Brain Arylamidase

PURIFICATION AND CHARACTERIZATION OF THE SOLUBLE BOVINE ENZYME

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1. An enzyme acting on aminoacyl- β -naphthylamides has been isolated from the soluble fraction of bovine brain and purified 205-fold by means of ammonium sulphate fractionation, hydroxyapatite adsorption and DEAE-Sephadex column chromatography. 2. Arylamidase requires thiol groups for retention of its activity, is heat-labile and is susceptible to freezing. *p*-Chloromercuribenzoate and *N*-ethylmaleimide inactivate the enzyme rapidly. 3. Metal ions are not required for its activity, but stimulation by Mn^{2+} and Mg^{2+} and inactivation by Co^{2+} and Zn^{2+} are observed. 4. Optimum pH 7.5 in phosphate buffer was exhibited for all substrates tested except L-leucyl- β -naphthylamide, for which optimum pH is 6.5. 5. K_m values for a number of substrates have been obtained and substrate inhibition at high concentrations was demonstrated. 6. The molecular weight is approx. 70000 as determined by Sephadex-gel filtration.

It is now well known that, in addition to well-defined proteases and peptidases, there is another group of hydrolytic enzymes that exhibit specific activity against aminoacyl- β -naphthylamides. These are generally known as arylamidases and their presence has been demonstrated in various tissues of different organisms. The arylamidase activities have been definitely shown to occur in human liver, intestine and pancreas (Behal, Asserson, Dawson & Hardman, 1965), ascites-tumour cells (Patterson, Hsiao & Keppel, 1963), cat and guinea-pig liver (Hopsu, Makinen & Glenner, 1966*a,b*), rat kidney (Hanson, Huetter, Mannsfeldt, Kretschmer & Sohr, 1967), rat liver (Hopsu, Makinen & Glenner, 1966*c*), central and peripheral nervous systems (Adams & Glenner, 1962), extracts of pituitary gland (Ellis & Perry, 1966), as well as in bacteria (Behal & Folds, 1967) and yeast (Tjeder, 1966). The distribution of arylamidase activity in various fractions of bovine brain was studied by Brecher & Barefoot (1967). None of the enzymes studied so far can be claimed to be identical with any other, except for the broad spectrum of aminoacyl- β -naphthylamides on which they act. We now report on the purification and properties of a bovine brain arylamidase, which appears to be as yet another distinct enzyme in the group.

Part of this work has been presented in preliminary form (Suszkiw & Brecher, 1968).

EXPERIMENTAL

Materials. All substrates were obtained from Mann Research Laboratories, New York, N.Y., U.S.A., and were

'Mann Assayed' grade. The $(NH_4)_2SO_4$ was 'Enzyme grade, Mann Assayed'. Hydroxyapatite, Bio-Gel HTP and Bio-Gel HT were obtained from BioRad Laboratories, Richmond, Calif., U.S.A. DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals Inc., Piscataway, N.J., U.S.A. All other reagents were Fisher Reagent grade, obtained from Fisher Scientific Co., Silver Spring, Md., U.S.A.

Determination of protein. Protein was determined by the Lowry, Rosebrough, Farr & Randall (1951) modification of the Folin phenol method throughout the purification sequence. Protein concentrations were expressed in mg./ml. from a standard curve obtained with bovine albumin (Cohn fraction V, lot no. 44049, Calbiochem, Los Angeles, Calif., U.S.A.).

Assay of enzyme. The hydrolysis of the aminoacyl- β -naphthylamide substrates was measured by the method of Goldbarg & Rutenburg (1958). Enzyme assays were conducted in a shaking water bath at 37°. All substrates were prepared in 0.1 M-potassium phosphate buffer, pH 7.5. Substrates insoluble in water were prepared in a mixture of 25% (v/v) methylCellosolve with 0.1 M-potassium phosphate buffer. A typical assay mixture contained 0.1 ml. of enzyme preparation, 1.0 ml. of substrate and 0.9 ml. of the buffer. The blank contained all the reagents with the phosphate buffer replacing the enzyme preparation. The length of incubation was usually 30–60 min., depending on the enzyme concentration used. The reaction was stopped with 1.0 ml. of 40% (w/v) trichloroacetic acid solution. β -Naphthylamine liberated was assayed colorimetrically by diazotization followed by coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution. The chromophore was read at 570 m μ . A standard curve was prepared by using β -naphthylamine solution and assaying appropriate amounts together with unknown samples. A unit of activity is defined as 1 μ mole of β -naphthylamine liberated/min./ml. of enzyme preparation. The specific activity is given in

terms of μ moles of β -naphthylamine liberated/min./mg. of protein.

