Measurement of 1-Aspartamido- β -*N*-Acetylglucosamine Amidohydrolase Activity in Human Tissues

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The activity of 1-aspartamido- β -*N*-acetylglucosamine amidohydrolase (aspartylglucosylaminase, EC 3.5.1.26) was measured in normal and diseased human liver, brain and kidney. Organs from patients with aspartylglucosaminuria show very little activity. Crude homogenates of human organs show a reaction catalysed by a complex enzyme system. With homogenate, the formation of product was linear with time up to about 6 h. Reaction times longer than 6–7 h resulted in a decrease in the total concentration of product. This phenomenon was not found with the partially purified enzyme fraction. Linearity of the enzyme activity with different protein concentrations was found, independent of the incubation time. Longer incubation of the crude homogenate resulted in the utilization of the product, *N*-acetylglucosamine. This phenomenon was not observed with the partially purified enzyme fraction. This amidase from human organs differs from that obtained from other sources and apparently represents a rather complex enzyme system.

Aspartylglucosaminuria was first described by Jenner & Pollitt (1967). Patients with this disease excrete large quantities of *N*-acetyl- β -aspartylglucosamine (1-*L*- β -aspartamido-2-acetamido-1,2-dideoxy- β -*D*-glucose) in urine and are mentally retarded (Pollitt *et al.*, 1968). The latter is considered as a secondary effect caused by a defect of the enzyme 1-aspartamido- β -*N*-acetylglucosamine amidohydrolase which is responsible for the breakdown of the β -aspartylglycosylamine linkage, the most common linkage involved in protein-carbohydrate bonding. This enzyme activity is considerably decreased in aspartylglucosaminuria patients (Pollitt & Jenner, 1969).

We have chosen human organs for the following reasons. (a) Although the presence of this enzyme activity in different sources has been demonstrated (Lee *et al.*, 1964; Kaverzneva, 1965; Murakami & Eylar, 1965; Roston *et al.*, 1965; Clamp *et al.*, 1966; Eylar & Murakami, 1966; Makino *et al.*, 1966, 1968; Mahadevan & Tappel, 1967; Plummer *et al.*, 1968; Conchie & Strachan, 1969; Kohno & Yamashina, 1969; Tarentino & Maley, 1969), hardly any attention has been given to human organs. (b) Some of the previous reports on the human enzyme (Palo *et al.*, 1971, 1972; Somer *et al.*, 1975) may be experimentally somewhat unsatisfactory and perhaps require reconsideration. The aim of the present paper is to examine some of the characteristics of this enzyme in human organs.

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Materials and Methods

Materials

The substrate 2-acetamido-1-(β -*L*-aspartamido)-1,2-dideoxy- β -*D*-glucose was obtained from Cyclo Chemicals, Los Angeles, CA, U.S.A.; K_2HPO_4 , KH_2PO_4 , $(NH_4)_2SO_4$, potassium borate, acetic acid, Triton X-100, glycerol and *p*-dimethylaminobenzaldehyde reagents were obtained from E. Merck, Darmstadt, W. Germany. Liver, kidney and brain specimens were obtained from aspartylglucosaminuria patients at autopsies carried out within 24–48 h after death and were frozen. Specimens of undiseased liver, kidney and brain were removed also 24–48 h after death and were used at once. It was, however, found that the frozen ($-20^\circ C$) undiseased specimens do not show any loss of enzyme activity even after 3 months.

Methods

Solutions. The substrate solution was made in 0.001 M- K_2HPO_4/KH_2PO_4 buffer, pH 7.7, and stored frozen. Borate buffer (0.8 M, pH 9.1) was prepared by dissolving potassium borate in water and adjusting the pH to 9.1 with HCl. About 10 drops of glycerol/100 ml of borate buffer were added to prevent the recrystallization of the borate salt. The solution was stored at room temperature ($23^\circ C$). All other solutions were stored at $4^\circ C$. *p*-Dimethylaminobenzaldehyde solution (10%, w/v) was prepared as described by Reissig *et al.* (1955). This

solution was diluted 1:10 with acetic acid immediately before use.

