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Separation and Properties of Two Arylamidases from Rat Cardiac-Muscle Extracts

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Two main arylamidase activities were separated from a particle-free supernatant of rat heart by chromatography on DEAE-Sephadex. Although both enzymes hydrolysed L-leucine 4-nitroanilide, only peak-II enzyme hydrolysed L-lysine 4-nitroanilide. A third minor peak (Ia) contained an enzyme that was active mainly on the L-lysine 4-nitroanilide. The mol.wts. of the enzymes in peaks I and II were approx. 257000 and 105000 respectively. The pH optimum was approx. pH 7.0 for peak-I enzyme and 7.0–8.0 for peak-II enzyme. Both enzymes were inhibited by addition of puromycin, *p*-hydroxymercuribenzoate, *o*-phenanthroline and bivalent metal ions. Addition of dithiothreitol resulted in stimulation of both activities. Dialysis against *o*-phenanthroline resulted in inhibition of peak-I and -II enzymes, but after dialysis against EDTA only peak-II enzyme was inhibited.

The term 'arylamidase' refers to enzymes that hydrolyse α-aminoacyl derivatives of 2-naphthylamine (Patterson *et al.*, 1963), 4-nitroaniline (Tuppy *et al.*, 1962; Ellis, 1963), aniline (Kleine *et al.*, 1973) or aminonitriles (Szewczuk *et al.*, 1965). Enzymes of related activity listed in the recommendations of the Commission for Enzyme Nomenclature (IUPAC–IUB, 1973) are classified as α-aminoacyl-peptide hydrolases (EC 3.4.11.–), e.g. aminopeptidase M of pig kidney (EC 3.4.11.2), arginine aminopeptidase of rat liver (EC 3.4.11.6) and aspartate aminopeptidase of rat kidney (EC 3.4.11.7). Arylamidase activity has been reported to be present in extracts of mammalian and non-mammalian muscle tissues, for example the skeletal muscle of various marine species (Bauer & Eitenmiller, 1974), man (Bury & Pennington, 1973) and rat (Pluskal & Pennington, 1973), the smooth muscle of rat (Kidwai *et al.*, 1971) and the cardiac muscle of man (Rehfeld *et al.*, 1967) and rat (Hopsu & Ekfors, 1969). In the skeletal muscle of both man (Bury & Pennington, 1975) and rat (Parsons & Pennington, 1976) the arylamidase activity was further separated by DEAE-Sephadex chromatography into three or four enzyme activities. Chromatography of rat cardiac-muscle supernatant on DEAE-cellulose revealed only one peak of activity towards L-leucine 2-naphthylamide (Hopsu & Ekfors, 1969). In a study to determine the role of rat cardiac-muscle arylamidases in proteolytic degradation of heart tissue we report the chromatographic separation and properties of two enzymes hydrolysing L-leucine 4-nitroanilide.

Experimental

Muscle

Male and female Wistar rats weighing 250–300g were killed by a blow on the head. The cardiac muscle from three rats was immediately obtained by dissection of the heart, followed by a thorough washing in ice-cold homogenization medium (see below).

Chemicals

Dithiothreitol, EDTA, *o*-phenanthroline and *p*-hydroxymercuribenzoate were obtained from BDH Chemicals, Poole, Dorset, U.K. L-Lysine 4-nitroanilide dihydrobromide was from E. Merck, Darmstadt, W. Germany. L-Leucine 4-nitroanilide and puromycin dihydrochloride were from Sigma Chemical Co., London S.W.6, U.K. DEAE-Sephadex (type A-50) and Sephadex G-200 were from Pharmacia (G.B.) Ltd., London W5 5SS, U.K.

Extraction and chromatography

All operations were carried out at 0–4°C. Muscle was homogenized with 10mM-Tris/HCl buffer, pH 7.2, containing 0.1mM-dithiothreitol and 0.2M-NaCl, in a Polytron homogenizer for 10s. Homogenization was continued for three or four periods of 30s by using a tight-fitting Potter–Elvehjem homogenizer. The volume was made up with the homogenization medium to 10ml/g of muscle. The homogenate was centrifuged for 45min at 100000*g* in a Beckman–Spinco rotor type 40 or 40.2 ($r_{av.}$ 5.9cm or 5.7cm

respectively). The clear supernatant was dialysed for at least 12h against the same medium. A portion (19.5ml) was applied to a column (1.5cm×25cm) of DEAE-Sephadex (type A-50) previously equilibrated with the medium. Elution of proteins was effected by 80–100ml of this medium followed by elution with a gradient of NaCl (0.2–0.5M) applied in 100ml of medium (Fig. 2). Fractions (2–3ml) were collected and every tube was assayed for hydrolysis of L-lysine 4-nitroanilide and L-leucine 4-nitroanilide. Fractions from each of the peaks I and II were separately pooled and used for subsequent experiments.

