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Solubilization and Characterization of Two Rat Brain Membrane-Bound Aminopeptidases Active on Met-Enkephalin†

Louis B. Hersh

ABSTRACT: Two aminopeptidases which hydrolyze Met-enkephalin at the Tyr–Gly bond have been solubilized from rat brain membranes and resolved by ion-exchange chromatography. These aminopeptidases are designated MI and MII based on the order in which they are eluted during ion-exchange chromatography. The two aminopeptidases can be distinguished kinetically; aminopeptidase MI hydrolyzes L-arginine β-naphthylamide 17 times faster than L-alanine β-naphthylamide, while only a 1.7-fold difference is exhibited by aminopeptidase MII. Aminopeptidase MII exhibits a higher affinity for amino acid β-naphthylamides, Met-enkephalin, Leu-enkephalin, and the inhibitor puromycin as compared to aminopeptidase MI. Greater than 90% of aminopeptidase MII activity is lost upon dialysis against ethylenediaminetetraacetate (EDTA) but can be reconstituted with $CoCl_2$ and $MnCl_2$. In contrast, aminopeptidase MI loses only 30% of its activity when dialyzed against EDTA. In addition to cleaving the Tyr–Gly bond of Met-enkephalin, aminopeptidase MII also cleaves the Tyr–Gly bond of α- and γ-endorphin. Hydrolysis of Met-enkephalin by intact membranes derived from whole rat brain occurs primarily by cleavage at the Tyr–Gly bond, with this activity attributable to aminopeptidase MII.

Recent studies suggest that the opioid peptides Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) may serve as neurotransmitters (Frederickson, 1977; Smith et al., 1976). Since neurotransmitters are generally inactivated shortly after they are released, recent attention has been focused on the mechanism of inactivation of Met- and Leu-enkephalin. Three mechanisms for the inactivation of enkephalins in brain have been observed: (a) cleavage at the Gly–Phe bond by a membrane bound dipeptidyl carboxypeptidase (Malfroy et al., 1978, 1979; Sullivan et al., 1978; Swerts et al., 1979; Guyon et al., 1979; Gorenstein & Snyder, 1979); (b) cleavage at the Gly–Gly bond by a membrane-bound dipeptidyl aminopeptidase (Gorenstein & Snyder, 1979), and (c) cleavage at the Tyr–Gly bond by soluble (Craves et al., 1978; Dupont et al., 1977; Craviso & Musacchio, 1978; Hambrook et al., 1976; Marks et al., 1977; Meek et al., 1977) and membrane-bound (Jacquet et al., 1976; Knight & Klee, 1978) aminopeptidases.

The soluble aminopeptidase which cleaves the Tyr–Gly bond of enkephalins has been identified as an arylamidase and has

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been purified from monkey brain (Hayashi, 1977; Hayashi & Oshima, 1978), rat brain (Schnebli et al., 1979), human brain (Traficante et al., 1980), and bovine brain (Hersh & McKelvy, 1980; Hersh et al., 1980). On the other hand, the membrane-bound aminopeptidase has not been previously characterized. In this paper the solubilization and characterization of two membrane-bound aminopeptidases from rat brain which are active toward enkephalins is described.

Methods

Preparation of Rat Brain Membranes. Male Sprague-Dawley rats were sacrificed by decapitation. The whole brain minus the cerebellum and lower brain stem was used to prepare the membrane fraction as described by Knight & Klee (1978).

Solubilization and Partial Purification of Aminopeptidases. Rat brain membrane fraction (25 mL) was washed twice by diluting it 15-fold with 50 mM Tris-HCl buffer, pH 7.5, and then centrifuging at 20000*g* for 20 min. The pellet was suspended in 25 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 0.05% Triton X-100 and stirred for 15 min at 4 °C. The suspension was centrifuged at 20000*g* for 30 min and the pellet discarded. The supernatant was applied to a column of Whatman DE-52 (2 × 14 cm) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5. After washing the column with 200 mL of starting buffer, a linear gradient (500-mL total volume) from 0 to 0.5 M NaCl in 50 mM Tris-HCl buffer, pH 7.5, was applied to the column. Fractions containing aminopeptidase activity were pooled and concentrated on an Amicon ultrafiltration apparatus using a PM-10 membrane.

