Endopeptidase 24.15 from rat testes

Isolation of the enzyme and its specificity toward synthetic and natural peptides, including enkephalin-containing peptides

Marian ORLOWSKI,*† Sandra REZNIK,* Julia AYALA* and Adrian R. PIEROTTI†

*Department of Pharmacology and †Research Center in Neurobiology, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029, U.S.A.

Endopeptidase 24.15, a metalloendopeptidase (EC 3.4.24.15) with an M_r of about 70 000, was purified to homogeneity from rat testes. The enzyme cleaves preferentially bonds on the carboxyl side of hydrophobic amino acids. Secondary enzyme–substrate interactions at sites removed from the scissile bond are indicated by the finding that a hydrophobic or bulky residue in the P_3' position greatly contributes to substrate binding and catalytic efficiency. The isolated enzyme is inhibited by metal chelators and by thiols. Loss of enzymic activity after dialysis against EDTA can be restored by low concentrations of Zn^{2+} and Co^{2+} ions. The rate of reaction of the Co^{2+} enzyme with a synthetic substrate was higher than that of the Zn^{2+} enzyme. These results are consistent with the classification of the enzyme as a metalloendopeptidase. *N*-Carboxymethyl peptides that fulfil the binding requirements of the substrate recognition site of the enzyme act as potent competitive inhibitors. Biologically active peptides such as luteinizing hormone-releasing hormone, bradykinin and neurotensin are cleaved at sites consistent with the specificity of the enzyme deduced from studies with synthetic peptides. Dynorphin A (1–8)-peptide, β -neoendorphin, metorphamide, and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ are rapidly converted to the corresponding enkephalins. The testis enzyme is catalytically and immunologically closely related to the previously identified brain enzyme.

INTRODUCTION

Previous work in this laboratory led to the identification and partial purification from rat brain of a metalloendopeptidase predominantly associated with the soluble fraction of homogenates [1]. A membrane-bound form of the enzyme constituting approx. 20 % of the total activity, and having properties similar to those of the soluble form, was found associated with brain particulate fractions, including synaptosomes [2]. The finding that endopeptidase-24.15 is highly active in brain, pituitary and testis, and that it does not apparently cleave proteins and large peptides containing more than 20 amino acid residues, suggests that the enzyme is involved in the metabolism of bioactive peptides.

Endopeptidase 24.15 together with endopeptidase 24.11 [3–6] seem to constitute the main two brain metalloendopeptidases. Unlike endopeptidase-24.11, also misleadingly called ‘enkephalinase’, because of its cleavage of the Gly–Phe bond in both Leu- and Met-enkephalin [7], endopeptidase 24.15 does not attack the two enkephalins. Indeed, experiments with purified preparations of the enzyme from rat brain and also experiments with synaptosomal membranes have shown that the enzyme converts some larger opioid peptides such as dynorphin A^{1–8}, β -neoendorphin and Met-enkephalin-

Arg-Gly-Leu into Leu- and Met-enkephalin respectively in reactions inhibited by cFE-AAF-pAB, a substrate-related specific inhibitor of the enzyme [2,8].

Isolation of a homogeneous preparation of endopeptidase 24.15 from rat brain by conventional enzyme purification methods presented difficulties [1]. Even the best enzyme preparations contained an inactive protein contaminant of almost the same isoelectric point and molecular mass, that could not be removed by ion exchange chromatography, preparative gel electrophoresis or chromatofocusing. Since rat testes are rich in endopeptidase 24.15, we directed our efforts towards isolation of the enzyme from this source. Most of the enzyme activity in rat testes is associated with the soluble protein fraction of homogenates, whereas the membrane-bound form of the enzyme constitutes only about 8 % of the total activity. Here we report the isolation of a homogeneous preparation of the enzyme, and the results of studies of its interaction with synthetic substrates, natural peptides, and inhibitors. Evidence is presented that the testis and brain enzymes are immunologically and catalytically closely related if not identical. The homogeneous testis enzyme, like its brain counterpart, converts dynorphin A^{1–8}, β -neoendorphin, metorphamide and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ into the corresponding enkephalins in a reaction inhibited by cFP-AAF-pAB.

Abbreviations used: Bz, *N*²-benzoyl; cFE-AAF-pAB, *N*-[(1*RS*)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB; cFP-AAF-pAB, *N*-[(1*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB; DFP, di-isopropyl fluorophosphate; DTT, dithiothreitol; LHRH, luteinizing hormone-releasing hormone; pAB, *p*-aminobenzoate; PCMB, *p*-mercuribenzoate; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis. The nomenclature proposed by Schechter & Berger [26] is used to describe the position (P) of the residues in the substrate and the corresponding subsites (S) in the active site of the enzyme.

† To whom correspondence and reprint requests should be addressed.

This is in contradiction with a report by Toffoletto *et al.* [9] that these reactions are catalysed by endo-oligopeptidase A but not by endopeptidase 24.15.

