Identification of Aminopeptidase M as an Enkephalin-Inactivating Enzyme in Rat Cerebral Membranes

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ABSTRACT: Two membrane-bound enkephalin-hydrolyzing aminopeptidase activities were partially purified from rat brain membranes. The first, which represents 90% of the total activity, was highly sensitive to both puromycin (K_i = 1 μM) and bestatin (K_i = 0.5 μM). The second was inhibited much more by bestatin (K_i = 4 μM) than by puromycin (K_i = 100 μM). The latter puromycin-insensitive aminopeptidase was found to resemble aminopeptidase M purified from rat kidney brush border membranes. Both displayed the same purification pattern and the same kinetic constants of substrates and inhibitors, and both were similarly inactivated by metal chelating agents. Moreover, antibodies raised in rabbits against rat kidney aminopeptidase M inhibited the aminopeptidase activities of both kidney and brain puromycin-insensitive enzymes at similar dilutions, while the brain puromycin-sensitive aminopeptidase activity was not affected. Thus, aminopeptidase M (EC 3.4.11.2) was found to occur in brain, and the role of this enzyme in inactivating endogenous enkephalins released from their neuronal stores is suggested.

Like other neurotransmitters, the enkephalins appear to be rapidly inactivated following their release from cerebral neurons. Recently much work has been devoted to identifying the peptidases involved [reviewed by Schwartz et al. (1981, 1982), Schwartz (1983), and Hui & Lajtha (1983)]. Since the opioid pentapeptides are substrates for a number of peptidases, some of which may not be involved in their physiological inactivation, inhibitors are important tools for identifying the true "neuropeptidases".

Selective inhibition of "enkephalinase", a membrane-bound peptidase that hydrolyzes the Gly^3-Phe^4 amide bound and which is found in brain (Malfroy et al., 1978) and peripheral organs like the kidney (Llorens et al., 1981; Almenoff et al., 1981; Malfroy & Schwartz, 1982, 1984; Fulcher et al., 1982), partially protects endogenous enkephalins from being extensively inactivated (Patey et al., 1981) and induces analgesia (Roques et al., 1980).

In addition, aminopeptidase involvement in enkephalin inactivation was suggested both by the analgesic effects of the inhibitor bestatin (Zhang et al., 1982; Hashisu et al., 1982; Chaillet et al., 1983; De la Baume et al., 1983) and by the complete protection of endogenous enkephalins released from brain slices in the presence of this compound together with thiorphan, an enkephalinase inhibitor (De la Baume et al., 1983). In contrast, puromycin (100 μM), another potent aminopeptidase inhibitor (Barclay & Phillips, 1978; Knight & Klee, 1978; Vogel & Alstein, 1979; Hersh et al., 1981; Cohen et al., 1983), did not significantly protect enkephalin from degradation, when studied in the same model (Patey et al., 1981). In order to determine whether a bestatin-sensitive, puromycin-insensitive aminopeptidase is in fact involved as suggested by De la Baume et al. (1983) and Schwartz (1983), it was necessary to study the properties of isolated enzymes.

Although soluble enkephalin-hydrolyzing aminopeptidases have been purified from mammalian brains (Hayashi, 1978; Schnebli et al., 1979; Hayashi & Oshina, 1980; Hersh, 1981a; Traficante et al., 1980; Vogel & Alstein, 1980; Hersh & Mc Kelvy, 1981; Wagner et al., 1981; Kelly et al., 1983), these are less likely than ectoenzymes to be involved in inactivating messengers released into the extracellular space, as previously discussed (Malfroy et al., 1978; Schwartz et al., 1981; Hui & Lajtha, 1983).

Two membrane-bound aminopeptidase activities from rat brain membranes were previously resolved by ion-exchange chromatography and characterized, although not identified (Hersh, 1981b). Interestingly, one of them, designated MI, was poorly sensitive to inhibition by puromycin, whereas the second and major one, designated MII, apparently similar to the aminopeptidase recently purified close to homogeneity by Hui et al. (1983), seemed highly sensitive to puromycin. The first one seemed a likely candidate for a neuropeptidase function but was neither extensively purified nor its specificity studied in details particularly regarding sensitivity to bestatin.

We have presently undertaken to purify membrane-bound enkephalin-hydrolyzing aminopeptidases from rat brain, trying particularly to identify an activity not inhibited by puromycin under conditions similar to those of release experiments with brain slices (Patey et al., 1981; De la Baume et al., 1983). After initial experiments suggesting that aminopeptidase M is present in brain, this possiblity was more extensively studied by undertaking parallel purification from kidney brush borders in which this peptidase represents about 5% of the proteins (Mc Donald & Schwabe, 1977; Kenny, 1977).

Materials and Methods

Chemicals. [*Tyr*-3,5-^{3}H]-Met^5-enkephalin (50 Ci/mmol) was purchased from Amersham International plc (Amersham, U.K.). DEAE-cellulose DE-52 was from Whatman Chemical Separation Ltd. (England), Concanavalin A–Sepharose, PBE 94 gel, and PB 74 buffer were from Pharmacia Fine Chemicals AB (Uppsala, Sweden), and polystyrene beads (Porapak Q) were from Waters Associates, Inc. (Milfor, MA). D-Mannitol was purchased from Serva Fine Biochemicals Inc. (New York); Leu^5- and Met^5-enkephalins and aminoacyl-β-naphthylamides were from Bachem, Feinchemikalein AG (Bubendorf, Switzerland). Bestatin was a generous gift from R. Bellon Laboratories (Neuilly/Seine, France). Thiorphan was kindly donated by Prof. B. P. Roques, and captopril was from Squibb Institute. Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

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Protein Determination. Protein content was determined with a Bio-Rad Protein Assay Kit using bovine serum albumin as standard. Triton X-100 at concentrations up to 0.05% did not interfere with protein measurements.