The recovery of aminopeptidase activity from ion-exchange chromatography was approximately 70% as assayed with L-alanine β-naphthylamide as substrate.

Enzyme Assays. The hydrolysis of amino acid β-naphthylamides was measured fluorometrically as previously described (Hersh & McKelvy, 1980). Reaction mixtures routinely contained 50 mM Tris-HCl buffer, pH 7.2, 0.02 mM L-alanine or L-arginine β-naphthylamide, enzyme, and water in a final volume of 0.5 mL. The hydrolysis of enkephalins and endorphins was assayed by measuring tyrosine release using a modification of the method Shoaf et al. (1974). Enzyme and substrate were incubated in 50 mM Tris-HCl buffer, pH 7.2. A 50-μL aliquot of the reaction mixture was taken and the reaction stopped by boiling for 1 min; 200 μL of the phosphate–amino acid oxidase–phenanthroline solution of Shoaf et al. (1974) was added. After a further incubation for 60 min at 37 °C, 200 μL of 56% sulfuric acid was added, and the absorbance at 530 nm was measured. The amount of tyrosine released was calculated from a standard curve prepared under identical conditions.

Analysis of Reaction Products. Reaction products were analyzed by high-performance liquid chromatography for peptides and thin-layer chromatography for amino acids. A Waters Associates radial compression module equipped with a Radial-Pak A (reverse-phase octadecylsilene) column was employed. Peptides derived from enkephalin were separated with an isocratic solvent system consisting of 60% aqueous 1.7 mM phosphoric acid–1.4 mM triethylamine and 40% acetonitrile. Peptides were detected by monitoring absorbance at 210 nm by using a Schoefel 770 detector system. As shown in Figure 1, this system separates the Tyr-Gly + Tyr-Gly-Gly, Gly-Gly-Phe-Met, Tyr-Gly-Gly-Phe, Phe-Met, and Met-enkephalin and can be used to identify cleavage sites in the enkephalin molecule.

Free amino acids liberated from Met-enkephalin were identified by two-dimensional thin-layer chromatography of their dansyl derivatives (Marks et al., 1977).

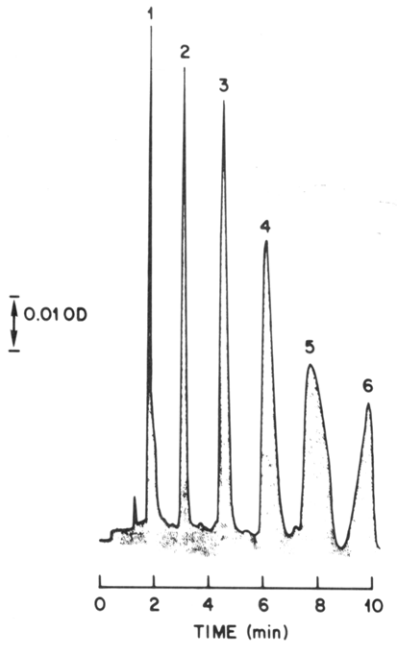

FIGURE 1: Separation of Met-enkephalin analogues by high-performance liquid chromatography. A mixture of potential Met-enkephalin metabolites was run on high-performance liquid chromatography as described under Methods: (1) tyrosine, (2) Tyr-Gly + Tyr-Gly-Gly, (3) Tyr-Gly-Gly-Phe, (4) Gly-Gly-Phe-Met, (5) Met-enkephalin, (6) Phe-Met.

