Purification and Characterization of an Enkephalin Aminopeptidase from Rat Brain†

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ABSTRACT: Rat brain enkephalin aminopeptidase was purified to apparent electrophoretic homogeneity. Enzyme activity was monitored during the purification by using ([3,5-$^3H_2$]Tyr)-Met-enkephalin and Tyr-β-naphthylamide as substrates. It was shown that the enzyme activities resulting in hydrolysis of the tyrosine residue of ([3,5-$^3H_2$]Tyr)Met-enkephalin and formation of β-naphthylamine from Tyr-β-naphthylamide copurified. The homogeneous enzyme had a specific activity of 10.5 μmol of β-naphthylamide hydrolyzed min^{-1} mg^{-1}. Hydrolysis of Met-enkephalin yielded the products L-tyrosine and the tetrapeptide Gly-Gly-Phe-Met. Subsequent removal of glycine from Gly-Gly-Phe-Met was not observed with the purified enzyme. The homogeneous aminopeptidase has an apparent molecular weight of 115 000 on Sephadex G-200 and a molecular weight of 102 000 as determined by electrophoresis in the presence of sodium dodecyl sulfate. The enkephalin-degrading enzyme had a pH optimum of 6.5–7.0 and exhibited maximal activity at 40 °C. Enzyme activity was inhibited by metal chelators, and it was found that 1 mol of Zn^{2+} was associated with 1 mol of enzyme (102 000 M_r). The enzyme hydrolyzes various neutral and basic amino acid β-naphthylamides but will not utilize acidic, D-amino acid, or N-terminal-blocked amino acid β-naphthylamides as substrates.

Since their isolation from pig brain (Hughes et al., 1975), enkephalins have come under intensive study because they afford an alternative potential method of pain relief. One of the major difficulties associated with use of these compounds as analgesics is their short half-life (Pert et al., 1976). Both Met- and Leu-enkephalin are rapidly inactivated by enzymes in serum and in brain, and the transient nature of the enkephalin activity noted in vivo has been attributed in part to enzymatic cleavage by an aminopeptidase which hydrolyzes the Tyr–Gly bond (Hambrook et al., 1976). This enzyme activity resembles that of other aminopeptidases which are known to remove the NH_2-terminal amino acid from proteins and polypeptides (Smith & Hill, 1960). Most efforts directed at obtaining longer acting enkephalins have centered on the synthesis of enkephalin analogues resistant to enzymatic degradation. For example, a simple substitution of Gly with D-Ala has resulted in a compound which is active via intracerebral or vascular administration (Pert et al., 1976).

Another approach to increase the effective action of enkephalins would be to limit their rate of degradation by blocking enzymatic pathways associated with their catabolism. This approach has proven to be of therapeutic value in treating diseases such as myasthenia gravis, where specific inhibitors of acetylcholinesterase are administered (Beeson & McDermott, 1975). Because enkephalin degradation in crude brain homogenates appears to be due in part to removal of the NH_2-terminal tyrosine and because substitution of D-amino acids for glycine result in longer acting enkephalin analogues in vivo, it was decided that an examination of the brain aminopeptidase(s) capable of enkephalin degradation was a logical first step in understanding how one might alter the catabolism of these peptides. This paper describes the purification and characterization of rat brain enkephalin aminopeptidase.

Experimental Procedures

Materials. ([3,5-$^3H_2$]Tyr)Met-enkephalin (58 Ci/mmol) was purchased from Amersham, and Met- and Leu-enkephalin were from Peninsula. DEAE[1]-cellulose (DE-52) was obtained from Whatman, DEAE-Sephadex (A-25-120) was from Sigma, Bio-Gel A-0.5m and hydroxylapatite (HTP) were from Bio-Rad, and Sephadex G-200 was from Pharmacia Fine Chemicals. The various amino acid β-naphthylamides were purchased from Bachem and β-naphthylamine was from Sigma. The 1,10-phenanthroline and diisopropyl fluorophosphate were obtained from Aldrich, the 1,5-phenanthroline was from ICN, the acrylamide was from Canalco, and the Porapak Q was from Waters Associates. Proteins used to calibrate the gel filtration columns or polyacrylamide gels were the highest quality available.

Assay for Enzyme Activity with Met-enkephalin. For removal of undesirable oxidation products, ([3,5-$^3H_2$]Tyr)-Met-enkephalin was reduced according to the method of Hoffmann et al. (1966). Briefly, 5 μL of 1% aqueous thioglycolic acid was added to 2 μCi of ([3,5-$^3H_2$]Tyr)Met-enkephalin. The incubation mixture was flushed with N_2 and incubated at 50 °C for 72 h. Chromatography on silica gel G plates showed a single spot of radioactivity coincident with authentic Met-enkephalin. The reduced substrate was stored in 50 mM dithiothreitol at −20 °C.

The brain extracts were assayed for activity with ([3,5-$^3H_2$]Tyr)Met-enkephalin by a procedure similar to that of Vogel & Altstein (1977). An aliquot of enzyme was placed in a solution containing 30 000 cpm of ([3,5-$^3H_2$]Tyr)Met-enkephalin, 10 μM unlabeled Met-enkephalin, and 10 mM Tris-HCl, pH 7.0, in a total volume of 100 μL and incubated at 25 °C for 2 min. After incubation, 25 μL of 7.5 M NH_4OH was added, and then the solution was placed on a column (a Pasteur pipet) containing 80 mg of Porapak Q which had been

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[1] Abbreviations used: Tyr-βNA, tyrosyl-β-naphthylamide; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetate, disodium salt; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; NA, β-naphthylamine; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-pressure liquid chromatography; Cbz, carbobenzyloxy.

