The N^{α} -acetylenkephalin carboxypeptidase activity of N -acetyltyrosine deacetylase from monkey kidney

Purification, characterization and substrate specificity

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N^{α} -Acetylenkephalin carboxypeptidase was co-purified with N -acetyltyrosine deacetylase from monkey kidney. Almost 90% of the activity from the homogenate was recovered in a high-speed supernatant without the use of detergents. The crucial steps in the purification were Cibacron Blue F3GA–Sephadex chromatography (involving negative and positive binding sequentially) and metal chelate affinity chromatography. The purified enzyme showed three bands on gel electrophoresis under non-denaturing conditions. All the three bands exhibited both N -acetyltyrosine deacetylase and N -acetylenkephalin carboxypeptidase activity, indicating their co-migration. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence and absence of 2-mercaptoethanol gave a single protein band of mol.wt. 34 000. The native enzyme was a dimer of mol.wt. 66 000 as observed on Bio-Gel P-300 gel filtration. The carboxypeptidase removed two amino acids from the C-terminal end of either N -acetyl[Met⁵]- or N -acetyl[Leu⁵]-enkephalin. Non-acetylated enkephalins were less active as substrates. Peptides with their carboxy end blocked were inactive as substrates. Models suggested for carboxypeptidase A [Hartsuck & Lipscomb (1971) *Enzymes* 3, 1–56] support the idea that the kidney N -acetylated aromatic amino acid deacetylase or acylase III [Endo (1978) *Biochim. Biophys. Acta* 523, 207–214] can act as a carboxypeptidase on peptides having hydrophobic amino acids at the C-terminal end.

The presence of N^{α} -acetylated forms of β -endorphin and enkephalin and the biosynthesis of N -acetylenkephalin in the mammalian brain and pituitary have been well documented (Smyth *et al.*, 1979; Mains & Eipper, 1981; Seizinger *et al.*, 1981). The biologically inactive N -acetylenkephalin has been shown to arise by the post-translational modification of the active non-acetylated peptide (Seizinger & Höllt, 1980; Mains & Eipper, 1981). The occurrence of N -acetylated [Leu⁵]enkephalin in rat neurointermediate pituitary was shown by Seizinger *et al.* (1981). Although acetylation may constitute a selective mechanism for the expression of activity associated with different regions of the precursor peptide of endorphin (Smyth *et al.*, 1979), another general hypothesis is that acetylation makes peptides less susceptible to peptidase action (Jornvall, 1975).

A number of enkephalin-degrading peptidases, such as aminopeptidases, endopeptidase and dipeptidyl carboxypeptidases, have been characterized

from the brain and kidney (Gorenstein & Snyder, 1980; Benuck & Marks, 1980; Traficante *et al.*, 1980; Benuck *et al.*, 1981; Hersh, 1981; Mumford *et al.*, 1981; Wagner *et al.*, 1981; Fulcher *et al.*, 1982). In particular a dipeptidyl carboxypeptidase that removes the dipeptide Phe-Met or Phe-Leu from the respective enkephalins has been noted in the rabbit kidney (Benuck *et al.*, 1981). In the present paper we describe the purification of a carboxypeptidase to homogeneity from monkey kidney that preferentially acts on N -acetyl-[Leu⁵]- or -[Met⁵]-enkephalin rather than the respective non-acetylated peptides to remove two amino acids sequentially from the C-terminal end. The use of N -acetyltyrosine as an alternative substrate for the assay of the carboxypeptidase during purification is also presented.

Experimental

Materials

N -Acetyltyrosine, trinitrobenzenesulphonic acid, *o*-phthalaldehyde and the peptide substrates were

Abbreviation used: SDS, sodium dodecyl sulphate.

obtained from Sigma Chemical Co. Amino acids were from Calbiochem. We thank Dr. N. Subramanian, Sandoz, Switzerland, for the kind gift of some [Leu⁵]enkephalin, Dr. Peter Böhlen, Salk Institute for Biological Studies, La Jolla, CA, U.S.A., for octadecasilyl-silica and Dr. A. N. Radhakrishnan, University of Hyderabad, Hyderabad, India, for methionine sulphoxide. Sepharose 6B was from Pharmacia, Uppsala, Sweden, and Bio-Gel P-300 was from Bio-Rad. Cibacron Blue–Sepharose (3 μ mol of dye/ml of gel) was prepared by coupling Cibacron Blue F3G-A (Ciba–Geigy) and Sepharose 6B (Ahmed *et al.*, 1977). The nickel and zinc chelate affinity columns were prepared from biscarboxymethylamino-Sepharose 4B and the metal chloride solutions (Porath *et al.*, 1975).

