Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 206-214

MEMBRANE BOUND PITUITARY METALLOENDOPEPTIDASE: APPARENT IDENTITY TO ENKEPHALINASE

June Almenoff, Sherwin Wilk and Marian Orlowski

Department of Pharmacology, Mount Sinai School of Medicine
of the City University of New York, New York, NY 10029

Received July 27,1981

SUMMARY

A membrane bound zinc-metalloendopeptidase from bovine pituitaries with a specificity toward bonds on the amino side of hydrophobic amino acids, cleaves Met- and Leu-enkephalin at the Gly-Phe bond, releasing Phe-Met and Phe-Leu respectively. The enzyme also hydrolyzes bonds on the amino side of hydrophobic amino acids in oxytocin, bradykinin, neurotensin and several synthetic substrates. A free carboxyl group on a dipeptide C-terminal to the hydrolyzed bond is not a requirement for activity. The enzyme is also present in brain membrane fractions. The regional distribution of this enzyme in brain, its specificity toward natural and synthetic substrates, and its sensitivity to inhibitors, suggest that the enzyme is identical to an activity referred to as "enkephalinase," which has been described as dipeptidyl carboxypeptidase. The data show that the enzyme is an endopeptidase with a specificity similar to that of a group of microbial proteases, one of which is thermolysin.

INTRODUCTION

The discovery of the opioid peptides Met- and Leu-enkephalin (1) has led to numerous studies of their metabolism. Several investigators have described in brain a membrane bound activity which hydrolyzes Met- and Leu-enkephalin at the Gly-Phe bond and differs from the angiotensin converting enzyme (2-6). The activity, which is strongly inhibited by thiols and metal chelators has been referred to as "enkephalinase" (3, 6-10) and classified by several authors as a dipeptidyl carboxypeptidase (5, 7, 8), because it cleaves the C-terminal dipeptide from the enkephalins. It has been suggested that the enzyme functions *in vivo* as a specific enkephalin inactivator, because its regional distribution in brain appears to parallel that of the opiate receptors (2, 11).

Abbreviations: DFP, diisopropylfluorophosphate; Cbz, N-benzyloxycarbonyl; 2NA, 2, naphthylamide or 2-naphthylamine; 4Me2NA, 4-methoxynaphthylamide; Bz, α-N-benzoyl; HPLC, high pressure liquid chromatography.

0006-291X/81/170206-09$01.00/0

Much of this work, however, was done with crude brain preparations, and detailed specificity studies have not been carried out.

We report here that brain "enkephalinase" is apparently identical to a metalloendopeptidase isolated in this laboratory from particulate fractions of bovine pituitaries (12). The enzyme is neither a dipeptidyl carboxypeptidase in the classical sense, nor is it specific for the hydrolysis of enkephalins. Its specificity resembles that of thermolysin (13).

MATERIALS AND METHODS

Aminopeptidase M (EC 3.4.11.2) was purchased from Boehringer, Mannheim, Inc. (Indianapolis, IN). Glutathione, N-ethylmaleimide, leupeptin, Leu-enkephalin, Met-enkephalin, neurotensin, EDTA, Cbz-Tyr-Gly-Gly and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). DFP was obtained from City Chemical Corp (New York, NY). Oxytocin was obtained from Bachem, Inc. (Torrance, CA). Bradykinin was obtained from U.S. Biochemicals (Cleveland, OH). Glutaryl-Ala-Ala-Phe-4Me2NA was obtained from Enzyme Systems Products (Livermore, CA). Bz-Gly-Arg-Arg-Leu-2NA, Bz-Gly-Arg-Arg-Gly-2NA and glutaryl-Ala-Ala-Phe-2NA were synthesized as described previously (12). Tyr-Gly-Gly was prepared from Cbz-Tyr-Gly-Gly by catalytic hydrogenation.

Enzyme preparations. Metalloendopeptidase was purified from particulate fractions of bovine pituitaries by the procedure described previously (12). The enzyme preparation had no aminopeptidase activity and did not cleave hippuryl-histidyl-leucine, a substrate used for the determination of angiotensin converting enzyme (EC 3.4.15.1). Membrane fractions containing enkephalinase activity were obtained from various brain regions of male Sprague Dawley rats weighing about 250 g by the procedure of Malfroy (3). Membranes from striata and substantia nigra were subjected to a single centrifugation at 200,000 g. Pellets were washed and suspended in 0.05 M Tris-HCl buffer (pH 7.6) to yield a protein concentration of about 1 mg/ml.