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equilibrated in water. The [3,5-$^3H_2$]Tyr was washed from the column with 2.5 mL of water and the eluate collected directly in scintillation vials. After the addition of a toluene-based scintillation fluid, the amount of radioactivity was determined.

Assay for Enzyme Activity with Tyr-β-naphthylamide. Enzyme activity was measured by monitoring the release of β-naphthylamine from the substrate Tyr-β-naphthylamide (Tyr-βNA).[1] The appearance of the fluorescent product was continuously measured in an American Instrument fluorescence spectrometer (Model 125) using excitation and emission wavelengths of 335 and 410 nm, respectively. The assay mixture contained a 2-mL solution composed of 200 μL of 500 μM Tyr-βNA in 25 mM acetic acid, either 50 or 5 μL of enzyme preparation, and 0.1 M Tris-HCl, pH 7.0, containing 5 mM 2-mercaptoethanol (referred to as buffer A). The increase in fluorescence was converted into nanomoles of β-naphthylamine (NA) by using a standard curve prepared with the latter compound. Assays were run at 37 °C unless specified otherwise.

Protein Determinations. Protein was determined by the Hartree modification (Hartree, 1972) of the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis. Electrophoretic analysis of native enzyme was carried out in 7.5% acrylamide gels according to the procedure of Rupnow et al. (1979). Electrophoresis of proteins in the presence of $NaDodSO_4$ was carried out in 4% acrylamide slab gels prepared according to the method of Weber et al. (1972).

Determining Enzyme Activity in Polyacrylamide Gels. Purified enzyme was subjected to electrophoresis as described above except the gel buffer contained 5 mM 2-mercaptoethanol. After electrophoresis the gels were immediately sliced into 2–3-mm sections and placed into 200 μL of 50 μM Tyr-βNA in buffer A. The gel slices were minced with a glass pestle, the suspensions were incubated for 24 h at 37 °C, and Tyr-βNA hydrolysis was measured fluorometrically.

Subcellular Localization of Enkephalin Aminopeptidase. Routinely, 4–6 g of fresh rat brain was homogenized with a Polytron at a setting of ~4 for 15 s in 10 mL of buffer A containing 250 mM sucrose. After homogenization, the suspension was centrifuged at 600*g* for 10 min. The pellet was washed twice with 1 mL of buffer A. The combined supernatants were centrifuged at 5000*g* for 5 min, and the pellet was washed and suspended in 0.5 mL of the above buffer. The supernatant was centrifuged at 100000*g* for 60 min. The pellet was washed once and resuspended in 0.5 mL of the above buffer. Sonication of the suspensions was performed with a Kontes cell disruptor at a power setting of 8 for 15 s/mL of liquid. Protein concentration and enzyme activities were measured as described above.

High-Pressure Liquid Chromatographic Identification of Products of Enkephalin Degradation. Met-enkephalin (1.0 mM) in 1.0 mL of 50 mM Tris-HCl, pH 7.0, containing 2-mercaptoethanol was mixed with 3 μL (0.1 mg/mL) of purified enzyme and incubated at room temperature. At various times, the enzyme reaction was stopped by adding 100-μL aliquots of the mixture to an equal volume of 10 mM ammonium acetate, pH 4.2. The mixture was analyzed on a Varian Model 5000 chromatograph with a C_{18} reverse-phase column (Varian, 0.635 × 25 cm). Elution was with 10 mM ammonium acetate, pH 4.2, containing 20% acetonitrile at a flow rate of 2 mL/min. Elution of the reaction products was monitored at 258 nm, and the amount of material eluted was calculated by a comparison with known concentrations of standards.

Thin-Layer Chromatographic Identification of Metabolites of ([3,5-$^3H_2$]Tyr)Met-enkephalin. The reaction mixture contained 0.1 mM Met-enkephalin, 30000 cpm of reduced ([3,5-$^3H_2$]Tyr)Met-enkephalin, and a 2-μL aliquot of enzyme in 100 μL of 0.01 M Tris-HCl, pH 7.0. Following incubation at 25 °C for 15 min, 350 μL of ethanol (99.5%) was added to stop the reaction, and the mixture was dried under N_2. After being dried, the residue was suspended in 25 μL of 0.2% 2-mercaptoethanol and spotted on silica gel G chromatography plates (Analabs). The plates were developed in ethyl acetate–pyridine–acetic acid–H_2O (100:43:11:25 v/v) containing 0.7 mM dithiothreitol or 1-butanol–H_2O–methanol (4:3:1 v/v) with 0.8 mM dithiothreitol. Authentic samples of Met-enkephalin and tyrosine were used as standards. The R_f values of the compounds following chromatography were determined by using fluorescamine. Corresponding 0.5 × 1 cm sections of plates which had not been subjected to fluorescamine were transferred to scintillation vials, and the radioactivity was determined by using a toluene-based scintillation fluor.