Assay mixture and procedure. The assay mixture (total volume 0.32ml) contained: 20 μ mol of K_2HPO_4/KH_2PO_4 buffer, pH 7.7 (0.02ml); 0.2ml of the enzyme fraction and 1.2 μ mol of 2-acetamido-1-(β -L-aspartamido)-1,2-dideoxy- β -D-glucose (0.1ml). The assay was carried out in sealed 10ml round-bottomed test tubes at 37°C. Incubation time was varied (see the Results section), but generally the enzyme extracts from undiseased specimens were incubated for 4–5h and that of diseased specimens for 6–7h. After the incubation, 56 μ mol (0.06ml) of borate buffer, pH 9.1, was added, the mixture boiled for 3min (Reissig *et al.*, 1955), cooled under running tap water and then 3ml of *p*-dimethylaminobenzaldehyde solution was added to each fraction. After further incubation of the reaction mixture for 20min at 37°C, it was again cooled in tap water and centrifuged for 10min at 2500 rev./min in a Sorvall GLC-1 bench centrifuge. The supernatant was carefully decanted into new tubes, and A_{585} was measured at room temperature against blanks in a double-beam Beckmann model 25 spectrophotometer. With careful pipetting this assay can also be used with a final total volume of 0.1ml.

At least two blanks with a standard *N*-acetylglucosamine solution were also incubated under similar conditions. It is recommended that at least one blank without substrate should be measured for every enzyme concentration used. The volumes were adjusted with water. The substrate alone shows no colour reaction under our experimental conditions.

Preparation of enzyme fractions. All the enzyme fractions were prepared as follows at 0–4°C.

Preparation of the crude homogenate. To 0.1M- K_2HPO_4/KH_2PO_4 buffer, pH 7.7, containing 0.1mM-EDTA (tetrasodium salt) was added Triton X-100, to a final concentration of 1%. This phosphate/EDTA/Triton buffer (pH 7.7) was used for the enzyme extraction from diseased and undiseased human liver, brain and kidney. The tissues were mixed with the buffer in the ratio 1:6 (w/v) and homogenized for 50s in a Waring blender at top speed. The extract was further homogenized in a Potter-Elvehjem homogenizer for 50s and stirred at 4°C for 2h. It was then centrifuged at 48000g for 30min (Sorvall SS34 rotor). The supernatant was carefully decanted. The pellet was subjected to a second extraction as above but showed hardly any activity. In all these fractions the enzyme activity was measured as described above.

Ammonium sulphate precipitation. For the results presented in Fig. 1, the crude homogenate was fractionated with solid $(NH_4)_2SO_4$. $(NH_4)_2SO_4$ was added to give 35% saturation (19.4g/100ml of enzyme extract), stirred for a further 5min, kept at –20°C for 30min and centrifuged at 48000g

for 30min (SS34 rotor). The pellet, which had no activity, was discarded. $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 60% (15.1g/100ml of supernatant), kept at –20°C for 60min and centrifuged at 48000g for 30min. The supernatant was discarded. The pellet (active enzyme) was dissolved in water and the pH of the solution was adjusted to pH 7.7 with K_2HPO_4 . The solution was dialysed for about 18h against 4 $\times$ 10 litres of water adjusted with K_2HPO_4/KH_2PO_4 buffer to pH 7.7. This dialysed fraction free of NH_4^+ ions (negative test with Nessler's reagent) was used to obtain the results presented in Fig. 1. In the undialysed fraction containing NH_4^+ ions (positive test with Nessler's reagent), no amidase activity was measured. The same inhibitory effect of NH_4^+ ions was observed during the studies with human liver, kidney and brain. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Fig. 1 shows the kinetics of aspartylglucosamine amidohydrolase from human liver in relation to the incubation time. A constant rate of reaction occurs initially. However, after about 6–7h the total

Fig. 1. Effect of incubation time on 1-aspartamido- β -*N*-acetylglucosamine amidohydrolase activity from human liver

Activity is measured as colour yield due to the formation of *N*-acetylglucosamine. For details of assay see the Materials and Methods section. Δ , Human liver crude homogenate; $\circ$, dialysed 30–60% satd.-(NH_4) $_2$ SO $_4$ fraction.

concentration of product in the reaction mixture actually decreases. In contrast, the enzyme activity in the fraction partially purified with $(\text{NH}_4)_2\text{SO}_4$ showed linearity up to 16h incubation time. After 16h incubation with the crude homogenate only about 10% of the product concentration obtained at 6h is found. Thus this behaviour with human liver differs markedly from that seen with other sources of 1-aspartamido- β -*N*-acetylglucosamine amidohydrolase (Lee *et al.*, 1964; Kaverzneva, 1965; Murakami & Eylar, 1965; Roston *et al.*, 1965; Clamp *et al.*, 1966; Eylar & Murakami, 1966; Makino *et al.*, 1966, 1968; Mahadevan & Tappel, 1967; Plummer *et al.*, 1968; Conchie & Strachan, 1969; Kohno & Yamashina, 1969; Tarentino & Maley, 1969).