Methods

Acetylation of [Met⁵]- and [Leu⁵]-enkephalins. To 10 mg of the peptide in 0.5 ml of 0.5% (w/v) NaHCO₃ under an N₂ atmosphere, 10 μ l of acetic anhydride was added and kept for 1 h at room temperature. The mixture was diluted with 2 ml of water and passed through a column (0.4 cm $\times$ 5 cm) of octadecasilyl-silica equilibrated with water (Böhlen *et al.*, 1980). After washing with 20 ml of water, the bound *N*-acetylenkephalin was eluted with 95% ethanol and the eluate was dried in a stream of N₂. The product gave a positive reaction with diazotized sulphanilic acid reagent (Wadman & deBree, 1976) but not with ninhydrin or trinitrobenzenesulphonate, which need a free amino group for reaction.

The acetylation of Try–Gly–Gly, Phe–Leu and phenylalanine was done in a similar manner, except that the reaction mixture was freeze-dried repeatedly instead of passing it through an octadecasilyl-silica column.

Preparation of ethyl esters. A stream of dry HCl gas was passed through the *N*-acetylenkephalin (2 mg) in 0.5 ml of absolute ethanol for 30 min at 50°C (Greenstein & Winitz, 1961a). After the reaction was over, the contents were dried in a stream of N₂.

Purification of *N*-acetyltyrosine deacetylase. All operations were done at 0–4°C unless otherwise mentioned.

Step 1 [(NH₄)₂SO₄ precipitation]. Frozen monkey (*Macaca radiata*) kidney (25 g) was minced and homogenized with 250 ml of Tris/HCl buffer, pH 7.2, in a Waring Blendor for 5 min. The homogenate was centrifuged at 55 000 *g* and the supernatant was subjected to a 33–60% (NH₄)₂SO₄ fractionation. The precipitate obtained was suspended in 50 ml of 20 mM-Tris/HCl buffer, pH 7.2, and dialysed for 24 h against 3 litres of the same buffer with three changes.

Step 2 (DEAE-Cellulose chromatography). The

dialysed (NH₄)₂SO₄ suspension was centrifuged at 12 000 *g* for 30 min and the supernatant was passed through a DEAE-cellulose column (25 cm $\times$ 2.2 cm) pre-equilibrated with 20 mM-Tris/HCl, pH 7.2. The column was washed with 6 litres of the same buffer and eluted with a linear gradient of 0–0.4 M-NaCl in the same buffer. The peak fractions showing *N*-acetyltyrosine deacetylase activity were pooled and dialysed for 24 h against 6 litres of 20 mM-tris/HCl buffer, pH 7.2, with three changes.

Step 3 (Cibacron Blue–Sepharose chromatography). A column of (7.5 cm $\times$ 2.7 cm) of Cibacron Blue–Sepharose was equilibrated with 20 mM-Tris/HCl, pH 7.2. The dialysed DEAE-cellulose eluate was centrifuged to remove a small amount of turbidity, passed through the column and the unbound enzyme was collected. The unbound enzyme (180 ml) was dialysed against 6 litres of 20 mM-phosphate buffer, pH 7.0, for 24 h with three changes and loaded on another Cibacron Blue–Sepharose column (7.5 cm $\times$ 2.7 cm) pre-equilibrated with the same buffer. The column was washed with 2 litres of 20 mM-phosphate buffer (pH 7.0)/0.05 M-NaCl and finally eluted with 20 mM-phosphate buffer (pH 7.0)/0.5 M-NaCl. The enzyme activity towards both *N*-acetyltyrosine and *N*-acetyl[Met⁵]enkephalin was co-eluted. The active fractions were pooled and dialysed against 10 mM-phosphate buffer, pH 7.0, concentrated by freeze-drying to 40 ml and redialysed against 20 mM-phosphate buffer, pH 8.0.

Step 4 (metal chelate chromatography). The metal chelate columns (5.2 cm $\times$ 1.4 cm) were equilibrated with 20 mM-phosphate buffer, pH 8.0. Cibacron Blue–Sepharose eluate (10 ml) was passed through the nickel-chelate column and 5 ml through the zinc-chelate column. Both columns were washed with 250 ml of 20 mM-phosphate buffer (pH 6.0)/0.5 M-NaCl and the enzyme was eluted with 20 mM-sodium acetate buffer (pH 5.0)/0.1 M-NaCl. The fractions (5 ml) were collected in 0.5 ml of 1 M-phosphate buffer, pH 8.0, and immediately dialysed against 20 mM-phosphate buffer, pH 7.0. Enzyme activity towards both *N*-acetyltyrosine and *N*-acetyl[Met⁵]enkephalin were monitored and were found to be co-eluted.

