α-galactosaminidase (EC 3.2.1.22) can be isolated from human liver using Sepharose 4B-concanavalin A chromatography, followed by CM-cellulose chromatography. Both forms hydrolyse not only *p*-nitrophenyl-*N*-acetyl-α-galactosaminide but also *p*-nitrophenyl-α-galactoside. These two forms were formerly known as α-galactosidase B and A-like α-galactosidase (Schram, A.W., Hamers, M.N., Brouwer-Kelder, B., Donker-Koopman, W.E. and Tager, J.M. (1977) Biochim. Biophys. Acta 482, 125—137; Schram, A.W., Hamers, M.N. and Tager, J.M. (1977) Biochim. Biophys. Acta 482, 138—144).

2. In fresh normal liver, most of the *N*-acetyl-α-galactosaminidase activity is found in the fractions containing α-galactosidase B and a minor portion in the fractions containing α-galactosidase A. However, the minor peak of *N*-acetyl-α-galactosaminidase activity does not coincide with the peak of α-galactosidase A activity. Both peaks of *N*-acetyl-α-galactosaminidase activity can be precipitated by antiserum raised against α-galactosidase B but not by antiserum raised against α-galactosidase A.

3. In fresh Fabry liver (which contains no α-galactosidase A) more than 98% of the *N*-acetyl-α-galactosaminidase activity was recovered in the fractions containing α-galactosidase B. Less than 2% was found in the fractions containing A-like α-galactosidase. Upon aging of the liver, the yield of the latter form increased (to almost 25% after 17 months storage at −20°C).

4. The conversion of *N*-acetyl-α-galactosaminidase to the second form upon aging was accompanied by a decrease in apparent molecular weight from 110 000 ± 5000 to 99 000 ± 3000, as measured by Sephacryl S-200 chromatography.

5. The kinetic properties of the enzyme change slightly upon aging. In contrast, there is no effect of aging on the pH optimum of the enzyme.

6. It is concluded that only one lysosomal *N*-acetyl-α-galactosaminidase is present in human liver and that one of the two forms of *N*-acetyl-α-galactosaminidase observed in extracts of liver is formed by chemical modification of the enzyme during storage of the liver or during storage of the purified preparation.

Introduction

N-acetyl-α-galactosaminidase (3.2.1.22) has been purified from a number of sources including snails [1], microorganisms [2,3], beef and pig liver [4], limpets [5] and human tissues [6—8]. The enzyme hydrolyses the *N*-acetyl-α-galactosaminidic linkage present in glycolipids like blood group A substance [1] and Forsmann hapten [5,7,9], in desialylated submaxillary mucins [4], and in the artificial substrate *p*-nitrophenyl-*N*-acetyl-α-galactosaminide [4—9]. The mammalian enzyme has an acid pH optimum [4—6] and appears to be located in the lysosomes [10,11]. So far, no deficiency of this enzyme has been described in man.

It has recently been shown by Schram et al. [8] and by Dean et al. [7,12] that *N*-acetyl-α-galactosaminidase from human liver is identical to α-galactosidase B, one of the two α-galactosidase isoenzymes that have been described in human tissues [13] (see ref. 14 for a review). Schram et al. [8] showed that the enzyme has a higher affinity for *p*-nitrophenyl-*N*-acetyl-α-galactosaminide than for *p*-nitrophenyl-α-galactoside, and a higher turnover number with the former substrate than with the latter. They therefore concluded [8] that the physiological role of the enzyme is to hydrolyse *N*-acetyl-α-galactosaminidic rather than α-galactosidic linkages.

The question arises of whether different isoenzymes of *N*-acetyl-α-galactosaminidase exist (see ref. 9). The occurrence of isoenzymes of several other lysosomal glycosidases, for instance hexosaminidase [16], is well documented (see ref. 15). Isoenzymes are often indentified on the basis of differences in physicochemical properties, for instance in electrophoretic mobility. However, lysosomal enzymes are glycoproteins [17] and a change in electrophoretic mobility may be due to a difference in the number of sialic acid groups present in the enzyme molecule [18,19]. The multiple electrophoretic forms appear to have the same kinetic properties [20].

We have recently shown [14] that human *N*-acetyl-α-galactosaminidase (formerly known as α-galactosidase B) changes on aging to a form behaving differently during ion-exchange chromatography on CM-cellulose. Furthermore, some of the kinetic properties of the new form appeared to differ from those of the original form.