MATERIALS AND METHODS

Materials

Endopeptidase 24.15 substrates and inhibitors were synthesized as described previously [1,10,11]. Frozen rat testes from Sprague-Dawley rats were obtained from Pel Freeze Inc. (Rogers, AR, U.S.A.). All other reagents were obtained from Fisher Scientific Co. or from Sigma Chemical Co.

Determination of enzyme activity

Enzyme activity was determined in a coupled enzyme assay in the presence of excess of aminopeptidase N (EC 3.4.11.2) using Bz-Gly-Ala-Ala-Phe-pAB (0.8 mM) as the substrate [1]. The enzyme cleaves the Gly-Ala bond yielding two products, Bz-Gly and Ala-Ala-Phe-pAB. The second of these products is hydrolysed by the aminopeptidase with the release of pAB which is then quantified after diazotization.

In crude enzyme preparations the Ala-Phe bond of the substrate can be cleaved by endopeptidase 24.11. Accordingly, in such preparations the activity was determined in the presence of 25 μ M-N-[(1*RS*)-carboxy-2-phenylethyl]-Phe-pAB, a specific inhibitor of endopeptidase-24.11 (K_i 7.1×10^{-8} M) [12]. To account for the possible substrate degradation by other enzymes present in crude tissue homogenates the degradation of the substrate was also measured in parallel reaction mixtures containing, in addition to the inhibitor of endopeptidase-24.11, also 25 μ M of cFP-AAF-pAB, a specific inhibitor of endopeptidase-24.15 [11]. The difference in the rate of substrate degradation between incubation mixtures containing both inhibitors and those containing only the inhibitor of endopeptidase-24.11 was taken as a measure of endopeptidase-24.15 activity. Activity is expressed in units, 1 unit being defined as the amount of enzyme which catalyses the release of 1 μ mol of product/h. Specific activity is expressed in units/mg of protein as determined by the method of Lowry *et al.* [13].

Determination of kinetic constants

The steady-state parameters, K_m and k_{cat} . ($= V/e$, where e = total enzyme concentration) were determined from initial velocity measurements at various substrate concentrations. Michaelis-Menten kinetics were observed with all substrates. K_m values were calculated from double-reciprocal plots by a linear-regression program. Correlation coefficients were generally 0.99 or better. In the calculation of data an M_r of 70000 was assumed, with one catalytic site per enzyme molecule.

K_i measurements were carried out by the method of Dixon [14] at three different substrate concentrations and at six different inhibitor concentrations (plots of $1/v$ versus $[I]$) using a computer program. Coefficients of determination (r^2) of better than 0.99 were generally obtained.

Polyacrylamide-gel electrophoresis

Disc PAGE was carried out under non-dissociating conditions in a 0.05 M-Tris/HCl buffer (pH 8.3). Enzyme protein (5–10 μ g) was layered on the top of the gel and a current of 4 mA/tube was applied for a time period

necessary for the tracking dye to reach the bottom of the gel. Preparative slab PAGE was carried out under similar conditions in gels of 3 mm thickness using a slab gel apparatus (model 100; Aquebogue Machine Shop, Box 205, Aquebogue, NY 11931, U.S.A.). Electrophoresis under dissociating conditions and M_r determinations were carried out in the same apparatus using a discontinuous buffer system [15]. Gels (8%) contained 0.1% SDS, and electrophoresis was run after the enzyme had been exposed to a 1% solution of SDS and 2% 2-mercaptoethanol and heated to 70 °C for 10 min. M_r standards contained 2.5 μ g of each protein (trypsinogen, ovalbumin and bovine serum albumin).

Isolation of the enzyme

All purification steps were carried out at 4 °C. De-ionized water was used for the preparation of buffers. Frozen rat testes (100 testes; 172 g) were defrosted, homogenized in 4 vol. of 0.01 M-Tris/HCl buffer (pH 7.6) containing 0.32 M-sucrose and 0.5 mM-2-mercaptoethanol (buffer A) and centrifuged at 30000 g for 120 min. The supernatant was collected and applied to the top of a DEAE-cellulose column (DE-52; 200 ml bed volume) equilibrated with buffer A (without sucrose). The column was washed with a total of 1000 ml of buffer A. Elution was then started with a linear gradient established between 400 ml of buffer A and 400 ml of the same buffer containing 0.3 M-NaCl. Fractions (8 ml) were collected and tested for protein by absorbance at 280 nm and for enzyme activity using Bz-Gly-Ala-Ala-Phe-pAB as the substrate. The enzyme emerged after 360 ml of the eluting buffer had passed through the column. Fractions within the central part of the activity peak were pooled and assayed for protein [13] and for activity as described above. The pooled fractions were concentrated to about 40 ml and then applied to the top of a Sephadex G-100 column (5.0 cm $\times$ 100 cm) equilibrated with a 0.05 M-Tris/HCl buffer, pH 8.0. Elution was carried out with the same buffer and fractions of about 20 ml were collected. The enzyme emerged, preceded by a large inactive protein peak, after about 780 ml of the eluting buffer had passed through the column. Fractions in the central portion of the active peak were pooled and applied to the top of a DEAE-Sephacel column (15 ml) equilibrated with a 0.02 M-Tris/HCl buffer (pH 8.0) containing 0.5 mM-2-mercaptoethanol. The column was washed with the same buffer (220 ml) and then eluted with a linear gradient established between 150 ml of the same buffer and 150 ml of the buffer containing 0.3 M-NaCl. Fractions of about 3 ml were collected. The enzyme emerged from the column after about 100 ml of the eluting buffer had passed through the column. A large amount of inactive protein was eluted before and after the main activity peak. Fractions containing enzyme with the highest specific activity were combined and desalted by passing through a Sephadex G-25 column (150 ml) equilibrated with 0.01 M-Tris/acetate buffer (pH 7.5).