Bovine brain fractionation. Ox brains were obtained at a slaughterhouse and were placed in ice within 10 min. of death. The brains were homogenized with a Teflon mortar and pestle, usually within 3 hr. of death, at a ratio of 1:4 or 1:9 (w/v) in 0.32 M-sucrose solution containing EDTA (0.1 mM) and adjusted to pH 7.4 with 0.1 M-NaOH. For large-scale preparative purposes, the homogenates were centrifuged at 44 000 *g* in a Spinco model L ultracentrifuge for 90 min. at 0°. The supernatant so obtained did not differ significantly in enzyme activity from a 100 000 *g* supernatant fraction.

Ammonium sulphate fractionation. The 44 000 *g* supernatant fraction was fractionated with $(\text{NH}_4)_2\text{SO}_4$ at 0–20%, 20–40%, 40–60% and 60–70% saturation, at pH 6.5 and at 0–5°. Each fraction was left overnight in the cold room and then centrifuged in a Servall refrigerated centrifuge at 17 000 *g* for 20 min. The sediments of the respective fractions were resuspended in 0.01 M-phosphate buffer, pH 6.5, at 0° and dialysed against at least three changes of the same buffer overnight at 0° before assay of activity or application to the next step in the purification sequence.

Hydroxyapatite fractionation. Hydroxyapatite HTP and HT (1:1, w/w) was washed several times with the equilibrating buffer (0.01 M-phosphate buffer, pH 6.5) and then resuspended in a volume of the buffer to make a suspension containing 0.88 g. of hydroxyapatite/ml. Before adsorption the amount of the suspension corresponding to the ratio of the adsorbent to the protein (1 g./3 mg.) was centrifuged at 5000 *g* and the supernatant discarded. The sedimented gel was resuspended in the active enzyme fraction and the mixture was centrifuged in the refrigerated Servall centrifuge for 10 min. at 5000 *g*. The adsorbed protein was then sequentially eluted with potassium phosphate buffers, pH 6.5, at the following concentrations: 0.01 M, 0.10 M, 0.15 M, 0.18 M, 0.20 M and 0.30 M. Each of the eluate fractions was tested for protein and enzyme activity. The buffer strengths required for elution varied somewhat depending on the batch of commercial hydroxyapatite employed.

Concentration step. To the active fraction from the preceding step $(\text{NH}_4)_2\text{SO}_4$ was added to 70% saturation at pH 6.5. The mixture was left overnight at 0–5° and then centrifuged in the cold at 27 000 *g* for 20 min. The sediment, containing activity, was resuspended in a desired volume of cold 0.01 M-phosphate buffer, pH 7.5, and was dialysed exhaustively against the same buffer at 0–5°. The concentrated fraction was then applied to the DEAE-Sephadex column.

DEAE-Sephadex chromatography. The size of the column was 25 cm. $\times$ 2.5 cm. All steps were carried out at 0–4°. The flow rate employed was usually 1–2 ml./min. and did not exceed 3 ml./min. The column was run under a pressure of 6 lb./in.² to maintain the desired rate of flow. It was initially charged with the enzyme preparation at 2 lb./in.² pressure. After the column was washed with the equilibrating buffer, it was eluted sequentially with potassium phosphate buffer, pH 7.5, at concentrations of 0.1 M, 0.12 M, 0.14 M and 0.16 M, and stripped of the remaining protein with 1.0 M buffer. Mercaptoethanol at 10 mM concentration was present throughout the purification sequence.

Disc electrophoresis. Polyacrylamide-gel disc electrophoresis of the enzyme preparations at various stages of purification was carried out by the method of Davis (1964).

Determination of properties of the purified arylamidase.

(a) pH optimum. This was studied in 0.1 M-potassium phosphate buffer. The substrates were made 0.913 M in water. Before the assay, equal parts of the substrate and 0.2 M buffer at a desired pH were mixed. The final incubation mixture at each pH contained: 1.0 ml. of substrate, 0.9 ml. of 0.1 M-phosphate buffer and 0.1 ml. of the enzyme preparation.