Determination of enzyme activity. Activity was determined with Bz-Gly-Arg-Arg-Leu-2NA, glutaryl-Ala-Ala-Phe-2NA or glutaryl-Ala-Ala-Phe-4Me2NA in a two stage reaction catalyzed in sequence by the metalloendopeptidase and aminopeptidase M. The enzyme cleaves these substrates on the amino side of the hydrophobic amino acids, leucine or phenylalanine. With Bz-Gly-Arg-Arg-Leu-2NA as substrate the reaction proceeds as follows:

$$\text{Bz-Gly-Arg-Arg-Leu-2NA} \xrightarrow{\text{Metalloendopeptidase}} \text{Bz-Gly-Arg-Arg} + \text{Leu-2NA} \quad (1)$$

$$\text{Leu-2NA} \xrightarrow{\text{Aminopeptidase M}} \text{Leu} + \text{2NA} \quad (2)$$

In the first reaction the metalloendopeptidase cleaves bonds on the amino side of a hydrophobic amino acid (reaction 1) releasing an amino acid naphthylamide. In the second reaction the amino acid naphthylamide is hydrolyzed to yield the free amino acid and naphthylamine, which can be determined by diazotization according to a modification (14) of the procedure described by Bratton and Marshall (15). Incubation mixtures contained substrate, enzyme or membrane preparation and buffer (0.05 M Tris-HCl; pH 7.6) in a final volume of 0.25 ml. Incubations were at 37° and 10 ug of aminopeptidase M and 25 ul of dithiothreitol (0.01 M) were added and the mixtures were incubated for 1 hr. After addition of trichloroacetic acid (0.25 ml of 10% solution), protein was removed by

centrifugation and 2NA was determined as described above. When glutaryl-Ala-Ala-Phe-4Me2NA was used for determination of activity, 4Me2NA was determined according to the procedure described by Barrett (16). Controls in which the metalloendopeptidase was omitted were included in each experiment. Enzyme assays were done under conditions in which the activity was proportional to both incubation time and amount of enzyme.

Identification of reaction products. Products of enzymatic hydrolysis of biologically active peptides were separated by HPLC on a Waters Associates liquid chromatograph equipped with a C_{18} uBondapak column (30 x 0.4 cm) and a variable wavelength detector; emerging peaks were monitored at 210 nm. Elution was carried out with a linear gradient established between a 0.1% aqueous solution of phosphoric acid and acetonitrile containing 0.1% phosphoric acid. The concentration of the organic solvent was increased from 5 to 60% during a period of 15 min. The flow rate of the solvent was 1.5 ml/min. Emerging peaks were collected and their amino acid composition was determined after removal of the solvent and acid hydrolysis in a Technicon-TSM amino acid autoanalyzer.

RESULTS

When the pituitary enzyme was incubated with several biologically active peptides the results shown in Table 1 were obtained. The enzyme cleaves both Met- and Leu-enkephalin at the Gly-Phe bond to yield Tyr-Gly-Gly and Phe-Met or Phe-Leu respectively. This specificity is similar to that reported for the "enkephalinase" (3-11) and is consistent with the specificity of the metalloendopeptidase, which is directed toward bonds on the amino side of hydrophobic amino acids (12). Incubation of the enzyme with oxytocin results in the formation of leucyl-glycinamide indicating that the enzyme is not a dipeptidyl carboxypeptidase, since hydrolysis can occur in the absence of a free carboxyl group. Bradykinin is rapidly cleaved by the enzyme at the Pro^7-Phe^8 bond, a reaction previously reported for brain "enkephalinase" (5), and also hydrolyzed at the Gly^4-Phe^5 bond. This confirms that the enzyme is an endopeptidase which can act as a dipeptidyl carboxypeptidase on peptides with a hydrophobic residue in the penultimate position at the C-terminus. Neurotensin is hydrolyzed at both the Tyr^{11}-Ile^{12} and Pro^{10}-Tyr^{11} bonds. Free tyrosine or leucine were not detected among the reaction products. This indicates that hydrolysis of the Pro^{10}-Tyr^{11} and Tyr^{11}-Ile^{12} bonds does not occur within the same molecule. That the tripeptide Tyr-Ile-Leu is not hydrolyzed shows that the enzyme cannot act as an exopeptidase.

