Walz, F. G., Jr., Vlasuk, G. P., Omiecinski, C. J., Bresnick, E., Thomas, P. E., Ryan, D. E., & Levin, W. (1982) *J. Biol. Chem. 257*, 4023–4026.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Weickmann, J. L., Elson, M., & Glitz, D. G. (1981) *Biochemistry 20*, 1272–1278.
Wojcieszyn, J. W., Wang, M. C., Lee, C. L., Murphy, G. P., & Chu, T. M. (1979) *J. Appl. Biochem. 1*, 223–234.
Yam, L. T. (1974) *Am. J. Med. 56*, 604–616.

Purification and Characterization of an Enkephalin Aminopeptidase from Rat Brain Membranes†

Koon-Sea Hui,* You-Jing Wang,‡ and Abel Lajtha

ABSTRACT: A membrane-bound aminopeptidase was purified from rat brain, and its activity was assayed by high-pressure liquid chromatography with Met-enkephalin as the substrate. The enzyme was extracted with 1% Triton X-100 and purified by chromatography, successively on DEAE-Sepharose CL-6B, Bio-Gel HTP, and Sephadex G-200 columns. The overall purification was about 1200-fold, with 25% yield. The purified enzyme showed one band on disc gel electrophoresis and two bands on sodium dodecyl sulfate electrophoresis with molecular weights of 62 000 and 66 000. The aminopeptidase has a pH optimum of 7.0, a K_m of 0.28 mM, and a V_{max} of 45 μmol (mg of protein)$^{-1}$ min^{-1} for Met-enkephalin. It releases tyrosine from Met-enkephalin, but it does not split the byproduct. It does not hydrolyze γ- or β-endorphin, or dynorphin, but it does hydrolyze neutral and basic aminoacyl β-naphthylamides. The enzyme is inhibited by the aminopeptidase inhibitors amastatin, bestatin, and bestatin–Gly. Its properties, such as its subcellular localization, substrate specificity, pH optimum, and molecular weight, distinguish it from leucine aminopeptidase, aminopeptidase A, aminopeptidase B, aminopeptidase M, and the soluble aminopeptidase for enkephalin degradation.

The enkephalins (Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu), which may serve as neurotransmitters (Hughes et al., 1975), are quickly inactivated. Efforts to obtain longer duration of activity have centered on the synthesis of analogues resistant to enzymatic degradation, such as the substitution of Gly with D-Ala at the 2-position of the peptide (Pert et al., 1976). Another approach toward increasing the action of enkephalins would be to block their degradative enzyme(s). Topographically, the synaptically released enkephalin first binds to the receptor and then is probably metabolized by a membrane-associated enzyme(s); reuptake of the peptide in the synapse does not play a significant role (Gorenstein & Snyder, 1980).

It has been suggested that the binding of enkephalin is coupled to subsequent aminopeptidase degradation (Knight & Klee, 1978), but the relationship between opioid receptor occupation and enkephalin hydrolysis remains to be clarified. Three enzymatic mechanisms for the inactivation of enkephalins in brain membrane have been observed: (a) cleavage at the Tyr–Gly bond by aminopeptidase (Jacquet et al., 1976; Knight & Klee, 1978), (b) cleavage of the Gly–Phe bond by a dipeptidyl carboxypeptidase and angiotensin-converting enzyme (Sullivan et al., 1978; Malfroy et al., 1978, 1979; Swerts et al., 1979; Guyon et al., 1979; Gorenstein & Snyder, 1979), and (c) cleavage at the Gly–Gly bond by a dipeptidyl-aminopeptidase (Gorenstein & Snyder, 1979). The first major product was tyrosine when enkephalin was incubated with neuroblastoma (Hazum et al., 1979) or glioma cells (Lazarewicz et al., 1981), resulting in inactivation, since tyrosine is required for enkephalins to exert their opiate effects (Coy & Kastin, 1980). Two membrane-bound aminopeptidases have recently been partially characterized (Hersh, 1981). We report here the solubilization, purification, and characterization of a membrane-bound aminopeptidase from rat brain with high activity in Met-enkephalin degradation.

Materials and Methods

Ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) was from Baker Chemical Co. (Philipsburg, NJ); dithiothreitol was purchased from Sigma; fluorescamine was from Roche; HPLC-grade acetonitrile was from Fisher (Pittsburgh, PA); captopril was a gift from Squibb and Son Inc. (Princeton, NJ); amastatin and bestatin were generously supplied by Dr. H. Umezawa, Microbial Chemistry Research Foundation (Tokyo, Japan); acrylamide was from Eastman Kodak (Rochester, NY); sodium lauryl sulfate ($NaDodSO_4$) was from BDH Chemical Ltd. (Poole, England). Except the enkephalins, from Boehringer Mannheim (Indianapolis, IN), all synthetic peptides were purchased from Peninsula (San Carols, CA). Other chemicals were obtained from Sigma.