Determination of Aminopeptidase Activity. Enzyme fractions were incubated at 37 °C in a final volume of 100 μL in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.5, in the presence of 40 nM [^{3}H]-Met^5-enkephalin, 2 μM captopril, an angiotensin-converting enzyme inhibitor (Cushman et al., 1977), and 0.2 μM thiorphan, an enkephalinase inhibitor (Roques et al., 1980). After periods of 5–30 min, the reaction was stopped by addition of 50 μL of 0.1 N HCl and enzyme activity measured according to Vogel & Altstein (1977): 100 μL of the reaction mixture was placed on a column containing 80 mg of polystyrene beads equilibrated in water. Whereas the intact substrate was retained on the column, [^{3}H]Tyr (and [^{3}H]Tyr-Gly) was eluted with 2 mL of water and the radioactivity of the eluate counted.

Results were expressed in moles of Met^5-enkephalin degraded per minute per milliliter of enzyme fraction.

The [^{3}H]-Met^5-enkephalin fragments obtained during incubation with enzyme fractions were identified by combined polystyrene beads chromatography, selective enzymatic decarboxylation of tyrosine, and thin-layer chromatography (TLC) (De la Baume et al., 1983). Spots were cut out, and their radioactivity was determined by liquid scintillation spectrophotometry.

The kinetic constants for enkephalins aminoacyl β-naphthylamides, and inhibitors were determined with purified aminopeptidase fractions.

In the case of aminoacyl β-naphthylamides, enzyme activities were determined by measurement of naphthylamine release using an Aminco-Bowman spectrofluorometer. The reaction was carried out at 37 °C in a final volume of 1 mL by using a substrate concentration from 10 to 100 μM and was stopped by adding 10 μL of 10^{-2} M bestatin.

Purification of Rat Membrane-Bound Aminopeptidase Activities. All purification steps were performed at 4 °C.

(*1*) *Preparation of Rat Brain Membranes.* Ten Wistar male rats were decapitated. The whole brains (minus cerebellum and lower brain stem) were homogenized in 10 volumes of 50 mM Tris-HCl buffer, pH 7.5, in a Teflon–glass homogenizer. The homogenate was centrifuged at 1000*g* for 10 min and the supernatant centrifuged again at 100000*g* for 20 min. The resulting pellet was washed 3 times by suspension in Tris buffer and centrifugation and was kept for solubilization.

(*2*) *Preparation of Rat Kidney Brush Border Membranes.* The kidneys of 10 Wistar male rats were rapidly collected after sacrifice and homogenized in 5 mM Tris-HCl buffer, pH 7.5, containing 125 mM D-mannitol and 12 mM $MgCl_2$ according to Biber et al. (1981). The homogenate was centrifuged at 1000*g* for 15 min and the supernatant decanted and centrifuged at 7000*g* for 120 min. The resulting pellet was resuspended in the above buffer diluted in water (D-mannitol concentration 62.5 mM) and left at 4 °C for 30 min. The suspension was then centrifuged at 1000*g* for 15 min and the resulting supernatant centrifuged again at 7000*g* for 120 min, yielding the microsomal pellet used for solubilization.

(*3*) *Solubilization of Membrane Fractions.* The cerebral and renal membrane fractions were solubilized by suspension in 50 mL of 50 mM Tris-HCl buffer, pH 7.5, containing appropriate amounts of Triton X-100. The suspension was stirred for 2–3 h at 25 °C and then centrifuged at 100000*g* for 30 min. The recovered supernatant was stored at 4 °C in the presence of 5 mM β-mercaptoethanol.

(*4*) *Con A–Sepharose Chromatography.* After solubilization, the supernatant was applied to a Con A–Sepharose column (2.5 × 15 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1% Triton X-100. After the column was washed with initial buffer, elution was carried out with 0.2 M methyl α-D-mannoside in Tris buffer and 0.1 % Triton X-100 at a flow rate of 10 mL/h, and 3-mL fractions were assayed for enzyme activity in the presence and absence of 10 μM puromycin.

(*5*) *DEAE-cellulose Chromatography.* The fractions with aminopeptidase activity were combined and loaded on a DEAE-cellulose DE-52 column (2.5 × 15 cm) equilibrated in 50 mM Tris-HCl buffer and 0.1 % Triton X-100 and eluted with a linear gradient (0–300 mM NaCl) in the same buffer at a flow rate of 30 mL/h. Fractions were tested for enzyme activity as previously described.

(*6*) *Chromatofocusing.* The active enzyme fractions from DEAE-cellulose chromatography were dialyzed against 25 mM histidine hydrochloride buffer, pH 7.0, containing 0.2 % Triton X-100 in order to eliminate traces of NaCl, then applied to a PBE 94 gel packed in a column (1.5 × 20 cm), and equilibrated with the above buffer. Elution was carried out with 12.5% polybuffer PB 74 adjusted to pH 4.0 with 0.1 N HCl in order to obtain a pH gradient from 4 to 7. Fractions (3 mL) were collected, 9 out of 10 immediately neutralized with 500 μL of 1 M ammonium carbonate and 1 of the 10 kept for pH measurement. Fractions with enzyme activity were pooled and concentrated on an Amicon PM10 membrane filter.

(*7*) *Production of Antibodies.* New Zealand female rabbits received an initial injection of purified rat kidney aminopeptidase emulsified in Freund's complete adjuvant followed by 10 injections at 10-day intervals in incomplete adjuvant. Doses (from 200 μg of immunogen per animal at the beginning to 50 μg at the end of immunization) were given subcutaneously in multiple sites. Bimonthly bleedings were obtained, and antibody production was controled as follows: increasing dilutions of antiserums were incubated in 50 mM Tris-HCl buffer, pH 7.5, with a known amount of rat kidney aminopeptidase. After 24 h at 4 °C, aminopeptidase activity was measured in the reaction mixture, and the presence of antibodies was detected by inhibition of enzyme activity. A purified IgG fraction was obtained from the serum by sodium sulfate precipitation and chromatography on DEAE-Sephadex A-50.

