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MEMBRANE-BOUND THERMOLYSIN-LIKE METALLOENDOPEPTIDASE: PURIFICATION AND CHARACTERIZATION OF THE ENZYME FROM RABBIT KIDNEY AND BRAIN. IDENTIFICATION OF A SIMILAR ENZYME IN HUMAN SERUM AND STUDY OF ITS ACTIVITY IN SARCOIDOSIS

City University of New York PH.D. 1983

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MEMBRANE-BOUND THERMOLYSIN-LIKE METALLOENDOPEPTIDASE

Purification and Characterization of the Enzyme from Rabbit Kidney and Brain. Identification of A Similar Enzyme in Human Serum and Study of its Activity in Sarcoidosis.

by

JUNE ALMENOFF

A dissertation submitted to the Graduate Faculty in the Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1983

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Abstract

MEMBRANE-BOUND THERMOLYSIN-LIKE METALLOENDOPEPTIDASE

Purification and Characterization of the Enzyme from Rabbit Kidney and Brain. Identification of A Similar Enzyme in Human Serum and Study of its Activity in Sarcoidosis.

by

June Almenoff

Adviser: Marian Orlowski, M.D., Professor of Pharmacology

A membrane-bound metalloendopeptidase from bovine pituitaries, rabbit kidney, and rabbit brain was shown to hydrolyze peptide bonds involving the amino group of hydrophobic amino acids in natural and synthetic peptides. Evidence is presented that this enzyme is identical with enkephalinase.

The enzyme [EC 3.4.24.11] was purified to homogeneity from rabbit kidney and partially purified from rabbit brain. It has a molecular weight of about 95,000 and is inhibited by metal chelators and by the active site directed inhibitors phosphoramidon and thiorphan.

N-(1-carboxy-2-phenylethyl) derivatives of phenylalanyl, alanyl, and glycyl-pAB were synthesized as potential

inhibitors of the enzyme. The two diastereomers of the phenylalanyl derivative were separated; the more potent isomer had a K_i of 2.9×10^{-8} M. The inhibitory potency of the alanyl and glycyl derivatives was lower by several orders of magnitude. The data suggest that a hydrophobic residue in the P_1' position and a carboxylate group coordinating with the active site zinc account for the inhibitory action of these compounds.

The enzyme from rabbit brain was compared with the rabbit kidney enzyme. The two enzymes had the same pH optimum, molecular weight, and specificity, however, minor but significant differences were found in their interactions with specific inhibitors and antisera.

N-(1-carboxy-2-phenylethyl) derivatives of phenylalanyl, alanyl, and glycyl-pAB were tested for antinociceptive activity. Intraperitoneal administration of all three compounds produced significant analgesia that lasted for 24 h. The data indicate, however, that the analgesia observed does not correlate with the K_i values of the inhibitors.

An enzyme with properties similar to the membrane-bound metalloendopeptidase was identified in human serum. The serum enzyme crossreacted with an antiserum to the rabbit kidney metalloendopeptidase, thereby suggesting that the two enzymes have common antigenic determinants.

Serum metalloendopeptidase activity was measured in 150 controls and in 95 sarcoidosis patients. The mean enzyme activity in the sarcoidosis group was more than 3-fold higher than that of the controls ($p < 0.001$). Highest activities were found in patients whose chest roentgenograms showed hilar lymphadenopathy and interstitial infiltrates. Enzyme activity in patients with active tuberculosis, pulmonary neoplasms, and interstitial pulmonary fibrosis did not differ significantly from that of controls.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Almenoff, J., Wilk, S., and Orlowski, M. (1981) Membrane Bound Pituitary Metalloendopeptidase: Apparent Identity to Enkephalinase. Biochem. Biophys. Res. Commun. 102, 206-214.

Almenoff, J., and Orlowski, M. (1983) Membrane-Bound Kidney Neutral Metalloendopeptidase: Interaction with Synthetic Substrates, Natural Peptides, and Inhibitors. Biochemistry 22, 590-599.

Almenoff, J., and Orlowski, M. (1983) Biochemical and Immunological Properties of a Membrane Bound Brain Metalloendopeptidase (Enkephalinase): Comparison with Thermolysin-Like Kidney Neutral Metalloendopeptidase. J. Neurochem. In Press.

Almenoff, J., Teirstein, A. S., Thornton, J., and Orlowski, M. (1983) Identification of a Thermolysin-Like Metalloendopeptidase in Serum and Study of its Activity in Normal Subjects and in Patients with Sarcoidosis. Submitted.

Antinociceptive Effects of N-(1-carboxy-2-phenylethyl) Derivatives of Amino Acid Amides of p-Aminobenzoate (In collaboration with Dr. Stanley Glick) Manuscript in Preparation.

DEDICATION

To Allen--my wonderful husband, friend, and colleague:
whose infinite love, and whose kindness, devotion and sense of humor have softened the setbacks and heightened the joys of scientific research.

And to my parents for their love and support.

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LIST OF ABBREVIATIONS

Boc	tert-butoxycarbonyl
Bz	benzoyl
CF_3CO_2H	trifluoroacetic acid
DCC	N,N'-dicyclohexylcarbodiimide
DFP	diisopropyl fluorophosphate
DMF	dimethylformamide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Hip	hippuryl
HPLC	high-pressure liquid chromatography
4-MeO2NA	4-methoxy-2-naphthylamide
2NA	2-naphthylamide or 2-naphthylamine
$NaDodSO_4$	sodium dodecyl sulfate
pAB	p-aminobenzoic acid
pNA	p-nitroanilide or p-nitroaniline
THF	tetrahydrofuran
Tris	tris (hydroxy-methyl) aminomethane

The nomenclature of Schechter and Berger (1967) is used to describe the interaction between substrate and enzyme. Amino acid residues and other residues are designated P_1, P_2, P_3, etc. in the N-terminal direction and P_1' etc. in the C-terminal direction from the bond undergoing hydrolysis. The corresponding subsites in the enzyme are identified with the letter S.

I. INTRODUCTION

A. BIOLOGICAL SIGNIFICANCE OF MEMBRANE-BOUND NEUTRAL PEPTIDASES

Membrane-bound neutral peptidases are of special interest because the subcelluar localization of these enzymes implies unique biological roles. These roles include limited proteolysis of secretory proteins and peptide hormones, degradation of biologically active peptides, and participation in membrane specific metabolic processes. In contrast to the soluble lysosomal proteases which participate in cellular catabolism, the biological functions of most membrane-bound enzymes are not well characterized.

The process of limited proteolysis, whereby inactive precursor proteins and peptides are converted to active forms by highly specific proteolytic enzymes, has been well established in many biological systems. One of the earliest studied reactions was the extracellular activation of pancreatic zymogens. This cascade is initiated by enterokinase (enteropeptidase, EC 3.4.21.9) a serine protease which is associated with both the brush border membranes of the intestine (for a review see Kenny, 1977). Enterokinase is highly

specific for the conversion of trypsinogen to trypsin. Once formed, trypsin can activate other molecules of trypsinogen as well as other pancreatic zymogens including procarboxypeptidase, proelastase, chymotrypsinogen and prophospholipase (Neurath and Walsh, 1976). Similar cascade mechanisms are known to regulate many physiological processes including blood coagulation and fibrinolysis, the complement cascade, and the kinin-kininogen pathways.

1. Processing of Proteins and Peptides

Limited proteolysis has recently been shown to be an important mechanism for intracellular post-translational modification of proteins. The "signal peptide hypothesis" put forth by Bloebel and Sabatini (1971) proposes that there is a membrane-bound protease which converts pre-prohormones to prohormones. Specifically, the hypothesis describes the sequence of events by which co-translational translocation of secretory proteins occurs. The process begins as the first 15-30 residues of the amino-terminus of a nanscent polypeptide, known as the signal peptide, are translated. The signal peptide, which contains a large percentage of hydrophobic amino acids, is believed to embed itself, via specific receptor interactions, in the rough endoplasmic reticulum membrane. As translation of the protein proceeds, the signal sequence is driven through the endoplasmic reticulum membrane and is subsequently cleaved by the "signal peptidase".

The signal peptidase, which is described as a membrane-bound endopeptidase that is specific for the post-translational processing of proteins, has not yet been purified. As described by Jackson and Bloebel (1980), the enzyme is insensitive to a wide variety of protease inhibitors including DFP, EDTA, pepstatin, and thiol blocking agents. Thus, the catalytic class to which this enzyme belongs remains unclear. Jackson and White (1981) have shown that phospholipid is required for activity of the solubilized enzyme. Other studies by this group have led to the discovery of two proteins, "Signal Receptor Protein" (Walter and Bloebel, 1981) and "Docking Protein" (Meyer and Dobberstein, 1980) which are thought to regulate the translocation process, but further characterization of the signal peptidase itself has not been reported.

Evidence for the signal hypothesis includes the observation that when messenger RNAs of secretory proteins are translated by in vitro membrane free systems, they contain hydrophobic pre-segments of varying lengths and sequences. Jackson and Bloebel (1980) proposed that there is a universal signal peptidase because dog pancreatic membranes have the capacity to correctly process such diverse proteins as bovine pre-prolactin and pre-growth hormone, angler fish pre-proinsulin, rat pre-lactalbumin, and even viral proteins. An alternative explanation, that the microsomal membranes contain numerous processing enzymes of varying specificities, is not addressed by this hypothesis.

It is currently accepted that after translocation through the rough endoplasmic reticulum and removal of the presegment by signal peptidases, peptide hormones and secretory proteins are further processed in the Golgi apparatus and in secretory granules. The first evidence for this phenomenon was the discovery by Steiner and co-workers (1967) of proinsulin, a larger, biologically inactive form of insulin. Pulse-chase studies revealed that the conversion is initiated in the Golgi and continues in newly formed secretory granules (Orci et al., 1971). The structure of proinsulin is such that correct processing requires proteolytic cleavages on the carboxyl terminal side of dibasic amino acid sequences followed by removal of carboxyl terminal arginine residues from the B chain.

It has been suggested that the enzymes responsible for this conversion consist of a trypsin-like and a carboxypeptidase B-like activity. Kemmler et al. (1971) have demonstrated that pancreatic trypsin and carboxypeptidase B are indeed capable of converting proinsulin to insulin <u>in vitro</u>. This group has observed that intact secretory granules from pancreatic islet cells can convert proinsulin to insulin (Kemmler et al., 1973) and identified low levels of trypsin-like and carboxypeptidase B-like activities in these granules. Fletcher et al. have recently characterized an activity in both the soluble and washed membrane fractions of these granules which converts

proinsulin, proglucagon, and prosomatostatin to their biologically active forms (Fletcher et al., 1980; Fletcher et al., 1981). The converting activity has a pH optimum of 4.5-5.5 which approximates the range of intragranular pH and of granular stability (Howell, et al., 1969; Kemmler et al., 1973). Activity was strongly inhibited by antipain, leupeptin, p-chloromercuribenzoate (PCMB), and dithiodipyridine but not diisopropyl fluorophosphate (DFP), chloroquine, EDTA, or soybean trypsin inhibitor. Inhibition by PCMB and dithiodipyridine was reversed in the presence of dithiothreitol. On the basis of these data Fletcher et al. have concluded that the converting activity present in pancreatic secretory granules is due to a unique thiol protease. A major weakness of this work, however, is that the granule preparations used were contaminated with lysosomes and the authors do not provide convincing evidence that the converting activity is distinct from cathepsin B, a lysosomal thiol protease which is optimally active in a similar pH range.

Recently, the primary structure of pro-opiomelanocortin was deduced from the sequence of a complementary DNA clone of pro-opiocortin messenger RNA (Nakanishi et al., 1979). The structure revealed that pro-opiocortin, a 32,000-dalton glycoprotein found in the anterior and intermediate lobes of pituitary, contains the amino acid sequences of numerous hormones including adrenocorticotropin (ACTH),

β-lipotropin (β-LPH), β-endorphin, and α-melanotropin (α-MSH). Pulse-chase studies have demonstrated that these hormones are indeed derived from the pro-opiomelanocortin molecule (Mains and Eipper, 1980).

As seen in the case of other prohormones, pairs of basic amino acids flank the various peptide hormone sequences present on pro-opiocortin. Thus, similar kinds of enzyme activities, with trypsin and carboxypeptidase B-like specificities, are thought to participate in the processing of pituitary hormones. A report by Loh and Gainer (1982) describes an activity present in both the soluble and particulate fractions of rat neurointermediate lobe secretory granules which converts labeled toad pro-opiocortin to ACTH, β-LPH, α-MSH and a β-endorphin related peptide. Pro-opiomelanocortin converting activity is inhibited by high concentrations of leupeptin, pepstatin A, and p-chloromercuribenzoate but not diisopropyl fluoro-phosphate, N-p-tosyl-L-lysine chloromethyl ketone, chloroquine, or EDTA. The activity, which is described by Loh and Gainer as an "acid thiol, arginyl protease" bears some resemblance to the prosomatostatin-proglucagon-proinsulin convertase described by Fletcher et al. (1980; 1981). It is likely that the pro-hormone converting enzymes identified in these crude preparations actually represent numerous proteolytic enzymes. Further purification and specificity studies are needed to establish the biological significance of these enzymes.

The enzymology of peptide processing is clearly in its infancy. Characterization of both the signal peptidase and the prohormone processing enzymes has just begun, and there are many enzymes yet to be discovered. Studies in this field are impeded by the cumbersome techniques of pulse-chase combined with immunoprecipitation, chromatography, and electrophoresis which are required to assay processing enzyme activity. Further difficulties are encountered in preparation of large amounts of highly purified granules, solubilization of membrane-bound activities and with the low specific activities of processing enzymes generally found in these preparations.

2. Degradation of Biologically Active Peptides

Many neuropeptides have been shown to act at specific membrane-bound receptor sites at which their actions must be terminated. The apparent absence of specific neuronal reuptake systems has led many investigators to explore the role of membrane-bound peptidases in inactivation of neuropeptides at their receptor sites. This discussion will focus on enzymes, derived from synaptic membranes, which have been reported to metabolize β-endorphin, substance P, and the enkephalins.

β-endorphin (β-LPH_{61-91}) is an opioid peptide derived from pro-opiomelanocortin, which has been shown by radioimmunoassay and immunocytochemical techniques to be concentrated in the anterior and intermediate lobes of

pituitary (Bloom et al., 1978; Gramsh et al., 1979). β-endorphin binds to u, $^{\kappa}$, and $^{\sigma}$ opiate receptors and produces naloxone reversible analgesia when administered centrally. Its sustained central effects, stability in plasma, and high concentrations in pituitary imply a neurohormonal rather than a neurotransmitter role. Although β-endorphin is relatively resistant to aminopeptidase action, removal of the amino terminal tyrosine abolishes opiate activity (for a review see Cox and Baizman, 1982).

γ-endorphin (β-LPH_{61-77}) and α-endorphin (β-LPH_{61-76}) were identified in hypophysial extracts by Ling et al. (1976) and were found to possess some morphinomimetic activity. Studies on the metabolism of β-endorphin have confirmed the presence of an endopeptidase in synaptic membranes which cleaves β-endorphin at the Leu^{77}-Phe^{78} bond to form γ-endorphin at both neutral and acidic pH, and a carboxypeptidase A-like enzyme which converts γ-endorphin to α-endorphin when membranes are incubated at pH 5. Unike β-endorphin, γ- and α-endorphin are extremely susceptible to the action of membrane-bound carboxypeptidases and aminopeptidases and they are thus readily degraded to numerous inactive fragments. It is noteworthy that at neutral pH, Met-enkephalin, whose sequence forms the amino terminal pentapeptide of β-endorphin, is not a significant metabolite (Austen et al., 1977; Burbach et al., 1980; Burbach et al., 1981). Studies by Graf

et al. (1979) have demonstrated that cathepsin D cleaves β-endorphin at the Leu^{77}-Phe^{78} bond to form γ-endorphin. These authors suggest that the presence of cathepsin D within brain slices or derived from lysosomal contamination of synaptic membranes may account for the results of Austen et al. (1977) and of Burbach et al. (1980). In addition, they suggest that this enzyme may play a physiologically significant role in β-endorphin metabolism.

Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH_2), a putative neurotransmitter which is distributed throughout the central and peripheral nervous systems, was first described by von Euler and Gaddum (1931) and more completely characterized in Leeman's laboratory (Chang and Leeman, 1970). Its pharmacological effects include increased salivation, increased gastrointestinal motility, and vasodilation. Immunocytochemical studies have revealed that substance P is present in neuronal cell bodies, processes, and terminals, and is highly concentrated in hypothalmus and mesencephalon (Elde and Hokfelt, 1978). It has also been localized in the myenteric plexus and endocrine-like cells of the gastrointestinal tract (Sundler et al., 1976).

The penultimate proline at the amino terminus and the amidated carboxyl terminus render substance P resistant to degradation by most exopeptidases. Its degradation can, however, be initiated by endopeptidase. Cytoplasmic

enzymes (for a review see Marks, 1978) including prolyl endopeptidase [EC 3.4.21.26] (Orlowski et al., 1979) can hydrolyse substance P.

A report by Lee et al. (1981) describes the biochemical properties and partial purification of a membrane-bound substance P inactivating enzyme from human brain. The enzyme, which has a neutral pH optimum, cleaves substance P at the Gln^6-Phe^7, Phe^7-Phe^8, and Phe^8-Gly^9 bonds giving rise to biologically inactive fragments. It has a molecular weight of 40,000-50,000 daltons, is strongly inhibited by metal chelators and is insensitive to high concentrations of captopril, a dipeptidyl carboxypeptidase inhibitor and to phosphoramidon, an inhibitor of thermolysin-like metalloendopeptidases. The enzyme hydrolyses biological peptides other than substance P. Its affinity for substance P, however, is markedly higher than for many other biologically active peptides. Furthermore, crude brain membrane extracts produce several metabolites of substance P which are the same as those formed by the purified enzyme. On the basis of these data, the authors suggest that this metalloendopeptidase might play an important role in the degradation of substance P at the synaptic cleft. This report will undoubtedly stimulate further specificity studies and biochemical characterization of this unique enzyme.

The discovery of the opioid peptides Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) (Hughes et al., 1975) has led to numerous studies of

their metabolism by synaptic membranes. Graf et al. (1982) have reported that incubation of Met-enkephalin with rat brain membranes releases the biologically inactive metabolites Tyr, Tyr-Gly, and Tyr-Gly-Gly in a molar ratio of 28:6:15. Guyon et al. (1979) have reported similar ratios. Thus, several membrane-bound enzymes have been implicated in enkephalin metabolism. These include aminopeptidases (Hersh, 1981a) and a dipeptidyl aminopeptidase (Gorenstein and Snyder, 1980) which catalyse the removal of a Tyr^1 and Tyr^1-Gly^2 respectively. The angiotensin converting enzyme (EC 3.4.15.1, peptidyl dipeptidase) and an enzyme designated as "enkephalinase" both hydrolyse the Gly^3-Phe^4 bond of Met- and Leu-enkephalin, thereby removing a C-terminal dipeptide (Arregui et al., 1979; Benuck and Marks, 1980; Gorenstein and Snyder, 1979; Malfroy et al., 1978). There is considerable controversy as to the extent of involvement of each of these enzymes in enkephalin metabolism.

The name "enkephalinase", which implies a specific, enkephalin degrading function, is associated with a membrane-bound metalloprotease that has been described as a dipeptidyl carboxypeptidase distinct from the angiotensin converting enzyme. Evidence supporting the role of this enzyme as a specific enkephalin inactivator includes the findings that the regional distribution of the enzyme in brain appears to parallel that of the opiate receptors (Malfroy et al., 1979; Sullivan et al., 1978) and that

thiorphan [(DL-3-mercapto-2-benzylpropanoyl)-glycine], a selective inhibitor of the enzyme, shows antinociceptive activity in the hot plate jump test in mice (Roques et al., 1980). Thiorphan was also shown to enhance the recovery of Met-enkephalin released by depolarized striatal slices, while puromycin, an aminopeptidase inhibitor, and captopril, an angiotensin converting enzyme inhibitor, did not have this effect (Patey et al., 1981). This result suggests that synaptically released enkephalins are primarily metabolized by enkephalinase. Furthermore, a recent study by Zhang et al. (1982) revealed that intracerebral administration of thiorphan increased striatal Met-enkephalin content by 30%, while bestatin, a potent aminopeptidase inhibitor, had no effect on striatal Met-enkephalin levels.

