Purification and Characterization of an Enkephalin-Degrading Dipeptidyl-aminopeptidase from Porcine Brain†

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ABSTRACT: A porcine brain dipeptidyl-aminopeptidase (DAP) has been purified more than 2400-fold from a crude mitochondrial fraction containing synaptosomes. This enzyme catalyzes the release of free Tyr-Gly from Leu-enkephalin (K_m = 2.5 μM) with an optimal activity between pH 6.0 and pH 8.0. The enzyme appears homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis devoid of detectable contaminating aminopeptidase activities. The native enzyme is a monomeric protein with a molecular weight of 51 000 ± 1000 and an isoelectric point of 4.6 ± 0.1. This enzyme cosediments with synaptosomes on a Ficoll–sucrose gradient and is partially associated with synaptic plasma membranes. Its activity is inhibited by the metal-chelating agents ethylenediaminetetraacetate and *o*-phenanthroline. It is not inhibited by the OH-reactive agent phenylmethanesulfonyl fluoride and SH-reactive agents such as *p*-(chloromercuri)benzoate and *N*-ethylmaleimide. Among the various biologically active peptides tested, the purified enzyme releases efficiently the N-terminal dipeptide moiety from enkephalins, Trp-Met-Asp-Phe-NH_2 (CCK_4), and Gly-Trp-Met-Asp-Phe-NH_2 (CCK_5). At variance, the native peptides CCK_8, substance P, neurotensin, and angiotensin II are not cleaved by the DAP. This enzyme is different from other unspecific DAPs, as well as from enkephalin-degrading DAPs previously reported, by its molecular weight and substrate specificity.

The enkephalins (Tyr-Gly-Gly-Phe-Leu and Tyr-Gly-Gly-Phe-Met), which are putative neurotransmitters (North, 1979), are hydrolyzed by various peptidases present in brain tissues. Inactivation of these opioid peptides occurs extremely rapidly both in vitro (Hambrook et al., 1976) and in vivo (Meek et al., 1977; Craves et al., 1978), accounting for their weak and transient antinociceptive action (Belluzzi et al., 1976).

Attention was first focused on aminopeptidases because the main metabolite liberated from enkephalins in brain tissue was tyrosine (Jacquet et al., 1976; Marks et al., 1977; Knight et al., 1978). It is believed that cleavage at the Tyr^1-Gly^2 level should be physiologically involved in enkephalin metabolism since (i) enkephalin analogues, protected from aminopeptidase degradation, elicit enhanced activity (Pert et al., 1976) and (ii) bestatin, a potent but not selective aminopeptidase inhibitor (Suda et al., 1976), potentiates the analgesic effect of coadministered Met-enkephalin (Carenzi et al., 1981; Yi et al., 1983).

The endogenous enkephalins are also inactivated by cleavage at the Gly^3–Phe^4 bond by a brain membrane-bound enzyme designated as enkephalinase (Malfroy et al., 1978). This metallopeptidase was shown to be identical with the neutral endopeptidase EC 3.4.24.11 (Matsas et al., 1983). Enkephalinase is competitively inhibited by thiorphan (Roques et al., 1980) and phosphoramidon (Fulcher et al., 1982) in the nanomolar range, and its biological relevance in the control of enkephalinergic transmission is clearly demonstrated by the antinociceptive effect of thiorphan, which is blocked by naloxone, a specific antagonist of opioid receptors (Roques et al., 1980).

A third possible pathway for the degradation of enkephalins could involve a dipeptidyl-aminopeptidase (DAP)[1] activity. The presence of an enzyme releasing Tyr-Gly from enkephalins was first suggested by Craves et al. (1978) and evidenced in rat brain by Gorenstein and Snyder (1979).

The assumption that inactivation of the enkephalins in vivo could involve three different peptidases is suggested by the high antinociceptive effect of kelatorphan, which is the first described almost complete inhibitor of these various peptidases (Fournié-Zaluski et al., 1984; Bouboutou et al., 1984). Indeed, kelatorphan potentiates the Met-enkephalin-induced analgesia. This effect is higher than the one produced by the association of bestatin and thiorphan (Fournié-Zaluski et al., 1984). This result could indicate the involvement of the DAPs in the metabolism of enkephalins.

In this study, we have purified to apparent homogeneity a dipeptidyl-aminopeptidase from porcine brain. This enzyme of an apparent molecular weight of 51 000 very likely belongs to the group of metallopeptidases and displays an optimal rate of degradation for Tyr-Gly-Gly-Phe, the enkephalins, and short cholecystokinin peptides such as CCK_4 and CCK_5.

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TEA, triethanolamine; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; PHMB, *p*-(hydroxymercuri)benzoate; Boc, *tert*-butyloxycarbonyl; Tyr-βNA, tyrosine-β-naphthylamide; DAP, dipeptidyl-aminopeptidase; AP, aminopeptidase; DEAE, diethylaminoethyl; CCK, cholecystokinin; HPLC, high-performance liquid chromatography; FPLC, fast-protein liquid chromatography.

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Experimental Procedures

Materials

[^{3}H]Leu-enkephalin was from New England Nuclear. Phenylmethanesulfonyl fluoride was purchased from Aldrich. Tyr, Tyr-Gly, Tyr-Gly-Gly, Met-enkephalin, Gly-Gly-Phe-Leu, *p*-(hydroxymercuri)benzoate, bacitracin, puromycin, pepstatin, *N*-ethylmaleimide, ovalbumin, and cytochrome *c* (type III) were from Sigma. Leupeptin, aprotinin, and antipain were from Serva (Heidelberg). Bovine serum albumin was purchased from Boehringer Mannheim (Germany). Iodoacetamide was from Merck. Neurotensin, substance P, Boc-enkephalin, and Tyr-β-naphthylamide (Tyr-βNA) were from Bachem (Switzerland). *N*-Ac-Leu-enkephalin was synthesized as described by Horsthemke et al. (1984). All other peptides were synthesized in the laboratory. Ficoll, Sephacryl S 200, AH-Sepharose 4B, phenyl-Sepharose, Mono P HR 5/20, and the FPLC system were from Pharmacia. All other reagents were of analytical grade.