Molecular-weight determination

Molecular-weight determinations (Andrews, 1965) were carried out on a column (1.5cm×35cm) of Sephadex G-200 (V_0 28ml) pre-equilibrated and eluted with the medium described above. A portion of peak-I enzyme (0.9ml), which had been previously concentrated 20-fold (in an Amicon Minicon B15 series of cells; Amicon Corp., Lexington, MA, U.S.A.), or of peak-II enzyme (2.0ml, not concentrated) was applied to the column. Fractions (2–3ml) were collected. Standard proteins used were horse heart myoglobin, chymotrypsinogen, ovalbumin, bovine serum albumin, γ-globulin and urease (mol.wts. 18650, 25000, 45000, 67500, 160000 and 490000 respectively). Flow rates for all column chromatography were 6–15ml/h.

Enzyme activities

Enzyme samples (10–350μl) were incubated at 37°C in a medium (final volume 1.0ml) containing Tris/acetate buffer (50mM), pH7.5, dithiothreitol (0.5mM) and either L-lysine 4-nitroanilide or L-leucine 4-nitroanilide (0.5mM) as substrate. The incubation was carried out for 0.5–4h, and the reaction was terminated by the addition of 0.5ml of 12.5% (w/v) trichloroacetic acid buffered to pH3.5 with sodium acetate buffer (50mM). The solution was centrifuged at 2000*g* for 10min and the A_{400} of the supernatant measured (ε for 4-nitroaniline is 11080). Control tubes, in which the enzyme was not added until after the trichloroacetic acid, were always included in the incubation. Protein concentration was determined from the A_{280} (Warburg, 1941). NaCl concentration in the column fractions was measured by flame photometry.

Results and Discussion

Rat heart extracts hydrolysed L-leucine 4-nitroanilide and L-lysine 4-nitroanilide at approx. 15 and 17nmol/h per mg fresh wt. respectively. This compares with the value of approx. 24nmol of L-leucine 2-naphthylamide hydrolysed/h per mg fresh wt. found by Hopsu & Ekfors (1969). In six separate experiments 64–92% of the arylamidase activity was released into the supernatant during homogenization. The pH optima for the hydrolysis of the 4-nitroanilides of leucine and lysine by the particle-free supernatant are shown in Fig. 1. The optima of pH7.0–8.0 are similar to those of other studies, e.g. arylamidase in human deltoid muscle (Bury & Pennington, 1973).

Chromatography

Chromatography of the supernatant on DEAE-Sephadex gave the results shown in Fig. 2. There was a recovery from the column of 70% of the enzyme activity applied in the supernatant. There were two main peaks of activity (I and II), with peak II having 80% of the total activity (against L-leucine 4-nitroanilide) recovered from the column. There was a trace of enzyme activity in peak Ia, most active with L-lysine 4-nitroanilide, but this peak has not been further investigated. Peak II, eluted during gradient elution at approx. 0.3M-NaCl, had the greatest purity (46-fold purification), and after electrophoresis on sodium dodecyl sulphate/polyacrylamide gels (10%; Weber &

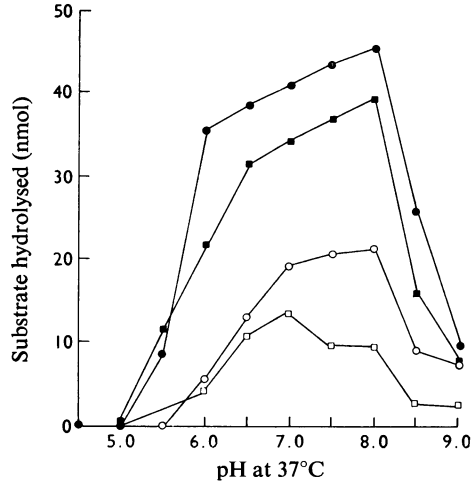

Fig. 1. *Variation with pH of the hydrolysis of the 4-nitroanilides of leucine and lysine by rat cardiac-muscle supernatant and enzymes in peaks I and II from the DEAE-Sephadex column*