The phenomenon observed with whole homogenate suggests that *N*-acetylglucosamine is being removed from the reaction system either by spontaneous decomposition or by some factor present in whole homogenate that is able to degrade this product. The results in Fig. 2, where *N*-acetylglucosamine was incubated with whole homogenate, demonstrate a time-dependent decrease in

concentration of this compound. Repeating this experiment with boiled homogenate caused no decrease in *N*-acetylglucosamine concentration over 16h (results not shown). Although the effect observed with whole homogenate must be explained primarily by the removal of product from the reaction system, an additional factor could be an inactivation during the assay of the 1-aspartamido- β -*N*-acetylglucosamine aminohydrolase enzyme. To examine this possibility, whole homogenate was incubated with all the ingredients of the assay system except substrate, and the reaction subsequently started after various time-periods at 37°C by substrate addition. The results (Fig. 3) show that for up to about 4h preincubation there is no effect on enzyme activity, but preincubation times of about 6h lead to an apparent activation of the enzyme, to a value some 18.7% higher than that of the non-preincubated control.

Similar preincubation of a partially purified sample of enzyme resulted in a similar though slightly greater activation (21%), but more prolonged preincubation (up to 16h) resulted in the loss of the activation and a return of the enzyme activity to the same value as that of the non-preincubated control. With whole homogenate, however, this more prolonged activity led to a marked decrease in the enzyme activity. Thus after 16h, the activity was decreased to

Fig. 2. Effect of crude liver homogenate (Δ) and the partially purified enzyme fraction ($\circ$) on the concentration of *N*-acetylglucosamine

Enzyme fractions (0.2ml) were incubated with 100nmol of *N*-acetylglucosamine, and the concentration of *N*-acetylglucosamine was measured at various times (see the Materials and Methods section). *N*-Acetylglucosamine ($\square$) incubated without any enzyme fraction does not show any change in concentration at 37°C.

Fig. 3. Effect of preincubation at 37°C of crude liver homogenate (Δ) and the partially purified fraction ($\circ$) for different times

Enzyme fractions (0.2ml) were preincubated at various times before the activity was measured as described in the Materials and Methods section.

37.5% of that of the non-preincubated control. It therefore seems that both catalysed removal of product from the reaction system and enzyme inactivation are involved in the unusual time-curve for the activity in the whole homogenate from human liver (Fig. 1).

Dialysis of the crude homogenates against water, pH 7.7, resulted in the same kinetics as shown in Fig. 1, suggesting that the inhibitory effect is not due to the presence of a loosely bound small molecule. Mixing the crude homogenates obtained from diseased and undiseased livers in the ratio of 1:1 (by wt.) did not alter the activity of the latter, indicating the absence of a loosely bound amidase inhibitor from the diseased liver.

No inhibitory effects were observed when the supernatant of the 60%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation was remixed with the pellet from this stage or when all three [35%-satd., 60%-satd. $(\text{NH}_4)_2\text{SO}_4$ pellets and the supernatant after 60% saturation with $(\text{NH}_4)_2\text{SO}_4$] were mixed together [in the proportions 1:1:1 (by wt. or by vol.)], suggesting that a complex enzyme system might be involved or that the factor(s) involved are unstable under the conditions of fractionation with $(\text{NH}_4)_2\text{SO}_4$. Incubation of the crude homogenate with 20 mM-substrate for 16 h did not change the enzyme kinetics presented in Fig. 1.

