Hydrolysis of Dipeptide 2-Naphthylamides by Human Muscle Enzymes

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Human muscle enzymes that hydrolyse dipeptide 2-naphthylamides were separated by chromatography on DEAE-Sephadex. Evidence was obtained that these substrates are hydrolysed by arylamidase as well as by dipeptidyl aminopeptidases.

An intracellular peptidase which hydrolysed the chymotrypsin substrate, glycyl-L-phenylalanine amide, was described by Gutmann & Fruton (1948) and termed cathepsin C. Subsequent studies by Fruton and his colleagues (see Metrione *et al.*, 1966) established that cathepsin C is a thiol-activated enzyme which catalyses the transfer of an *N*-terminal dipeptide; at pH 5 the reaction is entirely hydrolytic, but transamidation occurs at higher pH values. Glycyl-L-phenylalanine *p*-nitroanilide and the corresponding 2-naphthylamide also serve as substrates (Planta & Gruber, 1963; Vanha-Perttula *et al.*, 1965), and certain polypeptides are attacked by the enzyme (Planta & Gruber, 1964; McDonald *et al.*, 1969). The enzyme has a fairly broad specificity towards the dipeptide moiety, glycyl-L-arginine 2-naphthylamide being the most active substrate tested (McDonald *et al.*, 1969). It was established independently by Gorter & Gruber (1970) and McDonald *et al.* (1966a) that cathepsin C requires Cl⁻ for activity; certain other anions are also effective.

It was shown by McDonald *et al.* (1968) that anterior-pituitary extracts contain another enzyme which hydrolyses dipeptide 2-naphthylamides but the specificity of which differs from that of cathepsin C. It attacks the 2-naphthylamide of L-lysyl-L-alanine, which is not attacked by cathepsin C, but does not hydrolyse many of the substrates of the latter enzyme. It was proposed that cathepsin C should be renamed 'dipeptidyl aminopeptidase I' and the other enzyme called 'dipeptidyl aminopeptidase II'. A third enzyme, which hydrolyses only L-arginyl-L-arginine 2-naphthylamide, was also found in the pituitary (Ellis & Nuenke, 1967) and named dipeptidyl aminopeptidase III.

Meanwhile, Hopsu-Havu & Glenner (1966) had found, in rat liver, an enzyme which hydrolyses glycyl-L-proline 2-naphthylamide. This substrate is not hydrolysed by the other three enzymes, and Ellis and colleagues have proposed calling this enzyme 'dipeptidyl aminopeptidase IV'. Further information on these enzymes is given by McDonald *et al.* (1971).

Another group of enzymes, the so-called 'arylamidases', which hydrolyse 2-naphthylamides and *p*-nitroanilides of amino acids, are widespread in

animal tissues. Many also hydrolyse peptides, although, in general, the classification and specificity relationships among this group are ill-defined. It has been reported, however, that such enzymes from kidney (Vanha-Perttula *et al.*, 1965) and pituitary (McDonald *et al.*, 1966b) hydrolyse dipeptide 2-naphthylamides, splitting off the single amino acids sequentially.

We are studying peptide hydrolases in muscle in the hope of learning more of their role in protein breakdown in normal and diseased muscle. In a previous communication (Bury & Pennington, 1973) we reported on arylamidases in human muscle. The present paper describes the chromatographic separation from human muscle of enzymes which hydrolyse 2-naphthylamide derivatives of various dipeptides.

Experimental

Muscle. Human deltoid muscle was obtained within 24 h of death from persons without evidence of muscle disease. It was kept at -20°C until used.

Chemicals. 2-Naphthylamides of glycyl-L-arginine, L-arginyl-L-arginine, L-lysyl-L-alanine, L-seryl-L-tyrosine and glycyl-L-proline were obtained from Bachem Feinchemikalien A.G., CH4410 Liestal, Switzerland. 2-Naphthylamides of glycyl-L-phenylalanine and L-arginine were from Sigma Chemical Co., London S.W.6, U.K. Puromycin dihydrochloride was from Serva, Heidelberg, Germany, Hyamine 1622 (benzethonium chloride) from BDH Chemicals, Poole, Dorset, U.K., and Fluram (fluorescamine) from Roche Products Ltd., Welwyn Garden City, Herts., U.K.

Extraction and chromatography. Muscle was homogenized at 0–4°C with 10 mM-potassium phosphate buffer, pH 7.2, containing 1 mM-dithiothreitol. An Ultra-Turrax homogenizer was used and the muscle fibres were completely disintegrated, as seen by phase-contrast microscopy. The volume was finally made up with the medium to 10 ml/g of muscle. The homogenate was centrifuged (177 000g, 30 min) and the clear supernatant was dialysed overnight against potassium phosphate buffer (pH 7.2, 10 mM) containing dithiothreitol (1 mM) and NaCl (0.2 M). A

Fig. 1. Hydrolysis of 2-naphthylamides by fractions from DEAE-Sephadex chromatography of human muscle extract

For details of experiments see the text. (a) ●, Protein; ····, NaCl. (b) ○, L-Arginine 2-naphthylamide (50 μl of fraction; 0.5 h); ●, glycyl-L-arginine 2-naphthylamide (50 μl; 1 h); ◐, glycyl-L-phenylalanine 2-naphthylamide (50 μl; 2 h). (c) ○, As for (b); ●, L-lysyl-L-alanine 2-naphthylamide (50 μl; 3 h); ◐, L-arginyl-L-arginine 2-naphthylamide (100 μl; 2 h).

portion (approx. 20 ml) was applied to a column (0.9 cm × 60 cm) of DEAE-Sephadex (type A50) previously equilibrated with the same medium. Proteins were eluted with this medium followed by a linear gradient (0.2–0.5 M) of NaCl in the medium (100 ml). Fractions (approx. 3 ml) were collected.