Methods

Standard Assay of DAP. The assay of DAP measured the release of [^{3}H]Tyr-Gly from [^{3}H]Leu-enkephalin. The standard reaction mixture, final volume 200 μL, contained 2 pmol of [^{3}H]Leu-enkephalin in 0.05 M Hepes–KOH (pH 7.0). The reaction was initiated by addition of enzyme. Before use, commercial [^{3}H]Leu-enkephalin was purified on a Sep-Pak C_{18} cartridge to remove radioactive impurities eluting with water. For this purpose the radioactive solution (10 μL) was transferred on the cartridge previously equilibrated with distilled water, washed with 10 mL of water, and eluted by 2 mL of ethanol. During the early stages, in order to ensure a proper determination of the respective activities, specific inhibitors were used: bestatin, 10^{-5} M, was used to inhibit aminopeptidases (Suda et al., 1976), captopril, 10^{-5} M, to inhibit angiotensin converting enzyme (Ondetti et al., 1977), and thiorphan, 10^{-5} M, to inhibit enkephalinase A (Roques et al., 1980). For this purpose, the enzymatic fraction was incubated at 25 °C for 15 min with the required inhibitors in the buffer, and the reaction was initiated by addition of the substrate. The incubation performed at 25 °C, for 30 min or less, was stopped by heating for 3 min at 80 °C. An aliquot was applied to Sep-Pak C_{18} cartridges (Waters) previously washed with 10 mL of ethanol and 10 mL of water. [^{3}H]Tyr-Gly was directly eluted into scintillation vials with 3 × 2 mL of distilled water, and then [^{3}H]Leu-enkephalin was eluted with ethanol. This chromatographic separation on C_{18} columns allowed a quantitative separation of ^{3}H peptide fragments eluted by water from native enkephalin, retained on the resin (more than 99%). The radioactivity eluted with water was determined by liquid scintillation counting with Beckman Ready Solv MP as the scintillation liquid. Under these conditions, the assay was linear with time and enzyme concentration in the range of 0.08–0.7 pmol of hydrolyzed substrate. One unit of DAP released 1 pmol of Tyr-Gly from [^{3}H]Leu-enkephalin in 1 min.

Standard Assay of Aminopeptidases. Aminopeptidase activities were measured fluorometrically by a single time-point assay as described by Wagner et al. (1981). The standard assay mixture (200 μL) contained 1 nmol of Tyr-βNA in 50 mM Tris-HCl, pH 7.2. Incubations were performed at 37 °C and stopped by heating for 3 min at 80 °C. Assays were diluted 10-fold with distilled water, and measurements were performed with a Kontron SFM 23/B spectrofluorometer calibrated with standard amounts of β-naphthylamine (βNA) with excitation and emission wavelengths of 335 and 415 nm, respectively. Boiled enzyme controls were included as a blank to correct for any spontaneous hydrolysis of the substrate.

Protein Determination. The protein concentration was determined by the method of Bradford (1976) or by absorbance at 280 nm.

Molecular Weight Determination. A column (1.8 cm^2 × 90 cm) of Sephacryl S 200 was equilibrated with Tris-HCl, pH 7.4, and 0.5 M NaCl at a flow rate of 18 mL/h. The apparent molecular weight of the porcine dipeptidyl-aminopeptidase was determined from a comparison with standard proteins, bovine serum albumin, ovalbumin, pancreatic deoxyribonuclease, and cytochrome *c*, according to the method of Siegel and Monty (1966).

Mono P HR 5/20 Chromatography. A Mono P HR 5/20 column, which is beaded with a 10-μm spherical particle size of hydrophilic resin, was used as a strong anion exchanger. The Mono P column was equilibrated with triethanolamine [50 mM TEA–HCl, pH 7.7, 10% glycerol, 2 mM 2-mercaptoethanol (buffer A)]. Dialyzed enzyme was applied on the Mono P column and was eluted at a flow rate of 30 mL/h with a gradient of NaCl monitored by a GP 250 gradient programmer. The different slopes of the gradient were washing with buffer A, 0.2 M NaCl for 8 min, then a linear gradient of 0.2–0.35 M NaCl in buffer A for 76 min, and then 1 M NaCl in buffer A for 24 min.

Isoelectric Point Determination. The isoelectric point of porcine DAP was determined by using the chromatofocusing method with the FPLC system on a Mono P HR 5/20 column. The enzyme was loaded on the Mono P HR 5/20 column equilibrated with 0.025 M piperazine hydrochloride, pH 6.3. A linear gradient of 45 mL was generated with 10% Polybuffer 7-4/HCl, pH 4.3, with a flow rate of 60 mL/h. Start buffer and eluant Polybuffer were all degassed before use, to avoid fluctuation in the pH gradient due to the presence of bicarbonate ions.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out under dissociating conditions (2% SDS). Two milliliters of enzyme was lyophilized, redissolved in 0.05 mL of sample buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol), and denatured by briefly heating at 100 °C as already described (Leblanc et al., 1982). Electrophoresis was performed in a 10% polyacrylamide slab gel. Gels were stained by the silver-stain procedure (Switzer et al., 1979) and scanned with a Joyce-Loebl microdensitometer. The molecular weight was estimated by comparison with markers of known molecular weight (Pharmacia) as already described (Pierre & Laval, 1980).