Determination of pH and Temperature Optimums. The pH optimum of enkephalin aminopeptidase was determined by using both Tyr-βNA and ([3,5-$^3H_2$]Tyr)Met-enkephalin as substrates at 37 °C. The pH was determined at the beginning and end of each experiment to show stability of pH through the reaction. The temperature optimum was measured by using 2 mL of a 50 μM solution of Tyr-βNA in buffer A.

Atomic Absorption Spectrophotometry. Zinc was determined with a Perkin-Elmer Model 560 atomic absorption spectrophotometer equipped with a HGA-500 graphite furnace and an AS-1 auto sampling system. A monoelement hollow cathode lamp was used at 213.9 nm. Charring and atomization times and the corresponding ramp intervals were maximized for alcohol dehydrogenase standards. Triplicate 20-μL samples of alcohol dehydrogenase and $ZnCl_2$ in 10 mM Tris-HCl, pH 7.0, in the concentration range 0.01–1 μM were used to construct standard curves. Under these conditions, 20 μL of a 0.5 μM Zn solution, i.e., 10^{-11} mol of Zn, gave an absorbance reading of ~0.2. All protein samples were dialyzed before analysis against 10 mM Tris-HCl, pH 7.0.

Results

Subcellular Localization of Enkephalin Aminopeptidase. The enkephalin aminopeptidase activity was found divided between the 100000*g* soluble fraction (46%) and the 600*g* pellet (49%). Only 2.1 and 0.4% of enzyme activity was present in the 15000*g* and 100000*g* pellets, respectively. When the 600*g* pellet was suspended in buffer, no enzyme activity was measurable unless the suspension was first sonicated. Thus, this activity most likely originates from within unbroken cells or nuclei.

Purification of Enkephalin Aminopeptidase. The steps used in the purification of the enkephalin aminopeptidase from rat brain are shown in Table I. All steps were carried out at 4 °C. The Tyr-βNA and ([3,5-$^3H_2$]Tyr)Met-enkephalin assays were used to monitor the enzyme activity during purification. Because these two activities copurified (data not shown), the Tyr-βNA assay was used on a routine basis, and the activities cited in the purification scheme refer to this assay.

(*1*) *Extraction.* One hundred rat brains (150 g) were homogenized for 30–60 s in 300 mL of 50 mM Tris-HCl, pH 7.0, containing 5 mM 2-mercaptoethanol by using a Polytron homogenizer at setting 5. The resulting homogenate was centrifuged for 30 min at 10000*g* and the supernatant retained for further purification.

(*2*) *Ion-Exchange Chromatography on DEAE-cellulose.* The supernatant of (1) was applied to a DEAE-cellulose

Table I: Purification of Rat Brain Enkephalin Aminopeptidase

step	prepn	vol (mL)	total act. (μmol of NA/min)	total protein (mg)	sp act. (μmol of NA min^{-1} mg^{-1})	purification factor	recovery (%)
1	crude extract	400	141	27400	0.005		
2	centrifuged extract	280	91.6	7560	0.012	2.4	65
3	chromatography on DEAE-cellulose	220	104	946	0.11	22	74
4	gel filtration on Bio-Gel A-0.5m	55	70.0	73	0.96	192	50
5	chromatography on DEAE-Sephadex	105	50.9	8.5	6.00	1200	36
6	gel filtration on Bio-Gel A-0.5m	35	44.5	4.9	9.1	1820	32
7	chromatography on hydroxylapatite	30	40.0	3.8	10.5	2100	28

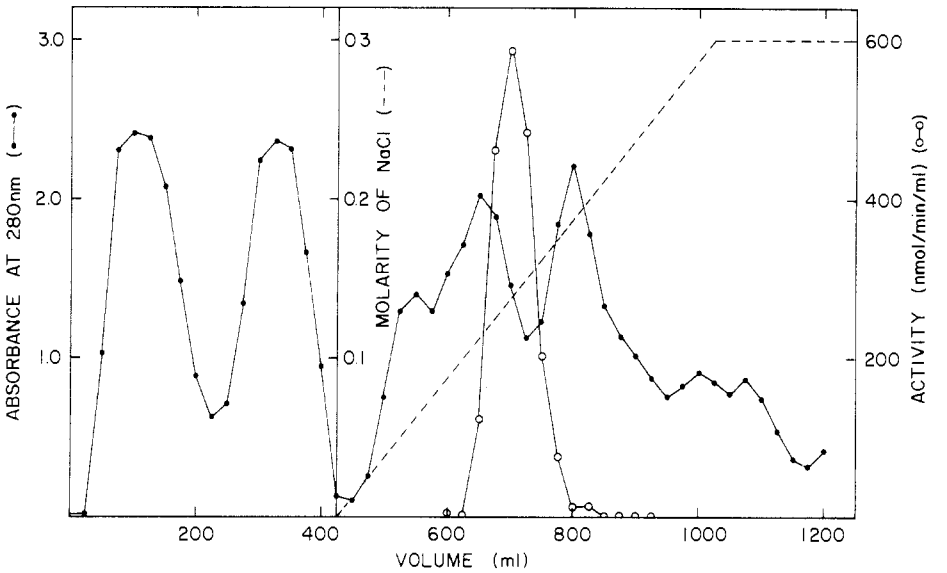

FIGURE 1: DEAE-cellulose chromatography. Absorbance at 280 nm and enzyme activity are represented by closed (●) and open (○) circles, respectively. The sodium chloride gradient is indicated by the broken line (- - -). The fractions between 625 and 775 mL were pooled and used in the following step of the purification.

column (2.5 × 20 cm) which had been equilibrated with 50 mM Tris-HCl, pH 7.0, containing 5 mM 2-mercaptoethanol. The column was washed with 450 mL of the same buffer. The protein was eluted at a flow rate of 50 mL/h with a 600-mL gradient of a 0–0.3 M NaCl in the buffer noted above. Fractions 130–160 contained the aminopeptidase (Figure 1).