Samples of the enzyme after step 4 of the purification procedure were subjected to PAGE as described above. Staining with Coomassie Brilliant Blue revealed the presence of two major protein bands (Fig. 1). Parallel unstained gels were cut into 2 mm slices, and the distribution of activity was examined by homogenizing the slices in 0.25 ml of Tris/HCl buffer (0.05 M, pH 7.0), and determining the activity in gel extracts as described above. Activity was associated with the slower-moving

protein band. For the isolation of homogeneous enzyme, aliquots of the enzyme after step 4 of the purification procedure containing about 1 mg of protein (Table 1) were concentrated to about 0.5 ml and subjected to preparative slab PAGE. The location of protein bands was visualized by staining gel strips from the edges and the centre of the slab gel with Coomassie Brilliant Blue. Good separation of the two protein bands was consistently obtained. The slab gel was sliced into 2 mm strips. The gel strips containing activity were placed in dialysis bags filled with 1.5 ml of Tris/HCl buffer (0.05 M, pH 8.3). The enzyme was electroeluted from the gel by placing the dialysis bags in a flat slab gel electrophoresis apparatus containing the same buffer and applying a current of 140 V for 5 h. The buffer containing the enzyme was recovered from the dialysis bag (1.35 ml) and the solution was tested for activity and protein by the method of Peterson [16]. Enzyme purity was determined by PAGE under non-dissociating and denaturing and dissociating conditions and also by testing the immunoreactivity toward an antibody raised in rabbits (see below). Enzyme samples obtained by this procedure (usually about 35–40 μ g of protein) were apparently homogeneous by several criteria (see below).

Raising of antibodies

Aliquots of the homogeneous enzyme, obtained after preparative slab PAGE, containing about 20 μ g of protein were used for immunization. The enzyme was mixed with complete Freund's adjuvant and injected intradermally at multiple sites into the back of a New Zealand white rabbit. The injections were repeated at least two or three times after 14–20 day intervals. Blood was collected into heparinized tubes from the ear vein 10 days after the last injection, at which time a rather high titre of anticatalytic antibodies was present. Aliquots (0.2 ml) of the plasma recovered by centrifugation were stored at -20°C .

Electrophoretic transfer of proteins from SDS/polyacrylamide gels was carried out essentially as described [17,18] using a Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories). A 5% bovine serum albumin solution (20 mM-Tris/HCl/500 mM-NaCl, pH 7.5) was used as the blocking solution. The first antibody was used in a 1:500 dilution in a buffer containing 1% bovine serum albumin and 0.05% Tween 20 in 20 mM-

Tris/HCl/0.5 M-NaCl (pH 7.5). An affinity-purified goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad) was used as the second antibody and visualization was accomplished according to the manufacturer's recommendations.

Fig. 1. PAGE of the enzyme from testis

Gel (b) (10 μ g of protein) represents the pattern obtained after step 4 of the purification procedure. Gel (a) and (c) (each containing about 5 μ g of protein) represent respectively the homogeneous enzyme and the impurity each isolated after preparative PAGE.

Table 1. Summary of purification of the enzyme from rat testes

Purification step	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Total (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
1. Supernatant	610	10.7	49	29890	4.58	—	100
2. DE-52 chromatography (pH 7.6)	77	6.6	194	14940	29.4	6.4	50
3. Sephadex G-100	130	0.96	83	10790	86.5	18.9	36
4. DEAE-Sephacel chromatography (pH 8.0)	35	0.38	129	4515	339	74	15
5. Preparative PAGE	1.35	0.025	30.4	41	1220	266*	0.14

* Aliquots of the enzyme after step 4 containing about 350 units were subjected to preparative slab PAGE. Only about 10% of homogeneous enzyme was recovered by electroelution as described in the Materials and methods section.

RESULTS

A summary of the purification procedure is given in Table 1. The specific activity of the enzyme fractions obtained after step 5 of the purification procedure (Table 1) represented a more than 260-fold purification. Disc PAGE after step 4 showed the presence of two well-separated protein components (Fig. 1). Activity was associated with the slower-moving protein band. Attempts to separate the two proteins by chromatography on various ion-exchange columns, chromatofocusing or chromatography on a hydroxyapatite column were not successful. The enzyme was therefore isolated in small batches by preparative slab PAGE. When the electrophoresis time necessary for the tracking dye to reach the bottom of the gel was doubled, the two bands were widely separated, allowing the excision of the band containing the enzyme, without contamination with the inactive protein. Attempts to obtain a similar separation in purified preparations from rat brain were not successful. The isolated enzyme solution gave a single band in PAGE under non-denaturing conditions (Fig. 1).