Identification of Cleavage Sites in Enkephalin and Other Peptides. The identification of enzyme products was performed by HPLC analysis using a Waters high-performance liquid chromatograph equipped with a C_{18} μBondapak column with a particle size of 9 μm (0.39 × 30 cm). A sample of 0.65 unit of enzyme preparation was incubated at 25 °C in 50 mM potassium phosphate buffer, pH 7.0, with a millimolar solution of the synthetic peptide in a final volume of 200 μL for 240 min or more. The reaction was stopped by heating for 3 min at 80 °C. An aliquot of sample was eluted at ambient temperature with a mixture of acetonitrile and 0.1 M phosphate buffer, pH 3.0, with a flow rate of 2 mL·min^{-1}. The peptides were measured by UV absorption at 210 nm. Various proportions of acetonitrile and phosphate buffer were used in order to separate the different products of enzymatic cleavage (1:99, 5:95, 15:85, 20:80) and to measure the reaction velocity. In order to separate Tyr, Tyr-Gly, and Tyr-Gly-Gly, a relatively high concentration of phosphate buffer (0.1 M) and low pH conditions were used for the mobile phase. For enkephalin

Table I: Purification of Enkephalin-Degrading Dipeptidyl-aminopeptidase from Porcine Brain

	fraction	vol (mL)	total protein (mg)	total act. (units/mL)[a]	sp act. (units/mg)	purification	recovery (%)
I	homogenate	800	11200	2400	0.2		100
II	washed pellet	300	3000	600	0.2		25
III	Triton extract	230	2530	782	0.3	1.5	32
IV	DEAE-cellulose	70	476	449	0.9	4.5	19
V	ammonium sulfate	68	235	313	1.3	6.5	13
VI	phenyl-Sepharose	20	15	212	14.1	70.5	9
VII	AH-Sepharose 4B	36	7.2	159	22.1	110.5	7
VIII	Mono P HR 5/20	4	0.22	32.4	147.3	736	1.4
IX	chromatofocusing	2	<0.005	2.4	>480	>2400	0.1

[a] One unit is defined as 1 pmol of Tyr-Gly liberated/min, under the conditions described in the text.

metabolites, the retention times were Tyr, 4.7 min, Tyr-Gly, 8.1 min, and Tyr-Gly-Gly, 7.0 min, with a 1:99 mixture of acetonitrile/phosphate buffer. To elute the largest or more hydrophobic peptides, a 10-min linear gradient of 20:80 to 30:70 was used in the same buffer.

Washed Pellet (P_2) Preparation. All the experiments described below and enzyme purification were performed at 0–4 °C unless otherwise specified. A fresh porcine brain was recovered on ice immediately after death. It was dissected and transected between the occipital cortex and the cerebellum. The cerebellum was discarded and the forebrain was used in all experiments. A 60-g portion was homogenized with a motor-driven glass–Teflon homogenizer at 400 rpm in 0.32 M sucrose containing 5 mM Tris-HCl, pH 7.4 (buffer B). The homogenate (fraction I) was centrifuged at 1000*g* for 5 min; the pellet was discarded and the supernatant was centrifuged at 10000*g* for 20 min. The pellet (P_2) was washed 3 times by resuspending in the same buffer and centrifuging at 10000*g* for 20 min (fraction II).

Isolation of Synaptosomal Fractions. The crude mitochondrial fraction (fraction II) was suspended in buffer B and applied to a three-step Ficoll–sucrose gradient consisting of successive 10-mL layers of 7.5%, 13%, and 17% (w/v) according to Cotman (1974). The preparation was centrifuged in a Beckman SW 28 rotor for 45 min at 68000*g*. The gradient was collected and 1-mL fractions were recovered. Each fraction was assayed for DAP. The incubation mixture was supplemented with bestatin (10^{-5} M), captopril (10^{-5} M), and thiorphan (10^{-5} M) in the absence or in the presence of kelatorphan (10^{-5} M). The active fractions were further characterized by HPLC.

Isolation of Synaptic Plasma Membranes. The DAP-containing fractions were collected at Ficoll interfaces (7.5/13.0 and 13.0/17.0), pooled, washed in 5 volumes of buffer B, and centrifuged at 68000*g* for 45 min. The pellet was lysed under alkaline conditions in 5 volumes of Tris-HCl, 0.005 M, pH 8.2, for 90 min at 4 °C (Cotman, 1974). After osmotic shock, the membranes were concentrated by centrifugation for 15 min at 76300*g*. The resulting pellet resuspended in buffer B was applied to a discontinuous sucrose gradient containing 5-mL layers of 0.7, 1.0, 1.1, 1.2, and 1.3 M sucrose. Centrifugation was carried out for 90 min at 68000*g*. The gradient was collected and assayed for DAP activity as described above.

Results

Purification

A dipeptidyl-aminopeptidase has been purified more than 2400-fold from a crude mitochondrial fraction. A summary of the purification is given in Table I.

Triton Extraction. The washed pellet P_2 (fraction II) prepared as described under Experimental Procedures was suspended in 10 volumes of 5 mM Tris-HCl (pH 8.1) with 0.5% Triton X-100 and extracted for 45 min at 4 °C under gentle stirring. This suspension was centrifuged for 40 min at 20000*g*. The supernatant was recovered, yielding the Triton extract (fraction III). The total dipeptidyl-aminopeptidase activity increased 1.3-fold after Triton X-100 treatment.

DEAE-cellulose Chromatography. The Triton extract was directly applied into a Whatman DE-52 column (12.5 cm^2 × 18 cm) previously equilibrated in 50 mM Tris-HCl buffer (pH 7.5), containing 0.1% Triton X-100 (buffer C). The column was washed with 2 bed volumes of equilibrating buffer, and the enzymes were eluted with a 500-mL linear gradient (0–0.5 M NaCl) in buffer C, at a flow rate of 50 mL/h (15-mL fractions were collected). Dipeptidyl-aminopeptidase (DAP) and aminopeptidase (AP) activities coeluted as a symmetrical peak at 0.2 M NaCl. The active fractions were pooled, yielding fraction IV.

