A Comparison of Aminoacyl- β -Naphthylamide Hydrolases in Extracts of Human Tissues

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1. The aminoacyl- β -naphthylamide-hydrolase activities of extracts of human liver, kidney, pancreas and small intestine were partially purified by chromatography on DEAE-Sephadex. 2. The enzymes from the different tissues showed slight variations in chromatographic behaviour and in mobility on starch-gel electrophoresis. 3. Michaelis constants for the hydrolysis of L-leucyl- and L-alanyl- β -naphthylamide by the partially purified enzyme fractions were determined by a spectrofluorimetric assay method. The enzymes from different sources gave similar K_m values. 4. L-Leucinamide and L-leucylglycine were competitive inhibitors of the hydrolysis of both substrates. Values of K_i (leucinamide) and K_i (leucylglycine) respectively were constant whatever combination of enzyme and substrate was used. 5. The relevance of these results to the identity or non-identity of the enzymes from different tissues is discussed.

Enzymes that catalyse the hydrolysis of L-aminoacyl- β -naphthylamides (L-aminoacyl- β -naphthylamines) are widely distributed among human tissues and are present in blood plasma and urine. It now seems clear that these enzymes are not identical with the Mn^{2+} -activated leucine aminopeptidase (EC 3.4.1.1) acting typically on L-leucinamide or L-leucylglycine (Behal, Hamilton, Kanavage & Kelly, 1963; Patterson, Hsiao & Keppel, 1963; Fleisher, Pankow & Warmka, 1964; Nakagawa & Tsuji, 1964). Increases in aminoacyl- β -naphthylamide-hydrolase activity in the blood plasma are observed in hepatobiliary disease and, as with other plasma enzyme estimations, the clinical value of these observations would be enhanced if the relative contributions of enzymes from different tissues could be assessed. In the present study, therefore, a sensitive spectrofluorimetric assay was developed, and certain electrophoretic and kinetic properties of aminoacyl- β -naphthylamide hydrolases from several human tissues have been compared. Preliminary reports of parts of this work have been presented (Panveliwalla & Moss, 1965*a,b*).

MATERIALS AND METHODS

Enzyme preparations. Specimens of human kidney, small intestine, pancreas and liver were obtained from the post-mortem room within 24 hr. of death. Each tissue was homogenized in cold water, first in a Waring Blendor, then in a Potter-Elvehjem homogenizer. The extracts were centrifuged and were then concentrated by dialysis against

a 30% (w/v) solution of Carbowax 20M, followed by dialysis overnight at 4° against a large volume of water. The aminoacyl- β -naphthylamide-hydrolase activities of the concentrates varied from as low as 17 units/l. for some pancreas extracts to as high as 2000 units/l. for some kidney extracts, when assayed under the conditions described below (1 unit of activity corresponds to the hydrolysis of 1 μ mole of L-leucyl- β -naphthylamide/min.).

Ion-exchange chromatography. The concentrated extracts were eluted at 4° from columns (42 cm. $\times$ 2.5 cm.) of DEAE-Sephadex A-25 (Pharmacia A.B., Uppsala, Sweden) by an increasing concentration of NaCl in 10 mm-tris-2 mm-succinic acid-0.5 mm-MgCl₂ buffer, pH 8.2 (Patterson *et al.* 1963). The enzyme activity, chloride concentration (Cotlove, Trantham & Bowman, 1958) and $E_{280\text{ m}\mu}$ were measured on each fraction (5 ml.). Fractions composing the enzyme peak from each chromatogram were pooled and concentrated by dialysis against Carbowax 20M followed by dialysis against water. Recovery of the enzyme activity was more than 90%.

Starch-gel electrophoresis. This was carried out on horizontal gels essentially as described by Smithies (1955) in trays (17 cm. $\times$ 10.5 cm.) with a gel thickness of about 0.7 cm., in a discontinuous tris-citrate-borate buffer system at pH 8.6 (Poulik, 1957). The applied voltage was 10 v/cm. for 18 hr. at room temperature. After electrophoresis the gels were sliced and enzyme activity was located in the following way: the gel was covered with 0.2 M-phosphate buffer, pH 5.8, to counteract the alkaline electrophoresis buffer and bring the pH to approx. 7. After a few minutes, the cut surface was covered with a piece of filter paper soaked in a solution of L-leucyl- β -naphthylamide hydrochloride (80 mg./100 ml. of phosphate buffer, pH 5.8) and incubated at 37° in a closed container for a period depending on the enzyme activity, during which the appearance of free β -naphthylamine could be judged by its blue