Purification of Membrane-Bound Aminopeptidase. All steps of the purification were performed at 4 °C. The enzyme was prepared by homogenization of five male Wistar rat brains (10 g) with 9 volumes of 0.32 M sucrose in a glass homogenizer with a motor drive. The homogenate was centrifuged at 800*g* for 10 min, and the cell debris was discarded. After centrifugation at 30000*g* for 20 min, the supernatant was discarded, and the pellet, resuspended in 50 mM Tris-HCl buffer of pH 7.5, was centrifuged and washed 2 additional times. The membrane pellet was solubilized by suspension in 20 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 1% (w/v) Triton X-100, and incubated at 37 °C for 45 min. The solubilized enzyme, obtained after centrifugation at 30000*g* for 10 min, was applied to a DEAE-Sepharose CL-6B column (Pharmacia; 1.5 × 25 cm) that was equilibrated with 25 mM

† From the Center for Neurochemistry, Rockland Research Institute, Ward's Island, New York 10035. *Received May 3, 1982; revised manuscript received November 12, 1982.* The investigation was supported in part by research grants from the National Science Foundation (NBS 78-26164), the National Institutes of Health (NB-3226), and the World Health Organization.

‡ World Health Organization International Fellow from the Institute of Acupuncture and Moxibustion, Academy of Traditional Chinese Medicine, Beijing, People's Republic of China.

0006-2960/83/0422-1062$01.50/0

Tris-HCl buffer, pH 7.5, containing 2 mM EGTA and 0.2 mM dithiothreitol (buffer A). The column was washed with 2 bed volumes of buffer A, and the enzyme was eluted with 500 mL of a salt gradient of 0–0.4 M NaCl in buffer A at a flow rate of 0.4 mL/min; 10-mL fractions were collected. The aminopeptidase activity was assayed by reverse-phase high-performance liquid chromatography (Waters HPLC Model 204), by measuring the disappearance of Met-enkephalin and by the generation of the tetrapeptide Gly-Gly-Phe-Met (Hui et al., 1981a,b).

The pooled enzyme fractions from ion-exchange chromatography were applied directly to a Bio-Gel HTP (hydroxylapatite) column (Bio-Rad; 0.9 × 12 cm) equilibrated with buffer A. The column was washed with 2 bed volumes of the same buffer, and the retained proteins were eluted at a flow rate of 0.4 mL/min with 500 mL of buffer A containing a 0–200 mM sodium phosphate gradient, pH 7.5.

The pooled enzyme fractions from the Bio-Gel HTP chromatography were concentrated to approximately 2 mL with an Amicon PM 10-membrane filter and applied to a Sephadex G-200 column (Pharmacia; 1.5 × 85 cm) equilibrated with buffer A. The protein was eluted with the same buffer at a flow rate of 3 mL/h. The eluate was collected in 2-mL fractions.

Protein was determined by UV absorption at 280 nm or by a Bio-Rad Protein Assay Kit. In the latter part of the purification, protein was assayed by tagging with fluorescamine and measuring the fluorescence (Stein, 1977). Bovine serum albumin was used as the standard for all the protein determinations.

Electrophoresis. Ten micrograms of the concentrated aminopeptidase was loaded on a 10% polyacrylamide gel (0.5 × 6 cm) that had been polymerized the day before and kept in the refrigerator overnight (Davis, 1964). The gels were run at 2.5 mA/tube with 25 mM Tris–Gly buffer, pH 8.3, for 2 h at 4 °C. After the run, the gels were stained with Coomassie Blue. For detection of enzyme activity, the gels were incubated with 10 mg of Met-enkephalin in 5 mL of buffer A and the released tyrosine was detected by reduction with tetrazolium salts (Sugiura et al., 1977). $NaDodSO_4$ gel electrophoresis was carried out as described by Laemmli (1970). The apparent molecular weight of the aminopeptidase was determined by $NaDodSO_4$ gel electrophoresis, with low molecular weight and high molecular weight $NaDodSO_4$–polyacrylamide gel electrophoretic standards (Bio-Rad) as markers run on separate gels according to the method of O'Farrell (1975).

Concentration of Enzyme Solutions. Solutions were concentrated by centrifugation with Amicon Centriflo membrane cones, type CF50A, unless otherwise stated.

Aminopeptidase Activity on Different Peptides and Aminoacyl-βNA. Peptide (33 μM) was incubated with the enzyme in a final volume of 150 μL of buffer A at 37 °C for 30 min. The reaction was terminated by adding 20 μL of 20% trichloroacetic acid. The mixture was centrifuged, and 50 μL of the supernatant was submitted to HPLC analysis on a Waters high-performance liquid chromatograph equipped with a Radial-PAK C_8 (5 × 100 mm) column with a particle size of 10 μm. The sample was eluted isocratically at ambient temperature with a mixture of acetonitrile and 0.1 M phosphate buffer, pH 3.0. The flow rate was 2 mL/min, resulting in a back-pressure of 500–1000 lb/in^2. The peptides were measured by UV absorption at 280 nm and/or 205 nm. The solvent system, acetonitrile–phosphate buffer, for Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg^6, and Met-enkephalin-Arg^6-Phe^7 was 20:80, for Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe, 10:90, for dynorphin-(1–13) and γ-endorphin, 27.5:72.5, and for β_h-endorphin, 42.5:57.5. In these systems, the retention time was 1.5 min for Met-enkephalin, 2.1 min for Leu-enkephalin, 3.0 min for Tyr-Gly-Gly-Phe, 3.5 min for Gly-Gly-Phe-Met, 1.38 min for Met-enkephalin-Arg^6, 4.2 min for Met-enkephalin-Arg^6-Phe^7, 2.8 min for dynorphin-(1–13), 2.6 min for γ-endorphin, and 1.8 min for β-endorphin. The released N-terminal Tyr residue was also detected by dansylation followed by two-dimensional thin-layer chromatography on polyamide plates (Hui et al., 1980c) or by HPLC on a LiChrosorb RP-18 column (250 mm × 4.6 mm, particle size 7 μm; Unimetrics, Westwood, NJ). The sample was eluted with acetonitrile and phosphate buffer (4:96). The flow rate was 1.5 mL/min. The retention time was 4 min for Tyr, which was resolved from Tyr-Gly (6.9 min) and Tyr-Gly-Gly (5.7 min). The hydrolysis of β-naphthylamides (βNA) of Tyr, Leu, Ala, Arg, and Glu was assayed by measuring the released β-naphthylamine with an Aminco spectrofluorometer (excitation at 360 nm and emission at 450 nm). The reaction was carried out in a 3-mL cuvette (1-cm width) at a constant temperature of 37 °C. Each incubation mixture contained 0.1 mM substrate, 0.1 mM dithiothreitol, 50–100 μL of enzyme, and 2 mL of 0.1 M sodium phosphate buffer, pH 7.0. β-Naphthylamine was used as the standard for the measurements, and the boiled enzyme was used as the blank for the assay.