Gel electrophoresis under non-denaturing conditions. Purified enzyme (20 μ g) was subjected to gel electrophoresis in gels (13 cm $\times$ 0.45 cm) by the method of Davis (1964) in 50 mM-Tris/glycine, pH 8.3. The electrophoresis was terminated when the tracking dye Bromophenol Blue emerged. The gels were either stained with Coomassie Brilliant Blue G (Holbrook & Leaver, 1976) or were cut into 0.9 mm slices for locating enzyme activities. The slices were introduced into dialysis bags containing 0.3 ml of 20 mM-phosphate buffer, pH 7.0, and dialysed exhaustively against the same buffer for 48 h. Enzyme activities towards *N*-acetyltyrosine and *N*-acetyl-

[Met⁵]enkephalin were estimated in the contents of the dialysis bags.

Molecular-weight determination by SDS/polyacrylamide-gel electrophoresis and gel filtration on Bio-Gel P-300. Purified enzyme (25 µg) containing 2% (w/v) SDS with or without 5% (v/v) 2-mercaptoethanol was boiled for 2 min and subjected to gel electrophoresis by the method of Laemmli (1970). The electrophoretic mobility was compared with the standards human immunoglobulin G, bovine serum albumin, ovalbumin and soya-bean trypsin inhibitor of mol.wts. 150 000, 68 000, 43 000 and 21 000 respectively.

Alternatively 1 ml of the purified enzyme was passed through a Bio-Gel P-300 column (26.5 cm × 2.1 cm) equilibrated with 20 mM-phosphate buffer (pH 7.0)/0.15 M-NaCl and 2 ml fractions were collected. The V_e/V_0 values of the enzyme were compared with those of the standard marker proteins mentioned above (Andrews, 1965).

Paper chromatography. One-dimensional or two-dimensional paper chromatography (descending) was done on Whatman no. 1 paper. The following solvent systems were used: solvent A, butan-1-ol/acetic acid/water (4:1:1, by vol.); solvent B, phenol/0.2 M-KCl/HCl (pH 2.0) (10:1, v/v); solvent C, pyridine/acetone/0.5 M-NH₃ (5:3:2, by vol.); solvent D, butan-1-ol/acetic acid/water (4:1:2, by vol.).

The spots were developed by spraying ninhydrin (0.4%, w/v) in acetone and heating the paper at 70°C for 30 min. Alternatively the copper/ninhydrin reagent of Ganapathy *et al.* (1981) was used for distinguishing amino acids from peptides. Diazotized sulphanilic acid (Wadman & deBree, 1976) was used to identify tyrosyl compounds.

Enzyme assays. *N*-Acetyltyrosine deacetylase was assayed by the method of Endo (1978a). The assay system consisted of 10 µmol of *N*-acetyltyrosine, 50 µmol of potassium phosphate buffer, pH 8.0, and the enzyme in a volume of 0.5 ml. Incubation was for 1 h at 37°C. The colour was developed with 0.067% (w/v) trinitrobenzenesulphonate. Blanks were devoid of the substrate.

N-Acetylenkephalin carboxypeptidase activity was determined by measuring the released amino acid by the *o*-phthalaldehyde fluorimetric method of Roth (1971). Under the conditions of this assay, free amino acids form fluorescent derivatives, but peptides do not. Fluorescence was measured in a Hitachi 204 A fluorescence spectrophotometer. The assay system had 160 nmol of enkephalin or its *N*-acetyl derivative, 10 µmol of potassium phosphate buffer, pH 8.0, and the enzyme in a total volume of 0.2 ml. After 30 min of incubation at 37°C, the reaction was terminated by keeping the tubes in boiling water for 1 min. In some cases (wherever mentioned) the enkephalin carboxy-

peptidase activity was measured by developing the colour of the reaction products with trinitrobenzenesulphonate as mentioned above.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard or by monitoring the absorption at 280 nm.