The experiments were carried out in buffers of 50mM concentration: sodium acetate, pH4.5–5.0; 2-(*N*-morpholino)ethanesulphonic acid/NaOH, pH5.5–6.5; Tris/acetate, pH7.0–9.0. The ionic strength was maintained constant at 50mM by addition of KCl. ●, L-Lysine 4-nitroanilide hydrolysis by supernatant (50μl for 30min); ■, L-leucine 4-nitroanilide hydrolysis by supernatant (50μl for 30min); ○, L-lysine 4-nitroanilide hydrolysis by peak-II enzyme (10μl for 1h); □, L-leucine 4-nitroanilide hydrolysis by peak-II enzyme (100μl for 1h).

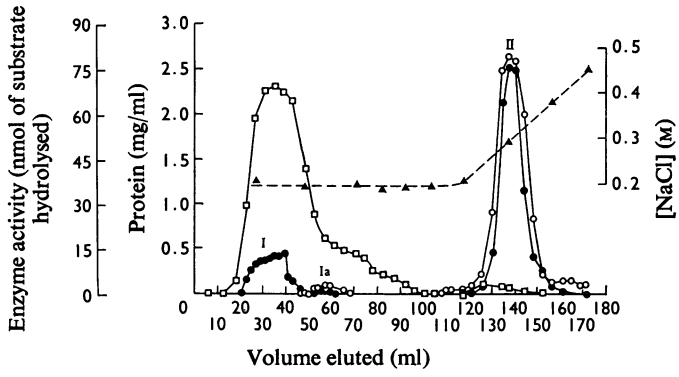

Fig. 2. *Hydrolysis of aminoacyl 4-nitroanilides by fractions from DEAE-Sephadex chromatography of rat cardiac-muscle supernatant*

Fractions (2–3ml) were collected and assayed for protein (□), NaCl (▲) and hydrolysis of the 4-nitroanilides of lysine (○) or leucine (●) by 100μl of fraction for 30min. For other details, see the text.

Osborn, 1969) only two protein bands were visible after staining with Coomassie Blue (results not shown). Enzymes from peaks I and II both hydrolysed L-leucine 4-nitroanilide, but only peak-II enzyme hydrolysed L-lysine 4-nitroanilide (and it did so at higher rates than for the L-leucine derivative). The properties of the enzymes of peaks were studied by using the 4-nitroanilides of leucine and lysine for peaks I and II respectively, unless otherwise stated.

pH optima

The pH optima were determined in 50mM buffers between pH4.5 and 9.0 (as shown in Fig. 1). Optima of approx. 7.0 and 7.0–8.0 were obtained for peak-I and -II enzymes respectively. There was still considerable activity at pH6.5 and 8.5 for both enzymes.

Molecular weights

Gel filtration of the enzymes from peaks I and II on Sephadex G-200 gave estimated mol.wts. of 257000± 5000 and 105000± 5000 respectively with L-leucine 4-nitroanilide as substrate in both cases. Peak-I enzyme shows a molecular weight of similar order to the ones reported for the pig kidney enzymes, aminopeptidase M (mol.wt. 280000; Wachsmuth *et al.*, 1966) and leucine aminopeptidase (EC 3.4.11.1, mol.wt. 255000; Melius *et al.*, 1970), the arylamidases of kidney from man (EC 3.4.11.–, mol.wt. 230000; Hutter *et al.*, 1973) or rat (EC 3.4.11.–, mol.wt. 207000; Kleine *et al.*, 1973). Connective-tissue peptidases (active on peptide substrates) have mol.wts. of 100000 (and 250000) (Schwabe, 1969) and the arylamidases of rat liver have mol.wts. between 65000 and 80000 (Patterson *et al.*, 1965) more closely resembling peak-II enzyme.

Effects of dithiothreitol, p-hydroxymercuribenzoate, bivalent metal ions, EDTA, o-phenanthroline and puromycin on the enzyme activities of peaks I and II

Representative results from a study of the effects of various compounds on the enzyme activities of peaks I and II are shown in Table 1. In each case the compound was preincubated for 5 or 10min at 0°C with the enzyme in the buffer medium before addition of substrate.

Dithiothreitol. Addition of 0.5mM- or 1.0mM-dithiothreitol (preincubation for 5min at 0°C) resulted in stimulation of both enzyme activities (compared with assays with no added dithiothreitol). Experiments with crude supernatant and peak-II enzyme showed that stimulation was maximal at 0.5mM-dithiothreitol, and this concentration was used as a routine. However, peak-I enzyme shows greater stimulation at 1.0mM-dithiothreitol.