(b) Effect of heating. Samples of enzyme solution in 0.01 M-phosphate buffer, pH 7.5, were heated for 10 min. at 50°, 60°, 70° and 80° in a controlled-temperature water bath and were chilled immediately in an ice bath for 20 min. The control and the heat-treated enzyme solutions were then assayed for enzyme activity against L-alanyl-, L-leucyl-, L-arginyl- and L-phenylalanyl- β -naphthylamide.

(c) *K_m*. These values were determined for several substrates. The substrates were prepared in 0.1 M-phosphate buffer in concentrations ranging from 1 to 0.01 mM. The initial velocities were obtained for at least four different substrate concentrations. In most cases a sufficient number of substrate concentrations was used to obtain information on the behaviour of the enzyme at very high and very low substrate concentration. All assays were conducted at pH 7.5.

(d) Effect of metal ions. The effect on enzymic activity was studied at 1 mM-, 5 mM-, 10 mM-, 15 mM- and 20 mM metal-ion concentrations. The solutions of chlorides of the respective metal ions were prepared in 0.05 M-sodium barbital buffer at twice the required final concentrations and equal parts of the enzyme solution and the metal-ion solution were preincubated for 10 min. Samples of the preincubation mixture were then assayed for enzyme activity.

(e) Effect of thiol reagents. Samples of fully active enzyme were preincubated for 10 min. with either *p*-chloromercuribenzoate or *N*-ethylmaleimide at the concentrations of 1, 5, 10, 15 and 20 mM, and then assayed for activity as described above.

(f) Effect of activating thiol reagents. The active enzyme was dialysed against three changes of 0.1 M-phosphate buffer, pH 7.5, thereby losing all of its activity. Cleland's reagent (dithiothreitol) was added to inactivated enzyme solutions to final concentrations of 2.5, 5 and 10 mM. Samples were withdrawn at 1, 5, 10 and 20 min. and tested for activity. Mercaptoethanol was tested in similar fashion.

(g) Molecular-weight estimation. A Sephadex G-200 gel column was calibrated with proteins of known molecular weight by the procedure of Andrews (1964), and the elution volumes were plotted against the molecular weight. From the plot, the molecular weight of arylamidase was estimated by extrapolation.

RESULTS

The sequence for the purification of the soluble arylamidase is given in Scheme 1. The summary of the results for each purification step is given in Table 1, and the electrophoretic patterns for the crude supernatant fraction and the DEAE-Sephadex eluate are shown in Fig. 1. Overall purification is 205-fold for the pooled peak, with 3.3% recovery of the starting material. The loss of activity is attributable to lability of the enzyme to

air oxidation. This loss of activity, however, appears to be reversible inasmuch as the enzyme can be reactivated by thiol reagents. Stability of the enzyme is greatly increased in the presence of 40% and higher saturations of ammonium sulphate, and the purified preparation can best be stored as an ammonium sulphate suspension. Alternatively, the purified enzyme can be stored for at least 2 weeks at 0–4°, in the presence of 10mM-dithiothreitol, without apparent loss of activity. Storage for even a short period of time at –18° is, however, detrimental and results in complete and irreversible inactivation. The enzyme also deteriorates rapidly at higher temperatures, whether in the presence or absence of thiol-protecting agents. Metal ions do not seem to have any effect on stability, nor does 0.1 mM-EDTA cause inactivation.

Ammonium sulphate fractionation. The 44 000 g supernatant solutions were fractionated with ammonium sulphate at pH 6.5. There was essentially no activity in 0–20%, 20–40% and 60–100%

saturation fractions. The recovery of activity in the 40–60% saturation fraction was generally greater than 100%, suggesting that some inhibitory protein molecule may have been removed. This conclusion is supported by the fact that ammonium sulphate had no direct effect on enzymic activity. The purification obtained in this step was 4.6-fold.

Batchwise purification on hydroxyapatite. When the enzyme preparation from the preceding step was equilibrated against 0.01 M-phosphate buffer, and was subsequently applied to the adsorbent, a considerable fraction of the total protein did not adsorb. However, only a small amount of activity is recovered in this fraction. The protein distribution in the eluates of 0.1–0.25 M buffer is basically of the same order, and much less protein is recovered in the 0.28–0.40 M eluates. Total recovery of protein was in the range 85–95%. Most of the arylamidase activity is distributed among the 0.20 M-, 0.25 M- and 0.28 M-phosphate buffer eluates. The specific activities of each fraction are very similar since the activity pattern parallels the protein pattern. The three peaks pooled together had a specific activity of 10. The purification obtained with respect to the applied material was 3.8-fold and the recovery was 24.2%.