The properties of these two forms of *N*-acetyl-α-galactosaminidase have now been examined further. The results are described in this paper.

Materials and Methods

α-Galactosidase A and *N*-acetyl-α-galactosaminidase (= α-galactosidase B) were purified from normal human liver using concanavalin A-Sepharose 4B and carboxymethyl cellulose chromatography as described [14]. Modified *N*-acetyl-

α-galactosaminidase (= A-like α-galactosidase) was isolated from Fabry liver using the same method [14].

To determine glycosidase activities, the enzyme preparations were incubated in a reaction mixture (final volume 0.5 ml) containing 0.5% bovine serum albumin, 100 mM sodium acetate (pH 4.6) and either 0.57 mM *p*-nitrophenyl-*N*-acetyl-α-galactosaminide or 12 mM *p*-nitrophenyl-α-galactoside. K_m and V values were determined by varying the concentration of *p*-nitrophenyl-α-galactoside from 2.8 to 18 mM and that of *p*-nitrophenyl-*N*-acetyl-α-galactosaminide from 0.03 to 1 mM.

After incubation at 37°C for 10—60 min, the reaction was stopped by adding 1 ml 0.3 M glycine/NaOH (pH 10.6). The liberated *p*-nitrophenol was estimated spectrophotometrically at 405 nm using a molar extinction coefficient of $18.5 \cdot 10^6\ M^{-1} \cdot cm^{-1}$ [21]. In control incubations either enzyme or substrate was omitted.

Antibodies against α-galactosidase A and B were prepared as described in [14]. Incubations with the antisera were carried out first for 30 min at 37°C and subsequently for 2 h at 0°C as described [14]. The incubation mixture was subsequently centrifuged at 10 000 × *g* for 4 min at room temperature. The resultant supernatant was assayed for glycosidase activity as described above.

Molecular weight determinations were carried out by exclusion chromatography using a Sephacryl S-200 (Pharmacia) column (1.1 × 103 cm) and 10 mM phosphate buffer (pH 6.5)/100 mM NaCl at a flow rate of 5 ml/h. The column was calibrated using aldolase, catalase, egg albumin, bovine serum albumin, cytochrome *c*, myoglobin and hexokinase as standards. Dextran blue was used as a marker of the void volume. Fractions of 1 ml were measured at 280 nm on a Zeiss spectrophotometer or, in the case of A-like α-galactosidase and α-galactosidase B, assayed for glycosidase activity as described above.

Results and Discussion

The relation between the α-galactosidase and *N*-acetyl-α-galactosaminidase activities present in isolated α-galactosidase A and B is illustrated in Fig. 1. Incubation of α-galactosidase A with anti-α-galactosidase A antibodies followed by centrifugation of the immune complexes leads to a decrease in α-galactosidase activity in the supernatant. In contrast, the *N*-acetyl-α-galactosaminidase activitity is not affected (Fig. 1). When α-galactosidase A is incubated with anti-α-galactosidase B antibodies, α-galactosidase activity in the supernatant does not change, whereas *N*-acetyl-α-galactosaminidase activity decreases. Thus, there is a relationship between *N*-acetyl-α-galactosaminidase present in the α-galactosidase A preparation and α-galactosidase B (see ref. 8). Incubation of α-galactosidase B with anti-α-galactosidase A antibodies effects neither the α-galactosidase nor the *N*-acetyl-α-galactosaminidase activity, whereas incubation with anti-α-galactosidase B reduces both activities (Fig. 1).

We have shown that α-galactosidase B can be converted to a form which behaves similarly to α-galactosidase A during carboxymethyl cellulose ion-exchange chromatography. The chromatographic behaviour of the *N*-acetyl-α-galactosaminidase and α-galactosidase activities on a CM-cellulose column has now been examined in more detail (Fig. 2). When the Sepharose 4B-concana-