Thiol reagents. Inclusion of *p*-hydroxymercuribenzoate (0.5mM, preincubation for 10min at 0°C) resulted in inhibition of both activities, even when 0.5mM- or 1.0mM-dithiothreitol was present. This suggests that *p*-hydroxymercuribenzoate reacts with thiol groups on the enzymes. As a univalent mercurial, it forms a strong complex with thiol groups, but does not under ordinary conditions promote the disruption of thioester or disulphide bonds (Jocelyn, 1972, p. 145). Hence the thiol groups complexed cannot be involved in maintaining structure or conformation by disulphide bridges. The enzyme catalytic process may utilize thiol groups at the active site, or they may be concerned with the binding of substrates or metal ions. Alternatively, inhibition may be due to a conformational change that occurs after binding *p*-hydroxymercuribenzoate (Jocelyn, 1972, p. 22).

Table 1. *Effect on enzyme activity of the additon of various compounds to the enzymes from peaks I and II from DEAE-Sephadex chromatography of rat heart supernatant*

All assays were carried out in duplicate and these agreed to within ±5% of the mean. Percentage changes in activity denote the stimulation (+) or inhibition (−) or no change (0) after addition of the compound under the conditions stated in the text. Where no dithiothreitol was added to the assay there was a residual amount present (from the enzyme samples) of 5 μM or 1 μM in the assays of peak-I or -II enzymes respectively. Where dithiothreitol was added to the enzyme in the presence of *p*-hydroxymercuribenzoate it was added 5min after the latter. The percentage change due to *p*-hydroxymercuribenzoate is relative to assays containing the same amount of dithiothreitol. Activities in control assays were 7–15 and 25–30nmol/h for peak-I and -II enzymes respectively.

Addition	Change in the hydrolysis of the 4-nitroanilides of: L-leucine by peak-I enzyme (%)	L-lysine by peak-II enzyme (%)
Dithiothreitol (1.0mM)	+70	+870
Dithiothreitol (0.5mM)	+39	+767
p-Hydroxymercuribenzoate (0.5mM)	−97	−37
p-Hydroxymercuribenzoate (0.5mM)+dithiothreitol (0.5mM)	−53	−67
p-Hydroxymercuribenzoate (0.5mM)+dithiothreitol (1.0mM)	−60	−33
Co^{2+} (0.1mM: 1.0mM)	−100; −100	−100; −100
Zn^{2+} (0.1mM; 1.0mM)	−52; −100	−59; −100
Mn^{2+} (0.1mM; 1.0mM)	−55; −90	−15; −32
Ca^{2+} (0.1mM; 1.0mM)	−19; −28	−11; −27
Mg^{2+} (0.1mM; 1.0mM)	−7; −9	0; −26
o-Phenanthroline (1.0mM)	−100	−89
EDTA (1.0mM)	+29	−33
Puromycin (1.0mM)	−31	−97
		−95 (L-leucine 4-nitroanilide)

Bivalent metal ions. The bivalent metal ions Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} were added as their chloride salts at either 0.1mM or 1.0mM final concentration (10min preincubation at 0°C). The general order of inhibition for both enzymes was: $Co^{2+} > Zn^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$. This inhibition may occur by interactions of these ions with free thiol groups on the enzymes in a manner which may cause denaturation (Vallee & Wacker, 1970, p. 144). Mn^{2+}, Mg^{2+} and Ca^{2+} were the least effective inhibitors and these ions bind more weakly to thiol groups than do Co^{2+} or Zn^{2+} (Jocelyn, 1972, p. 85).

Metal-ion chelators. EDTA (1.0mM, preincubated for 10min at 0°C) results in stimulation of peak-I enzyme and slight inhibition of peak-II enzyme activity. The stimulation by EDTA is not understood. Perhaps there are inhibitory ions associated with the enzyme preparation which are effectively removed by EDTA; alternatively there may be a direct interaction between EDTA and the enzyme, or through co-ordination with a metal ion, which causes a favourable conformational change. Also, higher concentrations of EDTA may be needed for inhibition, e.g. with aminopeptidase M 10mM-EDTA gave only 40% inhibition (Femfert & Pfleiderer, 1971). *o*-Phenanthroline (under the same conditions) appears to inhibit both enzymes potently. The stability constants for complexes of the above bivalent metal ions with EDTA are all greater than those for *o*-phenanthroline, in contrast with their efficiency as inhibitors of enzyme activity. Chelation by some of the six co-ordination sites of EDTA may be prevented by steric hindrance by the protein (Vallee & Wacker, 1970, p. 130), whereas *o*-phenanthroline with two ligands may not be hindered. Also, *o*-phenanthroline may interact with hydrophobic regions of the enzymes and cause denaturation (Vallee & Wacker, 1970, p. 142).