fluorescence in ultraviolet light. The filter paper was then replaced by one soaked in a solution of tetra-azotized *o*-dianisidine (Fast Blue B salt; about 1 mg./ml.), which coupled with the β -naphthylamine to give pink bands at the enzyme zones. The gel was then transferred to the methanol-acetic acid-water (4:1:5, by vol.) wash solution. For recovery of enzyme activity from the starch gel, the colour reaction was carried out on strips from the edges of the gels, and then segments were cut out corresponding to the zones thus located. The gel segments were frozen at -20° and thawed, and the liquid was expressed by squeezing them against a sintered-glass plate in a glass syringe. The recoveries of enzyme activity achieved in this way were very low, amounting to 2-3% only.

Spectrofluorimetric assay of enzyme activity. The fluorescence excitation spectrum of β -naphthylamine in tris-HCl buffer at pH 7.2 has three peaks, at 330, 278 and 238 m μ . When scanned in the presence of L-leucyl- β -naphthylamide (0.8 mm) only the peak at 330 m μ , now slightly shifted to 335 m μ , is observed, presumably owing to strong absorption by the amide at the other wavelengths. The emission spectrum is unaffected by the presence of the amide, and has a maximum at 415 m μ . Enzyme activity was determined by adding the enzyme solution (0.1 ml. or less, depending on its activity) to 3 ml. of a solution of L-leucyl- β -naphthylamide hydrochloride (0.8 mm) in tris-HCl buffer, pH 7.2 (0.2 M), and measuring the increase in fluorescence at 415 m μ with excitation at 335 m μ . A recording spectrofluorimeter in which the temperature of the cell-compartment could be maintained at 37° (Moss, 1962) was used, allowing reaction progress curves to be recorded. By varying the sensitivity of the instrument a wide range of enzyme activities could be assayed. The reproducibility of the method was ± 0.8 unit (s.d.) at the 20 units/l. level (eight replicates). Calibration curves of β -naphthylamine in buffer-substrate solution were linear over the range 0-40 μ moles of β -naphthylamine/3.1 ml., and the relationship between enzyme concentration and amount of β -naphthylamine produced/min. was linear at least up to 10 μ moles/min., equal to an activity of 100 units/l. with 0.1 ml. of enzyme solution.

Roth (1964) has described the fluorimetric measurement of L-leucyl- β -naphthylamide-hydrolase activity with similar excitation and emission wavelengths, but with a fixed period of incubation. The spectrofluorimetric method is applicable to measurement of the hydrolysis of other aminoacyl derivatives of β -naphthylamine, and was used in this work to assay L-alanyl- β -naphthylamide breakdown.

Kinetic studies. Michaelis constants were determined at 37° and pH 7.2 for the hydrolysis of L-leucyl- β -naphthylamide and L-alanyl- β -naphthylamide by enzyme fractions prepared both by column chromatography and by starch-gel electrophoresis. The sensitivity of the spectrofluorimetric method allowed initial velocity measurements to be made at substrate concentrations down to 0.002 mM with values of $V_{\max}$ as low as 1 unit/l. Values of K_m and $V_{\max}$ were derived from straight lines fitted to plots of $1/v$ against $1/s$ by the method of least squares. Inhibition of the hydrolysis of both substrates by L-leucinamide and L-leucylglycine was studied by adding these compounds (final concentrations 30 mM and 2 mM respectively) to the reaction mixtures over a range of substrate concentrations; the enzyme fractions from the chromatographic columns were used in these experiments. Incubation of the enzyme solutions overnight at 37° with L-leucinamide and L-leucylglycine, followed by paper chro-

matography in butan-1-ol-acetic acid-water (12:3:5, by vol.), showed that there was some release of L-leucine from these compounds. Since, however, the amount of L-leucine liberated was small, and since the incubation periods used in the inhibition studies were about 5-7 min. only, it was assumed that enzymic hydrolysis of the inhibitors during the latter experiments could be neglected.

The heat stabilities of the chromatographically prepared enzyme fractions were determined by measuring the residual activities against L-alanyl- β -naphthylamide and L-leucyl- β -naphthylamide after incubation in 0.2 M tris-HCl buffer, pH 7.2, at temperatures between 50° and 70° for periods up to 65 min.