Thiorphan has been shown to possess weak antinociceptive activity in the mouse hot plate jump test, however, it is inactive in the rat tail-flick test (Chipkin et al., 1982; Roques et al., 1980; Yaksh and Harty, 1982). It has been well established that thiorphan and phosphoryl-Leu-Phe, another enkephalinase inhibitor (Algeri et al., 1981) both potentiate the antinociceptive effects in the tail-flick test of [D-Ala^2]Met-enkephalin, an aminopeptidase resistant Met-enkephalin analogue (Chipkin et al., 1982; Roques et al., 1980; Yaksh and Harty, 1982; Zhang and Costa, 1982). This suggests that both aminopeptidase

and enkephalinase may play a role in the inactivation of exogenous enkephalins.

Accordingly, Zhang et al. (1982) have performed in vivo inhibition studies utilizing bestatin [(2S,3R)]-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine], an aminopeptidase inhibitor which is 1,000 times more potent than puromycin. Intracerebral administration of bestatin failed to change striatal Met-enkephalin concentrations or to produce analgesia, but it did potentiate the pharmacological effects of thiorphan. Similar potentiation was reported by Chaillet et al. (1983). In contrast to Zhang et al. (1982), however, they observed antinociceptive effects of bestatin itself. Despite this inconsistency, both studies provide data which suggest that enkephalinase and aminopeptidase both participate in the metabolism of enkephalins.

Two studies have explored the possibility that enkephalin degradation by synaptosomal enzymes is functionally or topographically coupled to enkephalin binding of opiate receptors. Knight and Klee (1978) studied the hydrolysis of Met-enkephalin by aminopeptidase at enkephalin concentrations well below the K_m of the enzyme, and surprisingly found that the rate of hydrolysis is independent of the relative amounts of enkephalin free in solution and bound to receptors. The authors speculate that the binding of enkephalins to opiate receptors acts as a concentrating

mechanism for membrane-bound aminopeptidases, which may be localized in the vicinity of the receptors.

Graf et al. (1982) have explored the possibility that a topographical relationship exists between enkephalin binding sites and enkephalin degrading peptidases. They reasoned that if receptor occupancy is coupled to the peptidase activities, then blocking of the receptor with other opiate ligands might alter enkephalin degradation patterns. They observed no significant effects of naloxone, morphine, or enkephalin analogues on the degradation of Met-enkephalin by rat brain membranes. They also showed that the dissociation curves of (^{3}H)Met-enkephalin from opiate receptors were the same in both the absence and presence of a mixture of peptidase inhibitors which completely protected Met-enkephalin from cleavage at the Tyr-Gly and Gly-Phe bonds. This result indicates that the process of dissociation of enkephalin from its binding sites is independent of the action of these peptidases.

The biochemical properties of these membrane-bound enkephalin degrading peptidases have been studied to varying degrees. Hersh (1981a) has isolated two membrane-bound, EDTA sensitive aminopeptidases from rat brain. Both enzymes are optimally active at pH 7, and both cleave Met-enkephalin at the Tyr^{1}-Gly^{2} bond. The two enzymes can be distinguished on the basis of their kinetic parameters for synthetic substrates and enkephalins.

Dipeptidyl aminopeptidase has been solubilized and partially purified by Gorenstein and Snyder (1979). It is sensitive to o-phenanthroline, a chelating agent, and also to p-hydroxymercuribenzoate, a thiol blocking agent, but its specificity and biochemical properties have not been extensively characterized.

There are two membrane-bound metalloproteases, angiotensin converting enzyme and enkephalinase, which cleave Met- and Leu-enkephalin at the Gly-Phe bond (Arregui et al., 1979; Benuck and Marks, 1980; Gorenstein and Snyder, 1979; Malfroy et al., 1978). The angiotensin converting enzyme is a dipeptidyl carboxypeptidase of broad specificity which has been purified to homogeneity from lung, one of its richest sources (Das and Soffer, 1975). The enzyme has been shown to convert circulating angiotensin I to angiotensin II (Ng and Vane, 1967), the latter having potent pressor effects. Angiotensin converting enzyme also acts as a kininase. By degrading bradykinin, a vasodilatory peptide, it reinforces the pressor effects of angiotensin II production (for a recent review see Ryan, 1982). Recently captopril [D-3-mercapto-2-methylpropanoyl-L-proline] (Ondetti et al., 1977) and MK-421 [N-(1-S-1-carboxy-3-phenylpropyl)-Ala-Pro] (Patchett et al., 1980), active site directed inhibitors of the converting enzyme, have been shown to have anti-hypertensive effects.

In 1978 Erdos and co-workers reported that the pulmonary angiotensin converting enzyme cleaves enkephalins at the Gly^3-Phe^4 bond, and they speculated that the enzyme might be involved in enkephalin metabolism. Studies by Benuck and Marks (1978) on a partially purified preparation of angiotensin converting enzyme from rat brain indicated that its biochemical properties were similar to those of the pulmonary enzyme. Subsequent investigations showed that the converting enzyme hydrolyzes enkephalins at the Gly-Phe bond (Arregui et al., 1978; Benuck and Marks, 1980; Gorenstein and Snyder, 1979; Swerts et al., 1979). The data of Schwartz and co-workers (Patey et al., 1981) suggest that angiotensin converting enzyme is not involved in the metabolism of synaptically released enkephalins. Graf et al. (1982) however, have observed that when Met-enkephalin is incubated with brain membranes, 1 mM captopril inhibits the release of 40% of the Tyr-Gly-Gly liberated. The latter result implies that the angiotensin converting enzyme may in fact be a biologically significant enkephalin degrading enzyme. Further studies are needed to delineate the metabolic processes regulated in brain by the angiotensin converting enzyme.

Enkephalinase was originally described by Malfroy et al. (1978) as a highly specific enkephalin degrading enzyme with a K_m for Met-enkephalin in the nanomolar range. Subsequent reports by this group and others indicate that

its K_m values for enkephalins are actually on the order of 10^{-5} M (Altstein et al., 1981; Benuck and Marks, 1980; Malfroy and Schwartz, 1982). Enkephalinase is inhibited by thiols and metal chelators, but not by captopril. Because it cleaves the C-terminal dipeptide from Met- and Leu-enkephalin, and also from bradykinin (Benuck and Marks, 1980) it was classified as a dipeptidyl carboxypeptidase. This classification, however, was not based on extensive specificity studies with a purified enzyme, but rather on limited studies with crude preparations. Blumberg et al. (1981) observed that benzyloxycarbonyl derivatives of amino acid hydroxamates, which inhibit the bacterial metalloendopeptidase thermolysin, also inhibited enkephalinase activity in crude brain membranes. This led them to postulate that enkephalinase might actually be a thermolysin-like endopeptidase rather than a true dipeptidyl carboxypeptidase.

In 1981, Llorens and Schwartz, and Benuck and co-workers reported on the exceptionally high specific activity of enkephalinase in peripheral tissues, particularly kidney and lung. Neither of these organs possesses significant numbers of opiate receptors, thus these findings cast doubt as to the specificity of this enzyme as an enkephalinase. Further characterization of enkephalinase may lead to the discovery of additional biological functions for the enzyme.

B. PROPERTIES OF A MEMBRANE-BOUND NEUTRAL METALLOENDOPEPTIDASE

A metalloendopeptidase which is optimally active at neutral pH was recently purified 10,000-fold from bovine pituitary membranes by Orlowski and Wilk (1981). The enzyme was solubilized by treatment with deoxycholate and papain and was found to have an apparent molecular weight of 90,000 as determined by gel filtration on Sephadex G-200 and G-100. Enzyme activity was not sensitive to inhibition by SH-blocking agents or by serine or carboxyl protease inhibitors, but was strongly inhibited by metal chelators. The enzyme was almost completely inhibited by dialysis against EDTA; activity was restored by addition of zinc and other divalent cations.

The specificity and kinetic parameters of the enzyme were studied with a series of synthetic peptide naphthylamides. The enzyme was found to have a primary specificity similar to thermolysin in that it cleaves peptide bonds on the amino side of hydrophobic amino acids. Relatively high reaction rates and specificity constants were obtained with substrates containing arginine residues in positions P_1 and P_2 thereby creating the impression of a trypsin-like activity. Differential centrifugation studies demonstrated that the highest specific activity of this enzyme was associated with the microsomal fraction. It was therefore suggested that the enzyme might play a role in

the processing of pituitary prohormones such as β-lipotropin, which require cleavage of a bond between a basic amino acid sequence and a tyrosine.

It was suggested by Orlowski and Wilk (1981) that the pituitary metalloendopeptidase might be similar to a thermolysin-like neutral metalloendopeptidase [EC 3.4.24.11] purified from kidney brush border by Kerr and Kenny (1974a and b). The enzyme, which is the first metalloendopeptidase to be isolated from mammalian tissue, was purified from rabbit and porcine kidney cortex. In contrast to thermolysin, which is a soluble microbial enzyme with a molecular weight of approximately 40,000 (Ohta et al., 1966), the solubilized kidney metalloendopeptidase was found to exist as a single polypeptide chain with a molecular weight of about 93,000. It was shown to contain stoichiometric amounts of zinc and to be strongly inhibited by chelating agents such as EDTA, o-phenanthroline and dithiothreitol. Kidney enzyme activity was inhibited with a K_i of 2×10^{-9} M by phosphoramidon [N-(α-L-rhamnopyranosyloxy-hydroxyphosphinyl)-L-leucyl-L-tryptophan], an active site directed inhibitor of thermolysin (Kenny, 1977). Kenny and co-workers demonstrated that the enzyme readily degrades peptides without significant secondary or tertiary structure such as insulin B chain (30 residues), glucagon (29 residues), and ACTH (24 residues) but that it has negligible activity toward proteins such as bovine serum albumin or

casein. The enzyme does not degrade native insulin. Kenny did not study the secondary specificity of the kidney enzyme, thus it is not possible to compare its kinetic properties to those described by Orlowski and Wilk. Like other brush border peptidases including γ-glutamyl transpeptidase (Orlowski and Meister, 1965) and aminopeptidase M (Wacker et al., 1971), which contain about 20% carbohydrate, the kidney metalloendopeptidase was found to contain about 15% carbohydrate (Kerr and Kenny, 1974b).

Varandani and Shroyer (1977) have also described a kidney brush border neutral metalloendopeptidase. Like the enzyme described by Kerr and Kenny, it degrades insulin B chain, glucagon, and ACTH. The enzyme, which was not purified to homogeneity, was found to have a molecular weight of approximately 200,000. Its sensitivity to phosphoramidon was not tested ñor was its primary specificity delineated, thereby making difficult a comparison of this enzyme to the one described by Kerr and Kenny.

More recently, Benyon et al. (1981) and Kenny et al. (1981) have described a brush border neutral metalloendopeptidase which degrades azocasein and also insulin B chain. The solubilized enzyme has a molecular weight of approximately 300,000-400,000 daltons. In contrast to the thermolysin-like neutral metalloendopeptidase, it is insensitive to inhibition by phosphoramidon, and it has a pH optimum (for azocasein) in the pH range of 8-9. Its

primary specificity and other biochemical properties await further characterization.

The kidney brush border is the surface on which much of the glomerular filtrate including water, ions, glucose, amino acids, and peptides, is reabsorbed. Many different enzymes, including phosphatases, glycosidases, and peptidases have been localized on the brush border. They have been proposed to participate in the metabolism of molecules being reabsorbed, but at present, this function remains only speculative. Accordingly, the kidney metalloendopeptidase [EC 3.4.24.11] is thought to play an important role in the degradation of filtered peptides, particularly insulin (Kerr and Kenny, 1974a; Varandani and Shroyer, 1977). Its presence however, in other tissues including intestinal brush border (Danielson et al., 1980) and pituitary (Orlowski and Wilk, 1981) indicates that it may have additional biological functions.

II. SPECIFIC AIMS

Specific aims of this work include:

1. Investigation of whether the membrane-bound neutral metalloendopeptidase is identical to enkephalinase.
2. Purification of the metalloendopeptidase from both rabbit brain and kidney.
3. Study of the interactions of the metalloendopeptidase with synthetic substrates, natural peptides and inhibitors. Comparison of kinetic and immunological properties of the brain and kidney enzymes.
4. Design and synthesis of active-site directed inhibitors.
5. Investigation of the in vivo effects of these inhibitors on nociception.
6. Identification and characterization of the metalloendopeptidase in human serum.
7. Study of serum metalloendopeptidase activity in healthy subjects and in patients with sarcoidosis.

III. EXPERIMENTAL METHODS

A. MATERIALS

Sodium phenylpyruvate, sodium cyanoborohydride, tert-butoxycarbonyl derivatives of amino acids, N,-N'-dicyclohexylcarbodiimide, N-hydroxysuccinimide, trifluoroacetic acid, triethylamine, hippuric acid, glutathione, dithiothreitol, 2-mercaptoethanol, p-mercuribenzoate, iodoacetamide, iodoacetic acid, N-ethylmaleimide, papain, antipain, chymostatin, EDTA, o-phenanthroline, L-Leu-pNA, Trizma base, phosphoramidon, bestatin, N-1-Naphthyl-ethylenediamine·2HCl, all dipeptides and biologically active peptides were obtained from Sigma Chemical Co. (St. Louis, MO). Barbital was obtained from Corning ACI (Palo Alto, CA), Noble agar from Difco (Detroit, MI), and agarose, N,-N' Methylene-bis acrylamide, and Coomasie Brilliant Blue were obtained from Bio-Rad (Richmond, CA). p-Amino-benzoic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI). DFP was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Silica gel plates (13181) for thin-layer chromatography and acrylamide were obtained from Eastman Kodak Co. (Rochester, NY). Sephadex G-200 and phenyl-Sepharose CL-4B were obtained from Pharmacia, Inc. (Piscataway, NJ). Aminopeptidase M [EC 3.4.11.2] was obtained from Boehringer/Mannheim Inc.

(Indianapolis, IN). Tyr-Gly-Gly was obtained by catalytic hydrogenation of Cbz-Tyr-Gly-Gly. Glutaryl-Ala-Ala-Phe-4MeO2NA was obtained from Enzyme Systems Products (Livermore, CA). Hip-Arg-Arg-Leu-2NA, Hip-Arg-Arg-Ala-2NA, Hip-Arg-Arg-Gly-2NA, and glutaryl-Ala-Ala-Phe-2NA were synthesized as previously described (Orlowski and Wilk, 1981). Thiorphan was obtained as a gift from Dr. J.-C. Schwartz, Unite de Neurobiologie, 75014 Paris, France. A 0.4 mM solution was freshly prepared with 2 mM DTT and diluted several-thousand-fold for inhibition studies. At these concentrations, DTT does not inhibit the enzyme. Gly-Pro-2NA, which was used to determine dipeptidyl aminopeptidase IV [EC 3.4.14.-] was obtained from Bachem (Torrance, CA). γ-Glutamyl-pNA was synthesized and used to determine γ-glutamyl transpeptidase [EC 2.3.2.2] as described (Orlowski and Meister, 1965).

All other reagents not specifically mentioned were obtained from either Fischer Scientific Co. (Springfield, NJ) or Sigma Chemical Co. (St. Louis, MO) and were of reagent grade purity.

Male Sprague-Dawley rats weighing about 250 g were used for all experiments.

B. METHODS

1. Synthetic Procedures and Identification of Reaction Products

Peptides were synthesized by using N-hydroxysuccinimide esters of Boc-amino acids (Anderson et al., 1964) in the

formation of the peptide bond, as described (Orlowski and Wilk, 1981). The purity of the compounds was determined by amino acid analysis, TLC, and HPLC. N-Carboxymethyl derivatives of amino acid amides of pAB were prepared by reductive amination of phenylpyruvate with either Phe-pAB, Ala-pAB, or Gly-pAB according to modification of the method described by Patchett et al. (1980). Proton NMR was used to confirm the structure of the diastereomers in addition to HPLC, elemental analysis, and TLC, which were used to determine purity.

A Perkin-Elmer Series 2 liquid chromatograph equipped with a variable-wavelength detector was used for analysis of all compounds whose syntheses are described here and for separation of the products of enzymatic hydrolysis of the biological peptides shown in Table 9. Emerging peaks were monitored at 210 nm. Samples were separated on a reverse-phase C_{18} column (4.5 x 250 mm, 5 um; IBM) by elution with a linear gradient established between KH_2PO_4 (0.05 M, pH 2) and acetonitrile. The starting acetonitrile concentration was 20%, and its concentration was increased at 1%/min at a flow rate of 1 ml/min.

The products of enzymatic hydrolysis of biological peptides shown in Table 1 were separated on a Waters Associates HPLC equipped with a C_{18} uBondapak column (30 x 0.4 cm). Elution was carried out with a linear gradient established between a 0.1% aqueous solution of

phosphoric acid and acetonitrile. 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.

The cleavage site of synthetic peptide naphthylamide substrates was determined by incubating the metalloendopeptidase, buffer, and aminopeptidase M as described in "Methods," Section 2. Free amino acids released during the course of the reaction were determined by amino acid analysis.

Ascending TLC was done on silica gel plates with fluorescent indicator in chloroform-methanol (60:40). Compounds were visualized by examination of plates in a viewing box equipped with a short wave UV light source. Amino acid analysis of peptides and the products of their acid and enzymatic hydrolysis was done as previously described by Orlowski and Wilk (1981).

Melting points are uncorrected. Analyses for carbon, hydrogen, and nitrogen were carried out by Schwartzkopf Microanalytical Laboratory (Woodside, NY) and the Microanalytical Service of the Rockefeller University (New York, NY).

Phe-pAB. N-Boc-Phe N-hydroxysuccinimide ester (5.5 mmol), prepared according to Anderson et al. (1964), was reacted in THF with pAB (5 mmol) in the presence of 1-hydroxybenzotriazole (5 mmol). The mixture was stirred overnight at room temperature after which time the solvent

was removd by flash evaporation. The remaining oil was dissolved in chloroform. The product crystallized from the solution within several hours. The crystals were collected on a sintered glass funnel, washed with chloroform, and dried. Treatment with CF_3CO_2H and ether yielded the CF_3CO_2H salt of Phe-pAB (61% yield). The salt can be converted to the free amide by dissolving it in ethanol and adding an equivalent amount of triethylamine. Phe-pAB precipitates within minutes, after which time it is washed with ethanol and dried: single peak on HPLC with a retention time of 9.2 min; mp 250° C dec.

Hip-Phe-pAB. A total of 0.5 mmol of Phe-pAB was dissolved in THF, along with an equivalent amount of triethylamine. Hippuryl N-hydroxysuccinimide ester (0.525 mmol) was added and the mixture stirred for 30 min, after which time an additional 0.3 mmol of triethylamine was added. After 24 h, the THF was flash evaporated; methylene chloride was then added, and the product readily crystallized. The crystals were filtered and washed with methylene chloride and dried (81% yield): HPLC, single peak with a retention time of 25.0 min; amino acid analysis, Phe 1.09, Gly 1.0; mp 242° C dec. Anal. Calcd for $C_{25}H_{23}N_3O_5 \cdot H_2O$: C, 64.79; H, 5.44; N, 9.07. Found: C, 65.00; H, 5.18; N, 8.81.

Glutaryl-Phe-pAB. One millimole of Phe-pAB was dissolved in THF with an equivalent amount of triethylamine.

The mixture was stirred in ice, and 3 mmol of glutaric anhydride was slowly added. After 1 h, the solvent was flash evaporated. The product crystallized from chloroform: HPLC, single peak with a retention time of 19.6 min.

Acetyl-Phe-pAB. A total of 1 mmol of Phe-pAB was dissolved in THF with an equivalent amount of triethylamine. The mixture was stirred in ice, and 3 mmol of acetic anhydride was slowly added. After 1 h, the solvent was flash evaporated, and the product was obtained as an amorphous white powder. It was then washed on a filter with diethyl ether: HPLC, single peak with a retention time of 17.2 min; mp 232° C dec.

Glutaryl-Gly-Phe-pAB. A total of 4.2 mmol of Phe-pAB was dissolved in THF and reacted with N-Boc-Gly N-hydroxysuccinimide ester (5 mmol) in the presence of 5 mmol of both 1-hydroxybenzotriazole and triethylamine. After 24 h, the solvent was evaporated, and the residue was dissolved in chloroform and washed twice with $NaHSO_4$ (0.02 M, pH 2.6) and twice with water. (The water phase was reextracted each time with a small amount of chloroform, which was then combined with the chloroform layer.) The organic phase was dried with anhydrous sodium sulfate and the solvent removed *in vacuo*. The residue was then treated with CF_3CO_2H and ether to yield $CF_3CO_2H \cdot$Gly-Phe-pAB, which was dried in a vacuum desiccator. A total of 0.5 mmol of the dried compound was dissolved without further purification in THF

with an equivalent amount of triethylamine. The mixture was stirred in ice, and DMF was added dropwise to keep the compound in solution. An equivalent amount of glutaric anhydride was slowly added, and after 1 h, the solvent was removed. The oily residue was dissolved in chloroform from which the product crystallized: HPLC, single peak with a retention time of 15.2 min; amino acid analysis, Gly 1.1, Phe 1.0; mp 170° C dec.