Phenyl-Sepharose Chromatography. For hydrophobic chromatography, fraction IV was supplemented with ammonium sulfate (40% saturated w/v). Seventy percent of the DAP activity did not precipitate while 51% of inactive proteins were discarded by centrifugation at 20000*g* for 20 min. The supernatant (fraction V) was applied to a phenyl-Sepharose column (0.8 cm^2 × 10 cm) equilibrated with 50 mM Tris-HCl (pH 7.6) and ammonium sulfate (40% saturated w/v). This column was eluted with a gradient of 80 mL of 50 mM Tris-HCl (pH 7.6) and ammonium sulfate (0% saturated w/v), at a flow rate of 20 mL/h. When fraction V was applied on the phenyl-Sepharose column, DAP and AP were both retained on the resin and eluted by lowering the ammonium sulfate concentration. They coeluted at 20% (saturated w/v) ammonium sulfate. The active fractions (5 mL each) were pooled and dialyzed against 10 mM Tris-HCl (pH 7.0) (buffer C) and applied to an AH-Sepharose 4B column (fraction VI).

AH-Sepharose Chromatography. Aminohexyl-Sepharose 4B has a very high binding capacity for DAP and AP enzymes. Fraction VI was applied to a column of AH-Sepharose 4B (0.8 cm^2 × 10 cm) previously equilibrated with buffer C. After the column was washed with 30 mL of buffer C, the enzymes were eluted with a 120-mL linear gradient of 0.15–0.5 M NaCl in buffer C. DAP and AP eluted at 0.37 and 0.43 M, respectively (Figure 1A). DAP-containing fractions were pooled and dialyzed against buffer A (fraction VII).

Mono P HR 5/20 Chromatography. Fraction VII was loaded on a Mono P HR 5/20 column, washed, and eluted as described under Experimental Procedures. DAP eluted at 0.22 M NaCl (DAP peak A) with a small discrete peak or shoulder at 0.23 M (DAP peak B). This pattern of elution was reproducibly observed. Fractions of peak A were pooled and dialyzed for 4 h against 2 L of buffer A. Bovine serum albumin was added to give a final concentration of 200 μg/mL (fraction VIII).

Chromatofocusing of Fraction VIII. Fraction VIII was loaded on a Mono P HR 5/20 column equilibrated with 0.025

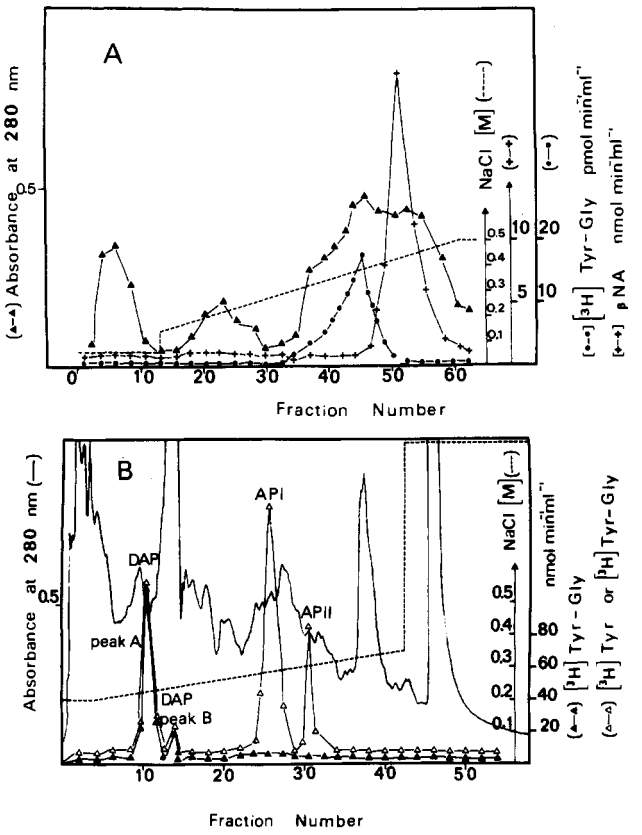


FIGURE 1: (A) Fractionation of aminopeptidase and DAP activities by AH-Sepharose 4B chromatography of fraction VI. The eluted fractions were assayed for absorbance at 280 nm (▲). DAP activity (●) in 5 μL of protein solution was assayed with [^{3}H]Leu-enkephalin as the substrate in the presence of bestatin (10^{-5} M) and aminopeptidase activity (+) in 5 μL of protein solution with Tyr-βNA as the substrate. For details see Experimental Procedures. (B) Fractionation of aminopeptidase and DAP activities by Mono P HR 5/20 chromatography of fraction IV. DAP activity (▲) in 3 μL of protein solution was assayed with [^{3}H]Leu-enkephalin as the substrate in the presence of bestatin (10^{-5} M). Aminopeptidase activity (△) in 3 μL of protein solution was assayed with [^{3}H]Leu-enkephalin as the substrate without inhibitor. When incubations were carried out without inhibitor, HPLC analysis showed that DAP and aminopeptidase activities were completely separated (contamination of DAP by aminopeptidase was less than 3‰).

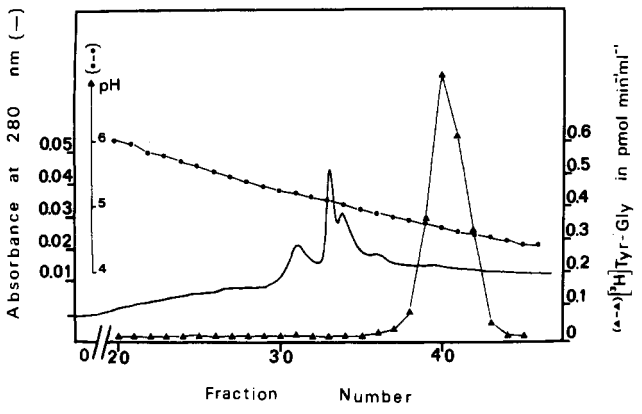


FIGURE 2: Chromatofocusing of fraction VIII on a Mono P HR 5/20 column. The pH was measured and enzymatic activity was determined on each fraction with [^{3}H]Leu-enkephalin as the substrate. For details see Experimental Procedures.