Table I: Distribution of Rat Brain Aminopeptidase[a]

	enzyme activity[b]			
	aminopeptidase		lactate dehydrogenase	
fraction	total units	%	total units	%
crude homogenate	9.2	(100)	420	(100)
soluble fraction	7.5	81.5	395	94.0
membrane fraction	1.9	20.6	3.4	0.8

[a] A homogenate of rat brain was centrifuged at 1000*g* for 10 min to yield the crude supernatant fraction. This was further fractionated into a soluble and membrane fraction by centrifugation at 20000*g* for 30 min. Aminopeptidase activity was determined as described under Methods by use of L-alanine-β-naphthylamide as substrate. Lactate dehydrogenase activity was assayed spectrophotometrically in 1-mL reaction mixtures containing 50 mM potassium phosphate buffer, pH 7.4, 0.1 mM NADH, and 3.0 mM pyruvate. [b] One unit is defined as the formation of 1 μmol of product/min.

Protein Determination. Protein concentration was determined by the method of Dulley & Grieve (1975).

Substrates. Met-enkephalin and α-, β-, and γ-endorphins were obtained from Pennisula Lab., Inc., San Carlos, CA.

Results

Solubilization and Resolution of Rat Brain Membrane-Bound Aminopeptidases. An extract from rat brain was prepared and fractionated according to the procedure of Knight & Klee (1978). When L-alanine β-naphthylamide is used as a substrate, approximately 20% of the aminopeptidase activity was found in the membrane fraction (Table I). As a control, the soluble enzyme lactate dehydrogenase was also measured. Less than 1% of the lactate dehydrogenase activity was found associated with the membrane fraction. Furthermore, when Triton X-100 was added to the membrane fraction to give a final concentration of 1%, no increase was observed in either the aminopeptidase or lactate dehydrogenase activities. Extraction of the membrane fraction with 50 mM

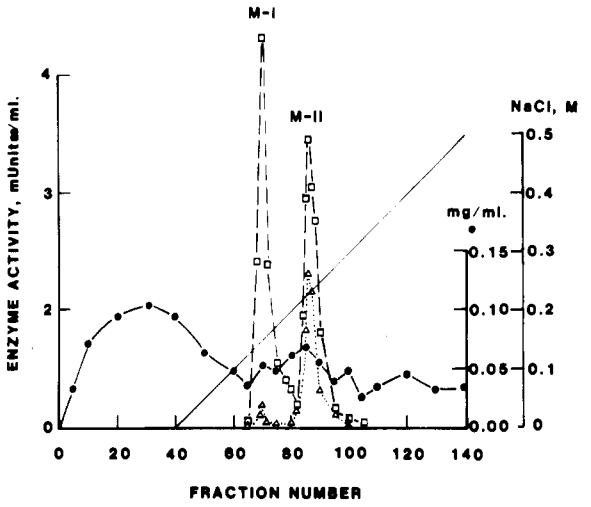

FIGURE 2: Separation of solubilized rat membrane aminopeptidases by ion-exchange chromatography. A rat brain membrane fraction was solubilized with 0.05% Triton X-100 and chromatographed on a column of DE-52 as described under Methods. Fractions of 5 mL were collected and assayed for aminopeptidase activity with L-arginine β-naphthylamide (□) and L-alanine β-naphthylamide (△) as substrate.

Tris-HCl buffer, pH 7.2, or Tris buffer containing 0.5 M sodium chloride did not solubilize the aminopeptidase activity. However, extraction with Tris buffer containing 0.05% Triton X-100 caused the release of over 80% of the aminopeptidase activity. These results serve to rule out the possibility that the aminopeptidase activity associated with the membrane fraction is due to occluded enzyme.

A partial purification of the membrane-bound aminopeptidase activity was achieved by chromatographing enzyme solubilized with 0.05% Triton X-100 on a column of DE-52 ion exchange resin. As shown in Figure 2, two aminopeptidase activity peaks were resolved, both of which reacted with L-alanine β-naphthylamide and L-arginine β-naphthylamide as substrates. In addition, both fractions hydrolyzed Met-enkephalin. These two aminopeptidase peaks were designated M-I and M-II according to the order in which they were eluted from the DE-52 column. Fractions 68–73 (MI) and fractions 84–90 (MII) were pooled and concentrated on an Amicon ultrafiltration apparatus using a PM-10 membrane for further characterization.