Scheme 1. Sequence for purification of soluble bovine brain arylamidase. All the steps were carried out at 0–4°. The presence of 2-mercaptoethanol was required to maintain activity; if the sequence was carried out without 2-mercaptoethanol, dithiothreitol was used to reactivate the enzyme. The equilibrating buffer is 0.01 M-potassium phosphate buffer at the pH indicated.

Fig. 1. Polyacrylamide-gel disc electrophoresis of crude and purified soluble bovine brain arylamidase. Polyacrylamide-gel disc electrophoresis was performed by the method of Davis (1964). A, 44 000 g supernatant; B, DEAE-Sephadex eluate.

Table 1. Purification of arylamidase from the bovine brain soluble fraction

Preparation	Specific activity (units/mg. of protein)	Purification	Total activity (units)	Recovery (% of total activity)
44 000 g supernatant	0.578	1	1421.5	100
40–60% saturated (NH ₄) ₂ SO ₄ , pH 6.5	2.66	4.6	1425.0	101
Hydroxyapatite	10.0	17.6	354.1	24.2
DEAE-Sephadex chromatography	116.5	205	47.3	3.3

Fig. 2. Estimation of molecular weight of bovine brain arylamidase by Sephadex G-200 gel filtration. ●, Trypsin; ■, hexokinase; △, glyceraldehyde 3-phosphate dehydrogenase; ○, lactate dehydrogenase; □, aldolase; ×, arylamidase. The molecular weight was assessed by the method of Andrews (1964). Each protein (100–500 μ g.) was applied to a 2.5 cm. $\times$ 30 cm. Sephadex G-200 column and eluted with 0.01 M-phosphate buffer, pH 7.5. Fractions of 5 ml. were collected.

Fig. 3. Optimum pH for purified bovine brain arylamidase activity. In each incubation mixture 1.5 μ g. of enzyme was utilized. Buffer: 0.1 M-potassium phosphate; incubation time: 1 hr. at 37°. Enzymic activity was measured, as described in the text, with: ●, L-alanyl- β -naphthylamide; ○, L-arginyl- β -naphthylamide; ▲, L-leucyl- β -naphthylamide; △, L-phenylalanyl- β -naphthylamide.

Fig. 4. Lineweaver-Burk plots of β -naphthylamides of (a) and (b) neutral, (c) basic and (d) aromatic amino acids. Assays of enzyme activity with each substrate were performed as described in the text. BNA, β -naphthylamide.

Fig. 5. Effect of metal ions on arylamidase activity: ●, Mn^{2+} ; ○, Mg^{2+} ; ▲, Co^{2+} ; △, Zn^{2+} . The chlorides of the metal ions were preincubated with enzyme at concentrations of 1 mM, 5 mM, 10 mM, 15 mM and 20 mM, after which assays of enzyme activity were performed as described in the text. Substrates: (a) L-alanyl-β-naphthylamide; (b) L-phenylalanyl-β-naphthylamide; (c) L-arginyl-β-naphthylamide; (d) L-leucyl-β-naphthylamide.

Table 2. Kinetic parameters of arylamidase-catalysed reactions

Abbreviation: BNA, β-naphthylamide.

Substrate	K_m (mM)	$10^6 V_{max}$. (mole/min.)
Gly-BNA	2.5	0.309
L-Ala-BNA	0.125	0.129
L-Leu-BNA	0.019	0.129
L-Arg-BNA	0.044	0.088
L-Lys-BNA	0.046	0.088
L-Phe-BNA	0.044	0.189
L-Tyr-BNA	0.100	0.119
L-Pro-BNA	0.245	0.014

The recovery can be significantly improved by shortening the procedure time or decreasing the amount of material fractionated. On a semi-

preparative scale, and with the total fractionation time not exceeding 6 hr., the recovery is up to 90% of the applied enzyme activity. It is evident that the time of exposure of the enzyme to the gel is critical for adequate recovery.

Concentration step. Since the pooled eluates from the batches obtained in the hydroxyapatite purification step constitute a several hundred-fold increase in volume over that of the starting material, the active fraction is concentrated by precipitation with ammonium sulphate to 70% saturation, followed by dialysis, before applying it to the DEAE-Sephadex column. Since the activity is lost irreversibly on freeze-drying, precipitation with ammonium sulphate is the method of choice. In this way, essentially 100% recovery of enzyme activity and of protein is obtained.