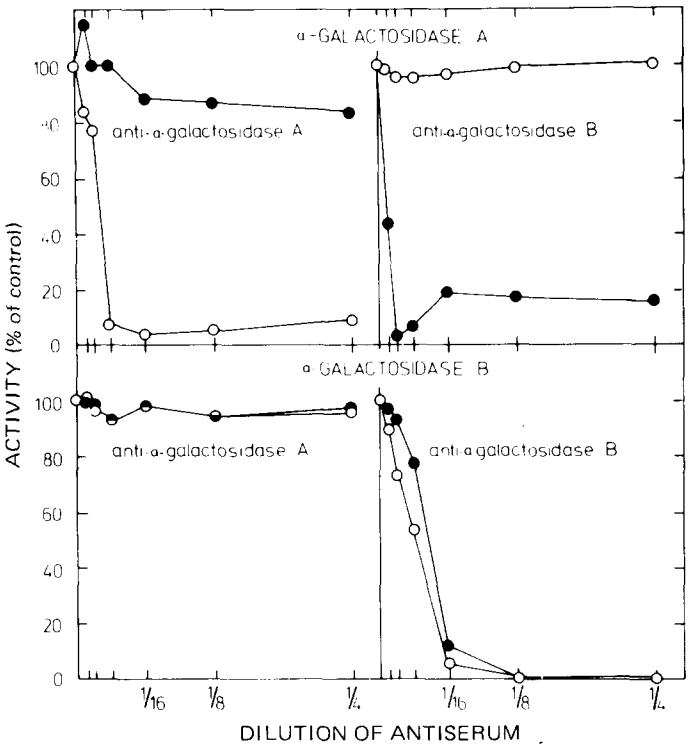

Fig. 1. Effect of preincubation with anti-α-galactoside A serum and anti-α-galactosidase B serum on the *p*-nitrophenyl-α-galactosidase (○———○) and *p*-nitrophenyl-*N*-acetyl-α-galactosaminidase (●———●) activity of human α-galactosidase isoenzyme preparations. Activities were measured in the supernatant after centrifugation of the enzyme-antiserum mixture.

valin A eluate of a normal liver extract is subjected to CM-cellulose chromatography, two peaks of *N*-acetyl-α-galactosaminidase are obtained (Fig. 2X), one coinciding with the smaller peak of α-galactosidase activity (referred to as α-galactosidase B [14]). A second peak of *N*-acetyl-α-galactosaminidase activity is observed in the same region as the main peak of the α-galactosidase activity (α-galactosidase A [14]). Significantly, however, the two peaks do not coincide exactly. A difference in effluent volume of 8 ml in peak fractions is observed. The elution pattern of a Fabry preparation (Fig. 2Y) also shows two peaks of *N*-acetyl-α-galactosaminidase activity, one coinciding with the main peak of α-galactosidase activity (α-galactosidase B [14]) and the other with the minor peak (A-like α-galactosidase). In contrast to the elution profile of a normal preparation, the second peak and the A-like α-galactosidase activity coincide exactly. These results, and those of Fig. 1 provide further evidence that the *N*-acetyl-α-galactosaminidase activity in the α-galactosidase A fraction from normal liver and the A-like α-galactosidase in Fabry liver are due to a modified form of *N*-acetyl-α-galactosaminidase.

The kinetic properties of the modified form of *N*-acetyl-α-galactosaminidase (referred to as A-like α-galactosidase) with the two artificial substrates are summarized in Table I. 5 separate isolations were carried out within a period of 16 months, using 50-g portions from the same Fabry liver. The mean value of the