Results

Characteristics of enzyme assays

Both *N*-acetyltyrosine deacetylase and *N*-acetylenkephalin carboxypeptidase activity in the purified enzyme exhibited an optimum pH of 8.0 in phosphate buffer. The activity was linear up to 5 h for the deacetylase and up to 1 h for the carboxypeptidase under standard assay conditions. The saturating substrate concentrations were 1.5 mM for *N*-acetyl-[Met⁵]enkephalin (apparent K_m 1.0 mM) and 20 mM for *N*-acetyltyrosine (apparent K_m 7.9 mM).

The amino acids released from [Met⁵]- or [Leu⁵]-enkephalins or their *N*-acetylated derivatives were always the two amino acids at the C-terminal end. At all time periods of incubation more free methionine or leucine than phenylalanine was released, suggesting the sequential release of amino acids from the carboxy end. The identification and quantifications of the individual released amino acids were made after paper-chromatographic separations by methods described below. The ninhydrin-positive spots were cut out, eluted with 50% ethanol and the colour read at 540 nm. At incubation periods of 30 min, 1 h and 2 h the molar ratio of released phenylalanine to methionine was 1:1.87, 1:1.63 and 1:1.44 respectively (with *N*-acetyl[Met⁵]enkephalin as substrate) and of phenylalanine to leucine was 1:1.33, 1:1.22 and 1:1.17 respectively (with *N*-acetyl[Leu⁵]enkephalin as substrate). For quantification of the released amino acids measured by the fluorimetric assay procedure a standard curve of a mixture of the respective amino acids in the appropriate ratio of 30 min of incubation was used.

Purification of *N*-acetyltyrosine deacetylase

The results of a typical purification of monkey kidney *N*-acetyltyrosine deacetylase and *N*-acetyl-[Met⁵]enkephalin carboxypeptidase are given in Table 1. The enzyme was eluted as a single peak of activity in DEAE-cellulose chromatography. The carboxypeptidase activity in the steps before Cibacron Blue-Sepharose chromatography could not be accurately determined due to the presence of other peptidases that could hydrolyse *N*-acetylenkephalin. Cibacron Blue-Sepharose chromatography was by far the most efficient step in the purification schedule because of the unusual property of both deacetylase and carboxypeptidase to bind to the gel in the presence of phosphate ions but

Table 1. Purification of *N*-acetyltyrosine deacetylase and *N*-acetylenkephalin carboxypeptidase

One unit of *N*-acetyltyrosine deacetylase releases 1 μ mol of tyrosine/h under standard assay conditions. One unit of carboxypeptidase releases 1 μ mol of a mixture of phenylalanine and methionine (in the ratio 1:1.87)/h under standard assay conditions. Values in parentheses indicate fold-purification and percentage recoveries over the preceding step. To avoid interference by Tris in the assay, portions of the homogenate, 55 000 g supernatant, (NH₄)₂SO₄ and DEAE-cellulose fractions were dialysed against 150 vol. of 20 mM-phosphate buffer, pH 7.0, for 24 h and used for assay of *N*-acetyltyrosine deacetylase.

Purification step	Total units		Protein (mg)	Specific activity (units/mg)		Deacetylase/ carboxy-peptidase activity ratio	Purification (fold)		Recovery (%)	
	Deacetylase	Carboxy-peptidase		Deacetylase	Carboxy-peptidase		Deacetylase	Carboxy-peptidase	Deacetylase	Carboxy-peptidase
Homogenate	234		2463	0.095					100	
55 000 g supernatant	208		1454	0.143			1.51		89.2	
33–60% (NH ₄) ₂ SO ₄ precipitation	199		362.5	0.549			5.79		85.2	
DEAE-Cellulose chromatography	146		41	3.56			37.6		62.6	
Cibacron Blue–Sephadex chromatography	115.5	36.86	3.78	30.5	9.75	3.12	322		49.4	100
Nickel-chelate chromatography	107	33.36	2.85	37.54	11.7	3.2	395 (1.24)	1.2	45.7 (92.4)	90.5
Zinc-chelate chromatography	60.3	18.61	1.87	32.3	9.95	3.3	340 (1.06)	1.02	25.8 (52.2)	50.5

not in the presence of Tris/HCl. Although the first Cibacron Blue–Sephadex chromatography in Tris/HCl did not yield any significant purification, the second chromatography of the unbound enzymes in phosphate buffer, pH 7.0, yielded remarkable purification. The eluate had only one major contaminant protein with low electrophoretic mobility. Both *N*-acetyltyrosine deacetylase and enkephalin carboxypeptidase activities co-eluted as a single peak with similar ratios of activities in the active fractions. The final step of purification by metal chelate affinity chromatography removed all detectable contaminating proteins. Since the enzyme lost activity rapidly in the presence of the elution buffer (20 mM-sodium acetate, pH 5.0), the eluate had to be immediately brought to pH 7.0 by phosphate buffer, pH 7.0. Nickel-chelate chromatography, which gave much higher yields of enzyme than zinc-chelate chromatography, has been used routinely in the purification of the enzyme. In both the metal chelate columns the deacetylase and carboxypeptidase activities co-eluted with constant ratios of activities.