On the basis of the activity of the original membrane fraction, this purification procedure resulted in an 11.6-fold purification of the aminopeptidase MI fraction. This fraction had a specific activity of 14 munits/mg protein with L-arginine β-naphthylamide as substrate, 0.6 munit/mg protein with L-alanine β-naphthylamide as substrate and 10.4 munits/mg protein with Met-enkephalin as substrate. The aminopeptidase MII fraction was purified 10-fold and had a specific activity of 12.3 munits/mg protein with L-arginine β-naphthylamide as substrate, 6.2 munits/mg protein with L-alanine β-naphthylamide as substrate, and 78.6 munits/mg protein with Met-enkephalin as substrate.

Both aminopeptidase fractions are relatively impure since each contains six to eight major protein bands as determined by disc gel electrophoresis. Attempts to identify the aminopeptidase band after disc gel electrophoresis have been unsuccessful to date. On the other hand, the only enkephalinase activity associated with these two fractions appears to be attributable to aminopeptidase activity. Dipeptidyl carboxypeptidase activity, assayed with hippurylhistidylleucine as substrate (Yang & Neff, 1972), was less than 0.5 munit/mg in the aminopeptidase MI fraction and less than 0.1 munit/mg in the aminopeptidase MII fraction. Analysis of the products

Table II: Kinetic Constants for Membrane Aminopeptidases[a]

	aminopeptidase MI	aminopeptidase MII
V_m(L-Arg-β-NA)/V_m(L-Ala-β-NA)	13.6	1.7
K_m(L-Arg-β-NA) (μM)	26	2.5
K_m(L-Ala-β-NA) (μM)	250	25
K_I(Met-enkephalin) (μM)	490 (510)	18 (16)
K_I(Leu-enkephalin) (μM)	2600 (2200)	23 (20)
K_I(puromycin) (μM)	1100	1.2

[a] Amino acid β-naphthylamide hydrolysis was measured as described under Methods. Inhibition studies were conducted at a constant concentration of amino acid β-naphthylamide (25 μM and 2.5 μM L-arginine β-naphthylamide for aminopeptidases MI and MII, respectively) and variable concentrations of inhibitor. Apparent K_I values were determined from plots of the reciprocal of the observed velocity vs the concentration of inhibitor. K_I values were calculated from the relationship apparent $K_I = K_I(1 + [S]/K_{ms})$. The values listed in the table are those obtained with L-arginine β-naphthylamide as a substrate while those in parentheses were obtained with L-alanine β-naphthylamide as a substrate.

obtained after hydrolysis of Met-enkephalin by high-performance liquid chromatography showed Gly-Gly-Phe-Met as the only product with either aminopeptidase MI or aminopeptidase MII as the source of enzyme. Cleavage at the Tyr–Gly was confirmed by identification of tyrosine, as its dansyl derivative, as the only amino acid product derived from Met-enkephalin hydrolysis.

Kinetics Properties of Membrane Aminopeptidases. The kinetic constants for the hydrolysis of L-alanine β-naphthylamide and L-arginine β-naphthylamide by aminopeptidases MI and MII are given in Table II. Although both substrates are hydrolyzed by aminopeptidase MI and MII, these two enzymes are clearly distinguishable. Aminopeptidase MI hydrolyzes L-arginine β-naphthylamide more than 13 times faster than it hydrolyzes L-alanine β-naphthylamide, while less than a 2-fold variation in maximal velocity is seen when the same substrates are hydrolyzed by aminopeptidase MII. Furthermore, the affinity of aminopeptidase MI for amino acid β-naphthylamides is an order of magnitude lower as compared to aminopeptidase MII.

Both aminopeptidases MI and MII are inhibited by puromycin; however, aminopeptidase MI is 1000-fold less sensitive to puromycin inhibition as compared to aminopeptidase MII. Both Met- and Leu-enkephalin are competitive inhibitors of aminopeptidase MI and MII catalyzed hydrolysis of amino acid β-naphthylamides (Table II). The affinity of aminopeptidase MII for enkephalin is 30–100-fold greater than that observed with aminopeptidase MI.