PAGE under denaturing and dissociating conditions (Fig. 2) also revealed the presence of a single protein band. The molecular mass of the enzyme calculated from its relative mobility with respect to marker proteins was approx. 70 000. This value is rather close to that obtained

for the brain enzyme (67 000) on the basis of measurements of the elution volume from calibrated columns of Sephadex G-100 [1]. Immunoblots also showed the presence of a single immunoreactive component in the isolated enzyme. These data taken together indicate the presence of a single polypeptide chain. Crude testis supernatants contained an additional, somewhat faster moving, immunoreactive band. The origin of this band and its significance is currently being investigated.

Double immunodiffusion experiments (Fig. 3) [19] gave single precipitation lines with the testis enzyme, suggesting the presence of a single antigenic component. The antiserum was also tested by the same method for its reactivity toward the enzyme in a crude rat brain extract. As shown in Fig. 3, patterns of identity were obtained for both the purified testis enzyme and the enzyme present in rat brain supernatant.

The effect of general protease inhibitors on the activity of the enzyme is summarized in Table 2. High concentrations of leupeptin weakly inhibited the enzyme. No inhibition was obtained with pepstatin, antipain and chymostatin. Indeed, some increase in enzyme activity was observed in the presence of pepstatin and chymostatin. The detailed mechanism of this activation has not been further studied. DFP and PMSF, irreversible inhibitors of serine proteases, had no effect on activity. Among several thiol blocking agents, PCMB and *N*-ethylmaleimide partially inhibited the enzyme, while iodoacetamide and iodoacetic acid at rather high (mM) concentrations had no effect. Consistent inhibition was obtained with the metal chelators such as EDTA and EGTA. Unlike crude enzyme preparations which were moderately activated by addition of DTT (0.4–1.0 mM), the isolated homogeneous enzyme was inhibited by DTT.

The profile of inhibition indicated that the enzyme belongs to the class of metalloendopeptidases. Consistent with this conclusion was the finding that dialysis of the enzyme against EDTA (Table 3) led to loss of activity. Activity could be restored by addition of some divalent metal ions. Complete reactivation was obtained after addition of zinc ions. The highest activity in reactivation experiments was obtained at Zn^{2+} concentrations as low

Fig. 2. PAGE and immunoblots of the enzyme after step 5 of the purification

Electrophoresis was carried out under denaturing and dissociating conditions as described in the Materials and methods section using 2.5 μ g of protein. Lanes (a) and (b) are immunoblots of the enzyme. Lane (c) contains the isolated enzyme stained with Coomassie Brilliant Blue and lane (d) contains the standard marker proteins: 1, bovine serum albumin; 2, ovalbumin; 3, trypsinogen.

Fig. 3. Ouchterlony double-immunodiffusion patterns of enzyme

The rabbit antiserum was placed in the centre well and allowed to diffuse toward the purified enzyme (wells 1, 2 and 4). Wells 3 and 5 contained a pH 5.0 supernatant of the soluble fraction of a rat brain homogenate after adjustment of pH to 7.6. The amounts of active enzyme are 0.14 units in (a) and 0.07 units in (b)

as 1 μM . Indeed, at this concentration enzyme activity was somewhat higher than that of the undialysed enzyme, suggesting that some of the enzyme was stripped of its metal content during purification. Higher Zn^{2+} concentrations led to a progressive decrease of activity. The extent of reactivation was greater after addition of Co^{2+} than of Zn^{2+} , although the concentration of Co^{2+} required for maximal activation was 100 times greater (100 μM) than that of zinc. These data indicate that the Co^{2+}

enzyme is more active than the Zn^{2+} enzyme toward Bz-Gly-Ala-Ala-Phe-pAB as the substrate. At an optimal concentration of each of the two metals, the Co^{2+} enzyme cleaved the substrate 2.5 times faster than the Zn^{2+} enzyme. The enzyme could also be reactivated by addition of Mn^{2+} , albeit at much higher concentrations than with cobalt or zinc (1.0–10 mM), while incomplete reactivation was obtained with calcium ions (0.05–2.0 mM). These data suggest that the enzyme is apparently a zinc-containing endopeptidase.

The specificity of the enzyme was studied with a series of synthetic substrates. The data summarized in Table 4 indicate that the apparent K_m and the turnover rate constant (k_{cat}), and accordingly the specificity constant (k_{cat}/K_m) are affected both by the structure of the amino acid residues that form the scissile bond as well as by the structure of residues beyond the immediate vicinity of this bond. The effect of replacing a Gly residue in the P_3' position by residues of increasing hydrophobicity is evident from the kinetic parameters obtained with substrates 1–3 (Table 4). Thus, replacing a Gly residue by a Leu or Phe residue causes a progressive decrease in the apparent K_m . This effect is also associated with an increase in the turnover rate constants and consequently the specificity constants. Replacement of the Gly residue by a Phe residue causes a 4-fold decrease in K_m , a 9-fold increase in k_{cat} , and almost a 40-fold increase in the specificity constant. These data suggest the presence of a hydrophobic binding pocket at the S_3' subsite of the active site.