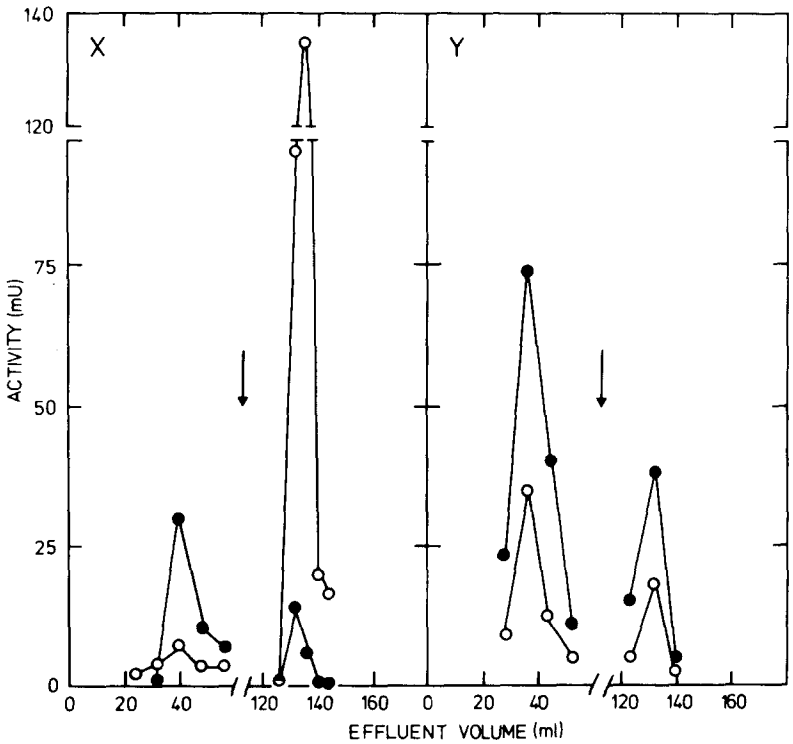

Fig. 2. CM-cellulose chromatography of a glycoprotein fraction from normal (X) and Fabry (Y) human liver. ○———○, *p*-nitrophenyl-α-galactosidase. ●———●, *p*-nitrophenyl-*N*-acetyl-α-galactosaminidase. The arrows indicate a change in buffer from 50 mM sodium acetate (pH 4.0)/50 mM NaCl to 100 mM sodium acetate.

TABLE I

PROPERTIES OF A-LIKE α-GALACTOSIDASE ISOLATED FROM A FABRY LIVER

Abbreviations: *p*NP-α-GAL, *p*-nitrophenyl-α-galactoside; *p*-NP-α-*N*AGA, *p*-nitrophenyl-*N*-acetyl-α-galactoside.

Preparation isolated on	K_m (mM) for		$V_{pNP\text{-}\alpha\text{-}NAGA}/V_{pNP\text{-}\alpha\text{-}GAL}$	Yield after CM-cellulose step (% of input) *
	pNP-α-NAGA	pNP-α-GAL		
26.8.1975	—	8.9	—	<2
29.8.1975	—	12.8	—	<2
28.7.1976	1.3	7.1	4.8	<2
18.1.1977	1.0	28.9	1.1	15
26.1.1977	2.8	25.0	0.7	23
α-Galactosidase B for normal liver				
Mean ± S.E.M.	0.9 ± 0.1 (n = 3)	22.3 ± 1.2 (n = 5)	2.0 ± 0.8 (n = 3)	

* Measured with *p*-nitrophenyl-α-galactoside.

K_m of the modified enzyme for *p*-nitrophenyl-*N*-acetyl-α-galactosaminide was 1.7 ± 0.6 mM (n = 3) whereas that of the unmodified enzyme was 0.9 ± 0.1 mM (n = 3). Thus no dramatic change in K_m value for *p*-nitrophenyl-*N*-acetyl-α-galactosaminide occurs during conversion. When *p*-nitrophenyl-α-galactoside was used as substrate a mean K_m value of 16.5 ± 4.4 mM was calculated for the modified enzyme, a value that does not significantly differ from that obtained with the unmodified enzyme. However, it should be noted that in the first three experiments the K_m values for *p*-nitrophenyl-α-galactoside were lower than in the last two experiments. Concomitantly. the yield of modified enzyme was much greater in the last two experiments. This can probably be explained as an effect of aging of the whole liver during the period between the experiments. The ratio between maximum velocities using the two substrates changed from a high value of 5 in Expt. 3 to values near 1 in the last two experiments, probably due to the same effect mentioned above. The mean value (2.2 ± 1.3) does not significantly differ from the value obtained with α-galactosidase B. Thus aging of the liver probably influences not only the extent of modification of *N*-acetyl-α-galactosaminidase, but also has minor effects on the kinetic properties of the enzyme.

Both forms of *N*-acetyl-α-galactosaminidase exhibit maximum activity at pH 4.5, measured either with *p*-nitrophenyl-α-galactoside or *p*-nitrophenyl-*N*-acetyl-α-galactosaminide (not shown).

The apparent molecular weights of modified and unmodified *N*-acetyl-α-galactosaminidase were determined as the Stokes radii by means of gel filtration on Sephacryl S-200. After calibration of the column with 7 different markers, molecular weights of 110 000 ± 5000 (n = 5) for the unmodified *N*-acetyl-α-galactosaminidase and of 99 000 ± 3000 (n = 5) for the modified enzyme were calculated. This could indicate a loss of approx. 10 000 in molecular weight. This loss does not influence the reaction of the enzyme with the antibodies against the unmodified form, nor does it dramatically change the kinetic properties; it results, however, in a change in chromatographic behaviour (see also ref. 14).