(*3*) *Gel Filtration on Bio-Gel A-0.5m.* The pooled enzyme from step 2 was concentrated to ~5 mL with an Amicon XM 50 membrane filter and then applied to a Bio-Gel A-0.5m column (2.5 × 120 cm) equilibrated with buffer A. Fractions 40–47 contained the aminopeptidase activity. The overall purification through step 3 was 192-fold with a 50% yield.

(*4*) *Ion-Exchange Chromatography on DEAE-Sephadex.* The fractions from step 3 were applied to a DEAE-Sephadex column (2.5 × 20 cm) which had been equilibrated with 50 mM sodium phosphate, pH 7.0, containing 5 mM 2-mercaptoethanol. The column was washed until the absorbance approached 0, and the protein was eluted with a 500-mL gradient of 50–250 mM sodium phosphate, pH 7.0, at a flow rate of 1.0 mL/min. The enzyme activity was eluted at ~0.15 M sodium phosphate. Fractions 40–56 were pooled and concentrated.

(*5*) *Repeat of Gel Filtration on Bio-Gel A-0.5m.* The pooled fractions from step 4, after concentration to ~5 mL, were passed over a Bio-Gel A-0.5m column as described for step 2.

(*6*) *Chromatography on Hydroxylapatite.* The enzyme preparation from step 5 was dialyzed overnight against 50 mM sodium phosphate, pH 7.0, containing 5 mM 2-mercaptoethanol and then applied to a Bio-Gel HTP hydroxylapatite column (1.0 × 5.0 cm) equilibrated with the same buffer. The column was washed and the bound protein eluted with a 150-mL gradient of 50–250 mM sodium phosphate, pH 7.0, containing 5 mM 2-mercaptoethanol (Figure 2). The protein and enzyme activity eluted as a single peak, and the specific activity of fractions 90–115 was constant (10.5 μmol min^{-1} mg^{-1}). The overall purification through step 6 was 2100-fold with a recovery of 28%.

Comparison of Tyr-βNA Hydrolysis Activity and Enkephalin Aminopeptidase Activity. The enkephalin aminopeptidase activity and the hydrolysis of Tyr-βNA were monitored throughout the purification procedure. Figure 3A shows the two activities are coincident after the first step in the purification procedure and also after the last step of the purification (Figure 3B). These results show that the isolated aminopeptidase hydrolyzes both compounds and that Tyr-βNA is a reliable substrate for use in purification of the rat brain enkephalin aminopeptidase.

Enzyme Storage and Stability. The enzyme was stored at −20 °C in sodium phosphate, pH 7.0, containing 5 mM 2-mercaptoethanol and 40% ethylene glycol. The enzyme is stable for 2–3 weeks but gradually loses activity and is totally

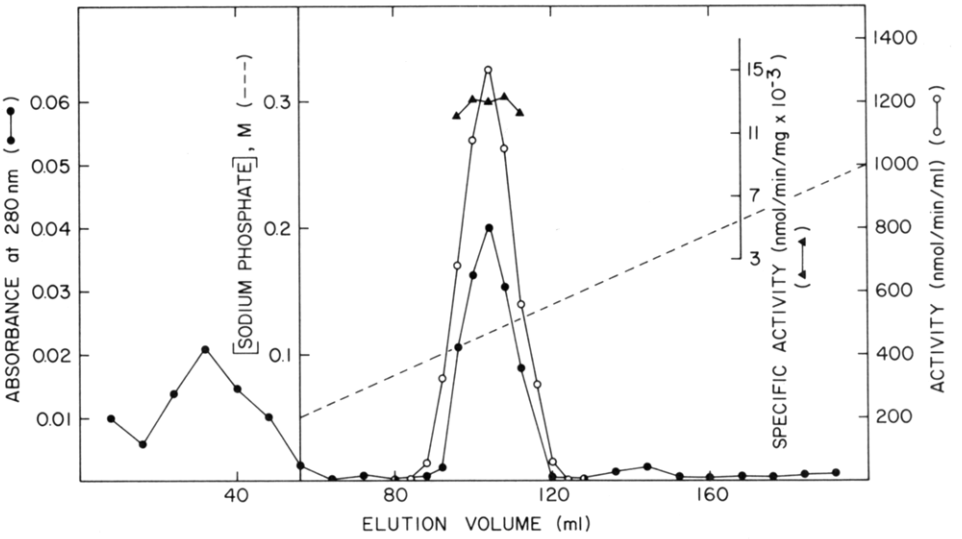

FIGURE 2: Hydroxylapatite chromatography. Absorbance at 280 nm and enzyme activity are represented by closed (●) and open (○) circles, respectively. The sodium phosphate gradient is indicated by the broken line (---), and specific activity of the fractions is represented by triangles (△). The fractions between 90 and 115 mL were pooled and analyzed for purity.