N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB was prepared by reductive amination of phenylpyruvate with Phe-pAB (Patchett et al., 1980). Phe-pAB (3.5 mmol) and sodium phenylpyruvate (17.6 mmol) were dissolved in 25 ml of water, and the pH was adjusted to 7.8 with 5 N NaOH. Sodium cyanoborohydride (14.8 mmol) was dissolved in 10 ml of water and added over a 5 h period with a syringe pump. After being stirred overnight at room temperature, the mixture was acidified under the hood to pH 2 with 6 N HCl. The precipitate was collected by filtration and washed with water. The dried material was then suspended in warm ethyl acetate, and the insoluble white solid was isolated by filtration. The material was dissolved in methanol after addition of triethylamine. Addition of CF_3CO_2H induced crystallization of a mixture of two diastereomeric products (49% yield): HPLC, two peaks with retention times of 21 (peak I) and 23.5 (peak II) min; TLC, two spots with R_f values 0.66 and 0.40. Anal. Calcd for $C_{25}H_{24}N_2O_5 \cdot 0.5H_2O$:

C, 68.02; H, 5.71; N, 6.35. Found: C, 68.27; H, 5.89; N, 6.58.

The two diastereomers were separated by preparative HPLC on two joined C_{18} uBondapak columns (7.8 x 30 cm) with a Waters Associates HPLC apparatus, by elution with a linear gradient, established between 0.28% triethylamine in water adjusted to pH 3.45 with trifluoroacetic acid and acetonitrile. The starting acetonitrile concentration was 10%, and its concentration was increased at a rate of 0.43%/min with a flow rate of 2.5 ml/min. A total of 10 mg of the diastereomeric mixture was injected per run, and emerging peaks were monitored at 285 nm. The two peaks, each of which contained a single diastereomer, were collected and evaporated under vacuum. Each compound was washed with tetrahydrofuran and ether and then dried at 100° C in a vacuum over calcium sulfate pellets for 24 h. Peak I showed the following: TLC, one spot, with R_f 0.66; mp 257-260° C dec; NMR (Me_2SO-d_6, δ downfield from Me_4Si) δ (AB quartet) 7.8-7.3 (J_{AB} = 8.7 Hz, 4 H, pAB), δ (aromatic) 7.2, 7.0 (10 H), δ (aliphatic) 2.9 (6 H, m). Peak II showed the following: TLC, one spot, with R_f 0.40; mp 248° C dec; NMR (Me_2SO-d_6, δ downfield from Me_4Si) δ (AB quartet) 7.9-7.6 (J_{AB} = 8.7 Hz, 4 H, pAB), δ (aromatic) 7.2, 7.1 (10 H), δ (aliphatic) 2.8 (6 H, m).

<u>N-[1-(R,S)-carboxy-2-phenylethyl]-Ala-pAB</u> was prepared by reductive amination of phenylpyruvate with Ala-pAB as

described above. After acidification to pH 2, the filtered precipitate was suspended in ethyl acetate, placed in a sintered glass filter, and washed extensively with ethyl acetate. A white solid was obtained (51% yield): HPLC, two peaks of equal height with retention times of 9.2 and 10.4 min; TLC, two spots with R_f values of 0.52 and 0.33; mp 220° C. Anal. Calcd with $C_{19}H_{20}N_2O_5 \cdot H_2O$: C, 60.95; H, 5.92; N, 7.48. Found: C, 61.64; H, 5.51; N, 7.46.

N-(1-carboxy-2-phenylethyl)-Gly-pAB was prepared by reductive amination of phenylpyruvate with Gly-pAB as described for the alanyl derivative. HPLC, one peak with a retention time of 8.1 min. Anal. Calcd for $C_{18}H_{18}N_2O_5$; C, 63.14; H, 5.26. Found: C, 62.94; H, 5.35. Mp 250° C.

2. Determination of Enzyme Activities

Metalloendopeptidase activity was determined either with Hip-Arg-Arg-Leu-2NA or glutaryl-Ala-Ala-Phe-2NA in a coupled enzyme assay, in the presence of an excess of aminopeptidase M [EC 3.4.11.2]. With glutaryl-Ala-Ala-Phe-2NA as substrate, the reaction proceeds as follows:

$$\text{glutaryl-Ala-Ala-Phe-2NA} \xrightarrow{\text{metalloendopeptidase}} \text{glutaryl-Ala-Ala} + \text{Phe-2NA} \quad [1]$$

$$\text{Phe-2NA} \xrightarrow{\text{aminopeptidase M}} \text{Phe} + \text{2NA} \quad [2]$$

In the first reaction the endopeptidase cleaves the bond on the amino side of the phenylalanine residue, releasing Phe-2NA, which is subsequently cleaved in the second reaction by the action of aminopeptidase M.

Incubation mixtures for determination of pituitary, brain, and kidney metalloendopeptidase activity contained substrate (0.4 mM; .02 ml of a 5 mM aqueous solution), enzyme (.01-.05 ml), aminopeptidase M (10 ug) and Tris-HCl buffer (0.05 M; pH 7.6) in a final volume of 0.25 ml. Incubations were for 30-90 min, depending on the concentration of enzyme. Reactions were terminated by addition of 10% trichloroacetic acid (0.25 ml). The 2-naphthylamine released during the reaction was quantitated by diazotization according to a modification (Goldbarg and Rutenberg, 1958) of the procedure by Bratton and Marshall (1939) or by the procedure of Barrett (1972). The pAB released by hydrolysis of pAB containing peptides was also determined by the diazotization procedure of Bratton and Marshall (1939). Some enzyme determinations were made in the presence of peptide inhibitors containing free amino groups. For these determinations, the enzyme, peptide, and substrate were incubated, and the reaction was terminated by boiling. 2NA was then released by adding 10 ug of aminopeptidase M and incubating for 30 min at 37° C before addition of trichloroacetic acid. This two-stage procedure was used to prevent degradation of unblocked peptides by aminopeptidase during the course of the endopeptidase reaction. Enzyme activity is expressed in units. One unit of bovine pituitary and rabbit kidney enzyme activity is defined as the amount of enzyme catalyzing the release of 1 umole of

Leu-2NA from Hip-Arg-Arg-Leu-2NA per h; one unit of rabbit brain enzyme activity is equivalent to the amount of enzyme catalyzing the release of 1 umole of Phe-2NA from glutaryl-Ala-Ala-Phe-2NA per h. Specific activity of the pituitary, kidney, and brain metallendopeptidases is in terms of units per milligram of protein as determined by the method of Warburg and Christian (1942).

Incubation mixtures (final volume 0.25 ml) for the determination of metalloendopeptidase activity in human serum contained glutaryl-Ala-Ala-Phe-2NA (0.8 mM; 0.040 ml of a 5 mM solution), Tris-HCl buffer (0.1 M, pH 7.6; 0.175 ml) aminopeptidase M (0.01 ml of a solution containing 1 mg/ml) and serum (0.025 ml). The samples were incubated at 37° C for 1-2 h at which time the reaction was stopped by addition of 10% trichloroacetic acid (0.25 ml). Precipitated protein was removed by centrifugation and 2-naphthylamine was determined in 0.3 ml aliquots of the supernatant. Diazotization was carried out by a modification of the procedure of Goldbarg and Rutenberg (1958). This involved addition of 0.25% sodium nitrite (0.2 ml), 1.25% ammonium sulfamate (0.2 ml) and 0.1% N-1-naphthylethylenediamine·2HCl (0.5 ml). One unit of human serum enzyme activity is defined as the amount of enzyme that catalyses the release of 1 nmole of product/min/ml serum under these conditions. Controls in which substrate or serum were separately omitted were also carried through the procedure.

Under conditions of the assay, a linear relationship was found between enzyme activity and either incubation time or amount of serum up to 0.025 ml. The reaction rate did not increase linearly when the amount of serum was increased up to 0.04 ml and above, apparently because of inhibition by serum components at higher serum concentrations. Sera with low enzyme activity were incubated for 2 h in order to generate amounts of product that could be reliably determined. The coefficient of variation for the assay was 3.5%.

Commercial preparations of aminopeptidase obtained from various suppliers are usually contaminated to varying degrees with a metalloendopeptidase activity, with a specificity apparently similar to the enzyme described in the present work. It was found that this contamination could be removed by hydrophobic chromatography on a phenyl-Sepharose column. This was carried out by adding a saturated solution of ammonium sulfate in 0.035 M Tris-HCl buffer (pH 7.6) to a solution of 25 mg of aminopeptidase M (Boehringer/Mannheim) to achieve a final saturation of 30% with respect to amminoum sulfate. The solution was applied to the top of a phenyl-Sepharose CL-4B column (15 ml) that had been previously washed with 30 ml of a 30% saturated ammonium sulfate solution in 0.05 M Tris-HCl buffer (pH 7.6). The column was washed with an additional 30 ml of the same solution, and the enzyme was eluted with a linear

gradient established between 150 ml of 30% saturated ammonium sulfate in 0.035 M Tris-HCl (pH 7.4) and 150 ml of 0.05 M Tris-HCl (pH 7.6). Fractions of about 3 ml were collected and tested for aminopeptidase activity with L-Leu-pNA as substrate and for metalloendopeptidase activity with Hip-Arg-Arg-Leu-2NA as described above. A virtually complete separation of aminopeptidase from the contaminating metalloendopeptidase was achieved, with the latter enzyme eluting after the aminopeptidase. The fractions containing the aminopeptidase were pooled and dialyzed against 0.05 M Tris-HCl buffer (pH 7.4). The protein concentration was adjusted to about 1 mg/ml, and the obtained solution was used in the experiments described here.

Aminopeptidase M (Pfleiderer et al., 1964) and dipeptidyl aminopeptidase IV (McDonald and Schwabe, 1977) activities were determined with Leu-pNA and Gly-Pro-2NA, respectively. Both enzymes were assayed in 0.05 M Tris-HCl (pH 7.6) at a substrate concentration of 0.4 mM. γ-Glutamyl transpeptidase activity was determined as described previously (Orlowski and Meister, 1965).

3. Isolation of Brain Membrane Fractions

The crude membrane preparations studied in Tables 3, 4, and 5 were obtained by the procedure of Malfroy et al. (1978). 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.

4. Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis was performed in 8% gels under dissociating (0.1% $NaDodSO_4$) and nondissociating conditions in 0.05 M Tris-HCl (pH 8.4) according to the method of Weber and Osborn (1969). A total of 5-20 ug of protein was layered onto each gel, and a current of 5 mA/tube was applied. Gels were either stained for protein with Coomassie Blue or sliced into 2-mm sections for determination of enzyme activity. Each slice was incubated in 0.3 ml of 0.05 M Tris-HCl (pH 7.65) overnight at 4° C, and 0.025 ml aliquots were then assayed for metalloendopeptidase activity as described above.

5. Immunodiffusion

Double radial immunodiffusion (Ouchterlony, 1958) was carried out in gels containing 0.15% agarose, 0.5% Noble agar, and 0.035% EDTA in 0.05 M sodium barbital (pH 8.6). Immunodiffusion was performed at 37° C in a humid atmosphere for 24 h after which time gels were washed overnight in saline and then soaked briefly in distilled water. Protein was detected by staining with Coomassie Blue.

6. Enzyme Purifications

The metalloendopeptidase was purified from bovine pituitaries as described by Orlowski and Wilk (1981). The kidney enzyme was purified from 100 g of frozen rabbit kidneys by the following modification of the method of Orlowski and Wilk (1981). A deoxycholate extract of the particulate fraction was prepared as described, and after

streptomycin precipitation, the supernatant was collected (step 1) and concentrated in an ultrafiltration cell (Amicon) to 66 ml. For each 25 mg of protein in solution, 1 mg of papain was added, followed by solid dithiothreitol to a final concentration of 5 mM. The mixture was incubated at 37° C for 90 min, centrifuged for 10 min at 3,000 g, and applied to a Sephadex G-200 column (5 x 90 cm) equilibrated with 0.05 M Tris-HCl (pH 7.6) (step 2). The column was eluted with the same buffer, and fractions of about 20 ml were collected. The enzyme emerged from the column in a single peak that was completely separated from dipeptidyl aminopeptidase IV (M_r 230,000), an abundant microvillus protein. Fractions containing metalloendopeptidase activity were pooled and concentrated by ultrafiltration to about 9 ml. A total of 6 ml of a saturated ammonium sulfate solution was added to the enzyme concentrate to yield 15 ml of a solution in 0.035 M Tris-HCl (pH 7.6) that was 40% saturated with respect to ammonium sulfate. This was applied to a phenyl-Sepharose column (25 ml) that was equilibrated with a 40% saturated ammonium sulfate solution in 0.035 M Tris-HCl (pH 7.6). The column was washed with 50 ml of the same buffer, and the enzyme was then eluted with a linear gradient established between 150 ml of the equilibrating buffer and 150 ml of 0.05 M Tris-HCl (pH 7.6). Fractions of 3 ml were collected and assayed for metalloendopeptidase activity (step 3). The enzyme was eluted from the column after about 200 ml of the eluting buffer passed

through the column and was well separated from an aminopeptidase activity that emerged in subsequent fractions. Active fractions were pooled, concentrated to about 30 ml and then dialyzed against two changes (4 L each) of 0.01 M sodium acetate buffer (pH 5.0). The enzyme was then applied to a carboxymethylcellulose column (CM-52, 7 ml) equilibrated with 0.01 M sodium acetate buffer (pH 5) and washed with 100 ml of this buffer. The enzyme was eluted with a gradient established between 150 ml of 0.01 M sodium acetate buffer (pH 5.0) and 150 ml of 0.01 M sodium acetate buffer containing 0.2 M NaCl. Fractions of 3 ml were collected and assayed for enzyme activity. The enzyme eluted as a single peak; active fractions were adjusted to pH 7.5 with 1 M Tris base.

The metalloendopeptidase was purified from the particulate fraction of rabbit brain homogenates as described above with minor modifications. After solubilization of the enzyme with deoxycholate and precipitation of excess deoxycholate with streptomycin sulfate, the supernatant was concentrated to about 45 ml. One mg papain was added per each 150 mg of protein in solution followed by solid dithiothreitol to a final concentration of 6 mM. The mixture was incubated at 37° C for 2 h, centrifuged for 10 min at 3,000 g and then subjected to Sephadex G-200 followed by phenyl-Sepharose chromatography as described above. Active fractions obtained after phenyl-Sepharose

chromatography were pooled, dialysed against 0.05 M Tris-HCl (pH 7.6) and stored in ice.

7. Immunization of Guinea Pigs

Antisera were raised to the rabbit kidney metalloendopeptidase at the Pocono Rabbit Farm & Laboratory, Canadenesis, PA. Pre-immune sera were obtained by cardiac puncture one week before immunization of guinea pigs with purified rabbit kidney metalloendopeptidase dissolved in 0.05 M Tris-HCl (pH 7.6) and emulsified with an equal volume of complete Freund's adjuvant. A total of 0.6 ml was injected intradermally at multiple sites along the dorsum at each immunization. Fifty to 60 ug of enzyme were initially injected followed by 25-30 ug booster doses at 4, 8, 11 and, in one animal, 13 weeks after the initial injection. Animals were bled 7-10 days after the final immunization, and the serum obtained was stored frozen. Antisera were raised to the enzyme both before and after CM-52 chromatography; all antisera gave similar results in the anticatalytic assay and in immunodiffusion experiments. The serum had no detectible metalloendopeptidase activity under the conditions of the anticatalytic assay and was thus used without further fractionation.

8. Anticatalytic Immunoinhibition Assay

Reaction mixtures (final volume 0.125 ml) containing 0.01 to 0.1 ml of rabbit kidney or brain enzyme, or of human serum after filtration on Sephadex G-200, varying

dilutions (1/300 - 1/5) of either pre-immune or immune serum, 0.05 mg of bovine serum albumin (omitted for incubation mixtures containing human serum) in 0.1 M Tris-HCl (pH 7.6) were pre-incubated at 37° C for 30 min. The enzymatic reaction was then initiated by adding 0.02 ml of glutaryl-Ala-Ala-Phe-2NA (5 mM), 0.095 ml 0.1 M Tris-HCl (pH 7.6) and 0.01 ml of aminopeptidase M (1 mg/ml) to each sample. Incubations were at 37° for 40 min. The reactions were terminated by adding trichloroacetic acid (0.25 ml of a 10% solution). Precipitated protein was removed by centrifugation and 2-naphthylamine was determined in 0.3 ml aliquots as described above.

9. Testing of Analgesia

The analgesic efficacy of N-[1-(R,S)-carboxy-2-phenylethyl] derivatives of phenylalanyl, alanyl, and glycyl-p-aminobenzoate was determined in the tail withdrawal test (Janssen et al., 1963). Each rat was initially placed in a plastic restrainer for 5 min. A baseline tail-withdrawal latency was then determined: the distal three inches of the tail was inserted into a beaker of water maintained at 56° C, and the time required for the rat to "flick" the tail out of the water was recorded with an electronic stopwatch. Each rat was then removed from the restrainer, injected intraperitoneally with either saline (1 ml/kg) or drug, and returned to its cage. Each rat was then retested at 1, 3, 6, 24 and 48 hours after treatment.

10. Acquisition and Handling of Serum Samples

Serum samples were obtained by venipuncture from healthy donors, sarcoidosis patients, and patients with other pulmonary diseases, and were stored frozen. No loss of metalloendopeptidase activity was observed with storage at -20° C for one year, and enzyme activity was stable through several cycles of freezing and thawing. Samples refrigerated at 4° for up to one week did not show any change in activity.

One hundred fifty control sera were obtained from male and female members of the Mount Sinai community and from anonymous blood bank donors. Subjects ranged in age from 18 to 70 years and had no known medical illnesses.

The patient population consisted of all sarcoid patients seen at The Mount Sinai Hospital Sarcoidosis Clinic or in the pulmonary division practice suite over a several-month period on whom a routine serum angiotensin converting enzyme (ACE) determination was made within two months of a chest roentgenogram. Metalloendopeptidase activity was then measured in the same serum from which ACE was determined. The diagnosis has been confirmed for all patients by either a positive Kveim-Siltzbach test or organ biopsy which shows non-caseating granulomas in a clinical setting which is consistent with sarcoidosis. Sarcoidosis patients with other diseases such as malignancies, diabetes, liver, or kidney disease were excluded from the study. The patient population consisted of individuals ranging in age

from 23-64 years, with inactive as well as active disease; 13 of the 95 patients studied were receiving steroid treatment at the time of metalloendopeptidase determination. Serum enzyme determinations were made with no knowledge of the patient's history, and radiographic and clinical assessment by the examining physician is made without knowledge of metalloendopeptidase levels. Radiographic stages were determined by Dr. Alvin Teirstein, Director of the Pulmonary Division of the Department of Medicine at The Mount Sinai Hospital according to the following criteria: Stage 0--Within normal limits; Stage I--Hilar lymphadenopathy; Stage II--Hilar lymphadenopathy with interstitial infiltrates; Stage III--Interstitial infiltrates.

Sera from patients with active tuberculosis and pulmonary neoplasms were provided by Dr. Marvin Lesser, Chief of the Pulmonary Division, Bronx Veterans Administration Hospital, New York. Sera from patients with idiopathic interstitial fibrosis were obtained from Dr. Alvin Teirstein. Statistical analyses were performed by Dr. John Thornton using SAS (Statistical Analysis System, 1983). The descriptive statistics were obtained from SAS's UNIVARIATE procedure. Enzyme distributions were compared by nonparametric analysis using SAS's NPAR1WAY procedure. The study was approved by the Research Advisory Committee of The Mount Sinai Hospital (#82-017 ME).

IV. RESULTS

A. COMPARISON OF THE MEMBRANE-BOUND PITUITARY METALLOENDOPEPTIDASE TO ENKEPHALINASE

Previous reports describing enkephalinase as a membrane-bound metalloprotease have led to the following investigation of whether the membrane-bound metalloendopeptidase isolated by Orlowski and Wilk (1981) is identical with enkephalinase. Although enkephalinase has been classified by several authors (Benuck and Marks, 1980; Fournie-Zaluski et al., 1979) as a dipeptidyl carboxypeptidase, this conclusion is based on work done with crude brain preparations on which detailed specificity studies were not carried out. The data presented here compare the purified pituitary metalloendopeptidase to enkephalinase with respect to their interactions with enkephalins, regional distribution in brain, and sensitivity to inhibitors.