(*8*) *Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE).* Polyacrylamide gel electrophoresis was carried out under denaturing conditions by using 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (Weber & Osborn, 1969). Prior to electrophoresis, samples were brought to a concentration of 2% SDS and 5% β-mercaptoethanol and heated at 95 °C for 5 min. The molecular weights of active fractions were roughly determined by comparison with migration of standard protein markers: myosin, M_r 200000; β-galactosidase, M_r 116000; phosphorylase B, M_r 92500; bovine serum albumin, M_r 66200; ovalbumin, M_r 45000. The proteins separated by SDS–PAGE were either stained with silver (Wray et al., 1981) or blotted (Towbin et al., 1979) onto nitrocellulose paper where they formed an immobilized replica of the original gel. Protein transfer was performed electrophoretically in 25 mM Tris and 192 mM glycine buffer, pH 8.3, containing 20% methanol. The blots were sliced into 0.5-cm strips which were individually assayed by immunological methods: they were soaked in 3% bovine serum albumin in PBS (9 mM phosphate, pH 8, and 140 mM NaCl) for 1 h to block unoccupied protein binding

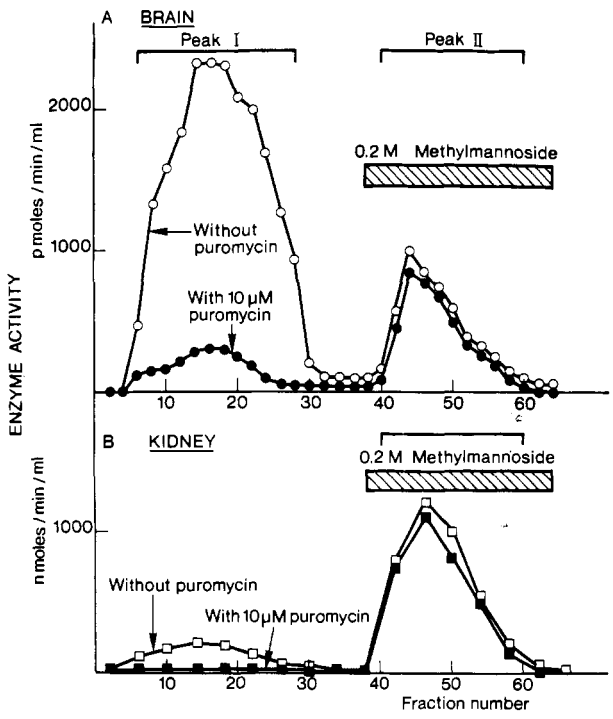

FIGURE 1: Concanavalin A–Sepharose chromatography of soluble extracts obtained after treatment by detergent of brain and kidney membranes. (A) The supernatant obtained after 1% Triton X-100 treatment of the rat brain membrane fraction was loaded onto a 2.5 × 10 cm column and then washed with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 % Triton X-100. Elution was initiated by 0.2 M methyl α-D-mannopyranoside. Aminopeptidase activities were evaluated in the absence (open circles) and presence (closed circles) of 10^{-5} M puromycin. Peak I and peak II fractions were pooled. (B) The supernatant obtained after 5% Triton X-100 treatment of rat kidney. The microsomal fraction was loaded onto a 2.5 × 10 cm column, and elution was performed as in (A). Open squares correspond to enzyme activity measurements without puromycin and closed squares to measurements in the presence of 10^{-5} M puromycin. Peak II fractions were pooled.

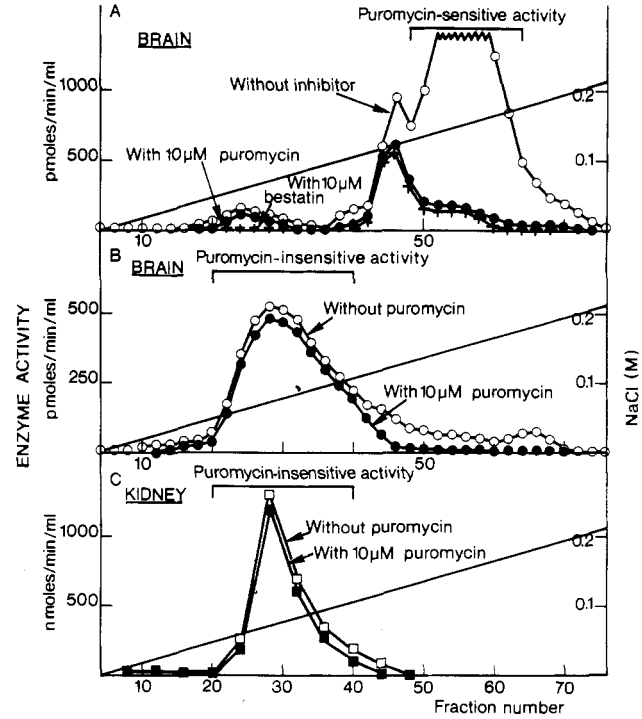

FIGURE 2: DEAE-cellulose DE-52 chromatography of active fractions pooled from Con A–Sepharose chromatography. (A) Rat brain peak I pooled fractions were applied to a 2.5 × 20 cm column, which was first washed with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 % Triton X-100, and then eluted with a linear NaCl gradient (0–300 mM) (transverse line) in the same buffer. Fractions (5 mL) were analyzed for enzyme activity without puromycin (open circles), in the presence of 10^{-5} M puromycin (closed circles), or in the presence of 10^{-5} M bestatin (+). (B) Rat brain peak II pooled fractions were treated as in (A). Fractions 20–40 were pooled, corresponding to the rat brain puromycin insensitive activity. (C) Rat kidney peak II pooled fractions were treated as in (A). Enzyme activity was determined in the absence of puromycin (open squares) or in the presence of 10^{-5} M puromycin (closed squares). Fractions 20–40 were pooled.

sites on the nitrocellulose sheet and then washed in PBS for 2 h. They were then incubated at 37 °C for 2 h with the antiserum in appropriate dilution, or with normal rabbit serum at the same dilution for controls, washed again in PBS (three changes) for 2 h, and reincubated for 1 h at 37 °C with the peroxidase–conjugated anti-rabbit IgG antibody in adequate dilution. After several washes as above, the color reaction was developed by soaking the blots in a diaminobenzidine solution (0.5 mg/mL 0.1 M Tris-HCl buffer, pH 7.6, and 0.03% H_2O_2) and was terminated by washing with water.