M piperazine hydrochloride (pH 6.3), and fractions of 1 mL were collected (Figure 2). A linear gradient of pH 6.0–4.3 was generated by using as eluant 45 mL of Polybuffer (pH 4.3) through the column. As shown in Figure 2, the DAP eluted as a symmetrical peak at pH 4.6 without absorbance

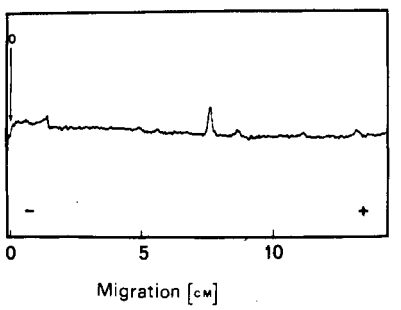


FIGURE 3: SDS–polyacrylamide slab gel electrophoresis of purified enkephalin-degrading DAP (fraction IX). A volume of 2 mL of protein solution was lyophilized, resuspended in 50 μL of sample buffer, briefly heated in the presence of 2-mercaptoethanol and SDS, and then analyzed by electrophoresis. The silver-stained gel was scanned in a Joyce-Loebl microdensitometer. The arrow shows the origin of migration.

detectable at 280 nm. The four active fractions were pooled and dialyzed against buffer A (fraction IX).

Comments on the Purification. Concerning the chromatofocusing elution, the determination of the specific activity of DAP was not possible as the amount of protein was too low to be detected either by absorbance at 280 nm or by the Bradford procedure. Using the UV absorption and the Bradford method for the determination of protein concentration, we assume that our detection limits are between 2 and 5 μg/mL. Therefore, we conclude that the protein concentration is less than 5 μg/mL and that the specific activity is higher than 480 units/mg of protein. This is probably an overestimation, since when we measured the purity of the enzyme by SDS–polyacrylamide gel electrophoresis (see below), the observed band was faint, although we used 2 mL of the preparation which was lyophilized and visualized by silver staining (Figure 3). It means that the amount of protein was probably less than 0.5 μg/mL.

If a preparation is required in which the DAP and AP activities are well separated, an alternative procedure is to use fraction IV after DEAE-cellulose chromatography and to directly purify this fraction by anion-exchange Mono P chromatography (Figure 1B). This procedure has some important features: (i) an excellent separation of DAP and AP enzymes was achieved (the cross-contamination of the two enzymes is less than 3‰ as quantified by HPLC analysis); (ii) the purification step was performed by using 102 mg of protein in 40 min with a recovery of 15%; (iii) the enzyme was concentrated 5 times; (iv) it shows a minor peak of DAP at 0.23 M (peak B), but no significant difference with protease inhibitors was noticed when peak A and peak B were tested (data not shown), and furthermore all the active fractions liberated Tyr-Gly by HPLC analysis. Due to the low amount of enzyme contained in peak B and its apparent similarity with peak A, it was not investigated further.

Subcellular Localization of the DAP

The distribution of the DAP on a Ficoll–sucrose gradient (Figure 4A) shows the presence of two peaks of enzymatic activity that sediment at the Ficoll–sucrose density corresponding to the distribution of synaptosomes. This preparation was examined by electron microscopy and found to be synaptosomes, barely contaminated with microsomes or mitochondria. The HPLC analysis of the DAP activity shows that more than 90% of the liberated radioactive fragments was [^{3}H]Tyr-Gly. This activity was potently inhibited by kelatorphan (10^{-5} M) (Figure 4A).

The distribution of the DAP on a sucrose gradient (Figure 4B) shows that the enzyme sediments in the lighter fractions,

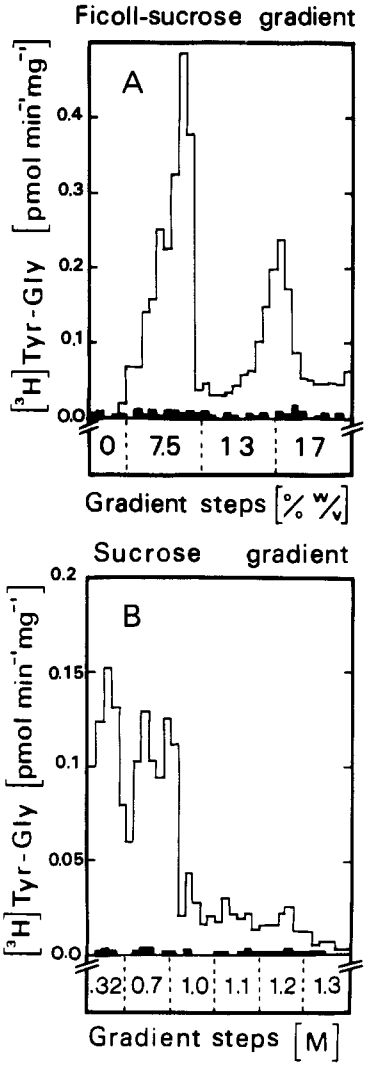


FIGURE 4: (A) Distribution on a Ficoll–sucrose gradient of the DAP activity from the crude mitochondrial extract. After centrifugation, fractions were recovered and assayed as described under Experimental Procedures in the absence (□) or in the presence (■) of kelatorphan (10^{-5} M). The active fractions were further characterized by HPLC. The top of the gradient is on the left. The two peaks of DAP activity cosediment with purified synaptosomes. For details see Experimental Procedures and text. (B) Distribution of the DAP activity on a sucrose gradient. The two peaks of synaptosomal activity purified on a Ficoll–sucrose gradient were pooled and submitted to an osmotic shock. The resulting synaptic plasma membranes were analyzed on a sucrose gradient and assayed in the absence (□) or in the presence (■) of kelatorphan (10^{-5} M).

partially with synaptic plasma membranes. The total recovery of DAP for the two successive gradients is 48%.

Physical Properties of the DAP

Criteria of Purity of the Preparation. The enzyme appears to be homogeneous as judged by polyacrylamide gel electrophoresis under denaturing conditions (Figure 3). When 0.65 unit was incubated for 240 min with 1 mM Leu-enkephalin, only Tyr-Gly was detectable after analysis of the products of the reaction by HPLC, showing that DAP activity was not contaminated by aminopeptidases or neutral endopeptidase (EC 3.4.24.11).