Table 1. Cleavage of Biologically Active Peptides by Metalloendopeptidase

Peptide	Site of cleavage	Products found
Met-enkephalin	Tyr-Gly-Gly↓Phe-Met	Phe-Met, Tyr-Gly-Gly
Leu-Enkephalin	Tyr-Gly-Gly↓Phe-Leu	Phe-Leu, Tyr-Gly-Gly
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro↓Leu-Gly-NH_2	Leu-Gly-NH_2, Cys-Tyr-Ile-Gln-Asn-Cys-Pro
Bradykinin	Arg-Pro-Pro-Gly↓Phe-Ser-Pro↓Phe-Arg	Arg-Pro-Pro-Gly, Phe-Arg, Phe-Ser-Pro, Arg-Pro-Pro-Gly-Phe-Ser-Pro
Neurotensin	pGLu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro↓Tyr↓Ile-Leu	Ile-Leu, Tyr-Ile-Leu, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr

Reaction mixtures contained substrate (0.87 mM Met-enkephalin, 1.4 mM Leu-enkephalin, 0.87 mM bradykinin, 1.0 mM oxytocin and 0.6 mM neurotensin), Tris-HCl buffer (0.05 M; pH 7.6) and enzyme (0.08 units) in a final volume of 0.2 ml. All incubations were for 21 h except for neurotensin, which was incubated for 3 h with 0.2 units of enzyme. Products of reaction were separated by HPLC and identified as described under Materials and Methods.

Met-enkephalin inhibited cleavage of the synthetic substrates glutaryl-Ala-Ala-Phe-2NA and glutaryl-Ala-Ala-Phe-4Me2NA by the metalloendopeptidase. The K_i values for these two substrates determined from Dixon plots (substrate concentrations were 0.4 to 1.2 mM; enkephalin concentrations were 0 to 0.5 mM) were 4.6×10^{-5} M and 3.3×10^{-5} M respectively. As expected, the two values are in close agreement. An average of these values (4×10^{-5}M) should be regarded as the K_m of the enzyme for Met-enkephalin, since $K_i = K_m$ when determined by treating the competing substrate as an inhibitor (17). This K_m value is close to the K_m value of Leu-enkephalin reported for "enkephalinase" (18). We have determined the k_{cat} for hydrolysis of Met-enkephalin by the enzyme to be 21.2 min^{-1}. The specificity constant k_{cat}/K_m is thus equal to 530. A com-

Table 2. Kinetic Parameters for Hydrolysis of Met-enkephalin and several Synthetic Substrates

SUBSTRATE	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Tyr-Gly-Gly-Phe-Met	0.04	21.2	530
Bz-Gly-Arg-Arg-Leu-2NA	0.18	240	1330
Glutaryl-Ala-Ala-Phe-2NA	0.59	592	1000
Bz-Gly-Arg-Arg-Ala-2NA	0.30	8.8	29
Bz-Gly-Arg-Arg-Gly-2NA	Negligible Hydrolysis		

The turnover rate constant (k_{cat}) for Met-enkephalin was determined by incubating the enzyme (0.08 units) with the substrate (0.04 mM) for 1-2 h in Tris-HCl buffer (0.05 M; pH 7.6) in a final volume of 0.25 ml. Aliquots of the reaction mixture (20 ul) were removed and analyzed quantitatively for the formation of Tyr-Gly-Gly by HPLC. The amount of peptide formed was determined by comparing the peak heights with standard solutions of Tyr-Gly-Gly analyzed in the same manner. Maximal velocity was calculated from initital velocity measurements by assuming a K_m of 0.04 mM. Data for other substrates are the same as those reported elsewhere (12).

parison of the kinetic parameters for Met-enkephalin with those of several model synthetic endopeptidase substrates is shown in Table 2. The data indicate that the rate of hydrolysis of Met-enkephalin is relatively low compared to some of these substrates. The specificity constant, however, is only about 2 to 2.5 times lower as a result of the low K_m value (0.04 mM).

Crude homogenates from rat and rabbit brain showed activity toward the metalloendopeptidase substrates glutaryl-Ala-Ala-Phe-2NA and Bz-Gly-Arg-Arg-Leu-2NA at pH 7.6 that was almost completely associated with particulate membrane fractions. The enzyme from rabbit brain could be purified by the same procedure used for purification of the pituitary metalloendopeptidase. Deoxycholate extraction of membrane fractions followed by removal of excess deoxycholate with streptomycin sulfate, treatment with papain and chromatography on Sephadex G-200 and DEAE cellulose columns yielded a preparation with a specific activity similar to that of the pituitary enzyme obtained after the same puri-

Table 3. Regional Distribution of Metalloendopeptidase in Rat Brain.