RESULTS

Purification of Rat Brain Membrane-Bound Aminopeptidase Activities. Since previous studies suggested that aminopeptidase activities are uniformly located in the rat brain, the whole brain minus cerebellum and lower brain stem was used as a source of enzyme. The membrane pellet was extensively washed in order to eliminate any contaminating soluble aminopeptidase activity, which is by far the largest enzyme activity in the homogenate. In preliminary experiments, the pellet was solubilized by using Triton X-100 in concentrations increasing from 0.05% to 10%. A 1% detergent treatment with stirring at 25 °C for 2–3 h yielded optimal recovery of aminopeptidase activity. At higher concentrations of detergent, solubilization was not improved, whereas contamination by enkephalinase (EC 3.4.24.11), which is less soluble in 1% Triton X-100, was increased. The yield of aminopeptidase activity was about 150%; 50 mM β-mercaptoethanol was added to the detergent extract in order to stabilize enzyme activity during storage. As shown in Figure 1A, with Con A–Sepharose chromatography it was possible to separate two kinds of aminopeptidase activities: the first, which represented 90% of the total activity, corresponded to unretained material and was almost completely inhibited by 10^{-5} M puromycin (peak I); the second, eluted by 0.2 M methyl α-D-mannopyranoside (peak II), was not affected by 10^{-5} M puromycin and corresponded to the remaining activity, about 10%.

Further purification of these two peaks was performed by DEAE-cellulose chromatography (Figure 2A,B). Peak I was resolved into three peaks of enzyme activity (Figure 2A). The first, eluting between 50 and 100 mM NaCl, presumably corresponded to contamination by a fraction of the puromycin-insensitive activity not retained on the Con A–Sepharose column. The second, eluting between 100 and 120 mM NaCl, was not inhibited by 10^{-5} M bestatin or 10^{-5} M puromycin. This peak corresponded to a dipeptidylaminopeptidase activity, as revealed by the formation of [^{3}H]Tyr-Gly (isolated by TLC) when incubated with [^{3}H]-Met^5-enkephalin. The third, which was the largest peak, eluted between 130 and 180 mM NaCl and was strongly inhibited by 10^{-5} M puromycin.

This puromycin-sensitive fraction was not further purified. Its enzymatic properties were compared to those of the puromycin-insensitive fraction.

Peak II was quantitatively retained on DEAE-cellulose and eluted between 50 and 100 mM NaCl in a single peak, the

Table I: Purification of Puromycin-Insensitive Aminopeptidase Activities Solubilized from Rat Brain and Kidney Membranes

fraction	total protein (mg)		total activity (pmol/min)		specific activity (pmol min^{-1} mg^{-1})		yield (%)		purification factor (x-fold)	
	brain	kidney	brain	kidney	brain	kidney	brain	kidney	brain	kidney
homogenate[a]	500	955	435	430000	0.9	450	100	100	1	1
Triton X-100 supernatant[a]	370	450	650	585000	1.7	1300	149	136	1.9	2.9
Con A–Sepharose (peak II)	19	74	330	481000	17	6500	76	112	19	14
DEAE-cellulose (50–100 mM NaCl)	1	11	250	325000	250	30000	57	76	278	67
chromatofocusing, pI = 5.0–4.5	0.4	3	195	180000	450	60000	45	42	500	133

[a] In order to selectively determine the puromycin-insensitive activity, enzyme determinations were performed in the presence of 10^{-5} M puromycin for homogenate and Triton X-100 supernatant fractions.

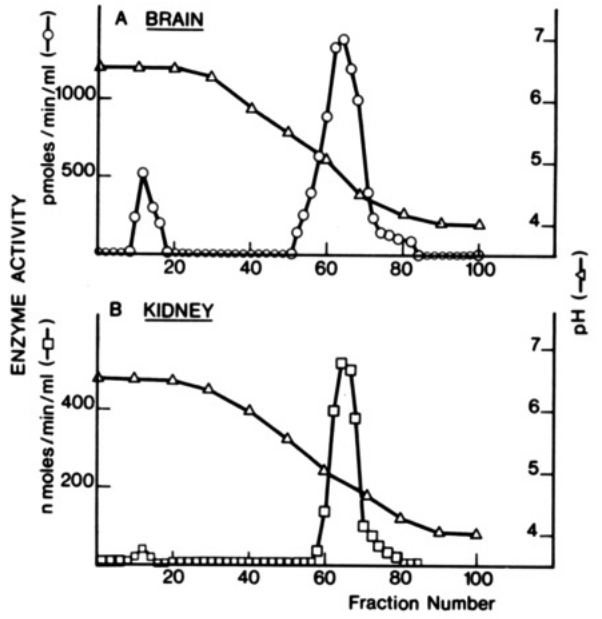

FIGURE 3: PBE 94 chromatofocusing of DEAE-cellulose chromatography active fractions. (A) Rat brain puromycin-insensitive pooled fractions were dialyzed against 25 mM histidine hydrochloride, pH 7.0, containing 0.2% Triton X-100, then applied on a PBE 94 gel column (1.5 × 20 cm), and eluted by 12.5% polybuffer 74–HCl buffer, pH 4.0, containing 0.2% Triton X-100. Fractions (5 mL) were rapidly buffered by 500 μL of 1 M ammonium carbonate, and the pH measurement (—) was performed as described under Materials and Methods. Enzyme activity (open circles) was determined on buffered fractions. (B) Rat kidney pooled fractions obtained after DEAE-cellulose chromatography were treated as described in (A). Enzyme activity (open squares) was determined on buffered fractions.