Results

Solubilization of Aminopeptidase from Rat Brain Membrane. The optimal time was 45 min for solubilizing the aminopeptidase from the rat brain membrane incubated with 1% Triton X-100 at 37 °C; beyond that, the yield declined. Almost all the aminopeptidase was extracted, since no measurable activity was detected in the extracted membrane. The total activity increased 3-fold after the Triton X-100 treatment.

The results of our purification of the enkephalin aminopeptidase from rat brain membrane are summarized in Table I. The complete purification took 4–5 days. Precipitation with $(NH_4)_2SO_4$ gave low yields and loss of activity. In addition, removal of $(NH_4)_2SO_4$ required time-consuming gel filtration, or dialysis, which resulted in further loss of activity. Chromatography on DEAE-Sepharose CL-6B proved to be a satisfactory step after the extraction, giving a 55% yield and 6-fold purification. The enzyme was eluted in a sharp peak in fractions 28–32 (280–320 mL) (Figure 1a). Less than 10% of the total activity was eluted at the void volume.

Enzyme fractions from step 3 were then applied to the Bio-Gel HTP. The enzyme was retained on the column, while most other proteins were not, and was eluted as a symmetrical peak between fractions 18 and 28 (180–280 mL). This step resulted in a 12-fold purification with a yield of 71% (Figure 1b). The pooled enzyme from step 4 was concentrated by ultrafiltration to approximately 2 mL and applied to a Sephadex G-200 column. Fractions 29–35 contained the aminopeptidase activity (Figure 1c). The overall purification through step 5 was about 1200-fold with a recovery of 74%, or 25% if the latent (Triton-X extraction activated) membrane-bound activity was also taken into consideration.

Comparison of Aminopeptidase Activity on Met- and Leu-enkephalin and Aminoacyl-βNA. The profiles of enzyme activity on Sephadex G-200 with Met-enkephalin, Leu-enkephalin, Ala-βNA, and Arg-βNA were identical. The rate of Met-enkephalin degradation was higher than that of Leu-enkephalin and higher with Arg-βNA than with Ala-βNA. The enzyme was insensitive to inhibition by Gly-Gly-Phe-Met.