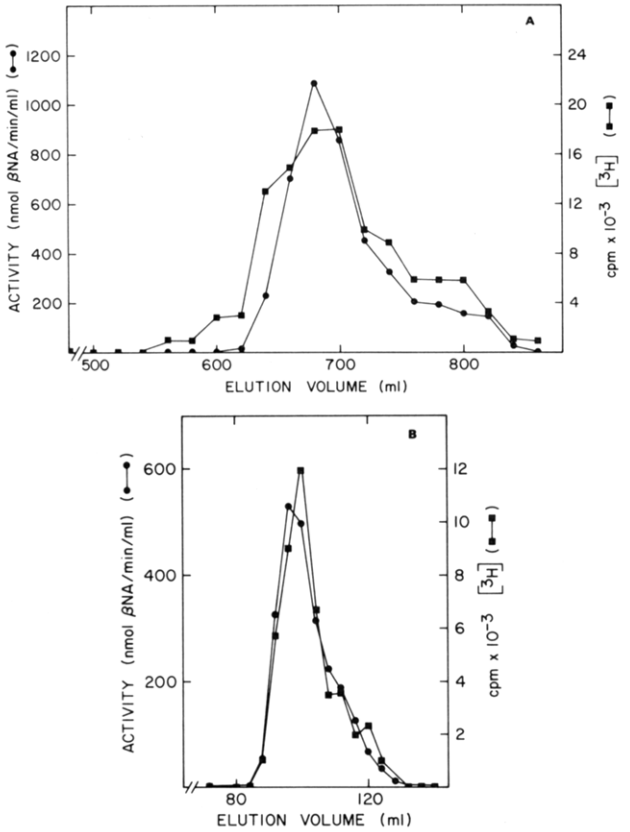

FIGURE 3: Tyr-β-naphthylamide hydrolysis (●) and enkephalin aminopeptidase (■) activities of fractions from (A) DEAE-cellulose chromatography and (B) hydroxylapatite chromatography.

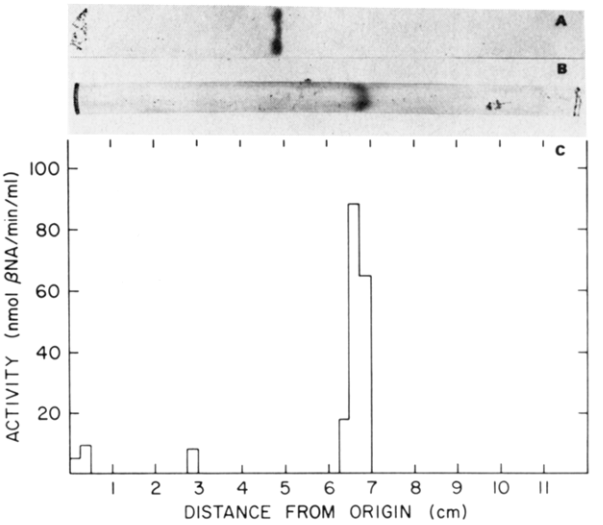

FIGURE 4: Polyacrylamide gels of (A) purified enkephalin aminopeptidase (20 μg) in the presence of $NaDodSO_4$ and 2-mercaptoethanol and (B) native purified enzyme (28 μg). The enzyme activity profile (C) obtained after electrophoresis of the purified enkephalin aminopeptidase.

inactive after 80 days. Storage of enzyme in the absence of ethylene glycol results in >60% activity loss within 24 h at 4 °C.

Evidence of Homogeneity and Molecular Weight. The purified protein was examined by 4% polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (Figure 4A). A single electrophoretic species was observed having a molecular weight of 102 000. Under nondissociating conditions a single protein was also observed (Figure 4B). The enzyme activity profile obtained from a polyacrylamide gel run under similar conditions (see Experimental Procedures) showed that the aminopeptidase activity (Figure 4C) and protein noted in Figure 4B were coincident. The molecular weight of the native enzyme was also estimated by gel filtration on a Sephadex G-200 according to the method of Andrews (1965). The column (110 × 1.6 cm) was calibrated with pyruvate kinase, lactate dehydrogenase, concanavalin A, and alcohol dehydrogenase. The enkephalin aminopeptidase elutes from this column like a globular protein of molecular weight 115 000.

Attempts to use an analytical ultracentrifuge to determine the molecular weight of the native form of the enzyme were unsuccessful. In order to obtain reliable data from the ultracentrifuge, it is necessary to remove the ethylene glycol from the protein solution. Under these conditions the enzyme retained only 42% of its initial activity over the 24-h run at 4 °C.

Metal Analysis. The purified enkephalin aminopeptidase was found to contain 0.98 ± 0.2 atom of Zn^{2+} per 102 000 M_r. Dialysis of the enzyme solution against buffers containing 5 × 10^{-3} M 1,10-phenanthroline for 24 h resulted in the loss of Zn^{2+} and a corresponding loss of activity.