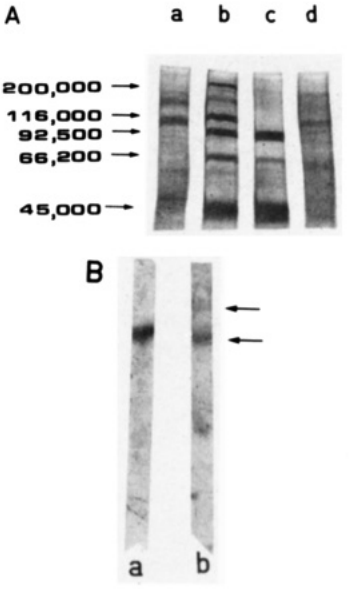

FIGURE 4: (A) Electrophoretic pattern of kidney aminopeptidase purified fraction (a) and brain puromycin-insensitive purified fraction (d). Electrophoresis was carried out according to Weber and Osborne in SDS under denaturing conditions. Molecular weight markers b and c were ovalbumin (M_r 45000), bovine serum albumin (M_r 68000), phosphorylase B (M_r 94000), β-galactosidase (M_r 116000), and myosin (M_r 200000). (B) Immunostaining of blots of rat brain puromycin-insensitive (a) and kidney (b) aminopeptidases. After electrophoresis was carried out as in (A), proteins were transferred onto nitrocelllose, and blots were soaked with rat kidney aminopeptidase antibodies (Towbin et al., 1979), then rinsed, and incubated with sheep peroxidase labeled anti-rabbit IgG antibodies (Institut Pasteur, Paris). Following a second rinse, the blots were developed by diaminobenzidine coloration.

activity of which was not affected by 10^{-5} M puromycin (Figure 2B). Further purification by chromatofocusing on a PBE 94 gel of this puromycin-insensitive fraction yielded two peaks (Figure 3A). The first one (10% of the applied activity) corresponded to unadsorbed material, and the second one (60%) was eluted at a pI range of 5.0–4.6. This fraction, concentrated on a PM10 Amicon membrane filter, was used to assess purity and to determine enzyme properties.

Purification of Kidney Membrane-Bound Aminopeptidase. Since the same microsomal fraction was used to extract both aminopeptidase and enkephalinase, and since the latter binds tightly to membranes, Triton X-100 at 5% final concentration was used to solubilize the enzyme activities. In this way, the recovery of aminopeptidase in the supernatant was about 135% of the activity initially evaluated in membranes.

Figure 1B shows the chromatographic pattern obtained after Con A–Sepharose chromatography of the resulting soluble fraction. About 85% of the enzyme activity was retained on the lectin gel and then eluted by 0.2 M methyl α-D-mannopyranoside. This active fraction was applied onto a DEAE-cellulose column, and elution with a NaCl gradient yielded a single peak of activity between 50 and 100 mM. This activity was insensitive to 10^{-5} M puromycin (Figure 2C). The active fractions were pooled, dialyzed against 25 mM histidine hydrochloride, pH 7.0, and 0.2% Triton X-100, and then loaded onto a PBE 94 chromatofocusing column. Elution with polybuffer 74, pH 4.0, resulted in a single peak of activity with a pI of 4.8 (Figure 3B).

This fraction was concentrated on a PM10 Amicon filter and used to investigate kidney aminopeptidase enzyme properties and to immunize rabbits. Table I summarizes the results obtained during the purification of kidney and brain puromycin-insensitive membrane-bound aminopeptidases.

Purity of Aminopeptidase Fractions. Both rat kidney and brain puromycin-insensitive aminopeptidase fractions were

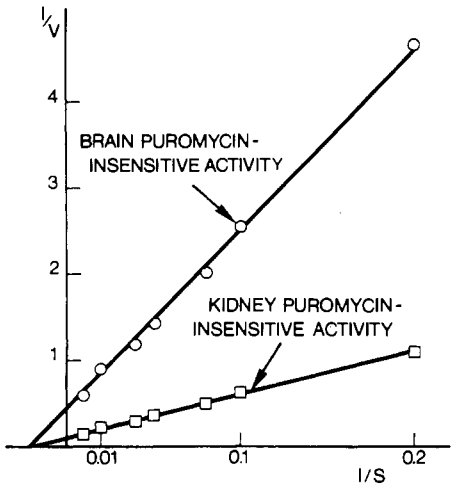

FIGURE 5: Lineweaver–Burk plots for kidney and brain puromycin-insensitive aminopeptidase activities. S, Met^5-enkephalin concentration (μM); V, enzyme activity (nmol min^{-1} mL^{-1}). The rate of Met^5-enkephalin degradation was measured with several concentrations of enkephalin (100–5 μM) with increasing specific activity (13.6–272 mCi mmol^{-1}), by incubating for 10 min at 37 °C and measuring ^{3}H-labeled metabolites after separation by chromatography on polystyrene beads.

Table II: Kinetic Constants for Substrates and Inhibitors of Membrane-Bound Rat Brain and Kidney Aminopeptidases

compounds	parameters (μM)	brain		kidney
		puromycin sensitive	puromycin insensitive	
Met^5-enkephalin	K_m	18	45	60
Leu^5-enkephalin	K_m	18	45	65
Leu β-NA	K_m	11	20	27
Ala β-NA	K_m	12	40	38
Gly β-NA	K_m	750	800	1000
Arg β-NA	K_m	7.5	27	25
Asp β-NA	K_m	>1000	>1000	>1000
puromycin	K_i	1	100	100
bestatin	K_i	0.5	4	4

examined by SDS–PAGE. Figure 4A shows a typical electrophoretic protein pattern: for the purified kidney aminopeptidase, two major bands were observed which represented about 70% of the total protein and which corresponded to approximate molecular weights of 130 000 and 100 000; the purified brain fraction was not homogeneous but contained the same two bands observed with kidney aminopeptidase.

Proteins of polyacrylamide gels were transferred onto nitrocellulose sheets and tested immunologically by using rat kidney aminopeptidase antibodies and a sheep anti-rabbit γ-globulin antiserum labeled with peroxidase. Figure 4B shows the coloration by diaminobenzidine of the treated blots. Two bands were obtained for the kidney preparation corresponding to the two major bands stained by the silver method. A third band weakly stained is obtained at M_r 45 000; this band is also obtained when the blots are incubated with normal rabbit serum instead of rat kidney aminopeptidase antiserum and must be due to nonspecific cross-reactivity of the second antibody with a contaminant protein present in the aminopeptidase preparation. With the brain puromycin-insensitive purified fractions only the M_r 100 000 band was clearly colored in most batches.

Substrate Specificity. The K_m values of Met^5- and Leu^5-enkephalins and various aminoacyl β-naphthylamides for the two brain and the kidney aminopeptidase preparations were determined from Lineweaver–Burk plots (Figure 5) using substrate concentrations from 1 to 100 μM. Very similar K_m values (45–65 μM) were obtained with kidney and brain puromycin-insensitive activities for the two pentapeptides; the puromycin-sensitive enzyme had a slightly lower K_m value (about 18 μM) for both enkephalins (Table II).