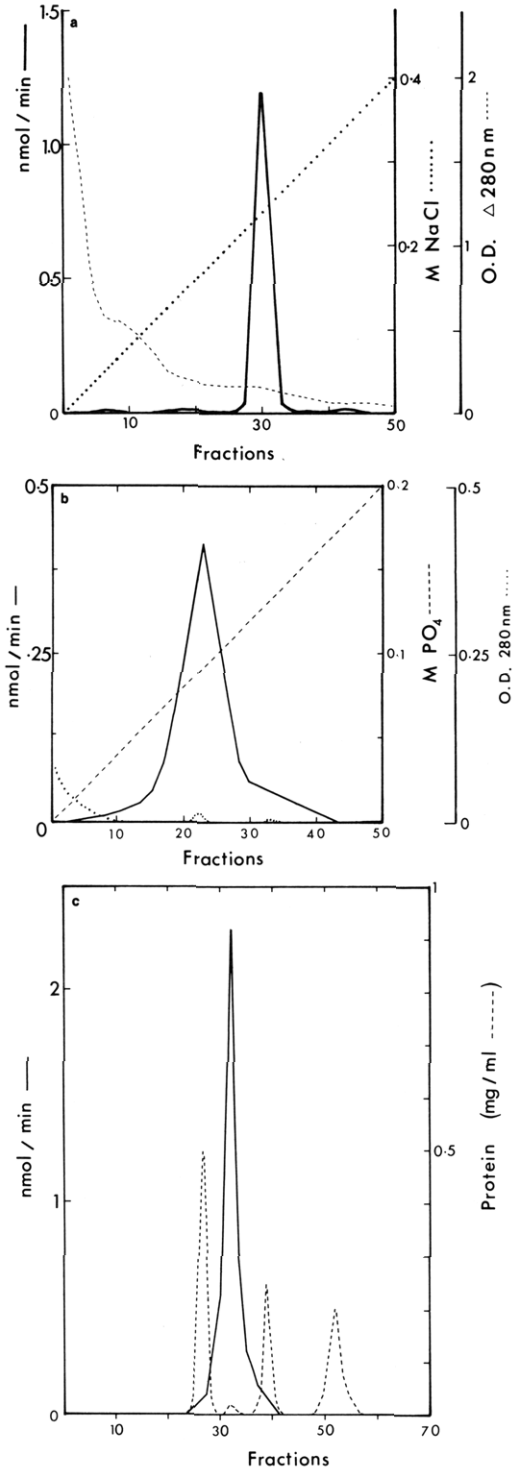

FIGURE 1: Elution profiles of membrane-bound aminopeptidase during various stages of purification. Chromatography was conducted as described under Materials and Methods. Enzyme activity was measured by high-pressure liquid chromatography with Met-enkephalin as the substrate. The volume of each fraction was 10 mL for chromatography on DEAE-Sepharose CL-6B (a) and chromatography on Bio-Gel HTP (b). The volume of each fraction was 2 mL for chromatography on Sephadex G-200 (c). The enzyme activity is expressed as nanomoles of Met-enkephalin breakdown per minute per 50-μL aliquot. The protein in chromatograms of DEAE-Sepharose CL-6B and Bio-Gel HTP columns was determined by UV absorption at 280 nm. The protein in the chromatogram of Sephadex G-200 column was determined by derivatizing with fluorescamine and measuring the fluorescence.

Storage. The purified enzyme lost activity upon freezing and thawing or in the absence of dithiothreitol and EGTA. The enzyme was stable for approximately 3 months when stored refrigerated in a solution containing 25 mM Tris buffer, pH 7.5, 0.2 mM dithiothreitol, and 2 mM EGTA, but it was inactive after 6 months of storage.

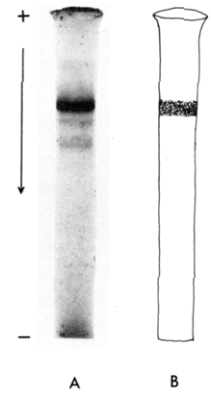

FIGURE 2: Disc polyacrylamide gel electrophoresis of purified aminopeptidase (10 μg of protein). The gel (10% acrylamide) was run with 25 mM Tris–Gly buffer, pH 8.3. After the electrophoresis, the gel was stained for protein with Coomassie Blue (A), and a parallel gel was stained for enzyme activity on Met-enkephalin breakdown and detected by reduction of tetrazolium salts. See details under Materials and Methods.

Homogeneity and Molecular Weight. The purified enzyme showed apparent homogeneity after disc gel electrophoresis on a 10% polyacrylamide gel (Figure 2a). There were one major and two trace protein bands. Only the major protein band was red after incubation with Met-enkephalin and staining with tetrazolium salts. The red color of the band (Figure 2b) was sensitive to light, and it easily faded out. Two electrophoretic species were observed in the presence of Na-DodSO$_4$ (Figure 3); they were estimated to be 66 000 and 62 000, in a ratio of 1:1, when detected with Coomassie Blue.

Metal Effects and pH Optimum Profile. When the purified enzyme was incubated with divalent metals (1 mM), its activity was increased more than 25% by Zn, Co, and Ca. Mg had no effect, and Cu, Fe, Cd, Ni, Hg, and Pb were inhibitory (less than 75% of the control). The enzyme lost its activity when dialyzed against 2 mM EDTA and then against buffer A. The activity could not be restored by any of the divalent metals. The enzyme was active between pH 4 and pH 9, with the maximal activity for Met-enkephalin at pH 7.0 (Figure 4).

Characteristics of the Aminopeptidase. The aminopeptidase in the membrane seems to be different from other exopeptidases and endopeptidases, since the substrate specificity of the crude Triton X-100 extract and that of the purified enzyme were different. With the Triton extract, the breakdown rate for Leu-enkephalin and Gly-Gly-Phe-Met was higher than that for Met-enkephalin. This reflects the presence of other aminopeptidases and dipeptidyl carboxypeptidases (Hui & Lajtha, 1982). The enzymes responsible for aminopeptidase activities are leucine aminopeptidase, aminopeptidase M, and membrane aminopeptidase M II (Hersh, 1981). The hydrolytic activity of the Triton extract on γ-endorphin, dynorphin-(1–13), and β_h-endorphin was due to the presence, in addition to the above, of aminopeptidases, carboxypeptidases, cathepsin D, and a neutral endopeptidase (Graf et al., 1979; Burbach et al., 1981).