When the pituitary enzyme was incubated with several biologically active peptides, the results shown in Table 1 were obtained. The enzyme cleaves Met- and Leu-enkephalin at the Gly-Phe bond to yield Try-Gly-Gly and Phe-Met or Phe-Leu respectively. This specificity is similar to that

Table 1. Cleavage of Biologically Active Peptides by the Pituitary 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-Try-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 or 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 "Experimental Methods".

reported for enkephalinase (Arregui et al., 1979; Benuck and Marks, 1980; Gorenstein and Snyder, 1979; Malfroy et al., 1978; Sullivan et al., 1980; Vogel and Altstein, 1980) and is consistent with the specificity of the metalloendopeptidase, which is directed toward bonds on the amino side of hydrophobic amino acids (Orlowski and Wilk, 1981). 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 (Benuck and Marks, 1980), 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, suggests that the enzyme cannot act as an exopeptidase.

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 (mean of two determinations) 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 (Cornish-Bowden, 1979). This K_m value is close to the K_m value of Leu-enkephalin reported for enkephalinase (Altstein et al., 1981; Benuck and Marks, 1980). The k_{cat} for hydrolysis of Met-enkephalin by the enzyme is 21.2 min^{-1}. The specificity constant k_{cat}/K_m is thus equal to 530. A comparison 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

Table 2. Kinetic Parameters[a] for Hydrolysis of Met-Enkephalin and Several Synthetic Substrates

Substrate P_3- P_2- P_1-P_1'-P_2'	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		

[a]The turnover rate constant (k_{cat}) for Met-enkephalin was determined by incubating the enzyme (0.08 units) with the substrate (0.4 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 the 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 initial velocity measurements by assuming a K_m of 0.04 mM. Data for other substrates are the same as those reported by Orlowski and Wilk (1981).

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 purification steps (step 4) (Orlowski and Wilk, 1981). The purified enzyme cleaved Met-enkephalin at the Gly-Phe bond, a reaction attributed to enkephalinase activity. 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 (Malfroy et al., 1979; Sullivan et al., 1978). 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

Table 3. Regional Distribution of the 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
Brainstem	0.186 ± .009 (4)	20
Prefrontal Cortex	0.156 ± .021 (4)	17

Activity was determined with the substrate glutaryl-Ala-Ala-Phe-2NA as described under "Experimental 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.

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

Inhibitor	Concentration (mM)	Percent Inhibition	
		Striatal Membrane Preparation	Pituitary Enzyme
Leupeptin	0.023	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 the substrate without preincubating the enzyme with the inhibitors. Data for the pituitary enzyme are those reported previously (Orlowski and Wilk, 1981). Membranes were prepared as described in "Experimental Methods."

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 enzymes to the same extent. A similar degree of inhibition of both enzymes was observed with phenobarbital, previously reported to inhibit enkephalinase activity (Altstein et al., 1981).

Table 5 shows the distribution of the metalloendopeptidase in rabbit tissues. The enzyme is most abundant in kidney, spleen and lung membranes, and has a relatively low specific activity in brain. These data are in agreement with previous reports (Benuck et al., 1981; Llorens and Schwartz, 1981) in which membranes of both kidney and lung have been shown to possess relatively high enkephalinase activity. Neither of these groups measured enkephalinase activity in spleen, which of the organs studied here, had the second highest specific activity.

B. PURIFICATION OF THE KIDNEY METALLOENDOPEPTIDASE AND DETERMINATION OF ITS PHYSICOCHEMICAL PROPERTIES

The specific activity of the metalloendopeptidase in brain and pituitary is very low, making it difficult to isolate the enzyme from these tissues. A convenient procedure is presented for purification of the enzyme to

Table 5. Specific Activity of the Metalloendopeptidase in Rabbit Tissues

Organ	Specific Activity	Relative Activity
Brain	0.13 ± 0.03	1.0
Heart	0.11 ± 0.08	0.8
Liver	0.16 ± 0.06	1.2
Testes	0.34 ± 0.09	2.5
Lung	2.77 ± 0.52	20.5
Spleen	5.50 ± 1.20	40.7
Kidney	84.20 ± 7.14	623.7

Membranes were prepared as described in "Experimental Methods" and activity was determined using 0.4 mM glutaryl-Ala-Ala-Phe-2NA as the substrate. 0.1 mg protein 0.25 ml reaction mixture was used, except in the case of lung and spleen, for which 0.01 mg protein was used, and kidney, for which 1 ug protein was used. Activity in all regions was inhibited approximately 90% by 2 mM DTT. Data are the mean values ± S.E. of 3-4 rabbits. Specific activity is expressed in umoles/h/mg protein. Relative specific activities are expressed with brain arbitrarily set at 1.

apparent homogeneity from rabbit kidney, an abundant source.

A summary of the purification procedure is shown in Table 6. Starting with the streptomycin-treated deoxycholate extract, the enzyme was purified 160-fold with a yield of approximately 19%. Like the pituitary metalloendopeptidase, the kidney enzyme was effectively released from the membranes by treatment with deoxycholate and papain. Papain (M_r 23,000) was completely separated from the metalloendopeptidase by chromatography on Sephadex G-200 as previously described (Orlowski and Wilk, 1981). A highly purified preparation was obtained after chromatography on phenyl-Sepharose (step 3); however, the enzyme eluted in a peak that coincided with the peak of γ-glutamyl transpeptidase activity. The two enzymes were separated in step 4. In 0.01 M sodium acetate (pH 5.0), metalloendopeptidase bound to carboxymethylcellulose, while most of the transpeptidase eluted with the starting buffer. The small amount of transpeptidase that bound to CM-52 under these conditions was eluted at the beginning of the sodium chloride gradient and was thus separated from the metalloendopeptidase, which was eluted in later fractions.

Electrophoresis of the enzyme carried out under nondissociating conditions revealed one major and one minor protein band (Figure 1A). Unstained duplicate gels were sliced into 2-mm sections and assayed with

Table 6. Summary of Purification of the Metalloendopeptidase from Rabbit Kidney[a]

Purification Step	Volume (ml)	Protein (mg/ml)	Activity		Sp Act. (Units/mg)	Recovery (%)	Purification (x-fold)
			Units/ml	Total			
(1) deoxycholate extract	260	13.8[b]	138	35,880	10	100	1
(2) papain treatment and Sephadex filtration	238	0.176	60.0	14,280	341	40	34
(3) phenyl-Sepharose chromatography	67	0.170	178	11,930	1,050	33	105
(4) carboxymethylcellulose chromatography	56	0.074	120	6,720	1,620	19	162

[a]For details and definition of units, see "Experimental Methods".

[b]Protein was determined for this step by the method of Lowry et al. (1951).

FIGURE 1. POLYACRYLAMIDE GEL ELECTROPHORESIS OF KIDNEY METALLOENDOPEPTIDASE.

Left (1A) nondissociating conditions (both bands have metalloendopeptidase activity); Right (1B) dissociating conditions (0.1% $NaDodSO_4$).

Hip-Arg-Arg-Leu-2NA and glutaryl-Ala-Ala-Phe-2NA; regions corresponding to both protein bands were found to contain metalloendopeptidase activity that was proportional to the intensity of the Coomassie blue staining. Electrophoresis under dissociating conditions (0.1% $NaDodSO_4$) revealed a single sharp band (Figure 1B). Treatment of these samples with 2% and 5% 2-mercaptoethanol gave a major band with a molecular weight of about 94,000 and a faint band with a molecular weight of approximately 85,000.

Given that the enzyme was released from membranes by papain digestion, it is possible that the faint band seen under reducing conditions may represent a fragment of the main polypeptide chain, which was generated by limited proteolysis during papain treatment. Similar results have been reported for other membrane-bound enzymes prepared by proteolytic digestion (Kenny et al., 1976). The diffuse quality of both active bands seen under nondissociating conditions (Figure 1A), which in the presence of $NaDodSO_4$ migrates as a single sharp band (Figure 1B), suggests that the enzyme is comprised of charge isomers.

The molecular weight of the enzyme was determined by gel filtration on a Sephadex G-200 column according to the method of Andrews (1965). Enzyme activity eluted as one peak with an apparent molecular weight of 98,000. These data, in conjunction with the $NaDodSO_4$ gels, suggest that the enzyme is a monomer with a molecular weight of about 95,000.

The pH optimum of the metalloendopeptidase for Hip-Arg-Arg-Leu-2NA (0.4 mM) was determined in 0.2 M Tris-HCl buffers, in the pH range between 7.0 and 8.8. A broad optimum between pH 7.5 and 8.0 was found, and enzyme activity was routinely determined at pH 7.6.

The effect of various inhibitors on enzyme activity is summarized in Table 7. Leupeptin, antipain, and chymostatin, transition-state aldehyde inhibitors of several thiol and serine proteases, have no effect on activity. That neither DFP, an irreversible serine protease inhibitor, nor any of the thiol blocking agents tested affected activity further indicates that the enzyme is neither a serine nor a thiol protease. Although moderate inhibition was noted with pepstatin, the concentrations required for inhibition (35% inhibition at 0.067 mM pepstatin) are by several orders of magnitude greater than those effectively inhibiting carboxyl proteases. Thus, for example, pepsin is inhibited with a K_i of about 10^{-10} M (Kunimoto et al., 1974), and cathepsin D, another carboxyl protease, is inhibited with a similar K_i value (Knight and Barrett, 1976). It is therefore concluded that the inhibition of the metalloendopeptidase is apparently due to nonspecific interaction of the enzyme with hydrophobic groups in the inhibitor. By contrast, the enzyme was inhibited by all thiols and metal chelating agents, in a manner similar to the inhibition of the pituitary metalloendopeptidase by the same reagents.

Table 7. Effect of Inhibitors on Kidney Enzyme Activity[a]

Inhibitor	Final Concentration (mM)	Inhibition (%)
Leupeptin	0.023	0
Antipain	0.013	0
Chymostatin	0.013	0
DFP	0.11	0
p-Mercuribenzoate	0.10	3
Iodoacetamide	1.0	0
Iodoacetic Acid	1.0	0
N-Ethylmaleimide	1.0	0
Pepstatin	0.067	35
Glutathione	1.0	76
Dithiothreitol	0.4	88
	1.0	98
EDTA	1.0	70
o-Phenanthroline	0.08	47
	0.2	73
2-Mercaptoethanol	2.0	39

[a]Activity was determined with Hip-Arg-Arg-Leu-2NA as described under "Experimental Methods". The enzyme was not preincubated with the inhibitors with the exception of DFP and EDTA, which were preincubated with the enzyme at 37° C for 20 min before addition of substrate. Controls in which the enzyme was preincubated in the absence of these inhibitors were included. At the concentrations tested, none of the inhibitors interfered with the aminopeptidase coupling reaction.

C. INTERACTION OF THE KIDNEY METALLOENDOPEPTIDASE WITH SYNTHETIC SUBSTRATES, NATURAL PEPTIDES, AND ACTIVE SITE DIRECTED INHIBITORS. DESIGN OF N-1-CARBOXY-2-PHENYLETHYL DERIVATIVES OF AMINO ACID AMIDES OF p-AMINOBENZOATE AS INHIBITORS OF THE ENZYME

The specificity of the enzyme toward various synthetic naphthlyamides is summarized in Table 8. The steady-state parameters K_m and k_{cat} (=V/e, where e = total enzyme concentration) were obtained from initial velocity measurements by a non-linear regression program in which the substrate concentration and velocity are fit to the Michaelis-Menten equation (Baing and Reid-Miller, 1980a). Strict Michaelis-Menten kinetics were observed for all substrates within the concentration ranges indicated. These data are consistent with a primary specificity directed toward bonds in which the amino group is provided by a hydrophobic amino acid residue (position P_1'). Replacement of the hydrophobic amino acid leucine (substrate 1) with an alanine residue (substrate 2) decreased the k_{cat} by a factor of 2 and the specificity constant (k_{cat}/K_m) by a factor of 10. Introduction of glycine (substrate 3) in this position was associated with negligible hydrolysis.

High k_{cat} values and specificity ratios were also observed with substrates 4 and 5, in which the P_1 and P_2 positions are occupied by alanine residues and P_3 by a glutaryl group. Although the enzyme will hydrolyze bonds on the N-terminal side of alanyl residues (substrate 2), no

Table 8. Kinetic Parameters for Hydrolysis of Several Peptide Naphthylamides by the Kidney Metalloendopeptidase[a]

	P_3- P_2- P_1- P_1'- P_2'	[S][b] (mM)	K_m[c] (mM)	k_{cat}[d] (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
(1)	Bz-Gly-Arg-Arg-Leu-2NA	0.08-0.4	0.18 ± 0.2	62.2	3.46×10^5
(2)	Bz-Gly-Arg-Arg-Ala-2NA	0.10-1.2	0.97 ± 0.03	31.8	3.28×10^4
(3)	Bz-Gly-Arg-Arg-Gly-2NA	0.2 -0.4		Negligible Hydrolysis[e]	
(4)	glutaryl-Ala-Ala-Phe-2NA	0.1 -0.8	0.27 ± 0.02	73.4	2.72×10^5
(5)	glutaryl-Ala-Ala-Phe-4MeO2NA	0.1 -0.6	0.23 ± 0.02	110.0	4.83×10^5

[a]Enzyme activity was determined as described under "Experimental Methods".

[b]Range of substrate concentrations used for the determination of K_m.

[c]Data are mean values ± S.E. of four determinations.

[d]Calculations based on a molecular weight of 95,000.

[e]Represents less than 1% of the rate for Hip-Arg-Arg-Leu-2NA at a concentration of 0.4 mM.

such cleavages were observed for substrates 4 and 5. No free alanine was detected in incubation mixtures containing enzyme, aminopeptidase, and substrates 4 and 5. Therefore, rapid cleavage of the Ala-Phe bond renders the glutaryl-Ala-Ala peptide unsusceptible to hydrolysis.

The primary specificity of the enzyme directed toward bonds on the amino side of hydrophobic amino acid residues was also evident when its action on several biologically active peptides was studied. The results summarized in Table 9 show that the enzyme has enkephalinase activity in that it cleaves both Met- and Leu-enkephalin at the Gly-Phe bond. Met-enkephalinamide is also hydrolyzed at the same bond, indicating that a free carboxyl group at the C-terminus of the enkephalin molecule is not a requirement for activity. This finding is similar to that observed with the pituitary metalloendopeptidase and indicates that the enkephalinase activity of the enzyme is that of an endopeptidase, rather than that of a dipeptidyl carboxy-peptidase.

The enzyme also degrades dynorphin, a Leu-enkephalin-containing opioid peptide, by hydrolyzing bonds on the amino side of hydrophobic residues Phe^4 and Ile^8. Cleavage proceeds preferentially at the Arg^7-Ile^8 bond with the formation of an N-terminal heptapeptide and a C-terminal hexapeptide. Longer incubation results in complete hydrolysis of the Gly^3-Phe^4 bond. It is this cleavage that

Table 9. Degradation of Biologically Active Peptides by the Kidney Neutral Metalloendopeptidase[a]

Peptide	Structure	Products Found
Met-enkephalin	Tyr-Gly-Gly↓-Phe-Met	Tyr-Gly-Gly, Phe-Met
Met-enkephalinamide	Tyr-Gly-Gly↓-Phe-Met-NH2	Tyr-Gly-Gly, Phe-Met-NH_2
Leu-enkephalin	Tyr-Gly-Gly↓-Phe-Leu	Tyr-Gly-Gly, Phe-Leu
Dynorphin	Tyr-Gly-Gly↓-Phe-Leu-Arg-Arg↓-Ile-Arg-Pro-Lys-Leu-Lys	Tyr-Gly-Gly, Tyr-Gly-Gly-Phe-Leu-Arg-Arg, Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys, Ile-Arg-Pro-Lys-Leu-Lys, Phe-Leu-Arg-Arg
Bradykinin	Arg-Pro-Pro-Gly↓-Phe-Ser-Pro↓-Phe-Arg	Arg-Pro-Pro-Gly, Phe-Arg, Phe-Ser-Pro
Angiotensin I	Asp-Arg↓-Val-Tyr↓-Ile-His-Pro↓-Phe-His-Leu	Asp-Arg-Val-Tyr, Ile-His-Pro, Asp-Arg, Val-Tyr, Asp-Arg-Val-Tyr-Ile-His-Pro, Phe-His-Leu

[a]Reaction mixtures contained substrate (0.8-1.0 mM), Tris-HCl buffer (0.04 M, pH 7.6), and enzyme (1 unit) in a final volume of 0.25 ml. All incubations were for 2-4 h. Products of the reaction were separated on the Perkin-Elmer HPLC system described under "Experimental Methods". In these experiments, the initial acetonitrile concentration was 10%, except for the dynorphin experiment in which the initial acetonitrile concentration was 3%.

terminates the opioid activity of dynorphin (Chavkin and Goldstein, 1981).

Bradykinin is hydrolyzed at the Pro^{7}-Phe^{8} and Gly^{4}-Phe^{5} bonds, again showing that the primary specificity is directed towards bonds on the amino side of hydrophobic residues. The hydrolysis of the Pro^{7}-Phe^{8} bond also shows that the enzyme can act as a dipeptidyl carboxypeptidase on peptides with hydrophobic residues in the penultimate position.

Angiotensin I is rapidly hydrolyzed at the Pro^{7}-Phe^{8} bond to form Phe-His-Leu and Asp-Arg-Val-Tyr-Ile-His-Pro. The latter peptide is further degraded at the Tyr^{4}-Ile^{5} bond, and the resulting tetrapeptide is slowly cleaved at the Arg^{2}-Val^{3} bond. This thermolysin-like pattern of cleavage is distinctly different from the activity of the angiotensin converting enzyme, which hydrolyzes angiotensin I at the Phe^{8}-His^{9} bond to form angiotensin II, a potent vasoconstrictor.

Although dipeptides are not substrates, the results in Table 10 (and Figure 2) show that the phenylalanyl, leucyl, and even alanyl dipeptides are inhibitory. By contrast, dipeptides such as Gly-Gly, Gly-Val, and Gly-Leu, with a glycine residue on the amino side, do not inhibit at concentrations as high as 2 mM. Free phenylanine was also without effect at these concentrations.

Table 10. Inhibition of the Metalloendopeptidase by Dipeptides and Peptide Derivatives of p-Aminobenzoate[a]

Peptide	$IC_{50} \times 10^{-4}$[b] (M)
(1) Gly-Gly	Negligible Inhibition[c]
(2) Gly-Val	Negligible Inhibition[c]
(3) Gly-Phe	Negligible Inhibition[c]
(4) Ala-Phe	5.9
(5) Phe-Phe	1.6
(6) Phe-Leu	4.4
(7) Phe-Leu-NH_2	Negligible Inhibition[c]
(8) Phe-Ala	0.21
(9) Phe-Gly	1.6
(10) Phe	Negligible Inhibition[c]
(11) Leu-Phe	0.23
(12) Leu-Trp	0.75
(13) Phe-pAB	0.19
(14) Acetyl-Phe-pAB	2.5
(15) Glutaryl-Phe-pAB	1.0
(16) Glutaryl-Gly-Phe-pAB	1.0
(17) Bz-Gly-Phe-pAB	0.18

[a]Inhibition studies were done by determining activity toward 0.4 mM glutaryl-Ala-Ala-Phe-2NA in the presence of varying concentrations of inhibitor. For these determinations, the enzyme, dipeptide, and substrate were incubated, and the reaction was terminated by boiling. 2NA was then released by adding 10 ug of aminopeptidase and incubating at 37° C for 30 min. The aminopeptidase reaction was then stopped by addition of fast garnet (Barrett, 1972). This two-stage procedure was used to prevent degradation of unblocked peptides by aminopeptidase during the course of the endopeptidase reaction.

[b]IC_{50} values were obtained with a computer program (Johnson, 1982) that performs iterative curve fits to the parameters (% inhibition vs. inhibitor concentration) to the logistic equation. Log dose response curves (Figure 2) with slopes close to unity were obtained for most compounds tested.

[c]No inhibition was observed at an inhibitor concentration of 2 mM.