The effect of structural changes in the amino acid residue at the P_1' position is indicated by the changes in kinetic parameters obtained with substrates 3, 4 and 5. Replacement of an Ala residue in this position by either a Phe or a Gly residue decreased both the rate of the reaction as well as the specificity constants.

The specificity of the enzyme toward the residue contributing the carbonyl moiety to the scissile bond is indicated by the kinetic parameters obtained with substrates 6 and 7. Substrates having a Phe residue in the P_1 position have markedly lower apparent K_m values and

Table 2. Effect of inhibitors on enzyme activity

Activity was determined with Bz-Gly-Ala-Ala-Phe-pAB (0.8 mM) as substrate. Activity in the presence of pepstatin was determined at 0.4 mM substrate concentration. Inhibitors were preincubated with the enzyme for 12 min and the reaction was then started by addition of substrate. Data are mean values obtained from two to four determinations.

Inhibitor	Final concentration (mM)	Relative activity (%)
None		100
Leupeptin	0.04	90
	0.12	68
Pepstatin	0.008	142
	0.24	171
Antipain	0.013	99
Chymostatin	0.013	108
DFP	1.0	102
PMSF	0.2	100
PCMB	1.0	35
Iodoacetamide	1.0	107
N-Ethylmaleimide	1.0	55
Iodoacetic acid	1.0	100
EDTA	1.0	40
EGTA	1.0	41
DTT	0.5	71
	1.0	48

Table 3. Effect of metal ions on enzyme activity after dialysis against EDTA

The enzyme after step 5 of the purification procedure was dialysed against three changes (1000 ml each) of a 1 mM solution of EDTA (pH 7.0) in deionized water, and then against three changes of a 1 mM solution of Tris/HCl (pH 7.0). Activity was then determined with Bz-Gly-Ala-Ala-Phe-pAB as the substrate. Activity was measured without preincubation with the metal ions.

Enzyme	Additions	Concn. (μM)	Activity (units/ml)	Relative activity (%)
Not dialysed	None	—	71	100
Dialysed	None	—	22.8	32
	ZnCl_2	0.5	45.6	64
		1	91.6	129
		5	48.3	68
		50	42.8	60
		500	16.1	23
	CoCl_2	1	80.9	114
		5	130.0	183
		50	212.2	299
		100	232.1	327
		500	192.7	271
		1000	151.2	213
		2000	98.5	139

Table 4. Kinetic parameters of endopeptidase-24.15-catalysed hydrolysis of synthetic substrates

Data are mean values $\pm$ S.E.M. obtained from four determinations. Data for substrate 2 are the mean from two determinations. The site of cleavage is indicated by an arrow.

	$P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'$	[S] (mM)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ ·M ⁻¹)
1	Bz-Gly↓Ala-Ala-Gly-pAB	0.96–4.8	3.45 $\pm$ 0.27	5.3	1.5 $\times 10^3$
2	Bz-Gly-Ala-Ala-Leu-pAB	0.64–3.2	1.54	17.0	1.1 $\times 10^4$
3	Bz-Gly-Ala-Ala-Phe-pAB	0.36–1.2	0.83 $\pm$ 0.037	48.3	5.8 $\times 10^4$
4	Bz-Gly-Phe-Ala-Phe-pAB	0.06–0.4	0.39 $\pm$ 0.13	19.2	4.9 $\times 10^4$
5	Bz-Gly-Gly-Ala-Phe-pAB	0.72–3.6	1.36 $\pm$ 0.21	4.54	3.3 $\times 10^3$
6	Boc-Phe-Ala-Ala-Phe-pAB	0.13–0.8	0.10 $\pm$ 0.01	94.1	9.4 $\times 10^5$
7	Bz-Gly-Phe-Ala-Ala-Phe-pAB	0.027–0.08	0.058 $\pm$ 0.007	179	3.1 $\times 10^6$
8	Bz-Gly-D-Phe-Ala-Ala-Phe-pAB	Inactive			
9	Bz-Gly-Phe-D-Ala-Ala-Phe-pAB	Inactive			
10	Bz-Gly-Ala-Phe-pAB	Inactive			

Table 5. Rate of degradation of natural peptides by endopeptidase 24.15

Conventional one-letter abbreviations are used for amino acids. Degradation rates were determined in incubation mixtures containing peptide (neurotensin 0.086 mM, all others 0.16 mM), enzyme and Tris/HCl buffer (0.1 M, pH 7.0). Aliquots (100 μ l) of the incubation mixtures (440 μ l) were subjected after various time intervals to h.p.l.c. on a C₁₈ μ Bondapak column and the rate of peptide degradation was calculated from the rate of disappearance of the substrate peak. Sites of cleavage are indicated by arrows. K_m values used for calculation of k_{cat} and k_{cat}/K_m values were those reported previously for the brain enzyme [8, 21], and should be therefore considered as an approximation, since the corresponding values for the testis enzyme have not been determined.