The enzyme degraded Met-enkephalin stoichiometrically into Gly-Gly-Phe-Met and Tyr. The hydrolysis was monitored simultaneously by UV absorption at 280 nm and at 205 nm. The disappearance of Met-enkephalin and the appearance of Gly-Gly-Phe-Met were in a 1:1 relationship, suggesting that there was no further degradation of Gly-Gly-Phe-Met. This was confirmed by separate, longer time (2 h) incubation of

Table I: A Typical Purification of Membrane-Associated Aminopeptidase for Met-enkephalin Degradation

step	preparation	total act. (μmol/min)	protein (mg)	sp act. [μmol (mg of protein)$^{-1}$ min^{-1}]	purification factor	yield[a] (%)
1	crude membrane	1.35	150	0.009	1	100
2	Triton X-100	4.08	120	0.034	3.8	302 (100)
3	DEAE-Sepharose CL-6B	2.25	11	0.2	22.2	167 (55)
4	Bio-Gel HTP	1.61	0.6	2.5	277	119 (39)
5	Sephadex G-200	1.0	0.1	10.6	1182	74 (25)

[a] Under the column "yield", the values in parentheses are calculated assuming that the starting material is the Triton X-100 extract of the membrane. Since there are more than one aminopeptidase in the crude membrane and we isolated only one of them, the exact yield of our purification will be higher.

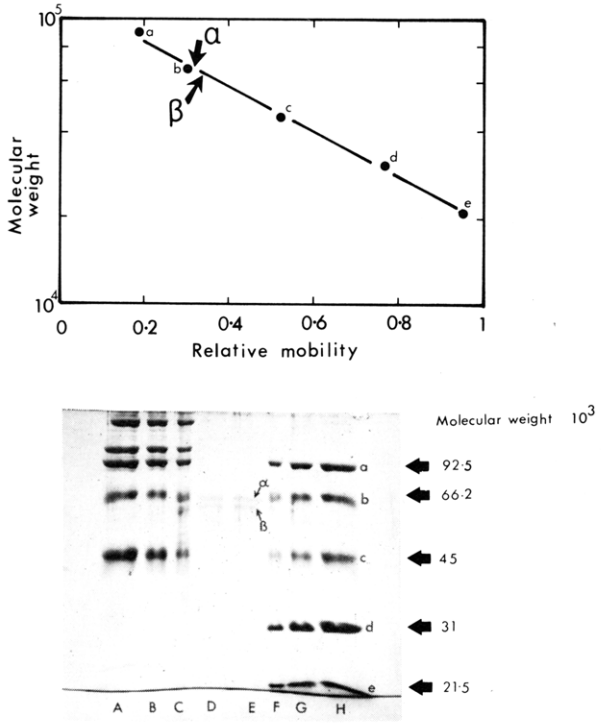

FIGURE 3: NaDodSO$_4$–polyacrylamide gel electrophoresis of membrane-bound aminopeptidase. The purified enzyme (D, 1.5 μg of protein; E, 3.0 μg of protein) was run in parallel with high molecular weight standards (A–C) and low molecular weight standards (F–H). The protein content in each band of A and H is 20 μg, in B and G is 10 μg, and in C and F is 5 μg. α = the heavier chain (66 000); β = the lighter chain (62 000); a = phosphorylase B (M_r 92 500); b = bovine serum albumin (M_r 66 200); c = ovalalbumin (M_r 45 000); d = carbonic anhydrase (M_r 31 000); e = soybean trypsin inhibitior (M_r 21 500).

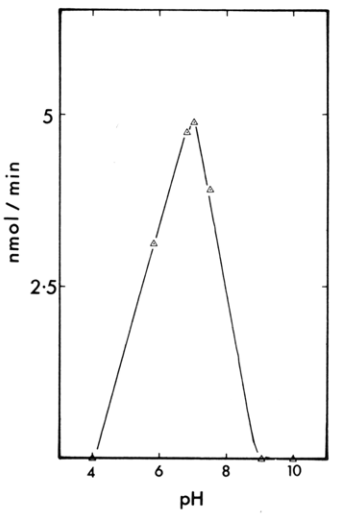

FIGURE 4: pH profile of the purified membrane-bound aminopeptidase. Each point represents the average of four experiments ± 5% error.

the latter compound with more enzyme (5 times) at 37 °C. Our HPLC method could detect a 5% change of the Gly-Gly-Phe-Met concentration. The amount of Tyr produced, measured by a different solvent system (4% CH_3CN in PO_4 buffer) and column (LiChrosorb RP-18), was equal to the amount of Gly-Gly-Phe-Met produced. The results of the HPLC assay were confirmed by amino acid analysis. Ion-exchange chromatography (Hui et al., 1980a) and dansylation (Hui et al., 1981b) showed that Tyr was the only amino acid released. Its generation and the disappearance of the Met-enkephalin were also in a 1:1 ratio. These results show that the purified membrane aminopeptidase specifically splits the Tyr-Gly bond with no further degradation of the resulting des-Tyr-tetrapeptide under the reaction conditions used.

The purified membrane aminopeptidase was active with Met-enkephalin. It also degraded Leu-enkephalin, Tyr-Gly-Gly-Phe, Met-enkephalin-Arg^6, and Met-enkephalin-Arg^6-Phe^7 but to a lower degree. It was inactive on the following substrates tried: Tyr-Gly, Tyr-Gly-Gly, γ-endorphin (Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu), dynorphin-(1–13) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys), and β_h-endorphin (Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu), demonstrating that the C terminal (size, composition, and configuration) of the substrate is important for the aminopeptidase. The purified enzyme split Tyr-βNA, Leu-βNA, Ala-βNA, and Arg-βNA but was inactive with Glu-βNA.