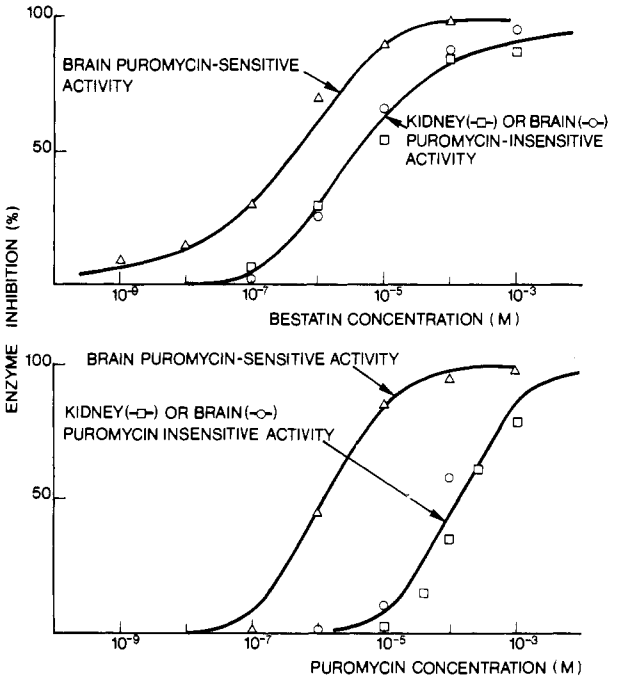

FIGURE 6: Effect of inhibitors in increasing concentrations on rat kidney and brain aminopeptidase activities. Enzyme activities were incubated at 37 °C in 50 mM Tris-HCl buffer, pH 7.5, in the presence of 40 nM [^{3}H]-Met^5-enkephalin and bestatin or puromycin in increasing concentrations. After 10 min, the reaction was stopped by acidification, and ^{3}H-labeled metabolites formed were separated by chromatography on Porapak Q beads; their radioactivity was measured by liquid scintillation spectrometry.

Table III: Effect of Metal Chelating Agents on Brain and Kidney Puromycin-Insensitive Aminopeptidases and Regeneration by Treatment with Divalent Metal Ions

treatment	aminopeptidase activity[a] (%)	
	brain	kidney
untreated enzyme	100	100
EDTA-treated enzyme	20	32
+1 mM $ZnCl_2$[a]	48	67
+1 mM $MnCl_2$[a]	106	
+1 mM $CoCl_2$[a]	100	130
+1 mM $CdCl_2$[a]	20	

[a] An aliquot of enzyme was dialyzed against 20 mM EDTA for 18 h, and then the aminopeptidase activity was determined in the presense of 1 mM metal ion.

Neutral and basic aminoacyl β-naphthylamides were hydrolyzed by the puromycin-insensitive enzyme from either kidney or brain with rougly similar K_m values, while aspartic acid β-naphthylamide was not attacked. Except for glycine β-naphthylamide, lower K_m values (about 10 μM) were obtained for the brain puromycin-sensitive aminopeptidase.

Whereas both the kidney and brain puromycin-insensitive aminopeptidases were inhibited by similar concentrations of either bestatin or puromycin, the inhibition patterns of the two brain enzymes were clearly distinct; differences in K_i values were 8-fold for bestatin and 100-fold for puromycin (Figure 6).

Effect of Metal Chelating Agents and Metal Ions. Both the kidney and brain puromycin-insensitive enzymes were 60–80% inactivated by treatment with 20 mM ethylenediaminetetraacetic acid (EDTA). After removal of EDTA by dialysis and incubation with divalent cations, enzyme activity

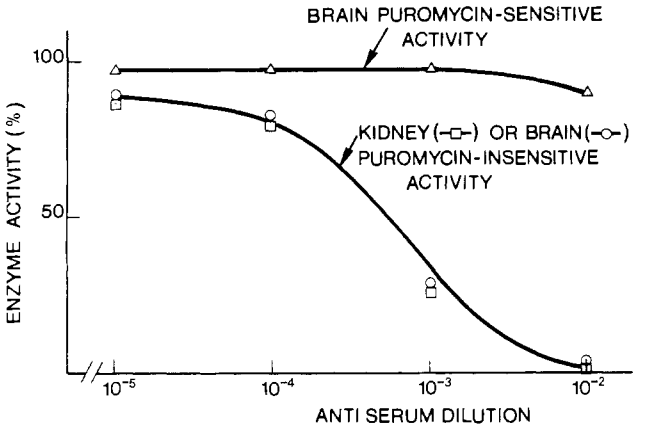

FIGURE 7: Effect of rat kidney aminopeptidase antibodies on rat kidney and brain aminopeptidase activities. The aminopeptidase preparations were preincubated at 4 °C for 24 h with the antiserum in increasing concentrations, and then enzyme activities were determined by incubation at 37 °C for 10 min in the presence of 40 nM [^{3}H]-Met^5-enkephalin and analysis of ^{3}H-labeled metabolites.

was restored. The most effective metal ions were Co^{2+} and Mn^{2+} (Table III).

Effect of Anti-Rat Kidney Aminopeptidase Antibodies. Antibodies raised against rat kidney aminopeptidase inhibited at similar concentrations the kidney and brain puromycin-insensitive aminopeptidase activities, while they had no effect on the brain puromycin-sensitive enzyme (Figure 7).