RESULTS

Each tissue extract gave a single major peak of enzyme activity on ion-exchange chromatography, with some evidence of heterogeneity (Fig. 1). There were some differences between the extracts in the chloride concentrations required to elute the enzyme activity. Rather diffuse enzyme zones showing variations in mobility from tissue to tissue were observed on starch gel (Fig. 2). Two zones of activity were present in some pancreatic extracts, but the appearance and relative proportions of these zones were not constant. The concentrated chromatographic fractions had mobilities on starch gel corresponding to those of the main enzyme zones in the original tissue extracts.

Plots of $1/v$ against $1/s$ were linear over a wide concentration range of either substrate, for both chromatographically and electrophoretically prepared enzyme fractions (Figs. 3 and 4). Values of K_m were similar for all the chromatographic enzyme fractions and for both L-leucyl- β -naphthylamide and L-alanyl- β -naphthylamide with a mean value of 0.068 mM (Table 1). Starch-gel electrophoresis resulted in a decrease in K_m values for the enzyme of small intestine, and for each tissue the K_m value after electrophoresis was similar to those after chromatography. The two zones seen on electrophoresis of certain pancreas extracts gave similar K_m values (Fig. 4). L-Alanyl- β -naphthylamide was hydrolysed about twice as fast as L-leucyl- β -naphthylamide by each enzyme preparation.

L-Leucinamide and L-leucylglycine were strictly competitive inhibitors of the hydrolysis of both substrates by the chromatographic enzyme fractions (Fig. 3). The values of K_i (leucylglycine) ranged from 0.45 to 0.77 mM and of K_i (leucinamide) from 5.4 to 8.4 mM (Table 1).

For a given tissue extract, the enzyme activities towards the two substrates declined in parallel when incubated at 50° , 60° , 65° and 70° at pH 7.2 (Fig. 5). The enzymes from different tissue sources behaved similarly on heating, with the possible exception of that of kidney extract, which appeared slightly less stable to heat than the others.

Fig. 1. Chromatography of tissue extracts on DEAE-Sephadex columns (42cm. $\times$ 2.5cm.) in tris-succinate buffer, pH 8.2. (a) Kidney; (b) small intestine; (c) pancreas; (d) liver. —, Aminoacyl- β -naphthylamide-hydrolase activity; ----, concn. of chloride.

Fig. 2. Aminoacyl- β -naphthylamide-hydrolase zones after starch-gel electrophoresis of tissue extracts at pH 8.6. (a) Kidney; (b) small intestine; (c) pancreas; (d) liver.

DISCUSSION

The slight variations in chromatographic behaviour between the enzymes from different tissues imply differences in net negative charge on the

enzyme molecules at pH 8.2. This implication is supported also by the different mobilities on starch-gel electrophoresis at pH 8.6, in that the enzyme fraction from small intestine, which migrated more slowly than the liver enzyme and thus had presumably a smaller net charge, was eluted at lower chloride concentration, whereas pancreatic enzyme, which exhibited a fast-migrating component, was also eluted at higher chloride concentration. The correspondence was not complete, however, in that kidney enzyme moved rapidly on starch gel and yet was eluted at low chloride concentrations; it is possible that molecular-size variations and hence different degrees of retardation in the gel are involved.

The K_m values for the hydrolysis of L-leucyl- β -naphthylamide do not vary significantly for enzymes from different tissues, and those with L-alanyl- β -naphthylamide as substrate are similar. The values reported here are of the same order as those reported by Behal *et al.* (1963) and Fleisher *et al.* (1964) for the hydrolysis of L-leucyl- β -naphthylamide by enzyme components of human plasma. If the leucyl- β -naphthylamide-splitting enzymes from different tissues are identical, and if these enzymes are responsible also for the hydrolysis of the alanyl substrate, the value of K_i (leucylglycine) should be constant, whatever combination of enzyme and substrate is used, as should the values of K_i (leucinamide). The mean value of K_i (leucylglycine) is 0.57mM and that of K_i (leucinamide) is 6.6mM; the variations from these means are random and probably within the limits of experimental error. Fleisher *et al.* (1964) have reported inhibitor constants of the same magnitude with

Fig. 3. Plots of $1/v$ against $1/s$ at pH 7.2 for chromatographically prepared enzyme fractions, showing competitive inhibition by L-leucinamide and L-leucylglycine. (a) Kidney; (b) small intestine; (c) pancreas; (d) liver. Substrate: —, L-leucyl- β -naphthylamide; ---, L-alanyl- β -naphthylamide. $\circ$, No inhibitor; $\bullet$, L-leucylglycine (2mM) added; $\square$, L-leucinamide (30mM) added. Values of K_m , K_i and $V_{\max}$ are collected in Table 1.