Fig. 1. Polyacrylamide-gel electrophoresis of (i) Cibacron Blue–Sephadex eluate, (ii) nickel-chelate eluate and (iii) zinc-chelate eluate (a) and SDS/polyacrylamide-gel electrophoresis of the purified enzymes (b)

(a) 20 μ g of each protein was used for electrophoresis. The arrow indicates the position of the major contaminating protein in the Cibacron Blue–Sephadex eluate. Migration was from top to bottom towards the anode. Details are given under 'Methods'. (b) (i) Zinc-chelate eluate under non-reducing conditions; (ii) nickel-chelate eluate under reducing conditions; 20 μ g of protein was used for (i) and 30 μ g each for (ii) and (iii).

Fig. 2. Polyacrylamide-gel electrophoresis of the purified enzyme and the profiles of enzyme activity in the gel slices. The electrophoresis was continued for 1 h after the tracking dye emerged to improve the resolution of the bands. ●, *N*-Acetyltyrosine deacetylase; ○, *N*-acetylenkephalin carboxypeptidase. The photograph of the gel shows the three protein bands.

Alternative methods, such as hydroxyapatite and CM-Sephadex chromatography or Sephadex G-200 gel filtration, were tried for purification of the enzyme during the course of our studies. The notable observation was that in all procedures both *N*-acetyltyrosine deacetylase and *N*-acetylenkephalin carboxypeptidase behaved identically in their elution profile and recovery.

Gel electrophoresis

The purified enzyme showed three closely moving bands on polyacrylamide-gel electrophoresis (Fig. 1). When electrophoresis was done in 13 cm-long 7% polyacrylamide gels for another 60 min after the tracking dye emerged, the three bands could be resolved sufficiently to be sliced separately. Both *N*-acetyltyrosine deacetylase and enkephalin carboxypeptidase activities in the slices corresponded to the three protein bands (Fig. 2). The products formed from *N*-acetyl[Met⁵]enkephalin by each of the three bands were verified by paper chromatography to be methionine and phenylalanine.

SDS/polyacrylamide-gel electrophoresis of the purified enzyme with or without 2-mercaptoethanol gave a single protein band (Fig. 1).

Molecular-weight determination

The molecular weight of the purified enzyme as determined by gel filtration on Bio-Gel P-300 by SDS/polyacrylamide gel electrophoresis was found to be 66 000 and 34 000 respectively.

Identification of products released by carboxypeptidase

On incubation of the purified enzyme with *N*-acetyl[Met⁵]enkephalin followed by one-dimensional paper chromatography in solvent A, two ninhydrin-positive spots that corresponded to methionine and phenylalanine were detected. It was evident that the products from *N*-acetylenkephalin were more intense than those from non-acetylated enkephalin. Use of the copper/ninhydrin reagent indicated that the spots were amino acids and not peptides. The areas corresponding to the spots (before ninhydrin spray) were cut out, extracted and hydrolysed in 6M-HCl at 110°C for 2 h. Rechromatography of the hydrolysed material again showed the products to be phenylalanine and methionine and an oxidation product of methionine, methionine sulphoxide.

Paper chromatography of the incubation mixture in two dimensions with solvents B and A in the first and second dimensions respectively again revealed only two ninhydrin-positive spots, which corresponded to methionine and phenylalanine. Similarly the products of incubation with *N*-acetyl[Leu⁵]enkephalin in two-dimensional paper chromatography gave two ninhydrin-positive spots that corresponded to phenylalanine and leucine. Incubation of the enzyme with as much as 750 nmol of *N*-acetyl[Met⁵]enkephalin for periods ranging from 15 min to 5 h failed to show any other ninhydrin-positive spot except methionine and phenylalanine.

Chromatography of the incubation mixture in solvent C followed by a second-dimension run in solvent D gave two spots on spraying with

Table 2. *Relative activities towards different substrates*

The fluorimetric assay procedure in Expt. 1 and the spectrophotometric assay method in Expt. 2 were as described under 'Methods'. In Expt. 1, 0.8 mM and in Expt. 2, 20 mM concentrations of substrates were used.