DEAE-Sephadex chromatography. Enzyme activity elutes at 0.14M buffer. The total recovery

Fig. 6. Effect of *N*-ethylmaleimide on enzyme activity. *N*-Ethylmaleimide at concentrations of 1 mM, 5 mM, 10 mM, 15 mM and 20 mM was preincubated with arylamidase for 10 min., after which time assays for enzyme activity were performed as described in the text. ●, L-alanyl- β -naphthylamide; ○, L-arginyl- β -naphthylamide; ▲, L-leucyl- β -naphthylamide; △, L-phenylalanyl- β -naphthylamide.

Fig. 7. Effect of thiols on enzyme activity: ▲—▲, 10 mM-dithiothreitol; △—△, 5 mM-dithiothreitol; ○—○, 2.5 mM-dithiothreitol; ▲—▲, 10 mM-mercaptoethanol; ●—●, 5 mM-mercaptoethanol; □—□, control enzyme inactivated by dialysis. Arylamidase was dialysed against 0.1M-phosphate buffer, pH 7.5. Portions of the dialysed enzyme were treated with the indicated concentrations of dithiothreitol or mercaptoethanol and subsequently tested for activity as described in the text.

of activity from the column averages 60%, the remainder apparently being irreversibly inactivated, as there is no activity detected in the following fractionations nor in the 1.0M buffer

eluate, where essentially all the remaining protein is eluted. The fractions with the highest specific activity were pooled, yielding the specific activity of 116.5 units/mg. of protein and 14% recovery with respect to the applied material. The purification obtained in this step was about 12-fold.

Molecular-weight estimation. Results in Fig. 2 show that a molecular weight of approx. 70 000 may be extrapolated for arylamidase.

Optimum pH. The results of pH optima studies are shown in Fig. 3. L-Alanyl-, L-arginyl- and L-phenylalanyl- β -naphthylamide substrates have optimum pH 7.5 for their hydrolysis. Hydrolysis of L-leucyl- β -naphthylamide gives optimum pH 6.5. The pH optimum obtained in 0.05M-sodium barbital buffer gave similar results.

Effect of heating on arylamidase activity. The enzyme preparation is inactivated to about 50% on heating at 50° for 10 min. At higher temperatures complete inactivation results. The enzyme behaved identically with respect to four substrates tested.

K_m studies. K_m values for several substrates were obtained to gain information about substrate specificity and enzyme-substrate interaction. The substrates investigated represent neutral, basic and aromatic amino acids, as well as the imino acid proline. The results are given in Fig. 4. A K_m of 0.245 mM was obtained for proline by plotting velocity against concentration of substrate, and obtaining the half-maximal velocity. Table 2 shows the kinetic parameters derivable from the Lineweaver-Burk plots. The substrate possessing the highest affinity is L-leucyl- β -naphthylamide, which is the most hydrophobic of the three neutral amino acid derivatives of β -naphthylamine tested. The striking similarity in the affinities of the two basic amino acid derivatives is also noteworthy. Inhibition by high substrate concentrations was seen with all substrates.

Effect of metal ions on activity. Fig. 5 shows the results of enzyme activity after the preincubation with various concentrations of Co^{2+} , Zn^{2+} , Mn^{2+} and Mg^{2+} ions. Co^{2+} and Zn^{2+} are completely inhibitory in the concentration range 0.1–10 mM. Mn^{2+} and Mg^{2+} either are without effect or they stimulate, except for the hydrolysis of L-phenylalanyl- β -naphthylamide, which is inhibited by Mg^{2+} to about 50% and shows decreased rate at higher concentrations of Mn^{2+} .

Effect of thiol reagents on arylamidase activity. The results (Fig. 6) show that the enzyme is 40% inhibited by 0.5 mM-*N*-ethylmaleimide. It is completely inhibited by the same concentration of *p*-chloromercuribenzoate.

Effect of dithiothreitol and 2-mercaptoethanol on arylamidase activity. Activity can be restored to the inactivated enzyme by addition of thiols (Fig. 7). The activation is a rapid process and a

Table 3. Comparison of some characteristics of leucine aminopeptidase and known arylamidases

Abbreviations: LAP, leucine aminopeptidase; AP, aminopeptidase; NA, naphthylamidase (arylamidase); BNA, β -naphthylamide; DFP, di-isopropylphosphorofluoridate; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetra-acetate; ME, 2-mercaptoethanol; MEA, 2-mercaptoethanolamine; MB, methylene blue; IAA, iodoacetate; PNA, *p*-nitroanilide; DTT, dithiothreitol.