Fig. 4. Plots of $1/v$ against $1/s$ at pH 7.2 for pancreas extract before electrophoresis ($\bullet$), and faster ($\square$) and slower ($\circ$) zones separated by starch-gel electrophoresis. Values of K_m (mM): whole extract, 0.055; faster zone, 0.052; slower zone, 0.060. Values of $V_{\max}$ (units/l.): whole extract, 17.3; faster zone, 1.45; slower zone, 1.35.

human plasma as the source of enzyme. The hypothesis that, in a particular tissue extract, the same enzyme hydrolyses both L-leucyl- and L-alanyl- β -naphthylamide is supported by the observation of a constant ratio of the two activities during thermal inactivation.

The kinetic evidence thus appears to favour the view that, within a given tissue, a single enzyme is responsible for the hydrolysis of both substrates, and that the enzymes from different tissues are identical, or at least have closely similar active centres. However, the variations in electrophoretic and chromatographic behaviour between the enzymes from different sources must be taken into account when considering the question of their identity or non-identity; though small, these differences are reproducible, and similar variations in electrophoretic and chromatographic behaviour among aminoacyl- β -naphthylamide hydrolases

Table 1. Values of $V_{\max}$, K_m , K_i (L-leucylglycine) and K_i (L-leucinamide) for enzyme fractions partially purified by ion-exchange chromatography, with L-leucyl- β -naphthylamide and L-alanyl- β -naphthylamide as substrates

$V_{\max}$ values are expressed as μ moles of substrate hydrolysed/min./l. of enzyme solution (units/l.); K_m and K_i values are expressed as mM.

Source of enzyme	L-Leucyl- β -naphthylamide				L-Alanyl- β -naphthylamide			
	$V_{\max}$	K_m	K_i (Leu-Gly)	K_i (Leu-NH ₂)	$V_{\max}$	K_m	K_i (Leu-Gly)	K_i (Leu-NH ₂)
Kidney	48	0.062	0.59	5.6	100	0.070	0.70	6.8
Small intestine	54	0.064	0.45	6.6	110	0.070	0.58	6.3
Pancreas	56	0.063	0.51	7.9	110	0.079	0.48	5.9
Liver	17	0.071	0.77	8.4	29	0.063	0.49	5.4

Fig. 5. Effect of incubation at different temperatures and pH 7.2 on activity of chromatographically purified enzyme fractions. (a) Kidney; (b) small intestine; (c) pancreas; (d) liver. Substrates: L-leucyl- β -naphthylamide (open symbols) and L-alanyl- β -naphthylamide (solid symbols). Temperatures: $\circ$ and $\bullet$, 50°; $\triangle$ and $\blacktriangle$, 60°; $\square$ and $\blacksquare$, 65°; ∇ and $\blacktriangledown$, 70°. Initial enzyme activities; L-leucyl- β -naphthylamide: (a) 7, (b) 59, (c) 55, (d) 44 units/l.; L-alanyl- β -naphthylamide: (a) 15, (b) 147, (c) 125, (d) 104 units/l.

from human tissues have been reported by other workers (Nakagawa & Tsuji, 1964; Behal, Asserson, Dawson & Hardman, 1965). There is some evidence also that tissue enzyme components may retain their chromatographic properties after release into the blood, since human plasma has been shown to contain several chromatographically distinguish-

able aminoacyl- β -naphthylamide-hydrolase components (Behal *et al.* 1963). When the distinction between enzymes from different sources rests only on the demonstration of small differences in net charge, as in the present case, the postulation of separate enzymes or isoenzymes must be made with caution, since such variations might result from the

attachment to the enzyme protein of non-enzymic entities such as carbohydrate or peptides, owing to environmental factors, without the presence of underlying differences in basic enzyme structure. In cases such as these the question of the identity or otherwise of the enzymes concerned can only be resolved by purification and structural studies.

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