Expt.	Substrate	Amino acid released ($\mu\text{mol}/\text{mg}$ of protein per h)
1	<i>N</i> -Acetyl[Met ⁵]enkephalin	13.8
	<i>N</i> -Acetyl[Leu ⁵]enkephalin	11.6
	[Met ⁵]enkephalin	8.0
	[Leu ⁵]enkephalin	6.2
	<i>N</i> -Acetyl[Met ⁵]enkephalin ethyl ester	0.5
	<i>N</i> -Acetyl[Leu ⁵]enkephalin ethyl ester	0.6
	Tyr-Gly-Gly	1.8*
	Phe-Leu	38.1†
	Phenylalanyl-leucinamide	0.2†
	<i>N</i> -Acetylated Phe-Leu	0†
	<i>N</i> -Acetylated Tyr-Gly-Gly	0*
2	<i>N</i> -Acetyltyrosine	43.8
	<i>N</i> -Acetylphenylalanine	238.0
	<i>N</i> -Acetylaspartic acid	2.1
	<i>N</i> -Acetyltyrosine ethyl ester	2.6
	<i>N</i> -Acetyltyramine	0

* Glycine released.

† Leucine released.

diazotized sulphanilic acid reagent that corresponded to *N*-acetyl[Met⁵]enkephalin (unchanged) and *N*-acetylated Tyr-Gly-Gly, the other product of carboxypeptidase action.

Incubation of the Cibacron Blue–Sephadex eluate with *N*-acetyl[Met⁵]enkephalin resulted in the release of only methionine and phenylalanine, demonstrating the absence of other peptidases even at this stage of purification.

Substrate specificity

As given in Table 2, both *N*-acetyl[Met⁵]enkephalin and *N*-acetyl[Leu⁵]enkephalin were hydrolysed approximately twice as fast as the unacetylated peptides. Blocking the free carboxy group as in the case of the ethyl esters of *N*-acetyl[Leu⁵]enkephalin and *N*-acetyl[Met⁵]enkephalin resulted in extremely low hydrolysis of these substrates, suggesting that the enzyme was a carboxypeptidase. Tyr-Gly-Gly exhibited only very low activity, indicating that glycine at the carboxy end is not favourable for enzyme action. Although the dipeptide Phe-Leu was hydrolysed very rapidly, blocking of its carboxy end as in phenylalanyl-leucinamide resulted in an almost complete disappearance of the activity, again suggesting that the enzyme is a carboxypeptidase. *N*-Acetylated Phe-Leu and *N*-acetylated Tyr-Gly-Gly were not hydro-

Fig. 3. *Effect of metal ions on N-acetyltyrosine deacetylase and N-acetyl enkephalin carboxypeptidase*

The metal chloride solutions were pre-incubated with the enzyme for 15 min before assay. The metal solutions were included in the appropriate reaction blanks. Values are expressed as percentage activation or inhibition over basal activity. Continuous lines indicate *N*-acetyltyrosine deacetylase activity and broken lines indicate *N*-acetyl-enkephalin carboxypeptidase activity. O, Co²⁺; Δ , Ni²⁺; $\bullet$, Zn²⁺; $\square$, Cu²⁺.

lysed at all, although their unacetylated counterparts were acted upon by the enzyme. The products of hydrolysis of these substrates were also identified by paper chromatography.

The lesser activity towards [Met⁵]enkephalin compared with *N*-acetyl[Met⁵]enkephalin was confirmed at different concentrations (from 0.08 to 1.6 mM) of both the substrates. The respective values for apparent K_m and V_{max} for enkephalin were 1.8 mM and 12.05 $\mu\text{mol}/\text{mg}$ of protein per h and for *N*-acetylenkephalin 1.0 mM and 18.0 $\mu\text{mol}/\text{mg}$ of protein per h.

N-Acetylphenylalanine was deacetylated 5.4 times faster than *N*-acetyltyrosine, demonstrating that the hydrophilic hydroxy group of tyrosine reduces the catalytic efficiency. *N*-Acetylaspartic acid, a hydrophilic amino acid, was deacetylated only to a very low extent. Blocking or removal of the carboxy group of *N*-acetyltyrosine, as in the case of *N*-acetyltyrosine ethyl ester or *N*-acetyltyramine,

Table 3. *Inhibition of the deacetylase and carboxypeptidase by metal chelators*

Inhibition of carboxypeptidase by EDTA was assayed by the fluorimetric method and that by 8-hydroxyquinoline by the trinitrobenzenesulphonic acid method as the latter inhibitor interfered in the fluorescence assay. The chelators were preincubated with the enzyme for 15 min before assay. Details of the assay are given under 'Methods'.