Molecular Weight. The molecular weight of DAP was estimated by electrophoresis in SDS–polyacrylamide slab gels and exclusion chromatography on Sephacryl S 200. From the electrophoresis experiments and by comparison with a series of marker proteins run in parallel, a molecular weight of 51 000 ± 1000 was determined. The Stokes radius was estimated by chromatography on the Sephacryl S 200 column as already described (Pierre & Laval, 1980). The enzyme eluted as a single symmetrical peak, and from the relation between the elution volumes of the proteins and their sizes, a Stokes radius of 32 ± 1 Å was evaluated, corresponding to M_r 51 000 ± 1000. These data, in conjunction with SDS–polyacrylamide gel results, indicate that the enzyme is a monomeric protein having molecular weight of 51 000 ± 1000.

Table II: Effect of Various Protease Inhibitors on Porcine Brain Dipeptidyl-aminopeptidase

inhibitor	K_i (mM)	inhibitor	K_i (mM)
o-phenanthroline[a]	0.02	bestatin	>0.1
EDTA[a]	7.5	puromycin	0.8
PMSF	>1	pepstatin	>0.1
PHMB	>0.5	antipain	>1
iodoacetamide	>0.1	leupeptin	>1
N-ethylmaleimide	>0.1	aprotinin	>1
bacitracin	>0.1	thiorphan	>0.1
captopril	>0.1		

[a] Each value represents the mean of three separate experiments (SEM less than 10% of the mean in all cases). The compound being tested was preincubated for 15 min at 25 °C.

Enzymatic Properties of the DAP

pH. The optimal pH for enzymatic activity was determined in 0.1 M Hepes–KOH between pH 5.5 and pH 8.5 at 25 °C. A broad maximum between pH 6.8 and pH 7.2 was found with 60% of maximal activity at pH 6.0 and 8.2. The enzyme activity was routinely determined at pH 7.0.

K_m. The initial reaction velocities were measured at different Leu-enkephalin concentrations with the standard assay. The Michaelis constant K_m was determined by using the Lineweaver and Burk representation. The K_m for Leu-enkephalin was 2.5×10^{-6} M. We did not observe inhibition by excess of substrate up to 10^{-3} M (data not shown).

Isoelectric Point. Its value was 4.6 ± 0.1 as measured by chromatofocusing on a Mono P HR 5/20 column (Figure 2).

Energy of Activation. The apparent energy of activation, calculated by using the Arrhenius equation, was found to be 8.2 kcal·mol^{-1} with Leu-enkephalin as the substrate. This value was determined from the initial velocity of the enzyme measured at 10, 25, 37, and 45 °C.

Effect of Protease Inhibitors. In an attempt to characterize the enzyme as a metallopeptidase, thiol peptidase, serine peptidase, or aspartyl peptidase, the action of inhibitors specific for these classes of peptidases was investigated (Table II). Among sulfhydryl reagents, *p*-(hydroxymercuri)benzoate, iodoacetamide, and *N*-ethylmaleimide had only a slight effect at high concentrations. PMSF and aprotinin, which are serine protease inhibitors, had no effect. Leupeptin and antipain, which inhibit serine or thiol proteases, had no effect. Pepstatin, a specific inhibitor of aspartyl proteases (Umezawa, 1977), did not decrease the enzyme activity. Among metallopeptidase inhibitors, EDTA and *o*-phenanthroline caused an effective inhibition. They both blocked the enzyme activity with 10 mM, but with 0.1 mM only *o*-phenanthroline was inhibitory. Bestatin (0.1 mM), thiorphan (0.1 mM), and captopril (0.1 mM) failed to inhibit the purified DAP activity. These three last inhibitors differentiated the DAP from aminopeptidases (Suda et al., 1976), neutral endopeptidase, EC 3.4.24.11 (Roques et al., 1980), and peptidyl-dipeptidase, EC 3.4.15.1 (Ondetti et al., 1977), respectively.

Effect of Different Metal Ions. The monovalent ions K^+ and Na^+ behaved in the same manner. They were poor inhibitors at high concentrations: 17% inhibition was obtained with 500 mM KCl and 12% with 1000 mM NaCl. However,

Table III: Rate of Degradation of Various Peptide Substrates by Dipeptidyl-aminopeptidase

	peptide	no. of cleavage sites	V^c
	Tyr-Gly-Gly	0[b]	0
	Tyr-Gly-↓Gly-Phe[a]	1	31
	Tyr-Gly-↓Gly-Phe-Leu	1	32
	Tyr-Gly-↓Gly-Phe-↓Leu-Arg	2	3.1
	Tyr-Gly-↓Gly-Phe-↓Met-Arg-Phe	2	1.6
	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu	0	0
	Gly-Phe-Leu	0	0
	Gly-Gly-↓Phe-Leu	1	3.1
	Gly-Tyr-Gly-Gly-Phe-Met	0	0
	Tyr-Phe-↓Phe	1	1.0
	Ac-Tyr-Gly-Gly-Phe-Leu	0	0
CCK_3	Met-Asp-Phe-NH_2	0	0
CCK_4	Trp-Met-↓Asp-Phe-NH_2	1	12.0
CCK_5	Gly-Trp-↓Met-Asp-Phe-NH_2	1	32
CCK_6	Met-Gly-↓Trp-Met-↓Asp-Phe-NH_2	2	2.4
CCK_7	Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2	0	0
CCK_{7S}	Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	0	0
CCK_{8S}	Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	0	0
	Tyr-Met-↓Gly-Trp	1	4.1
substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	0	0
neurotensin	*p*-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	0	0
angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	0	0

[a] Cleavage sites are indicated by an arrow. [b] No detectable degradation of the peptide is observed when incubated for 4 h with 0.65 unit of porcine brain DAP at 25 °C with 200 μL of a millimolar peptide solution. [c] Rate of the first cleavage site, in pmol·min^{-1} dipeptide.

complete inhibition was obtained when the enzyme was preincubated for 15 min at 25 °C with 1 mM $ZnCl_2$ or $CoCl_2$. When the enzyme was preincubated for 2 h with 10 mM EDTA and then dialyzed against buffer A, the enzyme activity was partially restored with 1 μM $ZnCl_2$ (7% restoration) and with 10 μM $CoCl_2$ (10% restoration). No restoration was observed with $NiCl_2$ by using concentrations between 0.1 and 1 mM.