Product Analysis of Enkephalin Aminopeptidase. The purified enzyme converts Met-enkephalin stoichiometrically

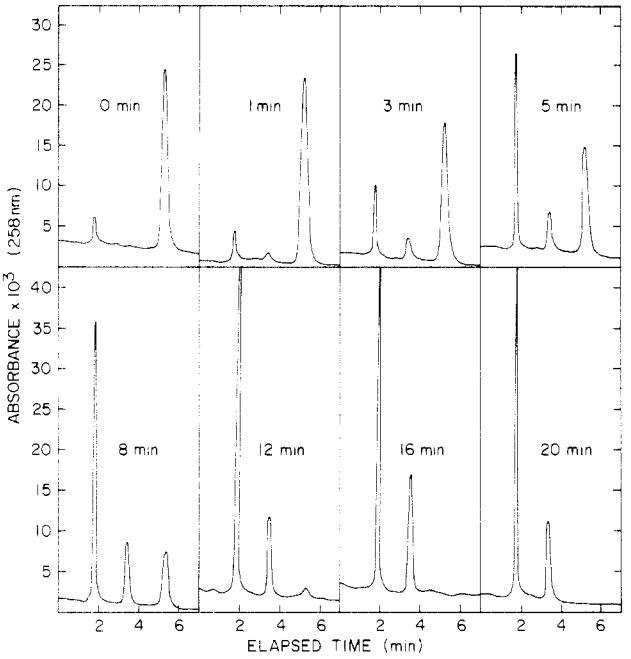

FIGURE 5: High-pressure liquid chromatogram of the degradation products of Met-enkephalin by enkephalin aminopeptidase. Reaction products were loaded onto a C_{18} reverse-phase column (Varian, 0.635 × 25 cm) and eluted with 10 mM ammonium acetate, pH 4.2, containing 20% acetonitrile at a flow rate of 2 mL/min. Retention times for tyrosine, Gly-Gly-Phe-Met, and Met-enkephalin were 1.7, 3.6, and 5.2 min, respectively.

into tyrosine and Gly-Gly-Phe-Met. The hydrolysis was monitored by HPLC (Figure 5). Only two products appeared in the chromatogram, suggesting that there is no further degradation of Gly-Gly-Phe-Met. This was confirmed by incubating the latter compound with the enzyme for 60 min at 37 °C. The identity of the products was confirmed by amino acid analysis. These results demonstrate that the purified enzyme specifically cleaves the Tyr–Gly bond with no further degradation of the resulting tetrapeptide under the reaction conditions employed. They are also consistent with the product analysis obtained by using ([3,5-$^3H_2$]Tyr)Met-enkephalin. Only one radioactive compound is observed on thin-layer chromatography. This compound comigrates in both solvent systems examined (see Experimental Procedures) with an authentic sample of tyrosine (data not shown).

Inhibition of Rat Brain Enkephalin Aminopeptidase. In order to demonstrate the particular class to which this protease belonged, we examined a number of inhibitors of the enzyme by using Tyr-βNA as substrate. Inhibitors (at the concentrations indicated) and enzyme were incubated in 0.25 M Tris-HCl, pH 7.0, for 5 min at 37 °C prior to the addition of substrate (total volume of 2 mL). Activity was monitored as previously described.

The enzyme was completely inhibited by 5×10^{-4} M 1,10-phenanthroline, while 1,5-phenanthroline showed only 37% inhibition at the same concentration (Table II). Other metal chelators like EDTA and EGTA also inhibited the enzyme, while iodoacetic acid affected enzyme activity to a lesser degree (61.5%). The serine protease inhibitors diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride showed 23.9% and 0% inhibition, respectively. Bacitracin and puromycin were both excellent inhibitors at the concentrations examined.

Kinetics of Enkephalin Aminopeptidase. The homogeneous aminopeptidase will hydrolyze a number of different α-aminoacyl β-naphthylamides (Table III). Substrates were dissolved in 10% Me_2SO in 25 mM acetic acid. Various

Table II: Effects of Inhibitors on a Rat Brain Enkephalin Aminopeptidase

inhibitor	concn (M)	% inhibition
EDTA	5×10^{-4}	78.3
EGTA	5×10^{-4}	69.8
1,10-phenanthroline[a]	5×10^{-4}	100
1,5-phenanthroline[a]	5×10^{-4}	36.7
iodoacetic acid	5×10^{-4}	61.5
N-ethylmaleimide	5×10^{-4}	9.9
phenylmethanesulfonyl fluoride[b]	5×10^{-4}	0
benzamidine	1×10^{-4}	15.2
diisopropyl fluorophosphate[c]	1×10^{-4}	23.9
bacitracin	5×10^{-4}	100
puromycin	5×10^{-5}	100

[a] Stock solution in ethylene glycol. [b] Stock solution in ethanol. [c] Stock solution in 2-propanol.