As shown in Table II, the inhibition constants obtained for Met-enkephalin or Leu-enkephalin as inhibitor are the same when either L-arginine β-naphthylamide or L-alanine β-naphthylamide are used as substrate. These data provide evidence that the aminopeptidase and enkephalinase activities associated with fractions MI and MII are due to a single enzyme.

On the basis of the observation that aminopeptidase fractions MI and MII hydrolyzed enkephalins by cleavage of the Tyr–Gly bond, the rate of tyrosine release from Met-enkephalin was quantitated by using the method of Shoaf et al. (1974) and compared to the rate of tyrosine release from α-, β-, and γ-endorphins. As shown in Table III, aminopeptidase MII not only hydrolyzed the Tyr–Gly bond of Met-enkephalin but also cleaved the same bond in α- and γ-endorphins, albiet at 18–22% of the rate. No hydrolysis of β-endorphin was detected under the assay conditions employed. Aminopeptidase MI hydrolyzed Met-enkephalin at about 13% of the rate observed with aminopeptidase MII. The hydrolysis of

Table III: Hydrolysis of Met-Enkephalin and Endorphins by MI and MII[a]

	V [nmol min^{-1} (mg of protein)$^{-1}$]			
	Met-Enk	α-endorphin	γ-endophorin	β-endorphin
aminopeptidase M-I	10.4	ND[b]	ND	ND
aminopeptidase M-II	78.6	14.4	17.0	ND

[a] Reaction mixtures containing 50 mM Tris-HCl buffer, pH 7.2. 0.4 mM substrate, enzyme, and water in a final volume of 175 μL were incubated at 37 °C. At various times, a 50-μL aliquot was withdrawn and boiled for 1 min, and liberated tyrosine was determined as described under Methods. [b] ND, not detectable.

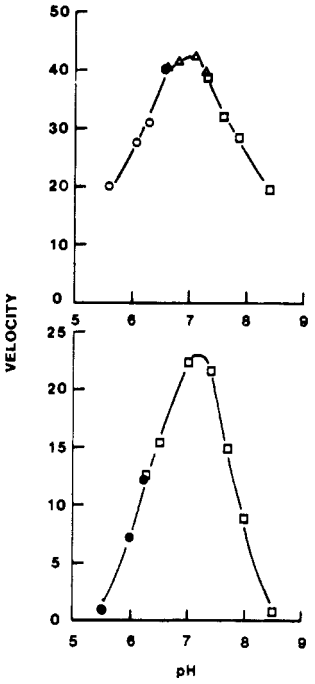

FIGURE 3: pH–rate profile for aminopeptidases MI and MIII. (Top): pH–rat profile for aminopeptidase MII using 0.05 mM L-arginine β-naphthylamide as substrate. (Bottom): pH–rate profile for aminopeptidase MI using 0.5 mM L-arginine β-naphthylamide as substrate. Velocity expressed as nmol min^{-1} (mg of protein)$^{-1}$. The following buffers were used at a final concentration of 50 mM: (○) morpholinoethanesulfonic acid; (△) morpholinopropanesulfonic acid; (□) Tris-HCl; (●) acetate. The pH of each assay mixture was measured after the initial velocity was determined.

endorphins by this aminopeptidase was not observed; however, if these substrates were hydrolyzed at ~20% the rate of Met-enkephalin, the amount of liberated tyrosine would not be detectable under our assay conditions.

At 50 μM puromycin the aminopeptidase MII catalyzed hydrolysis of 0.2 mM Met-enkephalin was inhibited more than 95% while less than 7% inhibition of Met-enkephalin hydrolysis by aminopeptidase MI was observed under identical conditions. This finding is consistent with the data presented in Table II in which puromycin was shown to be a potent inhibitor of aminopeptidase MII but a poor inhibitor of aminopeptidase MI. This finding provides additional evidence that both aminopeptidase and enkephalinase activities are associated with the same enzyme.

pH–Rate Profiles. The pH–rate profiles for aminopeptidases MI and MII were similar, both enzymes exhibiting a pH optima of around 7.0 (Figure 3). However, during the course of determining the pH optima for aminopeptidase MI, we consistently noted lower reaction rates in phosphate buffer