Substrate Specificity. In order to establish the substrate specificity, we tested different peptides as substrates. The rates of degradation were measured at four different incubation times with a substrate concentration of 1 mM. The reaction products were identified by HPLC analysis and quantified by using authentic standard peptides. Two different parameters have been considered: the cleavage sites of substrates and the rate of the enzymatic reaction (Table III). When the amino terminus of Leu-enkephalin was blocked with an acetyl group, no detectable hydrolysis of this peptide was observed when incubated for 4 h. Furthermore, this compound was not an inhibitor of the enzyme: both IC_{50} values of Boc-Leu-enkephalin and *N*-Ac-Leu-enkephalin were higher than 1000 μM when Leu-enkephalin was used as the substrate (data not shown).

The influence of the nature and the size of the peptide upon the enzymatic activity was investigated in two series: the enkephalin series and the cholecystokinin one. When CCK_4 was used as the substrate, the amount of Asp-Phe-NH_2 liberated in the incubation mixture was linear with time as shown in Figure 5. In the case of CCK_6, two sites of enzymatic cleavage were found and CCK_6 was sequentially degraded. In this case, Met-Gly first appeared and after a lag time Asp-Phe-NH_2 appeared, showing that CCK_6 had been split, liberating Met-Gly and Trp-Met-Asp-Phe-NH_2, which in turn was hydrolyzed to Trp-Met and Asp-Phe-NH_2. The first

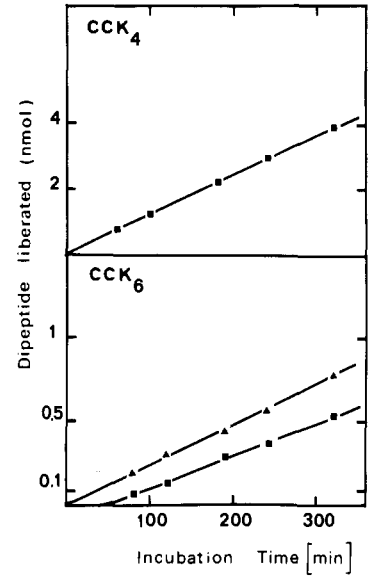
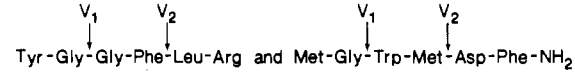


FIGURE 5: Hydrolysis of CCK_4 and CCK_6 by DAP. The products of the enzymatic hydrolysis of CCK_4 and CCK_6 by DAP were identified and quantified as Asp-Phe-NH_2 (■) and Met-Gly (▲) by HPLC analysis. For details see Experimental Procedures.

cleavage was always on the amino-terminal side of the peptide chain since this cleavage was the limiting factor for the second one. This was observed for



where V_2 was more than 10-fold lower than the velocity observed for the corresponding tetrapeptide cleavage. These experiments confirmed the free amino terminus requirement of a peptide in order to be a substrate for the enzyme.

In the enkephalin series, we found one site of enzymatic cleavage for the tetrapeptide and the pentapeptide, two sites for the hexapeptide and the heptapeptide, and no degradation of the octapeptide Try-Gly-Gly-Phe-Met-Arg-Gly-Leu. In the cholecystokinin series, the peptide chain was elongated on the amino-terminal side. We found similarly one site of cleavage for the tetrapeptide and the pentapeptide and two sites for CCK_6. No detectable degradation was observed after 4 h of incubation with nonsulfated CCK_7, CCK_7, and CCK_8. In the two series, the enzyme activity displayed an optimal rate of degradation for tetrapeptides and pentapeptides, i.e., for Tyr-Gly-Gly-Phe, Leu-enkephalin, CCK_4, and CCK_5 (Figure 6). No detectable degradation was observed for other peptides such as substance P and angiotensin II after incubation for 240 min. Additionally, as expected for a DAP, neurotensin, which has a blocked amino-terminal α-amino group, is not degraded by this porcine brain enzyme.

Discussion

In various mammalian tissues, four types of DAP activities have been described according to the dipeptide moieties liberated from unsubstituted amino termini of various peptides or dipeptide arylamides (Mc Donald et al., 1968, 1969a, 1971; Ellis et al., 1967). The specificity of these peptidases has not been ascertained with enkephalins as substrate since these enzymes were identified before the discovery of enkephalins. More recently, it has been shown that enkephalins are degraded by soluble (Lee et al., 1982; Van Amsterdam et al., 1983; Hazato et al., 1984) or membrane-associated brain DAPs (Gorenstein & Snyder, 1979), and one of these has been

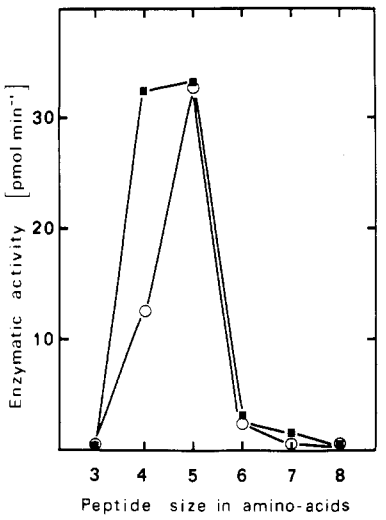


FIGURE 6: Initial rate of the first cleavage of various enkephalins and various cholecystokinins as a function of their peptide length. The initial degradation rate of the peptides was determined by HPLC analysis. Peptide size represents the number of amino acids of various substrates belonging to the two series: (■) enkephalins and (○) cholecystokinins.

characterized as DAP III (EC 3.4.14.4) (Lee et al., 1982).