Table III: Substrate Specificity of a Rat Brain Enkephalin Aminopeptidase

substrates (β-naphthylamides)	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
L-Leu	0.015	7.3
L-Arg	0.008	12.0
L-Phe	0.046	9.0
L-Ser	0.157	0.9
L-Pro	0.324	0.8
L-Ala	0.077	9.6
L-Tyr	0.090	8.1
Gly	0.676	1.2
L-Asp	not reactive	
Cbz-Arg	not reactive	
D-Ala	not reactive	
D-Leu	not reactive	

substrates were examined at the concentrations both above and below the K_m. The enzyme (5 μL) and 0.25 M Tris-HCl, pH 7.0, containing 5 mM 2-mercaptoethanol in a total volume of 2 mL was assayed as described under Experimental Procedures. The Michaelis constants (K_m) and maximal velocities (V_{max}) for the different β-naphthylamides were calculated by Lineweaver–Burk analysis. Basic α-aminoacyl β-naphthylamides have lower K_m and higher V_{max} values than hydrophobic analogues, while the acidic β-naphthylamide and those with a blocked NH_2 terminus or D-amino acid analogues are not cleaved by the enzyme. Gly-βNA is a poor substrate relative to either hydrophobic or basic amino acid β-naphthylamides (Table III). This result is of interest in light of the data from the product analysis which demonstrated that Gly-Gly-Phe-Met does not act as a substrate. It appears that the aminopeptidase is capable of hydrolyzing some β-naphthylamides which are not hydrolyzed when the same amino terminus is presented in a small peptide such as Gly-Gly-Phe-Met.

pH–Rate Profiles of Enkephalin Aminopeptidase. The enzyme is active between pH 5.25 and 9 with maximal activity for ([3,5-$^3H_2$]Tyr)Met-enkephalin at pH 7.0 (Figure 6). The pH–rate profile for Tyr-βNA hydrolysis is similar to although not identical with that of Met-enkephalin and shows a maximum near 6.5 (Figure 6). When the enzyme is incubated at pH 4.5 or 8.5 for 10 min and the pH then adjusted to 7.0 before assay with Tyr-βNA, activity is lost indicating irreversible alteration of the enzyme under these conditions.

Enkephalin Aminopeptidase Activity as a Function of Temperature. The maximum activity of the enzyme under our assay conditions was exhibited at ~40 °C. When the enzyme was held for 15 min at the elevated temperatures prior to the addition of substrate, it was found that at 60 °C the

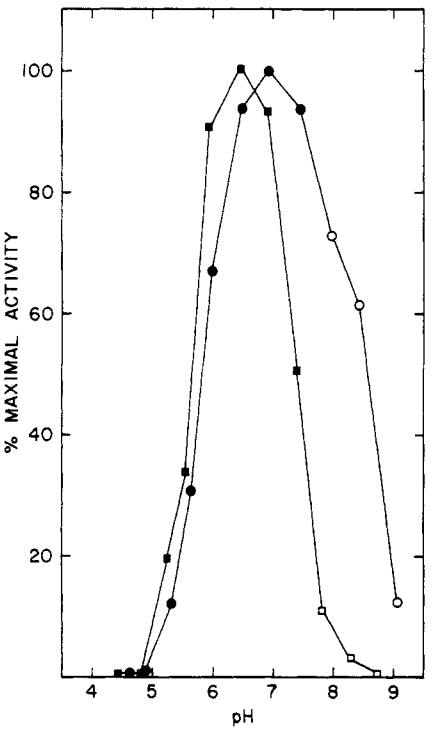

FIGURE 6: pH–rate profiles of enkephalin aminopeptidase activity using Tyr-βNA (■–□) or ([3,5-$^3H_2$]Tyr)Met-enkephalin (●–○) as substrates. The buffers used were 0.25 M sodium phosphate (closed symbols) or 0.25 M Tris-HCl (open symbols).

enzyme lost all activity while at 45 °C 87% of the activity remained.

Discussion

In the initial efforts made at purification of the enkephalin aminopeptidase, we observed that ammonium sulfate precipitation caused low yield and loss of enzyme activity. In addition, removal of the ammonium sulfate required dialysis which resulted in further losses of enzyme activity. Chromatography on DEAE-cellulose proved to be a satisfactory first step giving a 65% yield and 2.4-fold purification. The purification scheme outlined in Table I affords a final yield of 28% and can be performed in ~1 week.

The use of Tyr-βNA greatly facilitated the purification of enkephalin aminopeptidase. The assay is quick and sensitive and measures enkephalin-degrading activity as evidenced by the corresponding activities in chromatographic fractions. Product analysis clearly demonstrates that the purified enzyme hydrolyzes the amino terminal tyrosine residue of Met-enkephalin with no further degradation of the resulting Gly-Gly-Phe-Met. The cleavage of the Tyr–Gly bond was also the primary breakdown of Met-enkephalin noted in brain homogenates by Hambrook (Hambrook et al., 1976).

The rat brain enkephalin aminopeptidase has a molecular weight of 102 000. The enzyme contains 1 mol of Zn^{2+} per 102 000. Other aminopeptidases have been shown to be metalloproteins, and a number of these enzymes have been shown to contain zinc (Himmelhoch, 1969).

Kinetic and physical properties distinguish the rat brain aminopeptidase from aminopeptidases which have been previously described. The molecular weight of the rat brain enkephalin aminopeptidase is significantly different than that of leucine aminopeptidase (Melius et al., 1970) and shows different substrate specificity and pH optimum (Delange & Smith, 1971). Substrate specificity and molecular weight also distinguish the rat brain enzyme from aminopeptidases A, B, and M (Delange & Smith, 1971). The rat brain enzyme, however, shares some properties with similar enzymes isolated from other species. The kinin-converting aminopeptidase isolated from human serum (Guimaraes et al., 1973) is similar in molecular weight and substrate specificity to the rat brain enkephalin aminopeptidase. An arylamidase from monkey brain (Hayashi & Oshima, 1977) also has been isolated and characterized. The enzyme has an apparent molecular weight on gel filtration chromatography of 92 000 and is also inhibited by metal chelators. Recently, Traficante et al. (1980) described the purification of an enkephalin aminopeptidase from human brain. This enzyme appears to be sensitive to *o*-phenanthroline and has a molecular weight on $NaDodSO_4$–polyacrylamide gel and gel filtration electrophoresis of approximately 60 000–65 000. The exact relationship between the enzymes mentioned above and the enkephalin aminopeptidase from rat brain has not been examined in sufficient detail to indicate that they represent different species of the same enzyme.