Peptide and structure	Degradation rate (μ mol/h per mg)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ ·M ⁻¹)
Dynorphin A ¹⁻⁸ ↓ Y-G-G-F-L-R-R-I	2233	0.06	86.4	1.4 $\times 10^6$
β -Neoendorphin ↓ Y-G-G-F-L-R-K-Y-P	1647	0.038	37.9	9.98 $\times 10^5$
Bradykinin ↓ R-P-P-G-F-S-P-F-R	1126	0.067	33.8	5.0 $\times 10^5$
LHRH ↓ pGlu-H-W-S-Y-G-L-R-P-G-NH ₂	388	0.095	12	1.26 $\times 10^5$
Neurotensin ↓ pGlu-L-Y-E-N-K-P-R-R-P-Y-I-L	285	0.037	7.9	2.1 $\times 10^5$

greatly increased k_{cat} and k_{cat}/K_m values compared with those having a Gly residue in this position (substrate 3). This indicates preferential cleavage of substrates having a bulky hydrophobic group in the P₁ position.

The stereospecificity of the enzyme toward peptide bonds formed between L-amino acids is indicated by the finding that replacement of an L-amino acid residue in either the P₁ or P₁' position by a D-isomer renders the substrates (substrates 8 and 9, Table 4) resistant to degradation.

It is of interest that Bz-Gly-Ala-Phe-pAB (compound 10, Table 4), an analogue of Bz-Gly-Ala-Ala-Phe-pAB in which one of the Ala residues has been deleted, was completely resistant to hydrolysis. This finding indicates that hydrolysis requires the presence in the substrate of a minimum of five peptide bonds, and consequently suggests the presence of an extended substrate-binding site.

Like the rat brain enzyme the isolated testis enzyme was inhibited by a series of previously synthesized active-site-directed *N*-carboxymethyl peptide inhibitors [9, 10]. Both enzymes were inhibited in a competitive manner

with similar K_i values. Thus, for example *N*-(1*RS*)-carboxy-3-phenylpropyl-Ala-Ala-Tyr-pAB inhibited the partially purified brain enzyme and the isolated testis enzyme with K_i values of 0.016 and 0.026 μ M respectively.

It was important to determine whether the isolated testis enzyme, like its brain counterpart, is capable of generating enkephalins from several enkephalin-containing peptides (Table 5). Incubation of the enzyme with dynorphin A¹⁻⁸, β -neoendorphin, Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ and metorphamide led to a rapid conversion of these peptides to the corresponding enkephalins in a reaction completely inhibited by cFP-AAF-pAB, an active-site-directed inhibitor of the enzyme (K_i 0.027 μ M). The formation of Leu-enkephalin from dynorphin A¹⁻⁸ and Met-enkephalin from metorphamide (Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-NH₂) and inhibition of these reactions by cFP-AAF-pAB is shown in Fig. 4. Among the bioactive peptides studied dynorphin A¹⁻⁸ was one of the most rapidly cleaved. It can be calculated that this opioid peptide was converted to Leu-enkephalin with a V_{max} of about 74 μ mol/min per mg of enzyme. The

Fig. 4. Formation of Leu-enkephalin from dynorphin A¹⁻⁸ and Met-enkephalin from metorphamide by endopeptidase 24.15

Reaction mixtures contained dynorphin A¹⁻⁸ (0.16 mM; top panel) or metorphamide (0.2 mM; bottom panel), rat testis endopeptidase 24.15 (9 ng) and Tris/HCl (0.1 M, pH 7.0). After incubation at 37 °C (5 min for dynorphin A¹⁻⁸ and 15 min for metorphamide) reactions were terminated by addition of 5 μ l of glacial acetic acid and subjected to h.p.l.c. using a linear gradient between acetonitrile and 0.1 % phosphoric acid. The initial concentration of acetonitrile was 10 % and the concentration was increased to 35 % during 25 min for metorphamide, and to 30 % during 30 min for dynorphin A¹⁻⁸ (a) Control at zero time; (b) incubation with the enzyme; (c) incubation with enzyme in the presence of 25 μ M-cFP-AAF-pAB.

reaction with β -neoendorphin was somewhat slower; however, both these peptides had specificity constants in the order of 10^6 ($s^{-1} \cdot M^{-1}$). The enzyme also cleaved efficiently bradykinin, LHRH and neurotensin at sites consistent with its specificity deduced from experiments with model synthetic substrates. Substance P was cleaved by the testis enzyme with the release of six peptide products in a pattern similar to that previously obtained with the brain enzyme [1]. Although the rate of cleavage and the amino acid composition of the products has not been determined it can be assumed that both enzymes degrade the peptide in a similar manner.