Discussion

The present study identifies aminopeptidase M (EC 3.4.11.2) in rat cerebral membranes. This enzyme was originally isolated from the kidney of various mammals (Pfleiderer & Celliers, 1963; Wachsmuth et al., 1966; Wacker et al., 1971; Thomas & Kinne, 1973; George & Kenny, 1973); in the rat, it represents as much as about 5% of the total brush border proteins (Rankin et al., 1980). Therefore, we purified rat kidney aminopeptidase M almost to homogeneity in order to compare it to the puromycin-insensitive aminopeptidase purified from cerebral membranes. Although the latter preparation was not homogeneous (Figure 4), the enkephalin-hydrolyzing aminopeptidase it contains is likely to be identical with the renal aminopeptidase M. Both these enzymes, in contrast with the puromycin-sensitive aminopeptidase from cerebral membranes, are glycoproteins similarly retained on Con A affinity columns (Figure 1) and similarly inhibited by chelating agents and then reactivated by Zn^{2+} or Co^{2+} (Table III). That a Zn atom is essential for the activity of hog kidney aminopeptidase M is well established (Lehky et al., 1973). Also, both enzyme activities were purified in a closely parallel manner during the ion-exchange and chromatofocusing steps (Figures 2 and 3), indicating that the two proteins have similar properties. In addition, when their catalytic properties were compared, the K_m values of enkephalins and various aminoacyl β-naphthylamides were similar as were the K_i values of the inhibitors bestatin and puromycin (Table II).

Furthermore, the cerebral enzyme was immunologically indistinguishable from kidney aminopeptidase M with antibodies raised in rabbits against the latter. In contrast, the puromycin-sensitive aminopeptidase from cerebral membranes was immunologically distinct from kidney aminopeptidase M, as it was hardly affected by antiserum concentrations at which the activities of the puromycin-insensitive enzymes were abolished (Figure 7).

Finally with SDS–PAGE, only the two major bands (apparent M_r 130000 and 100000), which corresponded to about 70% of the proteins of the purified renal enzyme preparation, were recognized by the antiserum (Figure 4). This electrophoretic pattern is comparable to that of aminopeptidase M purified from rat kidney, obtained by Kozak & Tate (1982). Rat kidney aminopeptidase has been reported to be a dimer composed of M_r 130000 subunits (Thomas & Kinne, 1972); the heterogeneity we observed in subunit molecular weight could be due to proteolytic cleavage during the purification procedure. The most extensively studied aminopeptidase M, that of pig kidney and intestine, probably corresponds to a dimer of M_r ~300000 (Wachsmuth et al., 1966; Wachsmuth, 1967; Wacker et al., 1976; Booth & Kenny, 1976; Benajiba & Maroux, 1981; Svensson et al., 1982), whereas aminopeptidase M purified from rabbit kidney has a molecular weight of 136000 (Kenny et al., 1979; Ferraci & Maroux, 1980). However, the use of proteolytic enzymes like papain and trypsin to solubilize renal aminopeptidase before purification may result in an inaccurate determination of molecular weight [see Kenny & Maroux, (1982)]. On the other hand, these data may suggest the existence of species differences in renal aminopeptidase M, an idea consistent with our observation that antiserum raised against rat aminopeptidase M did not recognize the pig kidney enzyme (unpublished results).

From these results we can conclude that the puromycin-insensitive enzyme purified from rat cerebral membranes is physicochemically, catalytically, and immunologically indistinguishable from the renal brush border aminopeptidase, designated aminopeptidase M (or aminopeptidase N). Although aminopeptidase M is abundant in renal and intestinal brush border membranes (Maroux et al., 1973; Kim & Brophy, 1976; Noren et al., 1977; Sterchi & Woodley, 1980) and may also be present in various epithelia (Cassiman et al., 1981; Verlinder et al., 1981), its occurrence in brain has never been reported. However, cerebral aminopeptidase M may correspond to the MI enkephalin-hydrolyzing aminopeptidase of Hersh (1981b), as suggested by its low sensitivity to puromycin, although the K_i value of this compound was 1.1 mM, i.e., 10-fold higher than presently found (Table II), and the K_m value of Leu^5-enkephalin was 50-fold higher.

The present study confirms the existence in cerebral membranes of an enkephalin-hydrolyzing aminopeptidase sensitive to bestatin (K_i = 4 μM) but largely insensitive to puromycin (K_i = 100 μM). Aminopeptidase M's participation in inactivating endogenous enkephalins in brain slices is strongly indicated, since 20 μM bestatin, but not 100 μM puromycin, protects the released peptides (Patey et al., 1981; De la Baume et al., 1983). This conclusion is particularly noteworthy since only a minor fraction of the overall enkephalin-hydrolyzing aminopeptidase activity from cerebral membranes corresponds to aminopeptidase M. Most of the remaining activity, efficiently resolved at the lectin affinity column step, probably corresponds to the enzyme of Hui et al. (1983). Indeed, the specificity of the latter is very similar to that of our puromycin-sensitive aminopeptidase as well as, possibly, to that of the MII aminopeptidase (Hersh, 1981b) although there are differences as already noted by Hui et al. Interestingly, this puromycin-sensitive aminopeptidase (K_i = 1 μM) was even more sensitive to bestatin (K_i = 0.5 μM) than was aminopeptidase M. Additional studies with more selective inhibitors are needed to decide whether only aminopeptidase M is functionally important or whether both enzymes have to be completely inhibited to protect endogenous enkephalins.

Registry No. Met^5-enkephalin, 58569-55-4; Leu^5-enkephalin, 58822-25-6; Leu-β-NA, 732-85-4; Ala-β-NA, 720-82-1; Gly-β-NA, 716-94-9; Arg-β-NA, 7182-70-9; Asp-β-NA, 635-91-6; puromycin, 53-79-2; bestatin, 58970-76-6; aminopeptidase M, 9054-63-1; enke-

phalin aminopeptidase, 75496-63-8.

References

Almenoff, J., Wilk, S., & Orlowski, M. (1981) *Biochem. Biophys. Res. Commun. 102*, 206–214.

Barclay, R. K., & Phillipps, M. A. (1978) *Biochem. Biophys. Res. Commun. 81*, 1119–1123.

Benajiba, A., & Maroux, S. (1981) *Biochem. J. 197*, 573–580.

Biber, J., Stieger, B., Haase, W., & Murer, M. (1981) *Biochim. Biophys. Acta 647*, 169–176.

Booth, A. G., & Kenny, A. J. (1976) *Biochem. J. 159*, 395–407.

Cassiman, J. J., Verlinden, J., Van Der Schueren, B., Van Leuven, F., Saison, M., & Van Den Berghe, M. (1981) *Virchows Arch. A: Pathol. Anat. Histol. 37*, 179–189.