Measurement of enzyme activities. Enzymes were incubated in a medium (volume, including enzyme, 0.5 ml) containing buffer (50 mM), the appropriate 2-naphthylamides and dithiothreitol (1 mM). The following buffers were used (unless stated otherwise): potassium phosphate, pH 7.5, potassium phosphate,

pH 6.0, sodium citrate, pH 5.5, *N*-tris(hydroxymethyl)methylglycine, pH 9.0, and potassium phosphate, pH 7.7, respectively with the substrates for arylamidase and dipeptidyl aminopeptidases I, II, III and IV. The concentration of substrate was 0.25 and 0.50 mM for the L-arginine 2-naphthylamide and the dipeptide naphthylamides respectively. Stock solutions of glycyl-phenylalanine 2-naphthylamide and seryl-tyrosine 2-naphthylamide were prepared in dimethyl sulphoxide, giving a final concentration of 4% of this compound in the incubation medium. When the dipeptidyl aminopeptidase I substrates

Table 1. *Effect of benzethonium chloride on enzymic hydrolysis of glycyl-L-arginine 2-naphthylamide*

For details see the text.

Concn. of benzethonium chloride (mM)	Enzyme inhibition (%)		
	Peak A	Peak B	Peak D
0.1	0		55
1.0	53	74	100
1.0*	100	100	100

* pH 7.5 (50 mM-potassium phosphate buffer) instead of pH 6.0.

(glycyl-phenylalanine 2-naphthylamide, seryl-tyrosine 2-naphthylamide and glycyl-arginine 2-naphthylamide) were used the medium also contained 5 mM-NaCl. After shaking of the tubes at 37°C for the required time, 1 ml of ethanol was added and the tubes were centrifuged. The fluorescence of the liberated β -naphthylamine was measured (excitation 340 nm, emission 402 nm).

Protein was measured fluorimetrically with fluor escamine (Böhlen *et al.*, 1973) with bovine serum albumin as standard. The concentration of NaCl in the column fractions was monitored by measurement of sodium with a flame photometer.

Results and discussion

Fig. 1(b) shows the elution profile from DEAE-Sephadex of enzymes that hydrolyse the 2-naphthylamides of L-arginine, glycyl-L-arginine and glycyl-L-phenylalanine. It is clear that the greater part of the activity towards glycyl-L-phenylalanine 2-naphthylamide is associated with the peaks (C and D) of arylamidase activity. Peak A, which has no arylamidase, is presumed to be dipeptidyl aminopeptidase I. A similar pattern is shown with glycyl-L-arginine 2-naphthylamide as substrate, although, in this case, there is a greater proportion of the activity in peak A. Results with L-seryl-L-tyrosine 2-naphthylamide (not shown) were similar; over 90% of the activity was in the arylamidase peaks. For glycyl-L-arginine 2-naphthylamide an additional small peak is seen, in the same position as the smaller peak (B) of activity towards L-arginine 2-naphthylamide. In other experiments, however, it slightly preceded the latter.

The activity of the column fractions towards substrates of dipeptidyl aminopeptidases II and III is shown in Fig. 1(c). One peak of dipeptidyl aminopeptidase II activity without associated arylamidase is seen; this always followed slightly behind the dipeptidyl aminopeptidase I peak (A, Fig. 1b). The main

arylamidase peak (D) also showed activity towards L-lysyl-L-alanine 2-naphthylamide. For L-arginyl-L-arginine 2-naphthylamide, all of the activity was associated with the arylamidase peak (D).

No activity towards glycyl-L-proline 2-naphthylamide, the reported substrate for dipeptidyl aminopeptidase IV, could be detected in the human muscle extracts.

Table 1 shows the effect of benzethonium chloride (benzyl dimethyl-2-[2-(*p*-2,2,3,3-tetramethylbutylphenoxy)ethoxy]ethyl ammonium chloride) on the hydrolysis of glycyl-L-arginine 2-naphthylamide by individual peaks. This compound was shown by Mäkinen (1968) to inhibit arylamidases. The more pronounced inhibition with peak D is an additional indication that the activity in this peak is due to arylamidase. Further evidence was obtained with puromycin, which also inhibits arylamidases (Ellis & Perry, 1966). The activity in peak D towards the 2-naphthylamides of L-arginine, glycyl-L-phenylalanine and glycyl-L-arginine was inhibited 98, 91 and 97% respectively by 1 mM-puromycin, whereas there was no inhibition of the activity in peak A towards the latter two substrates. Moreover, with glycyl-L-arginine 2-naphthylamide as substrate, the activity in peak A was increased by 51% in the presence of 5 mM-NaCl, whereas that in peak D was unaffected.

It seems clear therefore from these results that none of the dipeptide 2-naphthylamides tested can be used as specific substrates for dipeptidyl aminopeptidases I, II or III in human muscle, since they are attacked also by arylamidase. The use of puromycin to inhibit arylamidases may, however, offer the possibility of specific dipeptidyl aminopeptidase assays in muscle preparations.

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