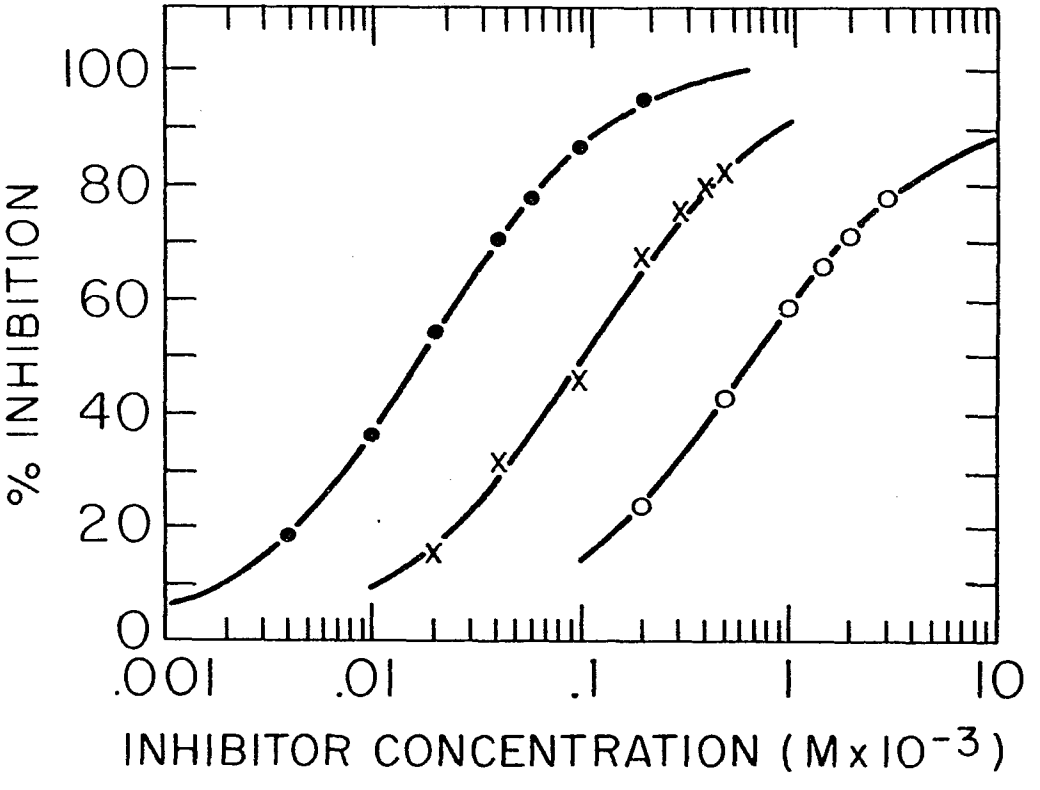


FIGURE 2. INHIBITION OF KIDNEY METALLOENDOPEPTIDASE.

Log dose response curves for inhibition of the metalloendopeptidase as described in Table 10. •, Hip-Phe-pAB; x, glutaryl-Phe-pAB; o, Ala-Phe.

On the basis of the specificity studies described in the preceding section, the inhibitory dipeptides, all of which contain a hydrophobic residue on the amino side, can be regarded as potential products of the metalloendopeptidase-catalyzed reaction. This interpretation is based on the finding that the enzyme acts as a dipeptidyl carboxypeptidase on those peptides having a hydrophobic residue in the penultimate position. It may therefore be assumed that the inhibitory dipeptides act by binding to the S_1' and S_2' subsites of the enzyme. By contrast, the noninhibitory dipeptides having a glycine residue on the amino side lack the ability to bind to these sites. The hydrophobicity of the amino residue on the carboxyl side of the dipeptide, as opposed to the residue on the amino side, does not seem to be a factor in determining inhibitory potency. For example, Phe-Gly, Phe-Phe, and Phe-Leu all inhibited to a similar extent, while the inhibition obtained with Phe-Ala was much greater. A free carboxyl group in the dipeptide seems to be of importance for inhibition, since the amide of Phe-Leu showed little inhibition in comparison with Phe-Leu.

It is of interest that replacement of the C-terminal amino acid residues in the phenylalanyl dipeptides by p-aminobenzoate yielded the amino acid arylamide Phe-pAB with an inhibitory potency equal to the best of the dipeptides tested (Phe-Ala). Substitution of the amino

group of Phe-pAB with an acetyl, a glutaryl, and a glutarylglycyl group diminished the inhibitory action, while a hippuryl group in the same position preserves the inhibitory potency, suggesting that a hydrophobic group in this position enhances binding.

Acylation of the amino group of Phe-pAB yields compounds that can serve as substrates of the kidney metalloendopeptidase. All four N-acyl derivatives of the Phe-pAB listed in Table 10 (peptides 14-17) are hydrolyzed by the enzyme, although at greatly different rates. Thus, N-acetyl-Phe-pAB (peptide 14) and glutaryl-Phe-pAB (peptide 15) are hydrolyzed about 1,000 times slower than glutaryl-Ala-Ala-Phe-2NA (measured at a substrate concentration of 0.4 mM, as described under "Experimental Methods"), and the hydrolysis of glutaryl-Gly-Phe-pAB and Bz-Gly-Phe-pAB proceeds at a rate about 65 and 35 times, respectively, slower than that of glutaryl-Ala-Ala-Phe-2NA. The great differences in the rate of hydrolysis of the different peptides could be related to the length of the peptide chain, with the longer peptides being hydrolyzed more rapidly. This suggests the presence of an extended substrate binding site in the enzyme.

The specificity of the kidney metalloendopeptidase toward bonds in which the amino group is contributed by hydrophobic amino acid residues, resembles that of thermolysin, a bacterial metalloendopeptidase. Thermolysin,

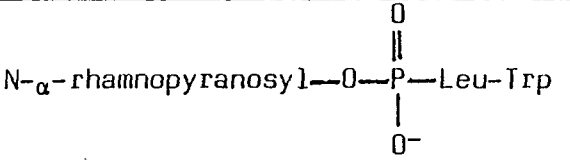
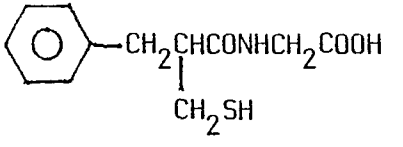
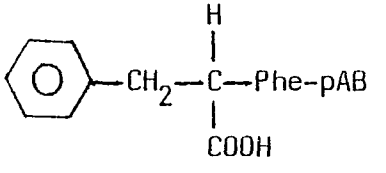
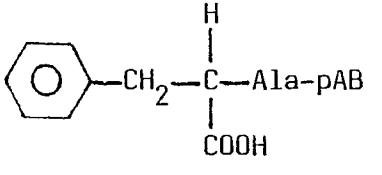
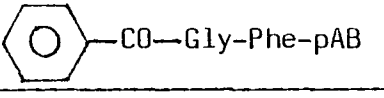
like other metalloendopeptidases, including angiotensin converting enzyme, and also carboxypeptidases A and B, contains a zinc atom in the active site that interacts with the carbonyl oxygen of the hydrolyzed peptide bond. Many of the synthesized inhibitors of this group of enzymes contain a ligand, which coordinates with the zinc atom in the active site. The ligand is usually attached to a peptide or amino acid capable of interacting with the substrate binding site of the enzyme. Due to the similarities in the mechanism of action of zinc-metalloendopeptidases, an inhibitor of one of these enzymes can inhibit other enzymes of the same group, when modified to conform to their specificity requirements. Thus, for example, D-3-mercapto-2-methylpropanoyl-L-proline (captopril), a potent inhibitor of angiotensin converting enzyme (Ondetti et al., 1977), was used as a model for the synthesis of thiorphan (Roques et al., 1980), an inhibitor of brain enkephalinase, having a benzylpropanoylglycine group in place of the propanoyl-L-proline group.

It was therefore of interest to examine the inhibitory effect of thiorphan and of phosphoramidon, a potent inhibitor of thermolysin, on the activity of kidney metalloendopeptidase. In addition, N-(1-carboxy-2-phenylethyl) derivatives of Phe-pAB and Ala-pAB were synthesized and tested as potential inhibitors of the kidney metalloendopeptidase. It was expected that these derivatives should

inhibit the kidney enzyme, since analogous N-(1-carboxy-3-phenylpropyl) derivatives of several dipeptides were found to strongly inhibit the angiotensin converting enzyme (Patchett et al., 1980), and a similar derivative of Leu-Trp was shown to be a potent inhibitor of thermolysin (Maycock et al., 1981). Studies by this group on the converting enzyme inhibitors indicate that the N-1-carboxylate group contributes significantly to the inhibitory potency.

The results of inhibition studies are summarized in Table 11. Reductive amination of phenylpyruvate with Phe-pAB or Ala-pAB to give the respective N-(1-carboxy-2-phenylethyl) derivatives generates a new asymmetric carbon, and accordingly, two diastereomers are formed during synthesis. The diastereomers derived from amination of phenylpyruvate with Phe-pAB were separated by HPLC as described under "Experimental Methods" (Figure 3). Their inhibitory action was determined separately and compared with that of the mixture of the two diastereomers. The N-(1-carboxy-2-phenylethyl) derivatives of Ala-pAB were examined only as a mixture of the two diastereomers. Due to the low K_i values of some of the inhibitors (see Table 11), the assumption that the concentration of free inhibitor is equal to the total inhibitor concentration ($[I] = [I]_{total}$) is not correct. The graphical method

Table 11. Inhibition Constants for the Kidney Metalloendopeptidase of Active Site Directed Inhibitors

Inhibitor	K_i[a] (M)	Structure
(1) Phosphoramidon	$(3.4 \pm 0.31) \times 10^{-9}$	
(2) Thiorphan (DL-3-mercapto-2-benzylpropanoylglycine)	8.0×10^{-8}[b]	
(3) N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB	$(7.1 \pm 0.66) \times 10^{-8}$	
(4) N-(1-carboxy-2-phenylethyl)-Phe-pAB (diastereomer I)	$(2.9 \pm 0.34) \times 10^{-8}$	
(5) N-(1-carboxy-2-phenylethyl)-Phe-pAB (diastereomer II)	$(2.4 \pm 0.36) \times 10^{-7}$	
(6) N-[1-(R,S)-carboxy-2-phenylethyl]-Ala-pAB	$(5.3 \pm 0.33) \times 10^{-6}$	
(7) Bz-Gly-Phe-pAB	$(3.0 \pm 0.48) \times 10^{-6}$	

[a] K_i values were determined by using Hip-Arg-Arg-Leu-2NA (0.3-0.7 mM) as substrate and several concentrations of inhibitor, according to the method of Dixon (1972). Incubations were for 30 min. Data are mean values ± S.E. of three to four determinations.

[b] This value represents the IC_{50}. For other details see the text.

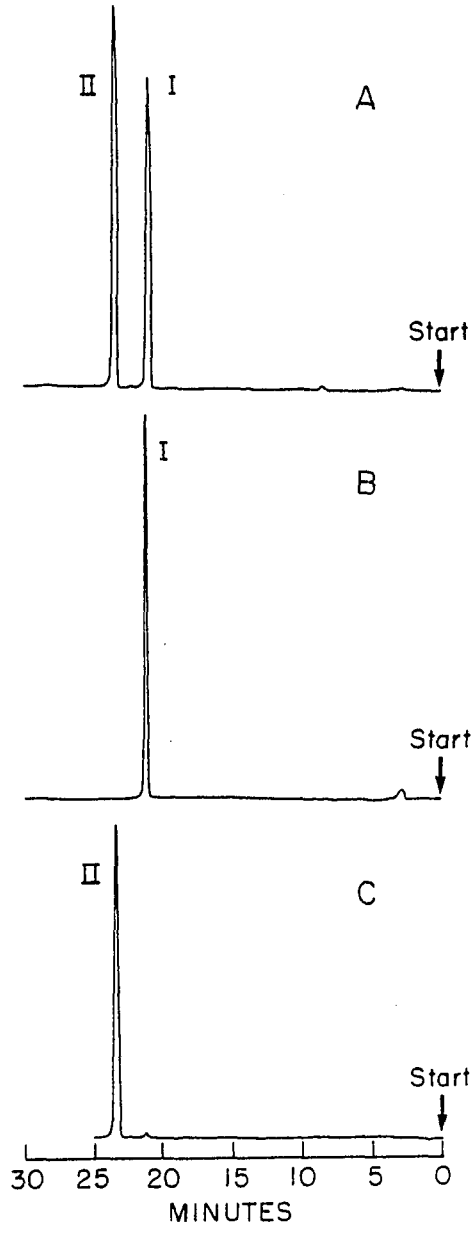


FIGURE 3. HPLC SEPARATION OF DIASTEREOMERS OF N-[1-(R,S)-CARBOXY-2-PHENYLETHYL]-Phe-pAB.

(A) N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB.
(B) Diastereomer (peak) I.
(C) Diastereomer (peak) II.

of Dixon (1972), valid for both tight binding and for less potent inhibitors, was therefore used for the determination of K_i values. Experiments were done by adding the enzyme to mixtures of substrate and inhibitor. Separate determinations in which the enzyme and inhibitor were preincubated for 15 min at 37° C before addition of the substrate showed no difference in the extent of inhibition by phosphoramidon, or by any pAB derivatives, thereby ruling out irreversible inhibition and slow binding phenomena (Kam et al., 1979). Inhibition by thiorphan, however, was observed to be potentiated by preincubation. This caused difficulties in attempts to reliably measure K_i values. Therefore, only an IC_{50} value for this inhibitor as determined in the presence of 0.4 mM substrate is given. For all compounds tested, inhibition was competitive in that it could be surmounted at high substrate concentrations.

The results in Table 11 show that phosphoramidon, a thermolysin inhibitor of bacterial origin, is the most potent of the inhibitors tested and that thiorphan is also highly inhibitory. These results further support the conclusion that the kidney metalloendopeptidase, like the analogous bovine pituitary enzyme, has thermolysin-like properties and that it is also similar to brain enkephalinase. The N-carboxymethyl derivatives of Phe-pAB all show high inhibitory potency. The most active disastereomer

(diastereomer I, Table 11) of N-(1-carboxy-2-phenylethyl)-Phe-pAB has a K_i of 2.9×10^{-8} M. This K_i is 2 orders of magnitude lower than that of Bz-Gly-Phe-pAB, a compound with an inhibitory potency similar to that of Phe-pAB (see Table 10). This indicates that N-alkylation of Phe-pAB by the carboxyphenylethyl group increases the inhibitory potency of Phe-pAB by almost 2 orders of magnitude. It is of interest that diastereomer II was less inhibitory by a factor of 10 and that the mixture of diastereomers had an intermediate potency, suggesting some stereoselectivity of inhibitor binding. A significant finding is that replacement of a phenylalanine residue in the inhibitor by an alanine residue decreased the inhibitory potency by a factor of 100. This indicates that the interaction between the aromatic ring of phenylalanine and a hydrophobic pocket in the S_1' subsite of the enzyme contributes significantly to binding, a conclusion consistent with the results of specificity studies described in a preceding section. A common feature of the more potent inhibitors listed in Table 11 is the presence of a group capable of interacting with the Zn^{2+} in the active site of the enzyme. This property is shared by the thiol group in thiorphan, the phosphoryl group in phosphoramidon, and the carboxyl group in the N-carboxymethyl derivatives of Phe-pAB and Ala-pAB.

D. BIOCHEMICAL AND IMMUNOLOGICAL PROPERTIES OF THE METALLOENDOPEPTIDASE FROM RABBIT BRAIN AND COMPARISON WITH THE RABBIT KIDNEY METALLOENDOPEPTIDASE

The kidney neutral metalloendopeptidase and enkephalinase have similar substrate specificity and physicochemical properties. It was therefore of interest to isolate the enzyme from rabbit brain and to study its interactions with substrates and inhibitors and with antisera raised against the rabbit kidney enzyme. The data are compared with those obtained for the kidney enzyme.

A summary of the purification is given in Table 12. A 374-fold increase in specific activity was obtained with respect to the deoxycholate extract. As with the rabbit kidney and bovine pituitary enzymes, the rabbit brain metalloendopeptidase was completely solubilized by treatment with deoxycholate and papain. The enzyme also had a similar elution volume on Sephadex G-200 chromatography suggesting a molecular weight of 90,000-100,000.

The pH optimum of the brain enzyme was tested with glutaryl-Ala-Ala-Phe-2NA using 0.2 M Tris-HCl buffers between pH 6.9-9.0. A broad pH optimum was observed in the tested range. Brain metalloendopeptidase activity was thus routinely determined at pH 7.6, the pH also used for the determination of the kidney enzyme.

Both the brain and kidney enzymes cleave Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) at the Gly-Phe bond. The rates of hydrolysis of

Table 12. Summary of Purification of the Metalloendopeptidase from Rabbit Brain[a]

Purification Step	vol (ml)	Protein (mg/ml)	Activity		Specific Activity (units/mg)	Recovery (%)	Purification (x-fold)
			Units/ml	Total			
(1) Deoxycholate extract	220	10.8[b]	0.534	117	0.49	100	1
(2) Papain treatment and Sephadex filtration	70	0.21	1.26	88.2	6.0	75.4	121
(3) Phenyl-Sepharose	50	0.08	1.48	74.0	18.5	63.2	374

[a]From 75 g of rabbit brains.

[b]Protein was determined for this step by the method of Lowry et al. (1951).

several synthetic substrates by the two enzymes is summarized in Table 13. The enzymes cleaved peptide bonds in substrates 1 to 3 on the amino side of phenylalanine, leucine, and alanine respectively. The highest reaction rates were obtained with substrates 1 and 2, having hydrophobic amino acid residues (leucine and phenylalanine) in the P_1' position. The reaction velocity decreased greatly when this position was occupied by an alanine residue (substrate 3); it was further reduced with substrate 4 which has a glycine at this position. The pattern of hydrolysis of the synthetic substrates indicates that the primary specificity of both enzymes is directed toward bonds on the amino side of hydrophobic amino acid residues. It is noteworthy that the relative rates of hydrolysis of the four substrates were almost identical for the two enzymes, indicating an identical specificity.

The specific activity of the kidney enzyme is more than 100 times greater than that of the brain enzyme. Kidney is a rich source of the enzyme; the specific activity of a deoxycholate extract from this organ (Table 6), is about 200 times higher than that from brain (Table 12). In order to achieve the same specific activity, the brain enzyme would have to be purified almost 50,000-fold, thereby requiring inordinate amounts of brain tissue.

Kidney metalloendopeptidase is specifically inhibited by thiorphan (DL-3-mercapto-2-benzylpropanoylglycine) an

Table 13. Rates of Hydrolysis of Several Synthetic Substrates by Brain and Kidney Metalloendopeptidase[a]

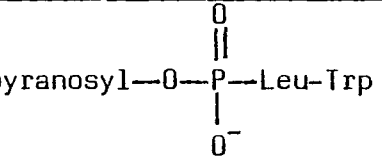
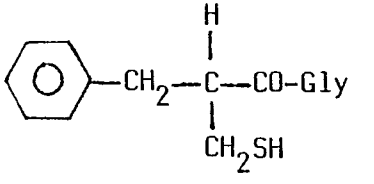
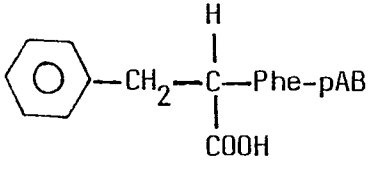
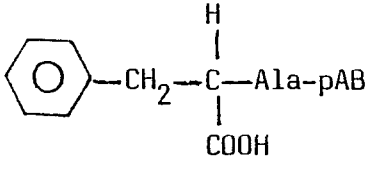
	Substrate P_3-P_2- P_1- P_1'-P_2'	Brain Enzyme Specific Activity (umol/hr/mg protein)	Kidney Enzyme Specific Activity (umol/hr/mg protein)
1.	glutaryl-Ala-Ala-Phe-2NA	19.1 (100)	2,450 (100)
2.	Hip-Arg-Arg-Leu-2NA	11.6 (60.7)	1,380 (56.3)
3.	Hip-Arg-Arg-Ala-2NA	2.26 (11.8)	182 (7.4)
4.	Hip-Arg-Arg-Gly-2NA	Negligible Hydrolysis[b]	Negligible Hydrolysis[b]

[a]Hydrolysis of all peptides was determined at a substrate concentration of 0.4 mM as described in "Experimental Methods". Initial velocities were used for all determinations. Values in parenthesis represent activities relative to those obtained with glutaryl-Ala-Ala-Phe-2NA arbitrarily set at 100.