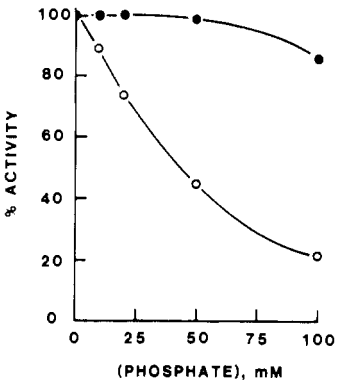

FIGURE 4: Inhibition of aminopeptidase MI by phosphate. Aminopeptidase MI activity was measured with 0.5 mM L-arginine β-naphthylamide as substrate in 50 mM Tris-HCl buffer, pH 7.2, in the presence and absence of the concentrations of potassium phosphate buffer, pH 7.2, indicated (○). After the assay was complete, the pH was measured and shown not to vary more than ±0.05 pH unit. The same experiment was conducted with aminopeptidase MI at 0.5 mM L-arginine β-naphthylamide and 0.05 mM L-arginine β-naphthylamide. Only the data for 0.5 mM L-arginine β-naphthylamide are shown (●).

Table IV: Reconstitution of Apoaminopeptidase MII Activity by Divalent Cations[a]

sample	activity (%)[b]
untreated enzyme	(100)
EDTA-treated enzyme	9.4
EDTA-treated enzyme + 1 mM $CoCl_2$	142.7
EDTA-treated enzyme + 1 mM $MnCl_2$	53.3
EDTA-treated enzyme + 1 mM $MgCl_2$	13.3
EDTA-treated enzyme + 1 mM $ZnSO_4$	2.7

[a] An aliquot of the aminopeptidase MII fraction was dialyzed against 100 volumes of 50 mM Tris-HCl buffer, pH 7.4 (untreated enzyme), or 50 mM Tris-HCl buffer, pH 7.4, containing 20 mM EDTA for 12 h, and then for an additional 12 h against 50 mM Tris-HCl buffer, pH 7.4. Activity was measured as described under Methods with 0.08 mM L-arginine β-naphthylamide as substrate. Reactivation of enzyme activity was accomplished by adding the metal ion directly to the assay mixture. No significant effect of any of the metal ions listed above was observed at a final concentration of 0.1 mM. [b] Activity is expressed as the percent of the activity of the untreated enzyme.

as compared to Tris buffer. As shown in Figure 4, aminopeptidase MI is inhibited by increasing concentrations of phosphate buffer while aminopeptidase MII is relatively insensitive to the concentration of phosphate buffer. The effect of phosphate buffer on aminopeptidase MI is not simply an ionic strength effect as the addition of 200 mM sodium chloride (equivalent to ~80 mM potassium phosphate at pH 7.2) has no inhibitory effect.

Effect of Metal Ions on Aminopeptidases MI and MII. In order to assess the importance of metal ions for the activity of aminopeptidases MI and MII, each enzyme was dialyzed against 50 mM Tris-HCl buffer, pH 7.2, containing 20 mM EDTA, and then against the Tris buffer alone. This treatment resulted in a loss of over 90% of the activity of aminopeptidase MII, while less than 30% of the activity of aminopeptidase MI was lost. As shown in Table IV, the addition of 1 mM $CoCl_2$ to the EDTA-treated aminopeptidase MII restored activity to 143% of the control value while $MnCl_2$ restored activity to ~50% of the control value. No effect of $MgCl_2$ was observed, while $ZnSO_4$ appeared to inhibit the enzyme. Although not shown, no significant effect of any of the metal ions listed in Table IV was observed at a concentration of 0.1 mM.

Hydrolysis of Met-Enkephalin by Intact Membrane. The relative contribution of aminopeptidase activity in hydrolyzing Met-enkephalin by intact rat brain membranes was examined.