Further characteristics of the enzyme activity were examined. Production of *N*-acetylglucosamine was proportional to the amount of protein present in the assays, whether homogenates or partially purified fractions were used. Similar linear dependence on protein concentration was observed with human liver, brain and kidney extracts. When human brain homogenates were incubated for 24 h, the activity was decreased to 4% of that observed in short incubations (Fig. 4). When fresh human serum was incubated for 5, 8 and 24 h, the results for normal subjects and for patients with about 1000 and 2000 times higher than normal aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) values (infective serum hepatitis) respectively showed linearity. No inhibitory effect was observed when fresh serum from patients with about 20 times higher alkaline phosphatase (EC 3.1.3.1) (neoplasma malignum prostatae) was incubated for different times. However, in both groups of patients, the serum amidase activity was increased about 2 and 4 times above normal values respectively. Furthermore, no decrease in amidase activity was observed when human plasma was incubated for 18–20 h (Pollitt & Jenner, 1969). It seems therefore that the inhibitory effect (Fig. 1) may be restricted to human tissues.

Similar linearity between activity and protein concentration was observed with the lower activity found in diseased (aspartylglucosaminuria) liver,

kidney and brain specimens. Only about 6%, 4% and 3% of the normal activity was observed in the diseased liver, kidney and brain respectively. As summarized in Table 1, the crude homogenate from diseased kidney shows the highest amidase activity, as with normal kidney, when compared with brain or liver crude homogenates.

To shed light on the complexities of this enzyme system, the amidase from human liver was purified to apparent homogeneity as judged by polyacrylamide-gel disc electrophoresis (performed as described by Maurer, 1968). The enzyme from human liver was extracted with Triton X-100, fractionated with solid $(\text{NH}_4)_2\text{SO}_4$, and the pellet between 35 and 65% $(\text{NH}_4)_2\text{SO}_4$ saturation was dissolved in water and again fractionated with solid $(\text{NH}_4)_2\text{SO}_4$. This second

Fig. 4. Relationship between enzyme activity and increasing protein concentration from human brain. Crude homogenate (Δ) and its supernatant (48000 g for 30 min) ($\circ$) were incubated for 5 h. The crude homogenate was also incubated for 24 h ($\square$). With increasing protein concentration and at different incubation times, linearity is observed, but the enzyme activity is greatly decreased after 24 h of incubation.

Table 1. Comparative study of normal and diseased human organs showing the total activity of the homogenate and of the supernatant (48000 g for 30 min)

Very low activity is observed in the diseased specimens. Activity is expressed as nmol of *N*-acetylglucosamine produced/h per mg of enzyme protein.

Specimen	Enzyme activity (nmol/h per mg)			
	Normal		Diseased	
	Homogenate	Super-natant	Homogenate	Super-natant
Liver	1.4	1.9	0.15	0.116
Kidney	4.2	4.6	0.19	0.194
Brain	3.5	4.7	0.17	0.14

pellet between 45 and 70% $(\text{NH}_4)_2\text{SO}_4$ saturation was extracted with satd. $(\text{NH}_4)_2\text{SO}_4$ solution in decreasing concentration (Dugal & Louis, 1974). The fractions between 57 and 45% $(\text{NH}_4)_2\text{SO}_4$ saturation were subjected to calcium phosphate gel adsorption, heat-treatment (10 min at 60°C) and acetone fractionation (active fraction at 75–100%, v/v). The pellet obtained after acetone treatment was dialysed and fractionated on a DEAE-cellulose column (2.5 cm $\times$ 25 cm). The active fractions, eluted with 0.06 M-NaCl, were concentrated (Amicon XM 50 filter) and purified further on a Sephadex G-200 column (2.5 cm $\times$ 80 cm), followed by a second heat-treatment (10 min at 60°C). The yield of purified enzyme with this method was 3% and the specific activity was 43 334 nmol/h per mg at pH 7.7. *p*-Hydroxymercuribenzoate, glutathione and cysteine (4 mM) all had no effect on the enzyme activity. Some 50% inhibition of the enzyme activity was obtained with 5 mM-*N*-acetylcysteine. Bivalent cations showed a concentration-dependent inhibition of the enzyme activity. These and other kinetics of the purified amidase may differentiate this enzyme from human liver from those obtained from other sources.