[b]Negligible hydrolysis represents less than 1% of the rate with glutaryl-Ala-Ala-Phe-2NA.

enkephalinase inhibitor (Roques et al., 1980), and by phosphoramidon (N-α-L-rhamnopyranosyloxyhydroxyphosphinyl-L-leucyl-L-tryptophan), an inhibitor of thermolysin (Suda et al., 1973). The enzyme is also inhibited by N-(1-carboxy-2-phenylethyl) derivatives of Phe-pAB and Ala-pAB. All of these inhibitors contain a peptide moiety that fulfills the requirements of the substrate binding site and a group capable of complexing with the zinc atom in the active site. Table 14 summarizes the K_i values of these inhibitors for the brain and kidney enzyme. All K_i values were determined by the method of Dixon (1972) which is valid for tight binding as well as for less potent inhibitors. Experiments were performed by adding enzyme to incubation mixtures containing buffer, Bz-Gly-Arg-Arg-Leu-2NA as the substrate (0.3-0.7 mM) and various concentrations of inhibitor. Preincubating the enzyme and inhibitor for 15 min at 37° C before addition of substrate did not potentiate inhibition by phosphoramidon and the pAB derivatives, and therefore suggests a reversible process. For these compounds, inhibition was overcome at high substrate concentrations and is thus consistent with a competitive mechanism. Thiorphan did, however, potentiate inhibition of the kidney enzyme, making it difficult to do kinetic experiments. Therefore, only an IC_{50} value for the thiorphan as determined at a substrate concentration of 0.4 mM is reported. The data in Table 14 show that phosphoramidon,

Table 14. Inhibition Constants of Several Inhibitors for the Brain and Kidney Metalloendopeptidase

Inhibitor	Structure	K_i[a] (M) Brain	K_i[a] (M) Kidney
Phosphoramidon		$2.0 \times 10^{-9} \pm 0.09$	$3.4 \times 10^{-9} \pm 0.31$
Thiorphan[b]		12.0×10^{-8}	8.0×10^{-8}
N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB		$3.1 \times 10^{-8} \pm 0.07$	$7.1 \times 10^{-8} \pm 0.66$
N-[1-(R,S)-carboxy-2-phenylethyl]-Ala-pAB		$2.0 \times 10^{-6} \pm 0.94$	$5.3 \times 10^{-6} \pm 0.3$

[a]Data are mean values ± S.E. of three to four determinations. K_i values of phosphoramidon and of N-[1-(R,S)-carboxy-2-phenylethyl]-phenylalanyl and alanyl-pAB are significantly different for the brain and kidney enzymes (t test, $p < 0.05$).

[b]Values for thiorphan represent the IC_{50}.

thiorphan and N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB are all potent inhibitors of both the brain and kidney enzyme. Replacement of phenylalanine in the last inhibitor by an alanine residue decreases the potency of inhibition of both enzymes by almost two orders of magnitude, showing the important contribution to binding of the hydrophobic residue in the P_1' position. The K_i values of phosphoramidon and the N-[1-(R,S)-carboxy-2-phenylethyl] derivatives of Phe-pAB and Ala-pAB were somewhat lower for the brain than for the kidney enzyme. These differences, although small, were statistically significant, suggesting that while the two enzymes are highly similar, they are not completely identical.

Further evidence for the similarity between the brain and kidney enzymes was demonstrated by showing close immunological crossreactivity in an anticatalytic immunoinhibition assay. Figure 4 shows the inhibition of the brain and kidney enzymes by anti-kidney metalloendopeptidase antiserum. Log dose response curves were generated by a computer program (Johnson, 1982) in which the parameters (% inhibition vs. microliters antiserum) are fit to the logistic equation. The data show two distinct curves which reach the same maximum. Transformation of the individual data points to double reciprocal plots (1/% inhibition vs. 1/microliters antiserum) gave two lines which, when tested by an analysis of covariance program

FIGURE 4. INHIBITION OF RABBIT KIDNEY AND RABBIT BRAIN METALLOENDOPEPTIDASE ACTIVITY BY ANTI-RABBIT KIDNEY METALLOENDOPEPTIDASE ANTISERUM.

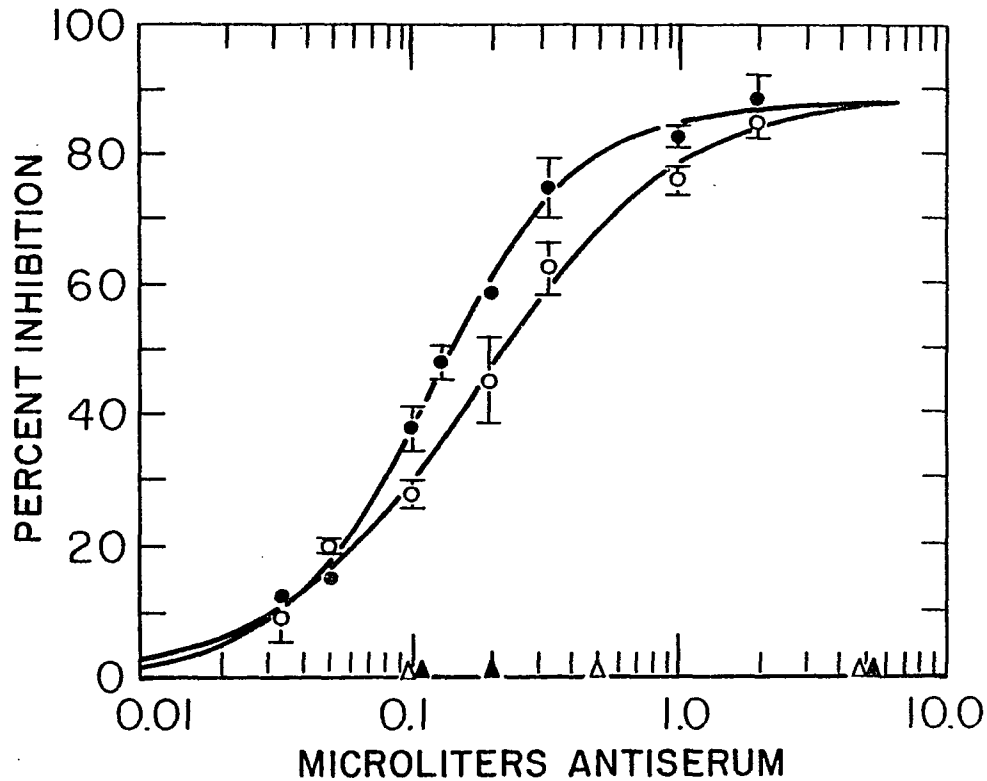
Data are the mean values ± S.D. of three to four determinations. Points without error bars represent the mean of two determinations.

-●- Rabbit kidney metalloendopeptidase incubated with anti-rabbit kidney metalloendopeptidase antiserum.

-○- Rabbit brain metalloendopeptidase incubated with anti-rabbit kidney metalloendopeptidase antiserum.

-▲- Rabbit kidney metalloendopeptidase incubated with rabbit pre-immune serum.

-△- Rabbit brain metalloendopeptidase incubated with rabbit pre-immune serum.


100
80
60
40
20
0
PERCENT INHIBITION
0.01
0.1
1.0
10.0
MICROLITERS ANTISERUM

(Baing and Reid-Miller, 1980b), were significantly different ($p < 0.05$). Clearly, the IC_{50} values for the two curves are quite similar, with the IC_{50} for the kidney enzyme being 0.120 ul and the IC_{50} for the brain enzyme being 0.180 ul of antiserum. The data therefore indicate that the two enzymes show strong immunological crossreactivity, but not complete identity.

The results of Ouchterlony immunodiffusion experiments led to a similar conclusion (Figures 5-7). All antisera gave a single precipitin line with the homogeneous kidney enzyme on Ouchterlony immunodiffusion plates. One antiserum against the kidney enzyme obtained after step 3 of the purification procedure (see Table 6) reacted with the brain enzyme on Ouchterlony plates by giving a single precipitin line. When the brain and kidney enzymes were incubated with this antiserum, a pattern of partial identity was obtained showing that the two enzymes share common antigenic determinants. The presence, however, of a spur associated with the precipitin line of the kidney enzyme, suggests that this enzyme contains antigenic determinants not present on the brain enzyme.

E. EFFECTS OF N-1-CARBOXY-2-PHENYLETHYL DERIVATIVES OF PHENYLALANYL, ALANYL, AND GLYCYL-p-AMINOBENZOATE ON NOCICEPTION.[1]

The role of the metalloendopeptidase as an *in vivo* enkephalin inactivator is controversial. Thiorphan, a

[1]All behavioral testing was carried out in the laboratory of Dr. S. Glick, Dept. of Pharmacology.

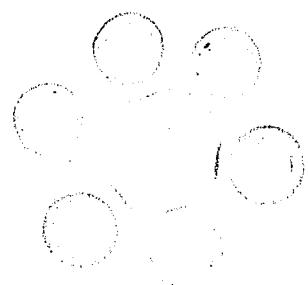


FIGURE 5. OUCHTERLONY IMMUNODIFFUSION OF RABBIT KIDNEY METALLOENDOPEPTIDASE.

Center well contains kidney metalloendopeptidase (0.37 ug), peripheral wells contain anti-kidney metalloendopeptidase antiserum (2.5 ul).

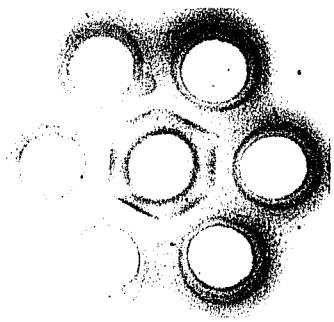


FIGURE 6. OUCHTERLONY IMMUNODIFFUSION OF RABBIT BRAIN METALLOENDOPEPTIDASE.

Center well contains brain metalloendopeptidase (0.8 ug), peripheral wells contain anti-kidney metalloendopeptidase antiserum (5 ul).

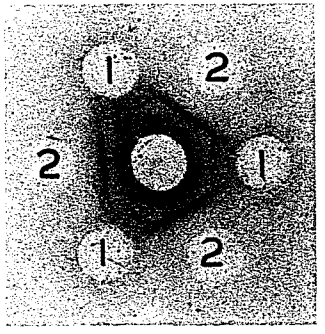
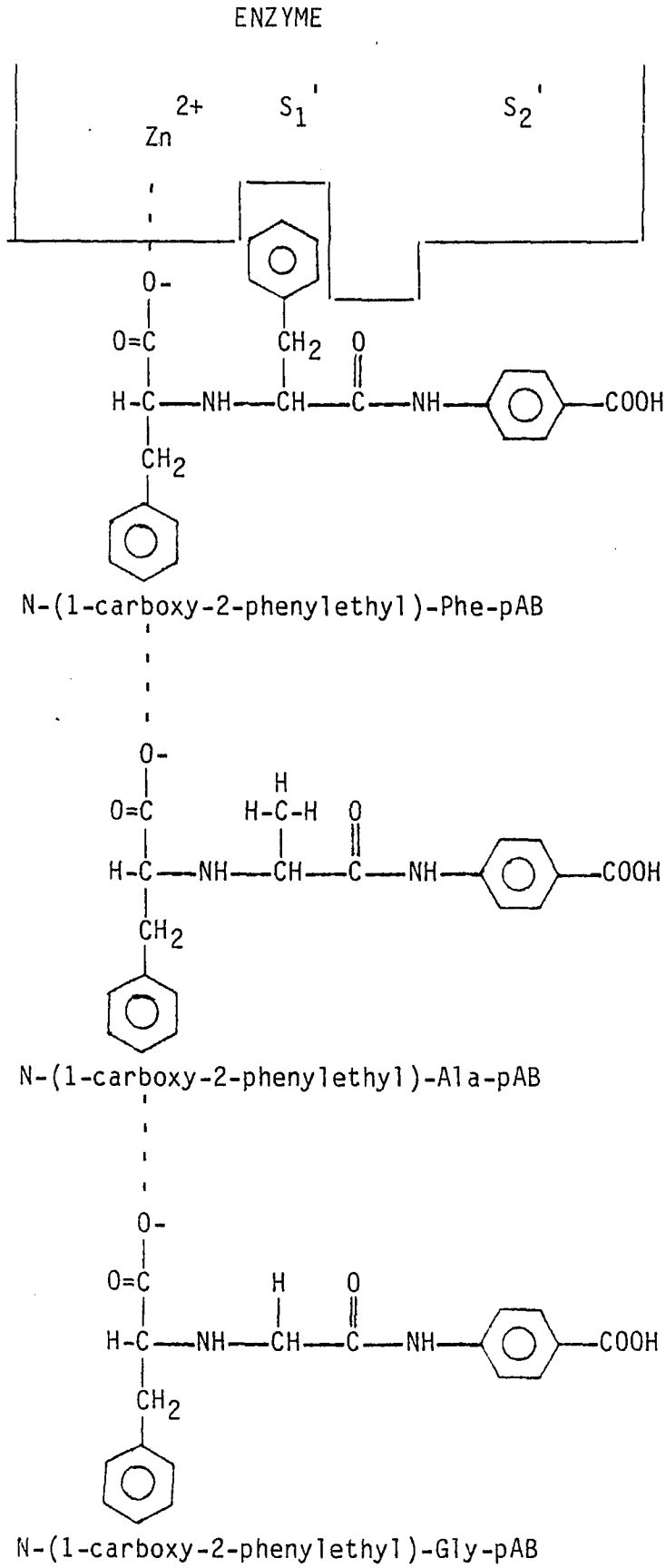


FIGURE 7. OUCHTERLONY IMMUNODIFFUSION OF RABBIT KIDNEY AND BRAIN METALLOENDOPEPTIDASE.

Center well contains antiserum (2.5 ul), peripheral wells contain (1) brain metalloendopeptidase (0.80 ug) and (2) kidney metalloendopeptidase (0.37 ug).

potent inhibitor of the enzyme, has been shown to have weak and inconsistent antinociceptive activity (see "Introduction", Part A). It was therefore of interest to test N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB, a novel and potent inhibitor of the enzyme for antinociceptive activity, and to compare its effects with the less potent inhibitors N-[1-carboxy-2-phenylethyl]-Gly-pAB and N-[1-(R,S)-carboxy-2-phenylethyl]-Ala-pAB.

Figure 8 summarizes the structures and K_i values for the kidney metalloendopeptidase of the inhibitors that were tested in vivo. As shown in Table 11, N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB has a K_i of 7.1×10^{-8} M. Its alanine analogue was less potent by almost two orders of magnitude. N-(1-carboxy-2-phenylethyl)-Gly-pAB was over 1,500-fold less potent than the phenylalanyl derivative. These data are consistent with the primary specificity of the enzyme, and demonstrate the importance of a hydrophobic residue at the P_1' position for effective binding. The glycyl derivative, which contains no such hydrophobic group, nonetheless weakly binds to the enzyme with a K_i of 1.15×10^{-4} M. This interaction is probably attributable to the carboxylate ion of the carboxy-phenylethyl group, which is capable of coordinating with the Zn^{2+} atom in the active site. Additional binding energy may be contributed by the hydrophobic phenylethyl group and the benzoate group on the inhibitor.



	K_i
N-(1-carboxy-2-phenylethyl)-Phe-pAB	(R,S) 7.1×10^{-8} M
N-(1-carboxy-2-phenylethyl)-Ala-pAB	(R,S) 5.3×10^{-6} M
N-(1-carboxy-2-phenylethyl)-Gly-pAB	1.1×10^{-4} M

FIGURE 8. INHIBITION OF METALLOENDOPEPTIDASE BY N-CARBOXYMETHYL DERIVATIVES OF AMINO ACID AMIDES OF p-AMINOBENZOATE.

Table 15 shows the dose response effects in the tail withdrawal test of N-(1-carboxy-2-phenylethyl) derivatives of phenylalanyl, alanyl, and glycyl-pAB. Two-way analyses of variance showed significant effects ($p < 0.05$-0.01) of dose and time for each of the inhibitors. Subsequent t-tests (Table 15) showed that all three compounds produce significant analgesia that was still apparent at 24 but not at 48 hours after administration. Analgesic effects were evident within one hour after administration of the phenylalanyl and glycyl derivatives and within three hours after the alanyl derivative. N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB was the most potent of the three inhibitors, producing significant effects at 3.125 and 6.25 umol/kg while the alanyl and glycyl derivatives were ineffective at up to 12.5 umol/kg. The three compounds had similar efficacies at 25 and 50 umol/kg. In a subsequent pilot experiment with three rats, the phenylalanyl derivative was administered intraventricularly via chronically implanted cannulae. A dose of 1.0 umol/kg was injected in a volume of 10 ul and significant analgesia was elicited for 24 h.

The data indicate that all three compounds are active when administered intraperitoneally. The relative potencies of the three compounds as metalloendopeptidase inhibitors, however, do not correlate with their antinociceptive activity.

Table 15. Tail Withdrawal Latencies[a] for N-(1-carboxy-2-phenylethyl) Derivatives of Phenylalanyl, Alanyl, and Glycyl-pAB

Time After Administration	Saline	N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB (umol/kg)					N-[1-(R,S)-carboxy-2-phenylethyl]-Ala-pAB (umol/kg)			N-(1-carboxy-2-phenylethyl)-Gly-pAB (umol/kg)		
		3.12	6.25	12.5	25.0	50.0	12.5	25.0	50.0	12.5	25.0	50.0
(n =)	(19)	(7)	(6)	(6)	(10)	(7)	(3)	(4)	(3)	(3)	(4)	(3)
0 h (Baseline)	3.00 ± 1.55	2.08 ± 0.85	2.12 ± 0.50	2.17 ± 0.52	3.00 ± 1.92	2.37 ± 0.68	2.63 ± 1.36	2.78 ± 1.05	3.03 ± 0.90	2.63 ± 1.40	2.85 ± 1.13	3.20 ± 0.50
1 h	2.38 ± 0.23	3.18* ± 1.59	3.33* ± 1.20	4.17* ± 1.73	4.41* ± 2.23	2.66 ± 0.83	1.67 ± 0.64	3.08 ± 1.57	3.40 ± 0.79	2.43 ± 0.21	5.43* ± 3.79	4.63* ± 0.16
3 h	2.12 ± 0.64	2.82* ± 0.93	3.47* ± 0.88	4.07* ± 0.90	5.26* ± 2.22	3.76* ± 1.92	2.83 ± 0.72	4.00* ± 1.34	6.44* ± 2.05	2.60 ± 0.72	6.13* ± 1.32	4.90* ± 0.30
6 h	2.10 ± 0.71	3.05* ± 1.32	3.72* ± 1.27	5.03* ± 1.53	5.50* ± 1.31	4.57* ± 2.03	2.24 ± 0.21	4.14* ± 1.41	6.70* ± 1.50	2.83 ± 1.29	5.13* ± 0.82	5.83* ± 1.25
24 h	2.38 ± 0.73	2.85 ± 0.65	3.17* ± 0.39	5.08* ± 2.56	6.12* ± 2.76	3.39* ± 1.79	2.50 ± 0.61	4.28* ± 0.56	3.57* ± 0.55	2.60 ± 0.36	4.48* ± 1.92	4.40* ± 0.90
48 h	2.49 ± 0.73	2.18 ± 0.41	2.18 ± 0.56	4.13 ± 1.75	3.21 ± 0.79	1.68 ± 0.46	3.00 ± 0.75	3.58 ± 1.38	3.10 ± 0.61	2.33 ± 0.68	3.43 ± 1.13	3.10 ± 0.66

[a]Latencies are in seconds ± S.D.

*Significantly different ($p < 0.05$-0.01) from saline (t-tests) and baseline (paired t-tests).

F. IDENTIFICATION AND CHARACTERIZATION OF THE METALLOENDOPEPTIDASE IN HUMAN SERUM

Angiotensin converting enzyme [EC 3.4.15.1] is a zinc-metallopeptidase which is similar to the metalloendopeptidase in its mechanism of action and relative abundance in lung membranes. Serum angiotensin converting enzyme has been extensively characterized (Das et al., 1977), and the enzyme has been reported to be a marker of active sarcoidosis, a systemic granulomatous disease of unknown etiology (Fanburg, et al., 1976; Lieberman, 1975; Rhogati et al., 1980; Rohrbach et al., 1979). It was therefore of interest to characterize the metalloendopeptidase in human serum and to study activity in patients with sarcoidosis.

Incubation of human serum with glutaryl-Ala-Ala-Phe-2NA in the presence of excess aminopeptidase M leads to the release of 2-naphthylamine. The reaction is dependent on the presence of aminopeptidase M, since omission of this enzyme causes a 70% to 80% decrease in 2-naphthylamine formation, and addition of bestatin (0.1 mM), a potent aminopeptidase inhibitor, results in its complete inhibition. This indicates that the hydrolysis of the substrate by the serum enzyme is associated with the exposure of a free amino group, and that the product of the reaction is susceptible to aminopeptidase action. In order to identify the site of cleavage, glutaryl-Ala-Ala-Phe-2NA was

incubated with aminopeptidase M and with active fractions obtained by subjecting serum to chromatography on a Sephadex G-200 column (see Figure 9). Amino acid analysis of the reaction mixture revealed the presence of phenylalanine as the only free amino acid. This indicates that the substrate is hydrolysed at the Ala-Phe bond, to yield glutaryl-Ala-Ala and Phe-2NA, the latter product being degraded to Phe and 2NA.