Kinetics. The Michaelis constants (K_m) and maximal velocities (V_{max}) for Met-enkephalin and Arg-βNA were calculated by Lineweaver–Burk analysis. The K_m and V_{max} for Met-enkephalin were 0.28 mM and 45 μmol (mg or protein)$^{-1}$ min^{-1}. For Arg-βNA the K_m was 0.05 mM and the V_{max} was 28 μmol (mg of protein)$^{-1}$ min^{-1}.

Inhibition of Membrane Aminopeptidase. Phe-Ala and captopril are inhibitors of enkephalin dipeptidyl carboxypeptidase (Patey et al., 1981) and angiotensin-converting enzyme (Ondetti et al., 1977). At a concentration of 900 μM neither of them had an effect on Met-enkephalin breakdown (Table II). Aminopeptidase inhibitors amastatin, bestatin, and bestatin–Gly were all inhibitory. The IC_{50} values were 0.17 μM for amastatin and 0.9 μM for bestatin.

The breakdown of Met-enkephalin was inhibited by other substrates for the enzyme, Leu-enkephalin, Met-enkephalin-Arg^6, and Met-enkephalin-Arg^6-Phe^7, but not Tyr-Gly-Gly-Phe. In addition, γ-endorphin, dynorphin-(1–13), and β_h-endorphin, with N termini resistant to the aminopeptidase, displayed a strong inhibitory effect on Met-enkephalin degradation by the enzyme (Table II). Gly-Gly-Phe-Met, itself not a substrate, had no effect. Most of the Tyr-dipeptides, and Tyr-Gly-Gly, did not inhibit Met-enkephalin breakdown.

Table II: Effect of Peptidase Inhibitors and Tyrosyl Peptides on Met-enkephalin Degradation by Purified Aminopeptidase[a]

compound	concn (μM)	act., % of control
amastatin	5	0
bestatin	5	0
bestatin-Gly	5	0
Leu-enkephalin	33	70
Met-enkephalin-Arg^6	33	60
Met-enkephalin-Arg^6-Phe^7	33	34
γ-endorphin	33	38
dynorphin-(1-13)	33	13
β_h-endorphin	33	0
Tyr	33	150
Tyr-Leu	33	126
Tyr-Phe	33	80
Tyr-Glu	33	134
Tyr-Val	33	124
Tyr-Ala	33	138

[a] The enzyme (10 mg of protein) and Met-enkephalin (75 μM) were incubated with the other peptides or inhibitors (their concentration is shown in the table in a final volume of 150 μL of buffer A), at 37 °C for 30 min. The breakdown of Met-enkephalin was then measured by high-pressure liquid chromatography. The values are averages of four to five experiments, with a variation within 5%. Phe-Ala (900 μM), captopril (1000 μM), Tyr-Gly-Gly-Phe (33 μM), Gly-Gly-Phe-Met (33 μM), Tyr-Arg (kyotorphin, 33 μM), Tyr-Tyr (33 μM), Tyr-Gly (33 μM), and Tyr-Gly-Gly (33 μM) did not affect the aminopeptidase in the Met-enkephalin hydrolysis (100% of control).

Discussion

We used enkephalin as the substrate and HPLC as the assay for the purification of a membrane aminopeptidase split by enkephalin. The assay of enzyme activity is rapid and sensitive; it takes only a few minutes to run a sample. Since the subcellular distribution of degradative activity is different for enkephalins from those for aminoacyl-βNA (Hui et al., 1981a), use of substrates other than enkephalin for enzyme characterization could be misleading. Triton X-100 treatment increased the enzyme activity 3-fold (Table I). This was probably due to the release by the reagent of the enzyme from the membrane matrix. Membrane fragments tend to form micelles after the homogenization. Incubation at 37 °C did not increase the enzyme activity. Membrane-bound aminopeptidases active on Met-enkephalin were also solubilized by using 0.05% Triton X-100 and stirring for 15 min at 4 °C (Hersh, 1981).

The purified membrane aminopeptidase released the amino-terminal tyrosine 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 intact neuroblastoma cells (Hazum et al., 1979) and glioma cells (Lazarewicz, 1981).

That the enzyme is an aminopeptidase was confirmed by its ability to hydrolyze aminoacyl-βNA and by its inhibition by the aminopeptidase inhibitors bestatin and amastatin. The enzyme activity detected in the polyacrylamide gel (Figure 2) was also strong proof for aminopeptidase, since the detection method is specific for the free tyrosine residue (Sugiura et al., 1977). Product analysis showed that there is no split at the Gly^3–Phe^4 bond, indicating that the enzyme is free of angiotensin-converting enzyme (Ondetti et al., 1977) and enkephalin dipeptidyl carboxypeptidase (Patey et al., 1981). This is confirmed by the lack of inhibition by captopril or Phe-Ala of the enzyme.