Puromycin. Addition of 1.0mM-puromycin (10min preincubation at 0°C) results in only moderate inhibition of peak-I enzyme but almost complete inhibition of peak-II enzyme. Puromycin was shown by Ellis & Perry (1964) to be a competitive inhibitor of L-lysine 4-nitroanilide hydrolysis in bovine pituitary extracts and has often been adopted as a specific inhibitory test for arylamidase.

Dialysis against metal-ion chelators

Three samples of enzyme from peak I or II (1.2–2.0ml) were placed in separate dialysis sacs (6.35mm diameter, thoroughly washed in EDTA solution, then water) and dialysed at 0°C for 13.5–21h against 0.5 litre of buffer alone (10mM-Tris/HCl, pH7.2 at 0°C, containing 0.1mM-dithiothreitol), buffer containing 1mM-EDTA or buffer containing 1mM-*o*-phenanthroline. Samples were removed for assay [(i) of Table 2]. The enzymes were redialysed at 0°C against 1 litre each of buffer only for 22–24h and samples taken again for assay [(ii) of Table 2]. The results of duplicate experiments with the same enzyme preparation are shown in Table 2. Dialysis against EDTA results in inhibition of peak-II enzyme but not of peak-I enzyme. This inhibition after the first dialysis can be partially reversed by addition of Mg^{2+} or Ca^{2+} but not Mn^{2+}, Co^{2+} or Zn^{2+} (1mM, results not shown) or by redialysis with buffer without chelator. The reversal by Mg^{2+} or Ca^{2+} suggests that the EDTA is binding a metal ion on the enzyme, since adding excess of these ions results in competition with the metalloenzyme for the bound inhibitor

Table 2. *Effect of dialysis against* 1 mM-*EDTA and* 1 mM-*o-phenanthroline on the enzyme activity of peaks I and II*

The percentage change in enzyme activity refers to the activity of the enzyme subjected to (i) dialysis against buffer with chelator then (ii) against buffer only, compared with an identical enzyme sample subjected to dialysis against buffer with no chelator, once [for (i)] then again [for (ii)] under the same conditions. Results under A and B are from separate experiments with the same preparation of peak-I or -II enzymes. Assays were carried out in duplicate and these agreed to within ±5% of the mean.

Enzyme sample and type of dialysis	Change in the hydrolysis of 4-nitroanilides of: L-leucine by peak-I enzyme (%) A	B	L-lysine by peak-II enzyme (%) A	B
EDTA				
(i) EDTA-dialysed enzyme	+40	+3	−100	−84
(ii) EDTA-dialysed enzyme after further dialysis	+40	+3	−45	−16
o-Phenanthroline				
(i) *o*-Phenanthroline-dialysed enzyme	−14	+8	−18	−25
(ii) *o*-Phenanthroline-dialysed enzyme after further dialysis	−70	−55	−63	−69

(Vallee & Wacker, 1970, p. 135). The reversal of inhibition by dialysis alone may be due to loss of EDTA but not of metal ion during dialysis and occurs if the enzyme binds more strongly to the metal ion than does EDTA. Adding 1 mM-bivalent metal ions to peak-II enzyme after the second dialysis did not restore the rest of the activity. The greater inhibition obtained by addition of EDTA by dialysis than by direct addition of EDTA to the assay may result from the longer preincubation time (i.e. during dialysis) of enzyme with inhibitor and thus a firmer binding. *o*-Phenanthroline causes little or no inhibition of either enzyme after dialysis against it. This is in contrast with the effect of its direct addition to the assay system. If *o*-phenanthroline binds to the enzyme very tightly and rapidly (as in direct addition), then a greater inhibition due to possible denaturation may be observed than if the inhibitor is gradually introduced (as in dialysis). That *o*-phenanthroline does bind more tightly to the metal ions of the enzymes than EDTA or than the enzymes themselves is shown by a further loss of enzyme activity during the second dialysis, when the chelator (and presumably the metal ion) has been removed. There was no restoration of activity by 1 mM-bivalent-metal ions after the first or second dialysis involving *o*-phenanthroline (results not shown).