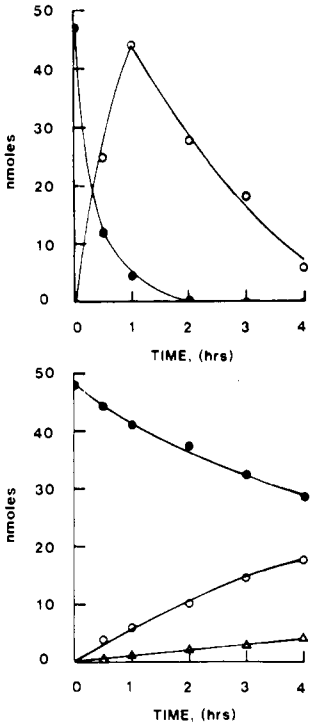

FIGURE 5: Hydrolysis of Met-enkephalin by rat brain membranes. Reaction mixtures containing 50 mM Tris-HCl buffer, pH 7.4, 0.33 mM Met-enkephalin, and 1.0 mg/mL of a rat brain membrane fraction were incubated at 37 °C. At the time periods indicated, a 400-μL aliquot was taken, boiled for 1 min, and filtered through a 0.22-μm Millipore filter. After lyopholization, the sample was taken up in water and analyzed by high-performance liquid chromatography as described under Methods. The data were quantitated by comparison to standard curves of peptides run under identical conditions. (●) Met-enkephalin; (○) Gly-Gly-Phe-Met; (△) Tyr-Gly-Gly. (Top) Met-enkephalin hydrolysis by intact membranes. (Bottom) Met-enkephalin hydrolysis by intact membranes in the presence of 100 μM puromycin.

Met-enkephalin was incubated with a membrane fraction for various time periods, and the formation of products was determined by high-performance liquid chromatography. As shown in Figure 5, the major product of Met-enkephalin degradation under these conditions is Gly-Gly-Phe-Met, which increases in concentration over the first hour and then decreases in concentration. In the presence of 100 μM puromycin, both Met-enkephalin degradation and Gly-Gly-Phe-Met appearance are inhibited approximately 90%. Under these conditions, Tyr-Gly-Gly appears as a product of Met-enkephalin hydrolysis. Although not shown, Gly-Gly-Phe-Met breakdown by the membrane fraction was not inhibited by puromycin. In addition, Phe-Met was rapidly hydrolyzed by the membrane fraction.

Discussion

Two membrane-bound aminopeptidase fractions have been solubilized and resolved by ion-exchange chromatography. Several lines of evidence have been presented which suggest that both enkephalinase activity and aminopeptidase activity are associated with the same enzymes: first, amino acid β-naphthylamide hydrolysis is competitively inhibited by enkephalins with the same inhibition constants obtained independent of the substrate used; second, amino acid β-naphthylamide hydrolysis and enkephalin hydrolysis show the same sensitivity to puromycin inhibition. The aminopeptidase designated M-II resembles the previously described soluble aminopeptidase from rat brain (Schnebli et al., 1979) in that both enzymes bind Met-enkephalin with an affinity constant in the range of 20–30 μM, both hydrolyze amino acid β-naphthylamides, both are metalloenzymes, and both are inhibited by puromycin in the micromolar range.

Although both of the aminopeptidase fractions described in this study hydrolyze Met-enkephalin, it appears that the low affinity of aminopeptidase MI for enkephalins (0.5 mM for Met-enkephalin and 2.4 mM for Leu-enkephalin) would preclude this enzyme from significantly contributing to enkephalin hydrolysis "in vivo". This conclusion is supported by the observation that Met-enkephalin hydrolysis by intact membranes is inhibited approximately 90% by 100 μM puromycin. Since puromycin inhibition of aminopeptidase MI has an inhibition constant of 1 mM, no significant inhibition of this aminopeptidase would be expected at 100 μM puromycin.

The pattern of Met-enkephalin breakdown by the whole rate brain membrane fraction differs from that reported by Guyon et al. (1979) who used a particulate fraction from mouse striatum. In the latter study there was a significant formation of Tyr-Gly-Gly attributable to a dipeptidyl carboxypeptidase activity which they named "enkephalinase". In agreement with the studies of Guyon et al. (1979), we found that the further degradation of Gly-Gly-Phe-Met is not inhibited by puromycin and appears to be attributable to the action of this "enkephalinase".