The molecular weight of the enzyme was estimated by exclusion chromatography on a Sephadex G-200 column to be 250000 with subunits of molecular weights 66000 and 62000. It must be emphasized that the enzyme was solubilized with Triton X-100 before its molecular weight was determined. The bound Triton X-100 may give falsely high values or it may yield subunits. The nonidentical protein subunits of the enzyme are of interest. It is not likely that they are due to the proteolysis or that one of the subunits is a contaminant, because the enzyme was stable at 4 °C; it showed a major band in the polyacrylamide gel electrophoresis, and the two proteins in the $NaDodSO_4$–polyacrylamide gel electrophoresis were in 1:1 ratio. The enzyme is a metal enzyme, as it is sensitive to EDTA. Its activity could not be restored by divalent metals after the EDTA treatment. Physical and kinetic properties distinguish our membrane aminopeptidase from a number of aminopeptidases that have been described, such as leucine aminopeptidase (M_r 255000), aminopeptidase A, aminopeptidase B (M_r 95000), and aminopeptidase M (M_r 280000) (Barrett, 1980). Leucine aminopeptidase and aminopeptidase B are located in the cytosol, whereas our enzyme is membrane bound. Aminopeptidase A splits aminoacyl-βNA with a pH optimum of 6.2, but our enzyme did not split Glu-βNA. Aminopeptidase M releases amino acids from Gly-Gly-Phe-Met in sequential order starting from the NH_2 terminus (Austen et al., 1979); our enzyme does not split des-Tyr-Met-enkephalin. Our rat brain membrane aminopeptidase has a higher molecular weight and greater stability in storage than the soluble aminopeptidase recently isolated from rat brain (Schnebli et al., 1979; Hui et al., 1980b; Hersh & McKelvy, 1980; Wagner et al., 1981). Our enzyme is very hydrophobic, as it is held tightly on a phenyl-Sepharose CL-4B column. The fact that it is not retained on a Con A column shows that it is different from the dipeptidyl-aminopeptidase (Kojima et al., 1980). The enzyme we purified shares some properties with the membrane aminopeptidase (MI) recently studied by Hersh (1981). They have the same pH optimum, kinetics, and specificity, and they do not split γ-endorphin or β_h-endorphin.

The membrane aminopeptidase has a high kinetic constant with Met-enkephalin [K_m is 0.28 mM and V_{max} is 45 μmol (mg of protein)$^{-1}$ min^{-1}]; this is a lower affinity than that of enkephalin dipeptidyl carboxypeptidase (enkephalinase A) to Met-enkephalin (Malfroy et al., 1979). This high K_m indicates that the enzyme is not easily saturated under most conditions. The enzyme degrades neutral and basic aminoacyl-βNA but not the acidic one. With peptides as substrates, membrane aminopeptidase shows high activity with Met-enkephalin (Table II). It is not inhibited by tyrosyl dipeptides Tyr-Gly-Gly or Tyr-Gly-Gly-Phe and is not active on Tyr-Gly and Tyr-Gly-Gly, showing that it is not a dipeptidase, tripeptidase, or tetrapeptidase. Tyr-Gly-Gly-Phe is a relatively good substrate; its degradation by the enzyme is 78% of that of Met-enkephalin. However, it is not a good inhibitor (IC_{50} > 50 μm) of Met-enkephalin degradation. This may be due to a difference in their affinity to the reactive site(s) of the enzyme. In fact, the longer the C-terminal extension of the enkephalin, the higher the affinity of the substrates to the enzyme (Hui et al., unpublished observation). The enzyme hydrolyzes Met-enkephalin-Arg^6 and Met-enkephalin-Arg^6-Phe^7, which have opiate properties (Inturrisi et al., 1980). The membrane aminopeptidase is inactive toward γ-endorphin, dynorphin-(1–13), and β_h-endorphin, in contrast to the aminopeptidase reported by Burbach et al. (1980). These larger peptides, which are not substrates, inhibit Met-enkephalin breakdown by the enzyme (Table II).

In distribution, membrane aminopeptidase activity does not correlate with the enkephalin-binding activity, but it is higher than the enkephalin dipeptidyl carboxypeptidase activity in

all the brain regions examined (Sullivan et al., 1978; Malfroy et al., 1978, 1979). The aminopeptidase has a lower affinity than the enkephalin dipeptidyl carboxypeptidase, which has a K_m of 1.4–27 μM (Schwartz et al., 1981; Altstein et al., 1981). However, this does not eliminate the possibility that, beside other functions, aminopeptidase is involved in the physiological inactivation of enkephalin. Analogously, acetylcholinesterase appears to be associated with cholinergic neurons in the brain, but it is also located in certain regions distinct from the cholinergic system (Gorenstein & Snyder, 1980). Although the membrane-bound enzyme is highly active and highly specific for Met-enkephalin, the physiological function of this new enzyme in enkephalin degradation needs further investigation.

Acknowledgments

We thank M. Hui for HPLC analysis and Dr. M. Banay-Schwartz and T. De Guzman for gel electrophoresis.

Registry No. Met-enkephalin, 58569-55-4; Leu-enkephalin, 58822-25-6; Ala-βNA, 720-82-1; Arg-βNA, 7182-70-9; Tyr-Gly-Gly-Phe, 60254-82-2; Met-enkephalin-Arg^6, 76310-14-0; Met-enkephalin-Arg^6-Phe^7, 73024-95-0; amastatin, 67655-94-1; bestatin, 58970-76-6; bestatin-Gly, 63642-42-2; γ-endorphin, 61512-77-4; dynorphin-(1–13), 72957-38-1; β-endorphin, 60617-12-1; enkephalin aminopeptidase, 75496-63-8.

References

Altstein, M., Mittman, S., & Vogel, Z. (1981) *Life Sci. 28*, 185–191.