DISCUSSION

Previous efforts to isolate a homogeneous preparation of endopeptidase 24.15 from rat brain failed because of the presence of an inactive contaminating protein having virtually the same molecular mass and isoelectric point. This led us to direct our efforts toward the isolation of the enzyme from rat testes, a tissue rich in endopeptidase 24.15 activity. The purification was modified from that reported previously [1] by eliminating the pH 5.0 pre-

cipitation step and hydrophobic chromatography and introducing two ion-exchange chromatography steps at two different pH values. An additional purification step consisting of preparative PAGE followed by electroelution of the enzyme was needed to obtain a homogeneous preparation of the enzyme. While the yield in the last step was rather low, it could be used repeatedly on fractions from a single purification run to obtain small batches of a homogeneous enzyme each containing about 40 μ g of protein. The isolated enzyme gave a single band in PAGE under non-dissociating and denaturing and dissociating conditions, and homogeneity was also suggested by Ouchterlony immunodiffusion experiments and Western blots using a polyclonal antiserum raised in rabbits. The electrophoretic pattern of the isolated enzyme indicated the presence of a single polypeptide chain with a molecular mass of about 70 kDa, similar to that estimated for the brain enzyme (67 kDa). The catalytic and immunological properties of the brain and testis enzyme are closely similar if not identical, although turnover rate constants for the testis enzyme were much higher as expected from the high purity of the isolated protein. Kinetic parameters determined with synthetic substrates having a minimum of five peptide bonds gave values for specificity constants ranging from 1.5×10^3 to $3.1 \times 10^6 s^{-1} \cdot M^{-1}$. The results indicated the presence of an extended binding site with major determinants of specificity being a hydrophobic residue in the P₁ position and a hydrophobic binding pocket in the S₃' subsite. The importance of these sites is indicated by the finding that replacement of Phe residues in the P₁ and P₃' positions by Gly residues caused a decrease in the specificity constants by more than three orders of magnitude (substrates 7 versus 1 in Table 4). While an Ala residue in the P₁' position seems to be favoured over a Gly or a Phe residue, the S₁' subsite seems to readily accommodate a basic amino acid residue as shown by the rate of cleavage of dynorphin A¹⁻⁸ and β -neoendorphin (Table 5).

The specificity of the enzyme determined with model synthetic substrates is clearly reflected in studies with bioactive peptides. Prominent among these is the rapid conversion of dynorphin A¹⁻⁸ and β -neoendorphin into Leu-enkephalin and metorphamide into Met-enkephalin. The cleavage of the Phe⁵-Ser⁶ bond in bradykinin, the Arg⁸-Arg⁹ bond in neurotensin and the Tyr⁵-Gly⁶ bond in LHRH also reflects the same enzyme specificity. Indeed, recent studies have shown that endopeptidase 24.15 is the primary enzyme responsible for cleavage of the Arg⁸-Arg⁹ bond in neurotensin [20], and the primary enzyme responsible for cleavage of the Tyr⁵-Gly⁶ bond in LHRH in membrane preparations from hypothalamus and pituitary [21]. Experiments have also shown that specific inhibitors of the enzyme [10,11] caused a dramatic increase in the half life of intravenously administered LHRH (A. Lasdun, S. Reznik, C. Molineaux & M. Orłowski, unpublished work).

Though we have not yet been able to determine the metal contents of the enzyme, several lines of evidence indicate that it is a metalloendopeptidase. This is based on the finding that metal chelators (*o*-phenanthroline, EDTA, EGTA) consistently inhibit the enzyme, that dialysis against chelators leads to loss of activity which can be restored by Zn²⁺ and Co²⁺ ions, that the Co²⁺ enzyme is more active than the Zn²⁺ enzyme, and finally that substrate-related *N*-carboxymethyl peptides act as specific and potent enzyme inhibitors [10,11]. All these

findings are characteristic for Zn^{2+} peptidases. An additional argument for classification of the enzyme as a metalloendopeptidase is inhibition of the isolated enzyme by low concentrations of DTT (0.4 mM). Crude enzyme preparations [1] from brain and testis are weakly activated by low concentrations of DTT (0.4–1 mM), but inhibited at higher concentrations (over 1.5 mM), whereas the isolated enzyme is inhibited even at low DTT concentrations. While the causes of this behaviour are not clear, we have observed that enzyme preparations after step 4 of the purification procedure have a tendency to form heterodimers with the contaminating impurity (our unpublished work). Accordingly the activating effect of DTT in crude preparations may result from preventing formation of such heterodimers.

Finally it is important to discuss the relationship of endopeptidase 24.15 to endo-oligopeptidase A, an enzyme described in rabbits by Camargo *et al.* [22–24]. While the two enzymes cleave bradykinin and neurotensin at the same sites, endo-oligopeptidase A was claimed to be a cysteine endopeptidase [22,24], to be inactive toward LHRH [25], not to hydrolyse the Gly–Ala bond in N^{α} -benzoyl-Gly-Ala-Ala-Phe-*p*-aminobenzoate (Bz-GAAF-pAB), a synthetic substrate used for the determination of endopeptidase 24.15 activity [1], nor to be inhibited by cFE-AAF-pAB [9]. Furthermore, it was recently claimed [9] that conversion of metorphamide and dynorphin A^{1-8} into Met- and Leu-enkephalin respectively is catalysed by endo-oligopeptidase A but not by endopeptidase 24.15, and that this reaction is not inhibited by cFE-AAF-pAB. In addition, the same authors report that an antiserum against endo-oligopeptidase A does not inhibit the cleavage of Bz-GAAF-pAB by endopeptidase 24.15. This led to the conclusion that endopeptidase 24.15 and endo-oligopeptidase A are two distinct enzymes.