Brain region	Specific activity	Relative activity
Striatum	0.937 ± .055 (4)	100
Substantia nigra	0.517 ± .053 (3)	55
Cerebellum	0.215 ± .026 (4)	23
Brain stem	0.186 ± .009 (4)	20
Prefrontal cortex	0.156 ± .021 (4)	17

Activity was determined with glutaryl-Ala-Ala-Phe-2NA as described under Materials and Methods. Data represent specific activities expressed in umoles of product/h/mg protein ± S.E. The number of determinations is given in parenthesis. Each determination represents results obtained from pooled tissue samples obtained from two rats.

fication steps (step 4) (12). The purified enzyme cleaved Met-enkephalin at the Gly-Phe bond, a reaction attributed to "enkephalinase" activity (6-10). Both the purified enzyme as well as crude membrane fractions from the various regions of rat brain cleaved several endopeptidase substrates at relative rates similar to those of the purified pituitary enzyme, as presented in Table 2. The regional distribution of activity in rat brain is shown in Table 3. The highest activity was found in striatum, while the activities in prefrontal cortex, cerebellum and brainstem were much lower. This distribution of activity is similar to that reported by other investigators for "enkephalinase" (10,11). Of interest is the rather high activity found in the substantia nigra, a region which has apparently not been studied by other groups.

The effect of inhibitors on the enzyme activity in preparations from rat striatal membranes is summarized in Table 4 and compared with the inhibition of the purified pituitary enzyme by the same inhibitors. Leupeptin, an inhibitor of several thiol and serine proteases, had no effect on either activity. Similarly, N-ethylmaleimide, a thiol blocking agent, and DFP, an inhibitor of serine proteases, had little effect on activity. Thiols (glutathione and dithiothreitol) and the metal chelators EDTA and phosphate inhibited both en-

Table 4. Effect of Inhibitors on Activity of Purified Pituitary Metalloendopeptidase and the Enzyme in Striatal Membrane Preparations.

Inhibitor	Concentration (mM)	Percent Inhibition	
		Striatal membrane Preparation	Pituitary enzyme
Leupeptin	10 ug/ml	0	0
N-ethylmaleimide	1.0	16	0
DFP	0.11	10	0
Dithiothreitol	2.0	90	93
Glutathione	2.0	61	57
EDTA	1.0	58	37
Phosphate	4.0	17	20
Phenobarbital	0.2	51	55

Activity was determined with Bz-Gly-Arg-Arg-Leu-2NA as described under Materials and Methods without preincubating the enzyme with the inhibitors. Data for the pituitary enzyme are those reported previously (12).

zymes to the same extent. A similar degree of inhibition of both enzymes was observed with phenobarbital, previously reported to inhibit "enkephalinase" activity (18).

DISCUSSION

The results reported here strongly suggest that purified metalloendopeptidase from bovine pituitaries is identical to an endopeptidase associated with rat and rabbit brain membrane fractions, and that these enzymes are identical to the enzyme referred to by several authors as "enkephalinase" (4-10). All of these membrane associated activities cleave the Gly-Phe bond in Met- and Leu-enkephalin, they share a similar spectrum of inhibition by thiols and metal chelating agents, and are distinct from the angiotensin converting enzyme. Furthermore, the pituitary metalloendopeptidase and the enzyme associated with striatal membranes are inhibited by phenobarbital (Table 4), reported by Altstein et al (18) to inhibit an "enkephalinase" activity in purified synapto-

somes. Because the enzyme releases a C-terminal dipeptide from the enkephalins, it is classified by several authors as a dipeptidyl carboxypeptidase (5, 7, 8). In these studies, however, only crude enzyme preparations were used and the specificity of the enzyme was not carefully tested with peptide subtrates blocked both at the N- and C-terminus. The results of specificity studies with both synthetic and natural peptides reported here, clearly indicate that the enzyme is an endopeptidase, and that it acts as a dipeptidyl carboxypeptidase only on those substrates having a hydrophobic amino acid in the penultimate position at the C-terminus. The specificity of the enzyme is directed toward bonds on the amino side of hydrophobic amino acids and resembles therefore in this respect the specificity of thermolysin (19), a bacterial zinc metalloendopeptidase (13).