Kinetic study of the inhibition of peak-II enzyme by puromycin

The results are shown in Fig. 3. The K_m for L-lysine 4-nitroanilide is 3.9×10^{-5} M. Puromycin inhibits in a competitive manner, with an inhibitor constant K_i of 8.3×10^{-7} M. These values are of the same order as found in studies of puromycin inhibition of L-alanine 2-naphthylamide hydrolysis by arylamidase from muscle of several marine species (Bauer & Eitenmiller, 1974). The molecular nature of the inhibition is unclear. The possibility exists that the inhibition is due to the presence of a cation, similar, for example, to the inhibition noted for dipeptidyl peptidase-II (EC 3.5.14.–) by puromycin (McDonald *et al.*, 1971). Inhibition of arylamidase in skeletal muscle (Bury & Pennington, 1975) occurred with puromycin as well as with the cationic detergent benzethonium chloride. The latter compound was shown to be a competitive inhibitor of arginine aminopeptidase B (Makinen, 1968). Alternatively, there may be a hydrophobic interaction, since the charged aminonucleoside moiety of puromycin alone did not inhibit the bovine brain arylamidase (Suszkiw & Brecher, 1970), but required the *p*-methoxyphenylalanine linked to the 3-aminoribose moiety of puromycin.

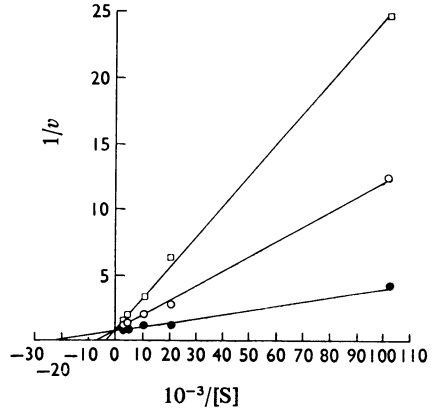

Fig. 3. *Kinetics of inhibition by puromycin of the hydrolysis of* L-*lysine 4-nitroanilide by peak-II enzyme*

Initial velocities were measured over a 5–10 min period in a Gilford model 252 spectrophotometer. A sample (75 μl) of peak-II enzyme, previously concentrated 4-fold, was incubated in the buffer medium as described, except that the total volume was 1.5 ml. Initial velocities (v) are in nmol of product formed/min per 75 μl of enzyme, without added puromycin (●) or with puromycin at concentrations of 2.17 μM (○) and 4.32 μM (□).

There appears to be a similarity between cardiac-muscle and skeletal-muscle tissues with respect to the arylamidase enzymes. Peaks resembling I, Ia and II in terms of elution order from DEAE-Sephadex columns and substrate specificity were obtained from extracts of skeletal muscle of man and rat (Bury & Pennington, 1973, 1975; Parsons & Pennington, 1976). Peak-I enzyme is very similar in many of its properties to the pig kidney microsomal amino-peptidase M (Femfert & Pleiderer, 1971), and studies on its intracellular distribution in cardiac muscle would be worth while. The evidence suggests that peak-I enzyme is probably not leucine amino-peptidase. For instance, although the classical leucine aminopeptidase property of stimulation by 1mM-Mn^{2+} of hydrolysis of leucyl-peptides has been demonstrated in cardiac muscle (of rabbit; Smith, 1948), peak-I enzyme from rat cardiac muscle is inhibited. Also, unlike peak-I enzyme, leucine amino-peptidase purified from pig kidney is not inhibited by 1m-puromycin (Marks *et al.*, 1968) or thiol-group reagents (Smith & Spackman, 1955). Peak-Ia enzyme may contain an arginine aminopeptidase-like enzyme similar to the arylamidase of bovine pituitary gland which preferentially hydrolyses substrates containing a basic amino acid (termed 'arginyl' arylamidase by Ellis & Perry, 1966). It may arise from extracellular spaces of heart tissue, having escaped, perhaps, from erythrocytes, which are known to contain an arginine aminopeptidase (Makinen & Makinen, 1971). Peak-II (and peak-I) enzymes may belong to a wider group of arylamidases termed 'arylamidase N' (Marks, 1970), since the nitroanilide derivative hydrolysed contains a neutral amino acid. Many arylamidases that hydrolyse these derivatives, however, also hydrolyse others, e.g. the derivatives of basic and acidic amino acids (Bury & Pennington, 1973).

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