Austen, B. M., Evans, C. J., & Smyth, D. G. (1979) *Biochem. Biophys. Res. Commun. 91*, 1211–1217.

Barrett, A. J. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 9–14.

Burbach, J. P. H., Loeber, J. G., Verhoef, J., Weigant, V. M., deKloet, E. R., & deWied, D. (1980) *Nature (London) 283*, 96–97.

Burbach, J. P. H., DeKloet, E. R., Schotman, P., & deWied, D. (1981) *J. Biol. Chem. 256*, 2463–2469.

Coy, D. H., & Kastin, A. J. (1980) *Peptides (N.Y.) 1*, 175–178.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Gorenstein, C., & Snyder, S. H. (1979) *Life Sci. 25*, 2065–2070.

Gorenstein, C., & Snyder, S. H. (1980) *Proc. R. Soc. London, Ser. B 120*, 123–132.

Graf, L., Kenessy, A., Patthy, A., Grynbaum, A., Marks, N., & Lajtha, A. (1979) *Arch. Biochem. Biophys. 193*, 101–109.

Guyon, A., Roques, B. P., Guyon, F., Descartes, A., Perdrisot, R., Swerts, J. P., & Schwartz, J. C. (1979) *Life Sci. 25*, 1605–1612.

Hazum, E., Chang, K. J., & Cuatrecasas, P. (1979) *Life Sci. 24*, 137–144.

Hersh, L. B. (1981) *Biochemistry 20*, 2345–2350.

Hersh, L. B., & McKelvy, J. F. (1980) *J. Neurochem. 36*, 171–178.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., & Morris, H. R. (1975) *Nature (London) 258*, 577–579.

Hui, K.-S., & Lajtha, A. (1982) in *Handbook of Neurochemistry* (Lajtha, A., Ed.) Vol. 4, Plenum Press, New York and London (in press).

Hui, K.-S., Cheng, K.-P., Banay-Schwartz, M., & Lajtha, A. (1980a) *Neurosci. Lett. 10*, 341–345.

Hui, K.-S., Cheng, K.-P., & Lajtha, A. (1980b) *Neurosci. Abstr. 6*, 767.

Hui, K.-S., Salshutz, M., Davis, B. A., & Lajtha, A. (1980c) *J. Chromatogr. 92*, 341–350.

Hui, K.-S., Wang, Y.-J., Wong, K.-H., Tsai, H., & Lajtha, A. (1981a) *Neuropeptides (Edinburgh) 1*, 167–173.

Hui, K.-S., Wang, Y.-J., Tsai, H., Wong, K.-H., & Lajtha, A. (1981b) *Peptides (N.Y.) 2*, 89–94.

Inturrisi, C. E., Umans, J. G., Wolff, D., Stern, A. S., Lewis, R. V., Stein, S., & Udenfriend, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5512–5514.

Jacquet, Y. F., Marks, N., & Li, C. H. (1976) in *Opiates and Endogenous Opioid Peptides* (Kosterlitz, H. W., Ed.) pp 411–414, North-Holland, Amsterdam.

Knight, M., & Klee, W. A. (1978) *J. Biol. Chem. 253*, 3843–3847.

Kojima, K., Hama, T., Kato, T., & Nagatsu, T. (1980) *J. Chromatogr. 189*, 233–240.

Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.

Lazarewicz, J., Wideman, J., & Kanje, M. (1981) *Proc. Int. Soc. Neurochem. 8*, 107 (Abstract).

Malfroy, B., Swerts, J. P., Guyon, A., Roques, B. P., & Schwartz, J. C. (1978) *Nature (London) 276*, 523–526.

Malfroy, B., Swerts, J. P., Llorens, C., & Schwartz, J. C. (1979) *Neurosci. Lett. 11*, 329–344.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Ondetti, M. A., Rubin, R., & Cushman, D. W. (1977) *Science (Washington, D.C.) 196*, 441–444.

Patey, G., De La Baume, S., Schwartz, J.-C., Cros, C., Roques, B., Fournie-Zaluski, M.-C., & Soroca-Lucas, E. (1981) *Science (Washington, D.C.) 212*, 1153–1155.

Pert, C., Pert, A., Chang, J., & Fong, B. (1976) *Science (Washington, D.C.) 194*, 330–332.

Schnebli, H. P., Phillipps, M. A., & Barclay, R. K. (1979) *Biochim. Biophys. Acta 569*, 89–98.

Schwartz, J.-C., Malfroy, B., & De La Baume, S. (1981) *Life Sci. 28*, 1715–1740.

Stein, S. (1977) in *Peptides in Neurobiology* (Gainer, H., Ed.) pp 9–37, Plenum Press, New York and London.

Sugiura, M., Ito, Y., Kirano, K., & Sawaki, S. (1977) *Anal. Biochem. 81*, 481–484.

Sullivan, S., Akil, H., & Barchas, J. O. (1978) *Commun. Psychopharmacol. 2*, 525–531.

Swerts, J. P., Perdrisot, R., Patey, G., De La Baume, S., & Schwartz, J.-C. (1979) *Eur. J. Pharmacol. 57*, 279–281.

Wagner, G. W., Tavianini, M. A., Herrmann, K. M., & Dixon, J. E. (1981) *Biochemistry 20*, 3884–3890.