Recent reports have described the presence of high "enkephalinase" activity in kidney membrane fractions (20, 21). We have recently found that rabbit kidney contains very high activities of a metalloendopeptidase with properties and specificity similar to those of the enzyme from bovine pituitary (12) and brain membrane fractions, further supporting the conclusion that these enzymes are identical to "enkephalinase." It should be noted that the pituitary enzyme (12) shows some resemblance to the neutral endopeptidase purified from rabbit kidney brush borders by Kerr and Kenny (22). The relationship and possible identity of this enzyme to "enkephalinase" needs to be investigated.

Several authors have suggested that the in vivo function of "enkephalinase" is to degrade enkephalins, because the distribution of the enzyme seems to parallel the distribution of opiate receptors (2, 11, 23). The finding, however, that the enzyme degrades not only enkephalins but other neuropeptides and that its specificity is not that of a dipeptidyl carboxypeptidase but of an endopeptidase, suggests the need for reevaluation of the function of the enzyme in vivo. the presence of high activities of the enzyme in peripheral tissues and especially in the kidney, makes it unlikely that the function of the enzyme is limited to the degradation of enkephalins.

While the data reported here strongly suggest that the purified metalloendopeptidase is identical to "enkephalinase," proof of complete identity must await isolation of the "enkephalinase" and comparison of its physicochemical properties with those of the purified metalloendopeptidase.

ACKNOWLEDGEMENTS

This work was supported by a grant from the NIH, AM-15377. June Almenoff is a trainee on Medical Scientist Training Grant GM-07280 from the NIH.

REFERENCES

1. Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A. & Morris, H.R. (1975) Nature, Lond. 258, 577-579.
2. Sullivan, S., Akil, H. & Barchas, J.D. (1978) Commun. in Psychopharm. 2, 525-531.
3. Malfroy, B., Swerts, J.P., Guyon, A., Roques, B.P. & Schwartz, J.C. (1978) Nature, Lond. 276, 523-526.
4. Arregui, A., Lee, C., Emson, P.C. & Iversen, L.L. (1979) Eur. J. Pharmacol. 59, 141-144.
5. Benuck, M. & Marks, N. (1980) Biochem. Biophys. Res. Commun. 95, 822-828.
6. Gorenstein, C. & Snyder, S.H. (1979) Life Sci. 25, 2065-2070.
7. Roques, B.P., Fournie-Zaluski, M.C., Soroca, E., Lecomte, J.M., Malfroy, B., Llorens, C. & Schwartz, J.-C. (1980) Nature, Lond. 288, 286-288.
8. Fournie-Zaluski, M.C., Perdrisot, R., Gacel, G., Swerts, J.P., Roques, B.P. & Schwartz, J.-C. (1979) Biochem. Biophys. Res. Commun. 91, 130-135.
9. Sullivan, S., Akil, H., Blacker, D. & Barchas, J.D. (1980) Peptides, 1, 31-35.
10. Vogel, Z. & Altstein, M. (1980) Progr. Biochem. Pharmacol. 16, 49-59.
11. Malfroy, B., Swerts, J.P., Llorens, L. & Schwartz, J.-C. (1979) Neurosci. Lett. 11, 329-334.
12. Orlowski, M. & Wilk, S. (1981) Biochemistry, In press.
13. Matsubara, H., Sasaki, R., Singer, A. & Jukes, T.H. (1966) Arch Biochem. Biophys. 115, 324-331.
14. Goldbarg, J.A. & Rutenburg, A.M. (1958) Cancer II, 283-291.
15. Bratton, A.C. & Marshall Jr., E.K. (1939) J. Biol. Chem. 128, 537-550.
16. Barrett, A.J. (1972) Anal. Biochem. 47, 280-293.
17. Cornish-Bowden, A. (1979) in Fundamentals of Enzyme Kinetics, Butterworths, pp. 82-85.
18. Altstein, M., Mittman, A. and Vogel, Z. (1981) Life Sci 18, 185-191.
19. Blumberg, S., Vogel, Z. & Altstein, M. (1981) Life Sci. 28, 301-306.
20. Llorens, C. & Schwartz, J.-C. (1981) Eur. J. Pharmacol. 69, 113-116.
21. Benuck, M., Berg, M.J. & Marks, N. (1981) Life Sci. 28, 2643-2650.
22. Kerr, M.A. & Kenny, A.J. (1974) Biochem. J. 137, 489-495.
23. Pert, C.B., Kuhar, M.J., & Snyder, S.H. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3729-3733.