Discussion

1-Aspartamido- β -*N*-acetylglucosamine amidohydrolase from human organs catalyses the hydrolysis of 2-acetamido-1-(β -L-aspartamido)-1,2-dideoxy- β -D-glucose. The behaviour of this enzyme in crude homogenate differentiates the human organ enzyme from that obtained from other sources (Lee *et al.*, 1964; Kaverzneva, 1965; Murakami & Eylar, 1965; Roston *et al.*, 1965; Clamp *et al.*, 1966; Eylar & Murakami, 1966; Makino *et al.*, 1966, 1968; Mahadevan & Tappel, 1967; Plummer *et al.*, 1968; Conchie & Strachan, 1969; Kohno & Yamashina, 1969; Tarentino & Maley, 1969). Thus with the whole homogenate from human liver, the build-up of product in the assay system during the reaction started to decrease after about 6 h and subsequently was actually reversed. This effect was not seen with

partially purified enzyme and has not been observed with whole homogenates of organs from other species. The decrease in the build-up of product would be consistent with enzyme inactivation. The fact that the partially purified enzyme from human liver does not show this effect argues against the theory that the enzyme is intrinsically unstable under the assay conditions, or that product inhibition is occurring. Preincubation of whole homogenate was associated initially with an activation effect, both for the partially purified enzyme and for whole homogenate. However, more prolonged times of preincubation led to marked falls in the enzyme activity of whole homogenate, but not with the partially purified enzyme. It seems reasonable to suggest that the enzyme activity in whole homogenate is somewhat unstable.

Although enzyme degradation could explain a decrease in the rate of product build-up in the assay system, the subsequent fall in product concentration must occur as a result of the removal of product from the system. The absence of this effect when partially purified enzyme is used demonstrates that spontaneous decomposition of the product during the assay cannot explain the effects observed with whole homogenate of human liver. It appears that the latter contains some factor able to remove *N*-acetylglucosamine from the system. Indeed, when *N*-acetylglucosamine was incubated with the whole homogenate, it seemed to disappear from the system, as is summarized in Fig. 5. The factor(s) responsible are heat-labile, since no effect was observed when the product was incubated with boiled homogenate. Thus the unusual form of the curve of product concentrations versus time seen with the whole homogenate of human liver may be explained as follows: initially, there is a smooth and constant production of product. This phase lasts for about 6–7 h, at which time enzyme inactivation becomes significant, presumably as a result of proteolytic digestion, and product is removed from the system, most likely as a result of metabolism by enzyme(s). These enzyme(s) are removed on partial purification

Fig. 5. Schematic interpretation of the results shown in Fig. 1.

S_1 (substrate for E_1 = enzyme₁ = amidase) is changed overall to P_2 [product of the second enzyme (E_2)-catalysed reaction], with E_2 utilizing P_1 (*N*-acetylglucosamine = product of E_1) as S_2 (substrate for E_2).

of the amidase, and seem to be limited to human tissues. This phenomenon was not observed when fresh human serum was incubated for different times, and furthermore no inhibitory effect has been reported with human plasma (Pollitt & Jenner, 1969). It seems therefore that the inhibitory effect (Fig. 1) is restricted to human tissues.

Aspartylglucosamine amidohydrolase activity of diseased organs (aspartylglucosaminuria) was also examined (Table 1). We could under no conditions reproduce the results published by Palo *et al.* (1971, 1972) and Somer *et al.* (1975) on this enzyme. Their high values, not only for normal but also for the diseased human organs, do not agree with the results given in the present paper. The reason for these discrepancies is not clear. However, it should be pointed out that low values should be obtained at a longer incubation time, but, if incubations are carried out without blanks, apparent high values are obtained. Furthermore, attempts were made to reproduce the assay used by Palo *et al.* (1971, 1972) for human tissues, but this did not prove possible. However, the results in the present paper suggest that the diseased organs show less than 6% of the activities found in the undiseased human organs. This could arise from (1) conformational change and/or deficiency of this enzyme protein, (2) the presence of some tightly bound inhibitor involved in this enzyme-catalysed reaction or (3) the substrate transport to the enzyme being disturbed under physiological conditions causing the aspartylglucosaminuria. However, our present knowledge of this enzyme cannot answer the question of how the defect and/or deficiency of this enzyme is related to the mental defect. The present paper may, it is hoped, stimulate the interest of other laboratories in the isolation of this enzyme from human brain and the comparison of it with that from human liver (see the Results section), so contributing to knowledge about this uncommon and incurable inborn error of metabolism, aspartylglucosaminuria.

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