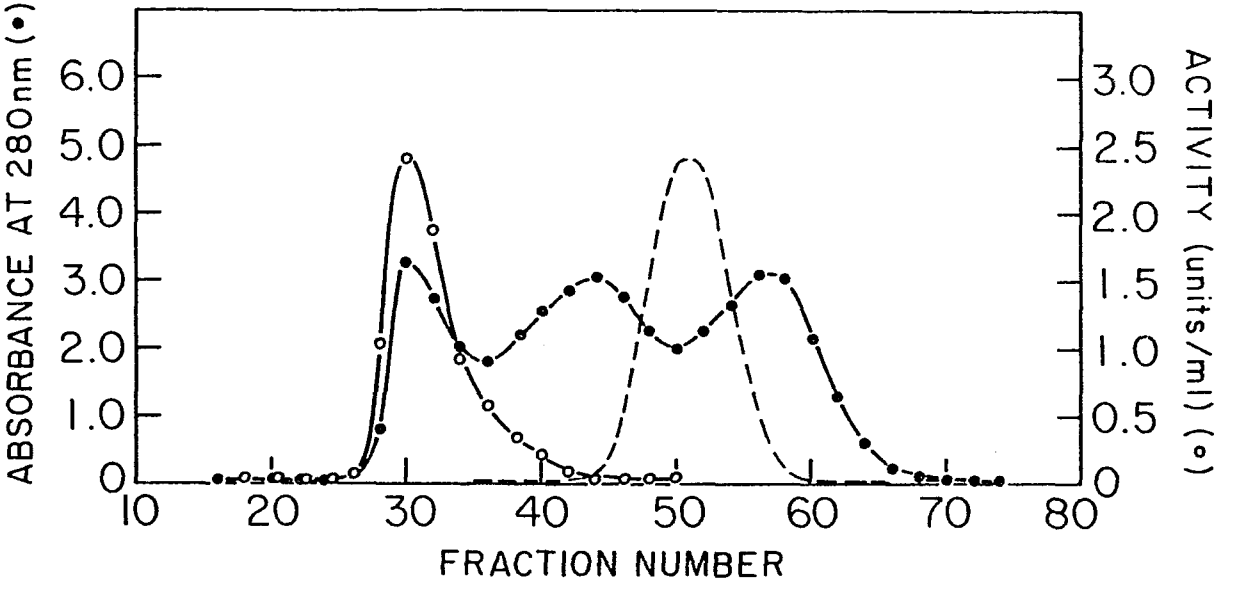
The pH dependence of the reaction was determined in 0.2 M HEPES buffer (pH 6.4 to 7.25) and 0.2 M Tris-HCl buffer (pH 7.0 to 8.8). Optimal activity was found in a broad range between pH 7.0 and 8.0. Routine determinations were therefore carried out at pH 7.6 in Tris-HCl buffer.

When serum was subjected to chromatography on a Sephadex G-200 column, a single peak of activity eluted in the void volume, together with the macroglobulin fraction (Figure 9), suggesting a molecular weight in excess of 200,000. This elution pattern is distinctly different from that of the rabbit kidney enzyme which, after solubilization from membrane fractions by deoxycholate and papain treatment, elutes with an apparent molecular weight of about 95,000 (Figure 9).

The enzyme obtained after Sephadex G-200 chromatography was used to determine a K_m for glutaryl-Ala-Ala-Phe-2NA. A value of 0.41 mM $\pm$ 0.04 (mean of four determinations $\pm$ S.E.M.) was obtained (Baing and Reid-Miller, 1980a). This

FIGURE 9. ELUTION PROFILE OF SERUM METALLOENDOPEPTIDASE FROM SEPHADEX G-200.

3 ml of serum from a patient with sarcoidosis were applied to the top of a 2.6 x 50.0 cm column equilibrated with a 0.05 M Tris-HCl buffer (pH 7.6) containing 0.15 M NaCl. Fractions of about 3 ml were collected and monitored for protein from absorbance at 280 nm (-●-) and for activity (-○-). The elution profile of purified kidney metalloendopeptidase corresponding to a molecular weight of approximately 95,000 is indicated by the broken line (---).


ABSORBANCE AT 280nm (•)
6.0
5.0
4.0
3.0
2.0
1.0
0
10
20
30
40
50
60
70
80
FRACTION NUMBER
3.0
2.5
2.0
1.5
1.0
0.5
0
ACTIVITY (units/ml) (∘)

value is similar to the K_m of 0.27 mM $\pm$ 0.02 obtained for the rabbit kidney enzyme (Table 8).

The effects of inhibitors on serum enzyme activity is summarized in Table 16. Leupeptin, antipain, and chymostatin, transition state aldehyde inhibitors of several thiol and serine proteases, had no effect on activity. The enzyme was also insensitive to inhibition by DFP, pepstatin, and the thiol blocking agents iodoacetamide and N-ethylmaleimide. Strong inhibition was observed by dithiothreitol, EDTA, and o-phenanthroline, all of which have metal chelating properties. These results suggest that the enzyme is a metalloprotease. Further evidence for this classification was derived from experiments in which enzyme obtained by Sephadex G-200 gel filtration was extensively dialysed against EDTA (1.0 mM; pH 7.5), and the metal chelator was then removed by dialysis toward several changes of 1.0 mM Tris-HCl buffer (pH 7.6). This procedure resulted in a more than 90% loss of activity. Complete reactivation was obtained by addition of $ZnCl_2$ (0.2 mM) to the incubation mixture (Figure 10). Cobalt chloride also completely reactivated the enzyme at a concentration of 0.8 mM. Addition of NaCl at the same concentration had no effect.

The hydrolysis of glutaryl-Ala-Ala-Phe-2NA on the amino side of the Phe residue, and the metal dependence of the reaction suggest that the serum enzyme is a thermolysin-like

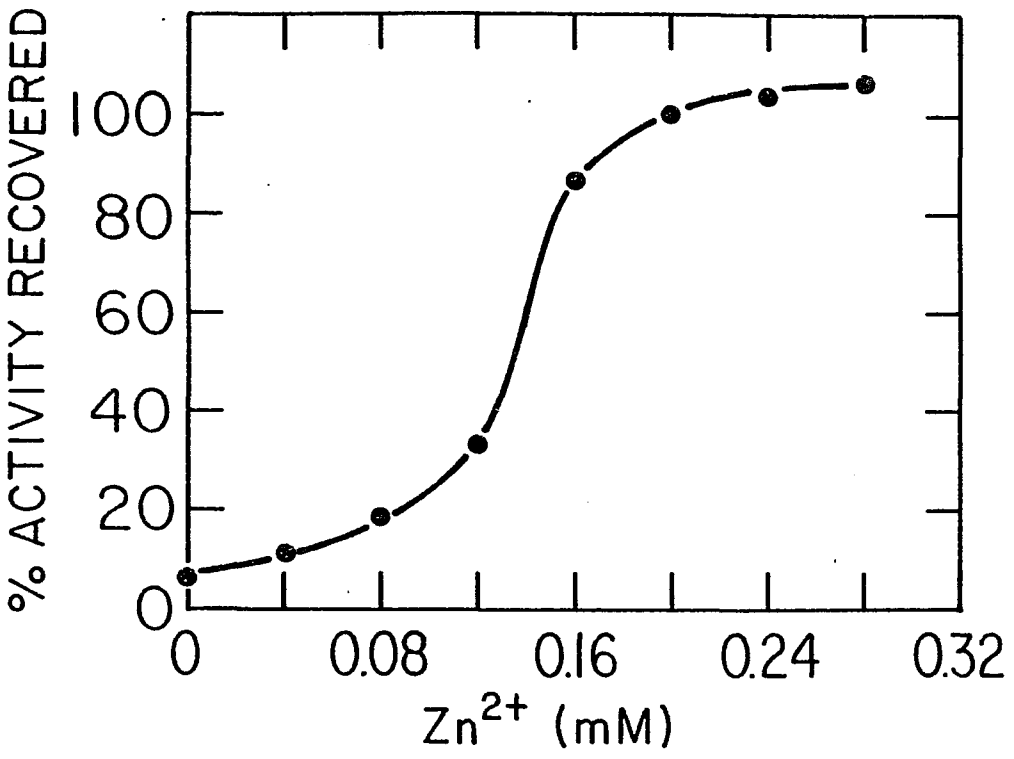
Table 16. Effect of Inhibitors on Serum Metalloendopeptidase Activity

Inhibitor	Final Concentration (mM)	% Inhibition[a]
Leupeptin	0.023	3.5 ± 1.8 (4)
Antipain	0.013	2.2 ± 2.0 (4)
Chymostatin	0.013	2.4 ± 2.0 (4)
DFP	0.11	1.0 ± 1.0 (4)
Pepstatin	0.06	5.7 ± 3.1 (5)
N-Ethylmaleimide	1.0	3.4 ± 3.4 (4)
Iodoacetamide	1.0	4.0 ± 2.4 (4)
Dithiothreitol	2.0	85.2 ± 2.2 (4)
EDTA	1.0	43.0 ± 2.2 (4)
	2.0	68.7 ± 3.5 (4)
o-Phenanthroline	0.1	58.0 ± 4.5 (3)
	0.2	86.0 ± 2.8 (3)

[a]Data are the mean values for % Inhibition ± S.E.M. The number of sarcoid patients from whom sera were tested is given in parentheses. Enzyme activity was measured in crude sera with glutaryl-Ala-Ala-Phe-2NA as the substrate (see "Experimental Methods"). The enzyme was not preincubated with inhibitors except for DFP and EDTA which were preincubated at 37° C for 20 min before addition of substrate. Controls in which the enzyme was preincubated in the absence of these inhibitors were included. At the concentrations tested, none of the inhibitors interfered with the aminopeptidase coupling reaction.

FIGURE 10. REACTIVATION OF AN EDTA-TREATED SERUM ENZYME BY ZINC IONS.

Serum was subjected to gel filtration on a Sephadex G-200 column (see Figure 9). Active fractions were pooled and concentrated to a protein concentration of about 6 mg/mL. An aliquot was dialyzed against two changes of 1.0 mM EDTA (pH 7.5) followed by dialysis against several changes 1.0 mM Tris-HCl (pH 7.5). A control serum aliquot was dialysed against the Tris-HCl buffer only. Enzyme activity was determined as described under "Experimental Methods" using 0.1 M Tris-HCl (pH 7.2). The percent activity recovered after addition of Zn^{2+} is expressed with respect to the control activity set at 100.


% ACTIVITY RECOVERED
100
80
60
40
20
0
0
0.08
0.16
0.24
0.32
Zn2+ (mM)

metalloendopetidase similar to the rabbit kidney metalloendopeptidase described in "Results", Section C, and to the bovine pituitary metalloendopeptidase isolated from bovine pitutiary (Orlowski and Wilk, 1981). It was therefore of interest to study the effects of active site directed inhibitors of the kidney metalloendopeptidase on the serum enzyme. These inhibitors interact with the substrate binding site on the enzyme and, in addition, have a group capable of coordinating with the active site zinc. Table 17 summarizes the results of these studies. Phosphoramidon, a potent microbial inhibitor of thermolysin and of the kidney and brain neutral metalloendopeptidase (see "Results", Sections C and D; Fulcher et al., 1982; Kenny, 1977; Suda et al., 1973), also inhibits the serum enzyme about 50% at a concentration of 80 nM. N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB, an inhibitor of rabbit kidney metalloendopeptidase, inhibited the serum enzyme about 50% at a concentration of 10 uM. The alanine analogue of this inhibitor was less potent by two orders of magnitude. Captopril, a potent inhibitor of angiotensin converting enzyme (Ondetti et al., 1977), which is used in the treatment of hypertension, did not inhibit the enzyme at concentrations as high as 1.0 mM. Thiorphan (Roques et al., 1980), an inhibitor related to captopril, but containing the more hydrophobic benzylpropanoyl group in place of the 2-methylpropanoyl group, had a strong inhibitory effect.

Table 17. Effect of Active Site Directed Inhibitors on Serum Metalloendopeptidase Activity

Inhibitor	Final Concentration	% Inhibition
Phosphoramidon (N-α-Rhamnopyranosyloxyhydroxy-phosphinyl)-Leu-Trp	8.0×10^{-8}M	52.8 ± 3.6
	8.0×10^{-7}M	86.0 ± 1.8
N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB	1.0×10^{-5}M	55.2 ± 1.3
	1.0×10^{-4}M	87.1 ± 1.3
N-[1-(R,S)-carboxy-2-phenylethyl]-Ala-pAB	1.0×10^{-3}M	46.2 ± 4.5
Captopril (D-3-mercapto-2-methylpropanoyl-L-proline)	1.0×10^{-3}M	2.8 ± 1.6
Thiorphan (DL-3-mercapto-2-benzylpropanoyl-glycine)	8.0×10^{-6}M	76.3 ± 1.6

Enzyme activity was determined as described in "Experimental Methods". Data are mean values ± S.E.M. resulting from determinations of inhibition in sera from four sarcoidosis patients.

Each of the inhibitors listed in Table 17 has a group capable of coordinating with the zinc atom in the active site of metalloendopeptidases. This property is represented by the phosphoryl group of phosphoramidon, the N-1-carboxylate groups of N-[1-(R,S)-carboxy-2-phenylethyl] derivatives of Phe-pAB and Ala-pAB and the mercapto groups of captopril and thiorphan. The inhibitory compounds share the common characteristic of having a hydrophobic group in the P_1' position, adjacent to the metal-chelating group. This includes the leucyl residue in phosphoramidon, the phenylalanyl residue in N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB and the benzylpropanoyl group of thiorphan. Enzyme activity is far less sensitive to inhibition by N-[1-(R,S)-carboxy-2-phenylethyl]-Ala-pAB and is unaffected by captopril, both of which lack this hydrophobic residue. This shows the importance of the hydrophobic residue in the P_1' position for the binding of these inhibitors to the serum enzmye, and are consistent with the data presented here on the kidney metalloendopeptidase (Table 11).

The results in Table 17 show that the concentrations of these inhibitors needed to inhibit the metalloendopeptidase in crude serum are approximately 10 times greater than those reported for inhibition of the purified kidney enzyme (Table 11). When serum was subjected to Sephadex G-200 gel filtration (see Figure 9), the sensitivity of the active fractions to inhibition by phosphoramidon and

N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB was similar to that of the kidney enzyme. This suggests that the lower sensitivity to inhibition of the enzyme in crude serum might be due to non-specific binding of the inhibitors to proteins (probably albumins) that are removed by Sephadex G-200 chromatography.

The similarity of the human serum and rabbit kidney metalloendopeptidases is also shown by their crossreactivity in an anticatalytic immunoinhibition assay. Both the serum and kidney enzymes were inhibited by an antiserum prepared against the purified rabbit kidney enzyme (Figure 11). The human serum enzyme was less sensitive to inhibition, and has a 50% inhibition point which is more than twice that of the rabbit kidney enzyme. At higher antibody concentrations, both enzymes were inhibited almost completely.

For the purposes of convenience, most of the biochemical studies on the properties of the serum enzyme were carried out on sera with elevated activities obtained from patients with sarcoidosis. In several experiments it was shown, however, that the enzyme activity in serum from healthy controls is inhibited by dithiothreitol, phosphoramidon, and anti-rabbit kidney metalloendopeptidase antiserum at the same concentrations and to the same extent as the enzyme in the serum of sarcoidosis patients. This indicates that the properties of the enzyme in controls and

FIGURE 11. INHIBITION OF RABBIT KIDNEY AND HUMAN SERUM METALLOENDOPEPTIDASE BY ANTI-RABBIT KIDNEY METALLOENDOPEPTIDASE ANTISERUM.

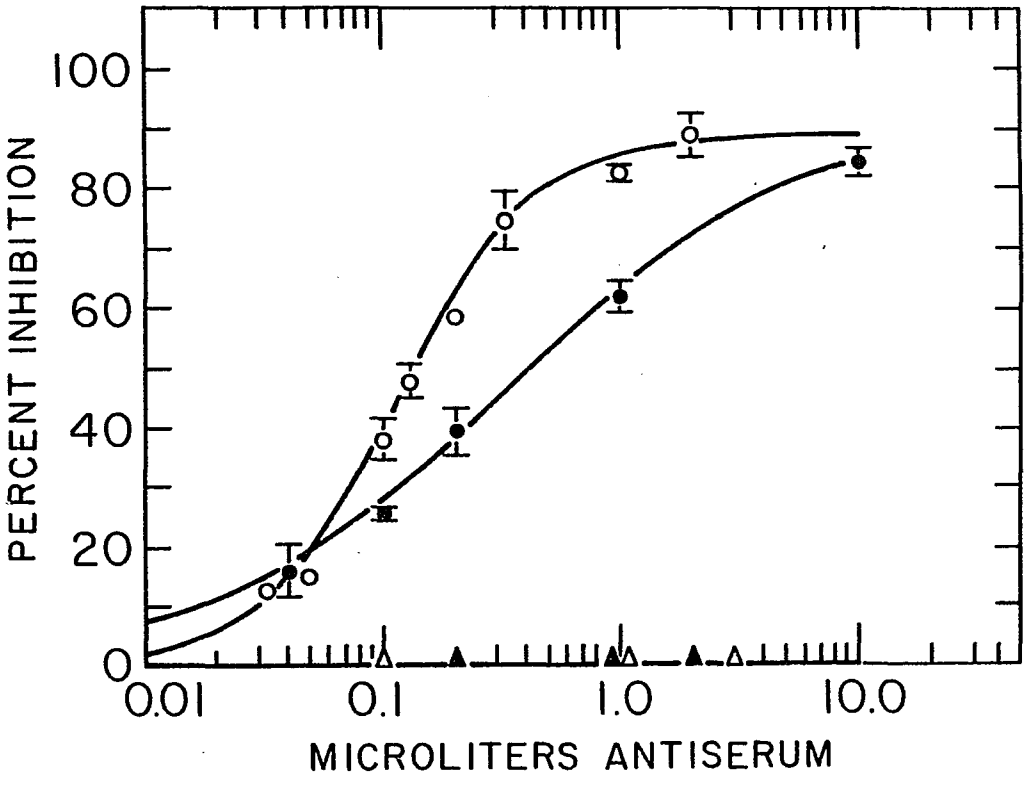
Rabbit kidney enzyme was prepared as described in "Results", Section B. Serum from a sarcoidosis patient was processed on Sephadex G-200 as described in Figure 9. Active fractions were pooled and used for measurements of enzyme activity. The log dose response curves were generated by a computer program which fits the parameters (% inhibition vs. microliters antiserum) to the logistic equation (Johnson, 1982). Data are the mean values ± S.D. of three to four determinations. Points without error bars represent the mean of two determinations.

-○- Rabbit kidney metalloendopeptidase incubated with anti-rabbit kidney metalloendopeptidase antiserum.

-●- Human serum metalloendopeptidase incubated with anti-rabbit kidney metalloendopeptidase antiserum.

-△- Rabbit kidney metalloendopeptidase incubated with rabbit pre-immune serum.

-▲- Human serum metalloendopeptidase incubated with rabbit pre-immune serum.


PERCENT INHIBITION
100
80
60
40
20
0
0.01
0.1
1.0
10.0
MICROLITERS ANTISERUM

in sarcoidcosis patients with elevated activity are the same. Incubations containing a serum mixture from a healthy control and from a sarcoidosis patient with elevated activity produced an activity which was the sum of the two. This result can be interpreted as showing both the absence of activators in serum from sarcoid patients and the absence of inhibitors in serum of healthy controls.

G. SERUM METALLOENDOPEPTIDASE ACTIVITY IN HEALTHY SUBJECTS, SARCOIDOSIS PATIENTS, AND PATIENTS WITH OTHER PULMONARY DISEASES

Serum metalloendopeptidase activities were determined in 150 healthy controls and in 95 patients with sarcoidosis. The descriptive statistics for the two groups are summarized in Table 18. The controls consisted of three groups: medical students, faculty and staff, and healthy, anonymous blood bank donors. No significant differences[1] were found among the groups using the Kruskal-Wallis k-sample test, and so the data therefore were pooled to form the control group. Of the 52 donors whose sex was known, no significant difference in enzyme activity was found between the 18 females and 34 males. Enzyme activities in the sarcoid group tended to be higher (mean = 9.06 units; median = 5.10) than in the control group (mean = 2.68 units; median = 1.80).

A stem-and-leaf display (Velleman and Hoaglin, 1981) of enzyme activities in the control and sarcoidosis groups

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[1]The level of significance for all statistical tests was 0.05.