Enzyme	Source	Substrate	Substrate specificity	Optimum pH	Activating agents	Inhibiting or inactivating agents	Agents with no effect	References
LAP	Pig kidney	L-Leu(NH ₂)	—	7.8-9.3	Mg ²⁺ , Mn ²⁺	—	DFP, PCMB, NEM, EDTA	Smith & Spackman (1955)
NA	Rat nervous system	L-Leu-BNA, DL-Ala-BNA, DL-Phe-BNA	—	7	—	IAA, PCMB, DFP, Mn ²⁺ , 60°	EDTA, Mg ²⁺	Adams & Glenner (1962)
NA	Ascites-tumour cells	L-Leu-BNA	—	II: 7	—	II: Mn ²⁺	II: Co ²⁺	Patterson <i>et al.</i> (1963)
NA	Human liver	L-Leu-BNA	Ala-, Phe-, Leu-, His-	IV: 6.5	Mg ²⁺	IV: Co ²⁺	IV: —	Behal <i>et al.</i> (1965)
NA	Human intestine	L-Leu-BNA	Ala-, Phe-, Leu-, His-	6	—	Co ²⁺ , PCMB, puromycin	Co ²⁺	Behal <i>et al.</i> (1965)
NA	Human pancreas	L-Leu-BNA	Ala-, Leu-, Phe-	7	—	Mg ²⁺ , Mn ²⁺ , Zn ²⁺ , PCMB, puromycin	Co ²⁺	Behal <i>et al.</i> (1965)
NA	Human liver	L-Leu-BNA	Ala-, Met-, Phe-, Leu-, Gly-	6.5	Co ²⁺ (slight) Zn ²⁺ (after EDTA)	Zn ²⁺ , Mg ²⁺ , Mn ²⁺ , PCMB, EDTA	NEM, IAA	Smith, Kaufman & Rutenburg (1965)
AP-B	Cat and guinea-pig liver	L-Lys-BNA, L-Arg-BNA	Arg-, Leu-	7.0	Cl ⁻	Hg ²⁺ , Pb ²⁺ , Cd ²⁺ , PCMB, MB, light	EDTA, DFP, cysteine, ME	Hopsu <i>et al.</i> (1966a,b)
NA	Rat liver	L-Lys-BNA, L-Arg-BNA	Arg-, Leu-	7.0	Cl ⁻	Mg ²⁺ , Zn ²⁺ , Mn ²⁺ , Hg ²⁺ , Pb ²⁺ , Ni ²⁺ , PCMB, IAA, EDTA, ME	—	Hopsu <i>et al.</i> (1966c)
NA	Rat kidney	L-Leu-BNA	Ala-, Phe-, Trp-, Leu-, Arg-, Thr-, Tyr-, Lys-, Ser-, Asp-, His-, Val-, Pro-	7-8	Co ²⁺	Mn ²⁺ , Mg ²⁺ , PCMB	—	Hanson <i>et al.</i> (1967)
NA	<i>Neisseria catarrhalis</i>	L-Ala-BNA	Ala-, Leu-, Met-, Asp-, Arg-, Gly-, Phe-, Ile-, Val-, Ser-	7.3	—	PCMB, puromycin	—	Behal & Folds (1967)
NA	Bovine anterior pituitary	L-Arg-PNA	Arg-PNA	6.5-8.5	Thiols	Di- and tri-peptides, polylysine, polyarginine, protamine, histones	—	Ellis & Perry (1966)
		L-Lys-BNA, L-Lys-PNA, Arg-PNA, Phe-PNA, Met-PNA, Leu-PNA, Ala ₃ , Ala ₄ , Ala ₅ , Ala ₆ , Lys ₄ , Lys ₅ , Lys ₆	Lys-PNA		Thiols, Mn ²⁺ , Co ²⁺ , Zn ²⁺	EDTA, MEA, urea, NEM, guanidine, PCMB, di- and tri-peptides, polylysine, polyarginine, protamine, histones, puromycin		
NA	Baker's yeast	L-Leu-BNA		8-8.5	Ba ²⁺	(NH ₄) ₂ SO ₄ , EDTA	Ca ²⁺ , Co ²⁺ , Mg ²⁺ , Mn ²⁺	Tjeder (1966)
NA	Bovine brain	L-Ala-BNA	Ala-, Arg-, Leu-, Phe-, Lys-, Val-, Pro-BNA, Lys-PNA, Leu-PNA	7.5	DTT, ME, Mn ²⁺ , Mg ²⁺	Co ²⁺ , Zn ²⁺ , PCMB, NEM, 60°	EDTA	This paper