In this study, we report the purification of an enkephalin-degrading DAP of porcine brain, starting from a crude mitochondrial fraction enriched in synaptosomes. The porcine brain DAP activity was measured by use of a specific and sensitive assay using [^{3}H]Leu-enkephalin as the substrate. The purified porcine brain DAP displays a high affinity for Leu-enkephalin with a K_m value of 2.5 μM. The affinity for Leu-enkephalin is in the same range as that of the rat brain DAP III (Lee et al., 1982), the calf brain striatal DAP (Van Amsterdam et al., 1983), and the monkey brain DAP (Hazato et al., 1984).

The molecular weight of the porcine brain DAP, which behaves as a monomeric unit, is estimated by exclusion chromatography and by SDS–polyacrylamide electrophoresis to be 51 000 ± 1000.

This molecular weight is different from those of other purified DAPs (DAP I, 200 000; DAP II, 130 000; DAP III, 80 000; DAP IV, 260 000) (Mc Donald et al., 1968, 1969a; Ellis et al., 1967; Kenny et al., 1976). All these enzymes are thiol or serine proteases. The porcine brain DAP is not inhibited by thiol or serine protease inhibitors, but its activity is blocked by chelating agents. Partial restoration of enzyme activity with divalent cations such as Zn^{2+} and Co^{2+} suggests that the enzyme is a metallopeptidase. Such an assumption is reinforced by an observed potent and competitive inhibition of this enzyme by a dipeptide hydroxamic acid and *N*-hydroxycarboxamide derivatives (Cherot et al., 1986).

The comparison of the substrate specificity of the porcine brain DAP and the four previously described DAPs is another important discriminating feature. DAP I (Mc Donald et al., 1969b) and DAP III (Lee et al., 1982) both hydrolyze angiotensin II whereas the porcine brain DAP does not degrade this peptide. DAP II is inhibited by cations such as Na^+, K^+, and Tris (Mc Donald et al., 1968), and its substrate specificity is restricted to tripeptides. It is inactive on longer peptides, unlike the porcine brain DAP. DAP IV is a post-proline DAP, i.e., an enzyme whose substrate requirements are a free amino terminus and a penultimate proline residue. For instance, substance P is a good substrate for DAP IV (Püschel et al., 1982). At variance, the porcine brain DAP does not degrade substance P.

Nevertheless, the substrate specificity of the porcine brain DAP is not restricted only to the enkephalins, a fact already observed with other peptidases (Turner et al., 1985). Indeed, the enzyme also cleaves fragments of the brain CCK_8 such as CCK_4 and CCK_5.

Furthermore, in two peptide series (enkephalins and cholecystokinins), a secondary substrate requirement appeared according to the peptide chain length. When the peptide chain was elongated either on the carboxyl terminus for the enkephalin series or on the amino terminus for the cholecystokinin series, the substrate specificity of DAP was restricted to short peptides of four or five amino acids. Such a strict specificity related to the molecular size of the substrate has been described for a peptidyl-dipeptidase (Neidle et al., 1984).

The subcellular localization of the DAP shows that this activity cosediments with synaptosomes on a Ficoll–sucrose gradient but does not seem to be tightly bound to synaptic plasma membranes as it is partially removed from this organelle.

The physiological relevance of DAP activity in the metabolism of enkephalins remains to be firmly established. Nevertheless, it should be recalled that a DAP activity has been detected in neuronal cell cultures whereas, strikingly, enkephalinase is only present in glial cells (Horsthemke et al., 1983). These results, coupled with the fact that the antinociceptive activity of kelatorphan is significantly higher than that of bestatin plus thiorphan, raise the question of the biological importance of this enzyme.

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Registry No. DAP, 72994-34-4; EDTA, 60-00-4; CCK_4, 1947-37-1; CCK_5, 18917-24-3; CCK_6, 21163-42-8; Tyr-Gly-Gly-Phe, 60254-82-2; Tyr-Gly-Gly-Phe-Leu, 58822-25-6; Tyr-Gly-Gly-Phe-Leu-Arg, 75106-70-6; Tyr-Gly-Gly-Phe-Met-Arg-Phe, 73024-95-0; Gly-Gly-Phe-Leu, 60254-83-3; Tyr-Phe-Phe, 105018-54-0; Tyr-Met-Gly-Trp, 105018-55-1; Leu-enkephalin, 58822-25-6; *o*-phenanthroline, 66-71-7; puromycin, 53-79-2.

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Decarboxylation of Oxalacetate by Pyruvate Carboxylase†

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ABSTRACT: The decarboxylation of oxalacetate by pyruvate carboxylase in the absence of ADP and P_i is stimulated 400-fold by the presence of oxamate, which is an inhibitory analogue of pyruvate. The observation of substrate inhibition when either oxamate or oxalacetate is varied at a fixed concentration of the other indicates that both molecules bind at the same site on the enzyme. The pH profiles for this reaction show no evidence of the involvement of an enzymic acid–base catalyst, suggesting that the proton and CO_2 units may be exchanged directly between the reactants (although CO_2 sequestered in the active site may be an intermediate in the process). The pH profiles of the full reverse reaction of pyruvate carboxylase in which oxalacetate decarboxylation is coupled to ATP formation and where P_i is the variable substrate do, however, indicate that such an acid–base catalyst is involved in the other partial reaction of the enzyme in proton transfer to and from biotin. The enzyme also displays two oxamate-independent oxalacetate decarboxylating activities, one of which is biotin-dependent and the other is independent of biotin.

Pyruvate carboxylase (EC 6.4.1.1) catalyzes the following reactions:

$$HCO_3^- + MgATP + \text{E–biotin} \xrightleftharpoons{\text{acetyl-CoA, } Mg^{2+}} \text{E–biotin–}CO_2^- + MgADP + P_i \quad (1)$$

$$\text{E–biotin–}CO_2^- + \text{pyruvate} \rightleftharpoons \text{E–biotin} + \text{oxalacetate} \quad (2)$$

These partial reactions are thought to occur at spatially

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