Inhibitor	Concentration (mM)	Inhibition (%)	
		Deacetylase	Carboxypeptidase
8-Hydroxyquinoline	5.0	95	78
	1.0	72	63
	0.1	36	20
	0.01	8	4
	0.001	0	0
EDTA	5.0	65	58
	1.0	46	39
	0.1	22	16
	0.01	6	0
	0.001	0	0

resulted in extremely low deacetylation (Table 2). This indicated that a free carboxy group in the substrate was a requirement for both the deacetylase as well as carboxypeptidase. The purified enzyme failed to hydrolyse *o*-nitroacetanilide, a substrate for arylacylamidase (George & Balasubramanian, 1980, 1981).

Effect of metals on enzyme activity

Co²⁺, Ni²⁺ and Zn²⁺ activated *N*-acetyltyrosine deacetylase activity in decreasing order (Fig. 3). Only Zn²⁺ showed significant activation of *N*-acetylenkephalin carboxypeptidase. Cu²⁺ was inhibitory to both enzymes. Cd²⁺, Mn²⁺, Mg²⁺ and Fe²⁺ had no effect on both enzyme activities.

Effect of potential inhibitors on enzyme activity

8-Hydroxyquinoline inhibited both enzymes considerably at 5 mM concentration, demonstrating the metalloprotein nature of the enzyme. EDTA was a less potent inhibitor of both enzymes (Table 3). Iodoacetamide and phenylmethanesulphonyl fluoride at 1 mM concentration had no effect on both enzyme activities, suggesting that the enzyme did not require thiol groups and was not a serine peptidase. Indol-3-ylacetic acid at 1 mM failed to inhibit both enzyme activities, a property distinct from the carboxypeptidase A of pancreas (Petra, 1970).

Mutual inhibition by N-acetyltyrosine and N-acetylenkephalin

N-Acetyltyrosine was inhibitory to the carboxypeptidase activity and *N*-acetyl[Met⁵]- and *N*-acetyl[Leu⁵]-enkephalin were inhibitory to the *N*-acetyltyrosine deacetylase activity. The inhibition by 10

and 20 mM *N*-acetyltyrosine of *N*-acetyl[Met⁵]-enkephalin carboxypeptidase was 40% and 60% respectively and of *N*-acetyl[Leu⁵]-enkephalin carboxypeptidase was 57% and 80% respectively. With 0.8 mM and 1.6 mM of either *N*-acetyl[Leu⁵]- or [Met⁵]-enkephalin, the inhibition of *N*-acetyltyrosine deacetylase was 8–10% and 17–22% respectively. These inhibition studies were done by separating the reaction products by paper chromatography and quantifying the released amino acids.

Discussion

The abundance of acylases and endopeptidases in kidney is well documented (Greenstein & Winitz, 1961*b*; Birnbaum *et al.*, 1952; Barrett, 1977; Endo, 1978*a*). Recently Endo (1978*a,b*) reported the presence and partial purification of an aromatic amino acid deacetylase in rat kidney that could deacetylate *N*-acetyl aromatic amino acids. This acylase has been classified as acylase III. Our interest in this deacetylase arose because of the possibility that this enzyme could deacetylate *N*-acetylenkephalin. However, during the course of this work it became apparent that the *N*-acetyltyrosine deacetylase activity was associated with *N*-acetylenkephalin carboxypeptidase activity.

A substantial fold-purification of the enzyme was achieved in the Cibacron Blue–Sephacel step by using Tris/HCl and phosphate buffers sequentially. It is likely that the binding of the enzyme in phosphate buffer was metal ion-promoted (Hughes *et al.*, 1982) because of its metalloprotein nature and that its inability to bind in Tris/HCl buffer was because of the chelating properties of Tris (Mahler, 1961). The identity of the carboxypeptidase and deacetylase activities was established from their co-purification with constant ratios of activities and fold-purification with similar recoveries in the different steps of purification. The final enzyme preparation on gel electrophoresis under non-denaturing conditions showed three closely moving protein bands and all the three bands showed both carboxypeptidase and deacetylase activities. However on SDS/polyacrylamide-gel electrophoresis the three species of enzyme merged into a single protein band of mol.wt. 34 000. This phenomenon is observed in carboxypeptidase A of bovine pancreas and is attributed to allotypic variants of the same enzyme, where amino acids may be substituted (Walsh *et al.*, 1966; Petra *et al.*, 1969). The mol.wt. of 66 000, as determined by Bio-Gel P-300 gel filtration, suggested that the native enzyme was a dimer of two identical subunits of mol.wt. approx. 34 000. The molecular weight of the native enzyme is roughly similar to the value of 55 000 obtained by Endo (1978*a*).