The question as to whether the membrane-bound aminopeptidase MII characterized in this study, the dipeptidyl carboxypeptidase described by several laboratories (Malfroy et al., 1978, 1979; Sullivan et al., 1978; Swerts et al., 1979; Guyan et al., 1979; Gorenstein & Snyder, 1979), or the dipeptidyl aminopeptidase described by Gorenstein & Snyder (1979) act as true enkephalinases in vivo remains to be elucidated.

It is well documented that tyrosine liberation from enkephalin represents the major degradative route of exogenously added enkephalin (Chang et al., 1976; Dupont et al., 1977; Jaquet et al., 1976). However, these results do not prove that endogenous enkephalins are metabolized by the same mechanism. Studies by both Sullivan et al. (1978) and Malfroy et al. (1979) demonstrated a correlation between the distribution of dipeptidyl carboxypeptidase activity and enkephalin binding in various brain regions. Aminopeptidase activity did not correlate with enkephalin binding activity but was more active than the dipeptidyl carboxypeptidase activity in all of the brain regions examined.

Although it is not clear as to whether membrane-bound aminopeptidases function "in vivo" as enkelphalinases in an analogous role to acetylcholinesterase, it is likely that these enzymes function in the conversion of β-endorphin to des-tyrosine α- and γ-endorphins as reported by Burbach et al. (1980). Clearly further studies are required before the precise role of these aminopeptidases in neuropeptide degradation can be delineated.

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Isolation and Identification of 24(*R*)-Hydroxyvitamin D_3 from Chicks Given Large Doses of Vitamin D_3†

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ABSTRACT: A new metabolite of vitamin D was isolated from the blood plasma of chicks given large doses of vitamin D_3. The isolation involved methanol–chloroform extraction and four column chromatographic steps. The metabolite was identified by high- and low-resolution mass spectroscopy, chemical derivatization, and comigration with authentic standard as 3β,24(*R*)-dihydroxy-9,10-seco-5,7,10(19)-cholestatriene [24(*R*)-hydroxyvitamin D_3]. No detectable 24(*R*)-hydroxyvitamin D_3 was recovered from 16 L of plasma from chicks receiving physiologic levels of vitamin D_3.

The toxicity of vitamin D in mammals when administered at elevated levels is well established (DeLuca, 1978). The mechanism of this toxic effect, however, has received little attention and is poorly understood.

Gross changes in the plasma concentrations of the known vitamin D metabolites in rats have been shown to occur on administration of toxic and subtoxic amounts of vitamin D_3 and 25-hydroxyvitamin D_3 (25-OH-D_3)[1] (Shepard & DeLuca, 1980). Use of this fact led to the recent identification of 25-OH-D_3-26,23-lactone (Wichmann et al., 1979). This metabolite is present in plasma at <1 ng/mL under physiologic conditions; however, at elevated vitamin D_3 levels, it becomes one of the major circulating metabolites.

The work described here was initiated to determine if other vitamin D_3 metabolites which normally are not present or are present at nondetectable levels are found in plasma of animals receiving large amounts of vitamin D_3. We wish to report the isolation and identification of 24(*R*)-hydroxyvitamin D_3 [24(*R*)-OH-D_3] from plasma of chicks receiving massive doses of vitamin D_3. This metabolite was not detected in plasma of chicks receiving physiologic levels of vitamin D_3.

Materials and Methods

Plasma Procurement and Extraction. Plasma Sample from Chicks Given High Doses of Vitamin D_3. Eighty 12-week-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, WI) raised on standard chicken mash were dosed intramuscularly with 10^5 IU of vitamin D_3 (Aldrich Chemicals, Milwaukee, WI) in 50 μL of ethanol daily for 3 days. The purity of the vitamin D_3 was found to be at least 99% by high-performance LC. On the fourth day, each chick received a total of 10^7 IU of vitamin D_3 dissolved in 500 μL of ethanol in four

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[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 24(*R*)-OH-D_3, 24(*R*)-hydroxyvitamin D_3; 24(*S*)-OH-D_3, 24(*S*)-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; LC, liquid chromatography.

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