plateau is reached after 10 min. preincubation with the activator. The degree of reactivation is dependent on the concentration of thiol and is maximal at 10mM under the conditions of experiment employed. Dithiothreitol is a better activating agent than 2-mercaptoethanol, presumably because

of better penetrability or lower redox potential, or both.

DISCUSSION

When properties of bovine brain arylamidase are compared with the reported properties of other

arylamidases (Table 3), it becomes evident that this enzyme is not identical with any of the other known arylamidases. On the other hand, it does share some of its characteristics with some of those of the other enzymes. The outstanding, although not unique, characteristics of brain arylamidase are: the requirement for thiol-protecting agents; lack of absolute requirement for a metal, although the activity can be modified by the presence or absence of certain metal ions, as shown; the pH optimum in the range for most hydrolytic enzymes. Whether this is simply a proteolytic enzyme or an enzyme with a more specific function remains to be seen.

The ox brain arylamidase is dissimilar from the classic leucine aminopeptidase (Smith & Spackman, 1955) in that the latter is unaffected by either *p*-chloromercuribenzoate or *N*-ethylmaleimide. Further, the pH optima of the two enzymes are different. The enzyme compares with that reported by Adams & Glenner (1962) in being inactivated by both *p*-chloromercuribenzoate and *N*-ethylmaleimide, but differs in being activated by Mn^{2+} , whereas the latter is inactivated by this ion. The enzymes isolated from ascites-tumour cells by Patterson *et al.* (1963) are different with respect to the effect of Co^{2+} , Mg^{2+} and Mn^{2+} on their activity and the activity of ox brain enzyme. Further, no specific requirement for thiol-protecting agent is reported by the authors. Enzymes characterized by Behal *et al.* (1965) are either unaffected by Co^{2+} , Mn^{2+} , Mg^{2+} or are inhibited by these metal ions. The action of *p*-chloromercuribenzoate and Zn^{2+} is analogous to that on brain arylamidase. Naphthylamidase isolated by Smith *et al.* (1965) exhibits properties almost completely dissimilar to the properties of brain arylamidase; however, it is also inactivated by *p*-chloromercuribenzoate. The enzymes reported by Hopsu *et al.* (1966a,b,c) show properties that diverge noticeably from the properties reported in the present paper. The bacterial arylamidase (Behal & Folds, 1967) has not been characterized sufficiently to draw a meaningful comparison; however, this enzyme appears to be inactivated by EDTA, whereas the brain arylamidase is not inactivated by EDTA.

It is tempting to speculate in view of the above that active proteins acting on aminoacyl- β -naphthylamides must have very similar if not identical active sites in spite of structural and conformation differences. It is possible that the main function of various compounds affecting activity, whether activators or inhibitors, is to make the active centre more or less accessible. However, it is a matter of conjecture whether the active sites are indeed identical.

Observations from the kinetic experiments that require attention are the following. (1) Increase in substrate affinity with increasing hydrophobic

character of neutral amino acid derivatives of β -naphthylamine. (2) Identical results for the two basic amino acids, arginine and lysine; the differences in their k_3 values suggest a lower rate of deacylation (assuming an acylation step to be indeed involved) for lysine. (3) A 50% decrease in affinity on introduction of the hydroxyl group on the aromatic ring of phenylalanine: this appears to be rather a steric hindrance than a polar effect, but the latter cannot be excluded entirely. (4) Distinctly different behaviour with respect to hydrolysis of prolyl- β -naphthylamide, which suggests that an α -amino group could be important in the mechanism. (5) Inhibition by high substrate concentrations, seen with all substrates. Inhibition by high substrate concentration may be due to: (a) the binding of two or more substrate molecules to the binding sites on the enzyme, thereby precluding proper orientation of a single substrate molecule and subsequent hydrolysis; (b) decrease in the effective water concentration, thereby slowing the velocity of the reaction; (c) allosteric effects of the substrate.

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