While this work was in progress, Schnebli (Schnebli et al., 1979) reported the isolation of an enkephalin-degrading aminopeptidase from rat brain. The observed concentration of the enzyme in the brain (~2 mg/100 g) is similar to our data (2.3 mg/100 g), and the enzymes show comparable molecular weights by gel filtration chromatography and similar kinetic parameters. It seems unlikely that the two reports describe different enzymes, especially when one considers that we could find no other Tyr-βNA-hydrolyzing activity in crude brain homogenates (data not shown). However, there are differences between the two enzyme preparations. Schnebli et al. (1979) report a subunit molecular weight of 48 000 and 50 000 while we observe a single polypeptide in the apparent molecular weight of 102 000. Schnebli et al. (1979) also report that their purified enzyme "ages". The authors do not give a reason for the aging of their enzyme, which occurs "3 days and up to 30 days later" and results in an increase in specific activity to 10 240 nmol min^{-1} (mg of protein)$^{-1}$. The enzyme prepared in our laboratory does not show any gain in specific activity similar to that reported by Schnebli et al. (1979). The reason for the apparent difference in these two reports is unclear, although the purification and storage procedures are quite different.

The putative role that rat brain enkephalin aminopeptidase plays in enkephalin degradation in vivo remains undefined. The possibility that this enzyme has functions in addition to enkephalin inactivation should not be overlooked. The purification and characterization of the enzyme described herein represent a first step in answering specific questions about the physiological role of this enzyme.

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Effects of Cations on Affinity of Calmodulin for Calcium: Ordered Binding of Calcium Ions Allows the Specific Activation of Calmodulin-Stimulated Enzymes†

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Appendix: Theoretical Approach to Study of Multiple Ligand Binding to a Macromolecule

Jacques Haiech

ABSTRACT: The acid stability of calmodulin has been used to devise a rapid and efficient method of decalcification based on trichloroacetic acid precipitation. Study of the competitive binding of K^+, Mg^{2+}, and Ca^{2+} to the Ca^{2+}-binding sites of calmodulin has allowed determination of the intrinsic binding constants of each of the three cations for the four Ca^{2+}-binding sites. The data are compatible with an ordered binding of Ca^{2+}. If the Ca^{2+} sites are labeled A, B, C, and D starting at the NH_2 terminus, the order of binding is postulated to be B, A, C, and D. The ordered binding properties support the suggestion that calmodulin translates quantitative Ca^{2+} signals into qualitatively different cellular responses.

Calmodulin, a heat-stable Ca^{2+}-binding protein responsible for the Ca^{2+} stimulation of cyclic nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi, 1970), is now known to play a central role in the Ca^{2+}-dependent regulation of eukaryotic cells. [For reviews, see Wolff & Brostrom (1979), Wang & Waisman (1979), Cheung (1980), Means & Dedman (1980), and Klee et al. (1980).] A basic scheme for the stimulation of enzyme by calmodulin was initially proposed for cAMP[1] phosphodiesterase

step 1

$$CaM + nCa^{2+} \rightleftharpoons CaM{\cdot}Ca_n^{2+} \rightleftharpoons CaM^*{\cdot}Ca_n^{2+}$$

step 2

$$CaM^*{\cdot}Ca_n^{2+} + E \rightleftharpoons E{\cdot}CaM^*{\cdot}Ca_n^{2+} \rightleftharpoons E^*{\cdot}CaM^*{\cdot}Ca_n^{2+}$$

where the asterisk indicates the active species. As a first step toward the understanding of the overall mechanism of calmodulin-mediated stimulation of enzymatic reactions, a study of step 1 was undertaken. The Ca^{2+}-binding properties of the purified proteins were first reported by Teo & Wang (1973) and subsequently examined by others (Lin et al., 1974; Watterson et al., 1976; Klee, 1977; Wolff et al., 1977; Jarrett & Kyte, 1979). Although all studies indicate the presence of four specific Ca^{2+}-binding sites, there are some discrepancies between different reports about the affinities of the different sites for Ca^{2+} and, more particularly, about the effect of Mg^{2+} on the binding constants. Therefore, Ca^{2+} binding to calmodulin was studied in the presence of various concentrations of H^+, K^+, and Mg^{2+} ions.

Materials and Methods

Trichloroacetic acid (Cl_3CCOOH) was obtained from Eastman-Kodak. All other reagents were as previously described (Klee, 1977; Haiech et al., 1979; Crouch & Klee, 1980). Calmodulin was purified from ram testes according to Autric et al. (1980). The calmodulin concentration was measured as previously described (Crouch & Klee, 1980).

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[1] Abbreviations used: cAMP, cyclic adenosine monophosphoric acid; CaM, calmodulin; Cl_3CCOOH, trichloroacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; UV, ultraviolet.

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