Our present study shows, in confirmation of our previous reports with less pure enzyme preparations and crude membrane fractions [2,8], that a homogeneous preparation of endopeptidase 24.15 converts several enkephalin-containing peptides to the corresponding enkephalins, and that these reactions are inhibited by specific inhibitors of endopeptidase 24.15. Indeed, conversion of dynorphin A^{1-8} to Leu-enkephalin by isolated endopeptidase 24.15 proceeds at a rate more than 10–20 times greater than that reported by Toffoletto *et al.* [9] for endo-oligopeptidase A. Furthermore, independent work by Checler *et al.* [20] showed that cleavage of the Arg⁸–Arg⁹ bond of neurotensin, attributed by Camargo and his group to endo-oligopeptidase A [9], is actually catalysed by endopeptidase 24.15 in a reaction inhibited by cFE-AAF-pAB.

Thus, the two enzymes catalyse the hydrolysis of the same bonds in neurotensin, bradykinin, dynorphin A^{1-8} , β -neoendorphin and metorphamide, but differ with respect to their mechanistic classification (cysteine versus metalloendopeptidase), activity toward LHRH and synthetic endopeptidase 24.15 substrates and inhibitors. In spite of these differences the possibility that the two enzymes are actually identical needs to be considered. The procedure used for the purification of brain endo-oligopeptidase A [24] also results in an enrichment of endopeptidase 24.15 activity, although the enzyme is still contaminated with the same inactive component men-

tioned above. Since endopeptidase 24.15 is a metallo-enzyme, the classification of endo-oligopeptidase A, claimed to be a thiol peptidase [9,22,24], would need to be re-examined, by studying activation of the enzyme by metal ions after dialysis under conditions of strict exclusion of traces of such ions. The claim that endo-oligopeptidase A is inactive toward LHRH [25], that it does not cleave synthetic endopeptidase 24.15 substrates, and that it is not affected by specific endopeptidase 24.15 inhibitors [9] would also require a thorough re-examination. Species differences in enzyme properties (especially rabbit versus rat) would also need to be examined.

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REFERENCES

1. Orlowski, M., Michaud, C. & Chu, T. G. (1983) *Eur. J. Biochem.* **135**, 81–88
2. Acker, G. R., Molineaux, C. J. & Orlowski, M. (1987) *J. Neurochem.* **48**, 284–292
3. Kerr, M. A. & Kenny, A. J. (1974) *Biochem. J.* **137**, 447–488
4. Almenoff, J., Wilk, S. & Orlowski, M. (1981) *Biochem. Biophys. Res. Commun.* **102**, 206–214
5. Matsas, R., Kenny, A. J. & Turner, A. J. (1984) *Biochem. J.* **223**, 433–440
6. Littlewood, G. M., Iversen, L. L. & Turner, A. J. (1988) *Biochem. Int.* **12**, 383–389
7. Malfroy, B., Swerts, J. P., Guyon, A., Roques, B. P. & Schwartz, J.-C. (1978) *Nature (London)* **276**, 523–526
8. Chu, T. G. & Orlowski, M. (1985) *Endocrinology (Baltimore)* **116**, 1418–1425
9. Toffoletto, O., Metters, K. M., Oliveira, E. B., Camargo, A. C. M. & Rossier, J. (1988) *Biochem. J.* **252**, 35–38
10. Chu, T. G. & Orlowski, M. (1984) *Biochemistry* **23**, 3598–3603
11. Orlowski, M., Michaud, C. & Molineaux, C. (1988) *Biochemistry* **27**, 597–602
12. Almenoff, J. & Orlowski, M. (1983) *Biochemistry* **22**, 590–599
13. Lowry, O. H., Rosebrough, A. L., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
14. Dixon, M. (1959) *Biochem. J.* **55**, 170–171
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
16. Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
17. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
18. Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
19. Ouchterlony, O. (1958) *Prog. Allergy* **5**, 1–78
20. Checler, F., Vincent, J. P. & Kitabgi, P. (1985) *J. Neurochem.* **45**, 1509–1513
21. Molineaux, C. J., Lasdun, A., Michaud, C. & Orlowski, M. (1988) *J. Neurochem.* **51**, 624–633
22. Camargo, A. C. M., Shapanka, R. & Greene, L. J. (1973) *Biochemistry* **12**, 1838–1844
23. Oliveira, E. B., Martins, A. R. & Camargo, A. C. M. (1976) *Biochemistry* **15**, 1967–1974
24. Carvalho, K. M. & Camargo, A. C. M. (1981) *Biochemistry* **20**, 7082–7088
25. Camargo, A. C. M., Fonseca, M. J. V., Caldo, H. & Carvalho, K. M. (1982) *J. Biol. Chem.* **257**, 9265–9267
26. Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162