Conclusions

The results suggest that only one lysosomal *N*-acetyl-α-galactosaminidase is present in human liver. However, during storage of the liver, *N*-acetyl-α-galactosaminidase is converted to a form with a different chromatographic behaviour and a slightly lower apparent molecular weight. The modified form is precipitated by antibodies raised against the unmodified enzyme. The kinetic properties are not dramatically changed as a result of the modification. The conversion of *N*-acetyl-α-galactosaminidase to a modified form also occurs during storage of isolated preparations [14].

The demonstration that multiple forms of *N*-acetyl-α-galactosaminidase can arise as an artifact stresses that caution is needed in interpreting the significance of the occurrence of multiple forms of lysosomal enzymes in general (see ref. 15). Although the presence of isoenzymes has been well-documented in some cases, stringent tests, including tests of the immunological cross reactivity are necessary in order to exclude the possibility that the occurrence of multiple forms of a lysosomal enzyme is an isolation artifact.

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References

1 Tuppy, H. and Staudenbauer, W.L. (1966) Biochemistry 5, 1742—1747
2 McGuire, E.J. and Roseman, S. (1967) J. Biol. Chem. 242, 3745—3747
3 Endo, Y. and Kobata, A. (1976) J. Biochem. 80, 1—8
4 Weissmann, B. and Hinrichsen, D.F. (1969) Biochemistry 8, 2034—2043
5 Uda, U., Li, S.-C., Li, Y.-T. and McKibbin, J.M. (1977) J. Biol. Chem. 252, 5194—5200
6 Callahan, J., Lassila, E., Den Tandt, W. and Phillipart, M. (1973) Biochem. Med. 7, 424—431
7 Dean. K.J., Sung. S.-S.J. and Sweeley, C.C. (1977) Fed. Proc. 36, 731
8 Schram, A.W., Hamers, M.N. and Tager, J.M. (1977) Biochim. Biophys. Acta 482, 138—144
9 Sung, S.-S.J. and Sweeley, C.C. (1976) in Advances in Experimental Medicine and Biology, Vol. 68, Current Trends in Sphingolipidoses and Allied Disorders (Volk, B.W. and Schneck, L., eds.), pp. 323—337, Plenum Press, New York
10 Weissmann, B., Rowin, G., Marshall, J. and Friederici, D. (1967) Biochemistry 6, 207—214
11 Mahadevan, S. and Tappel, A.L. (1968) Arch. Biochem. Biophys. 128, 129—132
12 Dean. K.J., Sung, S.-S.J. and Sweeley, C.C. (1977) Biochem. Biophys. Res. Commun. 77, 1411—1417
13 Beutler, E. and Kuhl, W. (1972) J. Biol. Chem. 247, 7195—7200
14 Schram, A.W., Hamers, M.N., Brouwer-Kelder, B., Donker-Koopman, W.E. and Tager, J.M. (1977) Biochim. Biophys. Acta 482, 125—137
15 Robinson, D. (1974) in Enzyme Therapy in Lysosomal Storage Diseases (Tager, J.M., Hooghwinkel, G.J.M. and Daems, W.Th., eds.), pp. 217—226, North-Holland Publ. Comp., Amsterdam
16 Robinson, D. and Stirling, J.L. (1968) Biochem. J. 107, 321—327
17 Bouma, J.M.W. (1974) in Enzyme Therapy in Lysosomal Storage Diseases (Tager, J.M., Hooghwinkel, G.J.M. and Daems, W.Th., eds.), pp. 197—206, North-Holland Publ. Comp., Amsterdam
18 Kint, J.A., Carton, D., Huys, A. and Hooft, C. (1974) in Enzyme Therapy in Lysosomal Storage Diseases (Tager, J.M., Hooghwinkel, G.J.M. and Daems, W.Th., eds.), pp. 227—237, North-Holland Publ. Comp., Amsterdam
19 Goldstone, A., Konecny, P. and Koenig, H. (1971) FEBS Lett. 13, 68—72
20 Romeo, G., DiMatteo, G., D'Urso, M., Li, S.-C. and Li, Y.-T. (1975) Biochim. Biophys. Acta 391, 349—360
21 Bergmeyer, H.U., Gawehn, K. and Grassl, M. (1970) in Methoden der Enzymatischen Analyse (Bergmeyer, H.U., ed.), Vol. 1, p. 457, Verlag Chemie, Weinheim