Chaillet, P., Marcais-Collado, H., Costentin, J., Yi, C. C., De La Baume, S., & Schwartz, J. C. (1983) *Eur. J. Pharmacol. 86*, 329–336.

Cohen, M. L., Geary, L. E., & Wiley, K. S. (1983) *J. Pharmacol. Exp. Ther. 224*, 379–384.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977) *Biochemistry 16*, 5484–5491.

De la Baume, S., Yi, C. C., Schwartz, J. C., Chaillet, P., Marcais-Collado, H., & Costentin, J. (1983) *Neuroscience (oxford) 8*, 143–151.

Feracci, M., & Maroux, S. (1980) *Biochim. Biophys. Acta 599*, 448–463.

Fulcher, S., Matsas, R., Turner, A. J., & Kenny, A. J. (1982) *Biochem. J. 203*, 519–522.

George, S. G., & Kenny, A. J. (1973) *Biochem. J. 134*, 43–57.

Hashisu, M., Nakamura, T., Kawashima, M., Shitoh, K., Fukatsu, S., Koeda, T., Sekizawa, Y., Manakata, M., Kawamura, K., Umezawa, H., Takeuchi, T., & Aoyagi, T. (1982) *Life Sci. 30*, 1739–1746.

Hayashi, M. (1978) *J. Biochem. (Tokyo) 84*, 1363–1372.

Hayashi, M., & Oshima, K. (1980) *J. Biochem. (Tokyo) 87*, 1403–1411.

Hersh, L. B. (1981a) *J. Neurochem. 36*, 1594–1596.

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

Hersh, L. B., & Mc Kelvy, J. F. (1981) *J. Neurochem. 36*, 171–178.

Hui, K. S., & Lajtha, A. (1983) in *Handbook of Neurochemistry* (Lajtha, A., Ed.) Vol. 4, pp 1–19, Plenum Press, New York and London.

Hui, K. S., Wang, Y. J., & Lajtha, A. (1983) *Biochemistry 22*, 1062–1067.

Kelly, J. A., Neidle, E. L., & Neidle, A. (1983) *J. Neurochem. 40*, 1727–1734.

Kenny, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., Ed.) pp 393–444, North-Holland Publishing Co., Amsterdam.

Kenny, A. J., & Maroux, S. (1982) *Phys. Rev. 62*, 91–128.

Kenny, A. J., George, S. G., & Aparicio, S. G. R. (1969) *Biochem. J. 115*, 18P.

Kim, Y. S., & Brophy, E. J. (1976) *J. Biol. Chem. 251*, 3199–3205.

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

Kosak, E. M., & Tate E. S. (1982) *J. Biol. Chem. 257*, 6322–6327.

Lehky, P., Lisowski, J., Wolf, D. P., Wacker, M., & Stein, E. A. (1973) *Biochim. Biophys. Acta 321*, 274–281.

Llorens, C., & Schwartz, J. C. (1981) *Eur. J. Pharmacol. 69*, 113–116.

Malfroy, B., & Schwartz, J. C. (1982) *Biochem. Biophys. Res. Commun. 106*, 276–285.

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

Maroux, S., Louvard, D., & Baratti, J. (1973) *Biochim. Biophys. Acta 321*, 282–295.

Mc Donald, J. K., & Schwabe, C. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., Ed.) pp 311–391, North-Holland Publishing Co., Amsterdam.

Noren, O., Sjöström, H., Svensson, B., Jeppesen, L., Staum, M., & Josefsson, L. (1977) *Ciba Found. Symp. 50*, 177–197.

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

Pfleiderer, G., & Celliers, P. G. (1963) *Biochem. J. 339*, 186–189.

Rankin, B. B., Mc Intyre, T. M., & Curthoys, N. P. (1980) *Biochem. Biophys. Res. Commun. 96*, 991–996.

Roques, B. P., Fournie-Zaluski, M. C., Soroca, E., Lecomte, J. M., Malfroy, B., Llorens, C., & Schwartz, J. C. (1980) *Nature (London) 288*, 286–288.

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

Schwartz, J. C. (1983) *Trends NeuroSci. (Pers. Ed.) 6*, 15–18.

Schwartz, J. C., Malfroy, B., & De La Baume, S. (1981) *Life Sci. 29*, 1715–1740.

Schwartz, J. C., De La Baume, S., Yi, C. C., Chaillet, P., Marcais-Collado, H., & Costentin J. (1982) *Prog. Neuro.-Psychopharmacol. 6*, 665–671.

Sterchi, E. E., & Woodley, J. F. (1980) *Clin. Chim. Acta 102*, 57–65.

Swensson, B., Sjöström, M., & Norêno, O. (1982) *Eur. J. Biochem. 126*, 481–488.

Thomas, L., & Kinne, R. (1972) *Biochim. Biophys. Acta 255*, 114–125.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Traficante, L. J., Rotrosen, J., Siekierski, J., Tracer, H., & Gershon, S. (1980) *Life Sci. 26*, 1697–1706.

Verlinden, J., Van Leuven, F., Cassiman, J. J., & Van Den Berghe, H. (1981) *FEBS Lett. 123*, 287–290.

Vogel, Z., & Altstein, M. (1977) *FEBS Lett. 80*, 332–335.

Vogel, Z., & Altstein, M. (1979) *FEBS Lett. 98*, 44–48.

Vogel, Z., & Altstein, M. (1980) in *Endogenous and Exogenous Opiate Agonists and Antagonists* (Leong Way, E., Ed.) pp 353–356, Pergamon Press, New York.

Wachsmuth, E. D. (1967) *Biochem. Z. 346*, 467–473.

Wachsmuth, E. D., Fritze, I., & Pfleiderer, G. (1966) *Biochemistry 5*, 169–174.

Wacker, H., Lehky, P., Vandermaeghe, F., & Stein, E. A. (1976) *Biochim. Biophys. Acta 429*, 546–554.

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

Weber, K., & Osborn, M. (1979) *J. Biol. Chem. 244*, 4406–4412.

Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.

Zhang, A. Z., Yang, H. Y. T., & Costa, E. (1982) *Neuropharmacology 21*, 625–630.