The enzyme was characterized as a carboxy-

peptidase releasing methionine or leucine and phenylalanine from *N*-acetyl-[Met⁵]- or [-Leu⁵]-enkephalin based on the following observations. (1) The products released from *N*-acetylenkephalins were identified as methionine or leucine and phenylalanine by one- and two-dimensional paper chromatography. (2) Blocking of the carboxy group of *N*-acetylenkephalin prevented the hydrolysis of the peptide. (3) Phenylalanyl-leucinamide was not hydrolysed by the enzyme but Phe-Leu was readily hydrolysed. (4) *N*-Acetylenkephalin was a better substrate than enkephalin, which is in accordance with the known properties of carboxypeptidase (Hofman & Bergman, 1940). (5) No di- or tripeptides (except Tyr-Gly-Gly or *N*-acetylated Tyr-Gly-Gly), *N*-acetyltyrosine or tyrosine could be detected in the incubation mixture, ruling out the possibility of endopeptidase or aminopeptidase activities. (6) The carboxypeptidase seemed to hydrolyse peptide bonds formed by a hydrophobic amino acid at the carboxy end, as there was very little release of glycine from Tyr-Gly-Gly and *N*-acetylated Tyr-Gly-Gly. This property is similar to that of carboxypeptidase A and different from carboxypeptidase B and N (Stahmann *et al.*, 1946; Folk, 1970; Plummer & Hurwitz, 1978).

The purified enzyme, though it could deacetylate *N*-acetyltyrosine, was incapable of deacetylating *N*-acetylenkephalin. This can be explained if we assume that the deacetylating activity and peptidase activity are functions of the same active site. Requirement of a hydrophobic amino acid and a free carboxy group in the substrate for both the deacetylase and carboxypeptidase activities (Table 2) support this assumption. By analogy with the carboxypeptidase A of pancreas (Hartsuck & Lipscomb, 1971). The positioning of the C-terminal amino acid of *N*-acetylenkephalin into the hydrophobic pocket of the active site will leave the *N*-acetyl bond five peptide lengths away from the cleavage site. However, with *N*-acetyltyrosine, the *N*-acetyl bond would be accurately positioned at the cleavage site. The same model would explain the enhanced deacetylation of *N*-acetylphenylalanine compared with *N*-acetyltyrosine, because of the greater hydrophobicity of the former compared with the latter. The inability of the enzyme to hydrolyse *N*-acetylated Phe-Leu may be because the more hydrophobic *N*-acetylphenylalanyl group (compared with the C-terminal leucine) is incorrectly positioned into the hydrophobic pocket and results in an unproductive binding (Hartsuck & Lipscomb, 1971).

The metalloprotein nature of the enzyme is evident from the inhibition of both enzyme activities by metal chelators and activation by Zn²⁺, Co²⁺ and Ni²⁺. The differential activation by Co²⁺ and Ni²⁺ of the carboxypeptidase and deacetylase activities may be because of the different substrates used.

N-Acetylation of peptides has been suggested to be a mechanism for their protection from proteolytic degradation (Jornvall, 1975), but as observed in the present work it may also result in their greater susceptibility to carboxypeptidase action. It is therefore unlikely that the *N*-acetylation of endorphin and enkephalin is a mechanism for their protection from peptidases.

We could not detect the presence of an *N*-acetyltyrosine deacetylase in the monkey brain, in agreement with the findings of Endo (1978a). The physiological importance of the enkephalin carboxypeptidase in the kidney is not clear because this organ has only a low content of the pentapeptide (Hughes *et al.*, 1977). The presence of this enzyme in kidney may be analogous to the role of this organ in the metabolism of other vasoactive peptides.

The actual role of *N*-acetyltyrosine deacetylase present in abundance in the kidney (Endo, 1978a) or other similar amino acid deacylases (Birnbaum *et al.*, 1952; Greenstein & Winitz, 1961b; Gade & Brown, 1981) remains doubtful because insufficient acetyl or formyl amino acids are present in the mammalian tissues to act as their substrates, except for *N*-acetylaspartate in the brain (D'Adamo *et al.*, 1973). The present study suggests that these deacetylases may indeed act as carboxypeptidases also.

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