Table 18. Descriptive Statistics for the Control and Sarcoidosis Groups[a]

Statistic	Group	
	Control (n = 150)	Sarcoidosis (n = 95)
Mean	2.68	9.06
Standard Deviation	3.09	12.56
Maximum	20.60	74.40
95th Percentile	8.47	33.93
90th Percentile	4.92	19.22
75th Percentile	2.78	10.85
50th Percentile (median)	1.80	5.10
25th Percentile	1.21	2.15
10th Percentile	0.75	1.29
5th Percentile	0.62	1.03
Minimum	0.15	0.49

[a]All values are in units of enzyme activity (nmol/min/ml serum).

is given in Table 19. The distribution of values has a similar pattern in both groups. Both distributions were skewed to the right; i.e., each distribution possessed some values which were large when compared with the majority of values within the distribution. The two groups were compared using the Wilcoxon Rank Test, and the difference in enzyme levels was found to be statistically significant ($p < 0.001$).

In the 95 sarcoidosis patients studied, enzyme activities were analyzed with respect to stage of disease as defined by chest roentgenogram. Table 20 summarizes the mean enzyme activity for patients within each stage, and gives the percentage of patients within each stage whose enzyme level was greater than the 90th percentile for the control group (i.e., > 5 units). A definition of the radiographic stages is given in the legend to this table (Siltzbach, 1967).

The highest mean enzyme activity was seen in patients with stage II x-rays. The stages 0, I and III groups had mean values which were less than half that of the stage II patients. Enzyme activities in the various groups were compared using the Kruskal-Wallis k-sample test and Fischer's protected least significant difference test (Ott, 1977). Stage II was found to be significantly different from stages 0, I, III, and controls, and stages I, II, and III (but not 0) were found to be significantly different from

Table 19. Stem and Leaf Display[a] of Enzyme Activity Distributions in the Control and Sarcoidosis Groups

CONTROL (n = 150)		SARCOIDOSIS (n = 95)
Units x 10^{-1}	Units	Units x 10^{-1}
999998887777766666654331	0	4679
99998888887777777766666665555555544333333222222222111111100	1	00012333455777899
977655444422222222111000000000	2	001124677889
9976444443321100	3	12245677
977633	4	3666
8742	5	001122446688
4	6	348
2	7	3
63	8	01124
7	9	03
	10	1258
5	11	236
7	12	338
	13	38
	14	35
	15	
	16	1
	17	
85	18	447
	19	9
6	20	
	>21	[b]22.2,26.8,29.0,32.2, [b]40.2,56.4,67.6,74.4.

[a]The table is a modified histogram which shows the individual data points. Each digit in the rows labeled CONTROL or SARCOIDOSIS represents a single individual with an activity equal to the number of units shown in the center column (UNITS) plus the number of units x 10^{-1} shown in the rows labeled CONTROL or SARCOIDOSIS. For example, the row whose center column contains the digit 6 represents one healthy control with 6.4 units (left side) and three sarcoidosis patients having 6.3 units, 6.4 units, and 6.8 units (right side).

[b]These rows display all activities (in units) that were above 21 units. These extreme values were found only in the sarcoidosis group.

Table 20. Serum Metalloendopeptidase Activities in Various Radiographic Stages of Sarcoidosis

Stage[a]	Number of Patients	Mean Enzyme (Units)	% Elevated[b]
0	11	4.1	36
I	29	6.3	41
II	44	12.9	61
III	11	6.2	45

[a]Radiographic stages are defined as follows: 0 = Within normal limits; I = Hilar lymphadenopathy; II = Hilar lymphadenopathy with interstitial infiltrates; III = Interstitial infiltrates.

[b]This column gives the percentage of patients in each stage in whom enzyme activity is above the 90th percentile for the control group.

controls ($p < 0.05$). It is noteworthy, however, that although stage II was significantly different from stages 0, I, and III, approximately 40-60% of patients within each group have enzyme levels of more than 5 units.

Metalloendopeptidase activity was also measured in 19 patients with primary pulmonary neoplasms (5 of whom had known metastases), 10 patients with active, sputum culture positive tuberculosis, and 9 patients with idiopathic interstitial fibrosis. The mean activity level was 3.1 units $\pm$ 2.9 (S.D.) for the patients with neoplasms, 2.5 units $\pm$ 1.0 for the patients with tuberculosis and 2.1 units $\pm$ 1.2 for the patients with interstitial pulmonary fibrosis. None of these groups significantly differed from the controls (Kruskal-Wallis k-sample test).

V. DISCUSSION

A. MEMBRANE-BOUND METALLOENDOPEPTIDASE

The isolation and characterization by Orlowski and Wilk (1981) of a thermolysin-like metalloendopeptidase present in the membrane fractions of bovine pituitaries prompted studies on the properties of this enzyme and on its action on biologically active peptides. The finding that the enzyme cleaves the Gly-Phe bond in enkephalins posed the question of whether this enzyme is identical with enkephalinase, a membrane-bound peptidase suggested to function as a specific enkephalin inactivator. The data presented here show that the two enzymes have similar sensitivity to inhibitors, regional distribution in brain, and specificity. The low specific activity of this enzyme in brain and pituitary make it extremely difficult to isolate as a homogeneous protein; the 10,000-fold purification described by Orlowski and Wilk (1981) results in a preparation containing an inactive protein contaminant. Various tissues were therefore screened with the purpose of finding a rich source of the enzyme. After finding that rabbit kidney had the highest specific activity of all tissues studied, a purification procedure was developed for

isolation of the enzyme from this tissue. This facilitated studies of the interaction of the enzyme with synthetic substrates, natural peptides, and inhibitors. The rabbit kidney metalloendopeptidase was also used to raise specific antisera which allowed comparison of the immunological properties of this enzyme with metalloendopeptidase from rabbit brain and from human serum.

Because the enzyme has been proposed to act *in vivo* as a specific inactivator of enkephalins, it was of interest to design active site directed inhibitors which could be used to test the *in vivo* function of the enzyme. This led to the synthesis of N-(1-carboxy-2-phenylethyl)-Phe-pAB, an inhibitor with a K_i in the nanomolar range. This compound and several less potent analogues were tested for antinociceptive activity in the rat tail-flick test.

The results reported here strongly suggest that the highly purified metalloendopeptidase from bovine pituitaries is identical with an endopeptidase associated with rat and rabbit brain membrane fractions, and that these enzymes are apparently identical with the enzyme referred to by several others as enkephalinase (Arregui et al., 1979; Benuck and Marks, 1980; Fournie-Zaluski et al, 1979; Gorenstein and Snyder, 1979; Roques et al., 1980; Sullivan et al., 1980; Vogel and Altstein, 1980). 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 chelators, 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. (1981) to inhibit enkephalinase activity in purified synaptosomes. Because the enzyme releases a C-terminal dipeptide from the enkephalins, it was classified by several authors as a dipeptidyl carboxypeptidase (Benuck and Marks, 1980; Fournie-Zaluski et al., 1979; Roques et al., 1980). In these studies however, only crude enzyme preparations were used, and the specificity of the enzyme was not tested with peptide substrates blocked at both the N- and C-terminus. The results of specificity studies presented here with synthetic peptide naphthylamides and with oxytocin, bradykinin, neurotensin, and enkephalinamide 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 in this respect resembles the specificity of thermolysin (Blumberg et al., 1981), a bacterial zinc metalloendopeptidase (Matsubara et al., 1966).

The data in Table 5 show that rabbit kidney is a far more abundant source of the enzyme than rabbit brain. This is consistent with reports by Llorens and Schwartz (1981) and by Benuck et al. (1981) which have described the presence of high enkephalinase activity in kidney membrane fractions. The particulate fractions of rabbit kidney were therefore used to conveniently obtain large quantities of a homogeneous enzyme. The kidney enzyme can be efficiently solubilized after preparation of a deoxycholate extract, removal of excess deoxycholate with streptomycin sulfate, and a subsequent short (90-min) treatment with papain. The solubilized enzyme can be readily separated from other membrane-bound kidney peptidases on the basis of molecular size, hydrophobic interactions, and charge differences. Thus, chromatography on Sephadex G-200 columns removed dipeptidyl aminopeptidase IV, an enzyme having a molecular weight more than twice that of the metalloendopeptidase (McDonald and Schwabe, 1977); hydrophobic chromatography on phenyl-Sepharose eliminated the contamination by aminopeptidase M; and chromatography on carboxymethylcellulose eliminated the accompanying γ-glutamyl transpeptidase, an enzyme closely associated with kidney brush border membranes (Albert et al., 1961; Orlowski and Meister, 1965).

Electrophoresis of the enzyme under non-dissociating conditions gave one major and one minor protein band, both of which were found to contain metalloendopeptidase

activity. Electrophoresis under dissociating conditions (0.1% $NaDodSO_4$) revealed a single sharp band (Figure 1). It was therefore concluded that the enzyme was purified to apparent homogeneity, and that it is comprised of charge isomers which appear as diffuse bands under non-dissociating conditions. It is possible that these charge isomers result from differences in the carbohydrate composition of different enzyme molecules.

The kidney enzyme had a molecular weight of approximately 95,000 as determined by gel filtration on Sephadex G-200 and electrophoresis in the presence of $NaDodSO_4$, and seemed to be composed of a single polypeptide chain. It was sensitive to inhibition by thiols and metal chelators and resistant to inhibition by thiol blocking agents, DFP, and several thiol, serine, and carboxyl protease inhibitors of bacterial origin such as leupeptin, chymostatin, antipain, and pepstatin. Activity was strongly inhibited by thiorphan and phosphoramidon, both of which are known to strongly inhibit brain enkephalinase and kidney neutral metalloendopeptidase (Fulcher et al., 1982; Hudgin et al., 1981; Roques et al., 1980).

The specificity of the rabbit kidney metalloendopeptidase toward peptide naphthylamide substrates closely resembles the specificity of the enzyme purified from membrane fractions of bovine pituitary (Orlowski and Wilk, 1981; see also Table 2). A hydrophobic residue on the P_1'

position is required for activity of both these enzymes, and replacement of this residue by small neutral amino acids such as alanine or glycine greatly diminishes or virtually eliminates activity. It is of interest that high (k_{cat}/K_m) ratios are obtained with those substrates having either arginine or alanine residues in positions P_1 and P_2. Apparently, the S_1 and S_2 subsites of these enzymes accommodate well either small neutral amino acids or basic amino acid residues. Thus, synthetic chromogenic substrates having a hydrophobic residue in the P_1' position and either small neutral or basic residues in positions P_1 and P_2 can be used for the convenient and sensitive determination of enzyme activity in a coupled enzyme assay with aminopeptidase M, as described here. Such substrates are particularly useful for kinetic studies and should also find application for the determination of activity of bacterial metalloendopeptidases including thermolysin.

Kidney metalloendopeptidase also showed the same specificity as the pituitary enzyme with respect to hydrolysis of naturally occurring peptides. Both enzymes hydrolyzed the Gly-Phe bond in Met- and Leu-enkephalin and in Met-enkephalinamide and also readily hydrolyzed bonds on the amino side of hydrophobic residues in several other biologically active peptides.

There is a growing interest in inhibitors of zinc metallopeptidases, because of their potential pharmacological

effects, which are related to the function of these enzymes in vivo. For example, captopril (D-3-mercapto-2-methylpropanoyl-L-proline), an inhibitor of angiotensin converting enzyme, has found application in the treatment of hypertension (Antonaccio and Cushman, 1981); and N-carboxymethyl dipeptides, a new class of converting enzyme inhibitors, may find the same application (Patchett et al., 1980). With the potential role of the metalloendopeptidase described here in the degradation of enkephalins and other neuropeptides, inhibitors of this enzyme are of interest because of their potential pharmacological effects. Indeed, study of such effects may provide clues to the physiological function of this enzyme in vivo. There are clear similarities in the basic mechanism of action of the angiotensin converting enzyme, the kidney metalloendopeptidase described here, thermolysin, and also carboxypeptidases A and B. All of these enzymes have a zinc atom in the active site that participates in catalysis by polarizing the carbonyl group of the bond undergoing hydrolysis. Differences between the enzymes relate to substrate specificity and binding. Accordingly, principles of design of potent inhibitors of these enzymes are based on the need for the presence of a group capable of interacting with the zinc atom in the active site, in analogy to the interaction of the carbonyl oxygen of the hydroylzed bond with this zinc atom, and in the presence of a group with optimal binding characteristics to the substrate binding site of the enzyme.

Thermolysin and the kidney metalloendopeptidase are closely related with respect to specificity in that a hydrophobic residue in the P_1' position is needed for effective binding. Although both of these enzymes can act as dipeptidyl carboxypeptidases on peptides having a hydrophobic residue in the penultimate position, unlike the angiotensin converting enzyme, a free carboxyl group is not required for activity. Several potent synthetic thermolysin inhibitors have been described (Kam et al., 1979; Maycock et al., 1981; Nishino and Powers, 1979). Each of these inhibitors contains a peptide capable of interacting with the substrate binding site of the enzyme and a group such as a hydroxamic acid, phosphoramidate, thiol, or carboxyl capable of coordinating with the zinc atom of the active site. It was anticipated that similar compounds would be effective inhibitors of the kidney metalloendopeptidase. Indeed, inhibition of the enzyme by phosphoramidon, a naturally occurring analogue of a series of phosphoramidates synthesized by Kam et al. (1979), supports this conclusion. The use of phosphoramidates in <u>in vivo</u> experiments presents difficulties due to their susceptibility to enzymatic degradation. Similarly, thiol-containing inhibitors such as thiorphan may present problems related to the facile oxidation of the sulfhydryl group.

N-carboxymethyl derivatives of amino acid amides containing p-aminobenzoate in amide linkage were therefore

synthesized as potential inhibitors of the enzyme. This approach was based on the finding that the enzyme is inhibited by dipeptides having a phenylalanyl residue on the amino side and that Phe-pAB itself is a potent inhibitor with a K_i in the micromolar range. It was expected that addition of a zinc-binding group to Phe-pAB should greatly increase its inhibitory potency. Indeed, N-(1-carboxy-2-phenylethyl) derivatives of Phe-pAB have proved to be potent inhibitors of the enzyme, with the most active diastereomer having a K_i of 2.4×10^{-8} M. As expected, the alanyl and glycyl analogues of this inhibitor were far less potent than the phenylalanyl derivative, thereby reflecting the lower binding affinity of alanine and glycine to the hydrophobic pocket of the enzyme. On the basis of the available information, the mode of binding of the inhibitor to the enzyme can be presented schematically as shown in Figure 12. This simplified scheme does not consider the contributions to binding provided by the carbonyl group of the Phe-pAB bond and by the carboxylate group of p-amino-benzoate. That these groups contribute to binding is suggested by the finding that Phe-pAB is strongly inhibitory while free phenylalanine is not. This binding mode presented in Figure 12 is similar to that presented for several inhibitors of thermolysin (Holmes and Matthews, 1981; Kam et al., 1979; Nishino and Powers, 1979). Another factor thought to contribute to the inhibitory potency of the

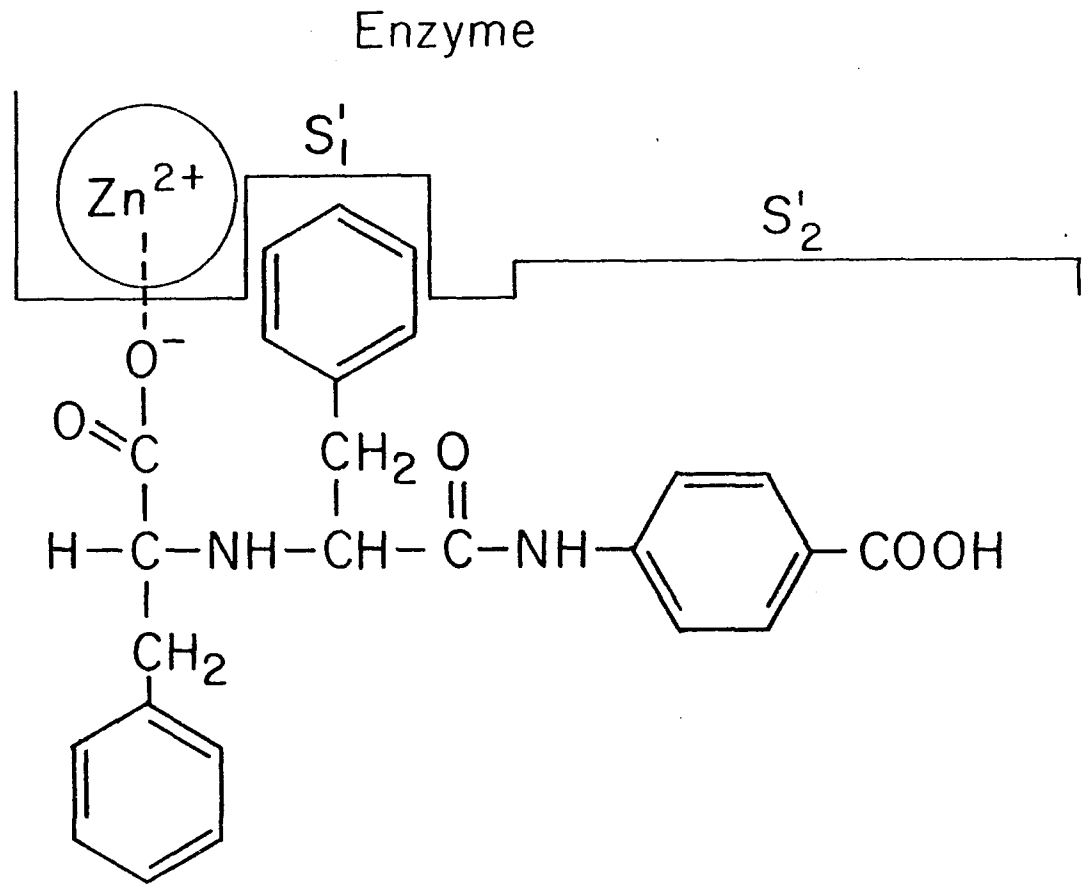

FIGURE 12. MODE OF BINDING OF N-(1-carboxy-2-phenylethyl)-Phe-pAB TO THE METALLOENDOPEPTIDASE. (Schematic Drawing)

N-carboxymethyl derivatives is their resemblance to the tetrahedral transition state involved in the hydrolysis of peptide substrates (Patchett et al., 1980).

The procedure described for purification of the kidney metalloendopeptidase has also facilitated isolation of the enzyme from brain. A 374-fold purification of the brain enzyme was attained. Although the specific activity of the brain enzyme is much lower than that of the kidney enzyme, this reflects the fact that the specific activity of a deoxycholate extract of brain membranes is only 0.5% that of kidney membranes. Nevertheless, the brain enzyme was free of contamination with aminopeptidase, an enzyme interfering in studies of enkephalin degradation by the metalloendopeptidase.

Experiments showed that the brain and kidney enzymes are identical with respect to pH optimum, molecular weight (as judged from elution volumes recorded during Sephadex G-200 chromatography) and substrate specificity. Both enzymes hydrolyze several synthetic substrates with the same relative velocities (Table 13) and both hydrolyze the Gly-Phe bond in Met- and Leu-enkephalin. In addition, the same purification procedure can be used for the isolation of both enzymes.

The close similarity, if not identity, of the two enzymes is supported by studies on the effect of active site directed inhibitors. The K_i values obtained for

phosphoramidon are in agreement with those reported by Kenny (1977) for the kidney neutral metalloendopeptidase and more recently by Malfroy and Schwartz (1982) for rat kidney and mouse striatal membranes. Thiorphan, an enkephalinase inhibitor (Roques et al., 1980) inhibited the brain and kidney enzymes with a similar K_i, again suggesting that two enzymes are closely related and apparently identical with enkephalinase. The inhibition of both enzymes by N-[1-(R,S)-carboxy-2-phenylethyl] derivatives of Phe-pAB and Ala-pAB, two specific inhibitors, again supports the strong similarity of the enzymes.

Similar conclusions can be derived from immunological crossreactivity observed between the kidney metalloendopeptidase and the brain enzyme. Anticatalytic immunoinhibition techniques have been a valuable tool for studying the relationship of proteolytic enzymes from different tissues. Andrews et al. (1982) and Hersh (1981b) have used this approach to analyze the properties of prolyl endopeptidase from various tissues and species. Das and Soffer (1976) have used this technique to study the properties of the angiotensin converting enzyme from various sources. Kenny and his co-workers (Danielsen et al., 1980) raised antisera to hog kidney microvillar membranes and demonstrated crossreactivity between the kidney neutral metalloendopeptidase and a similar enzyme found in intestinal membranes.

Despite the close similarity of the brain and kidney metalloendopeptidases, the differences between the two enzymes cannot be neglected. These differences are expressed in the somewhat lower K_i values of phosphoramidon and N-[1-(R,S)-carboxy-2-phenylethyl] derivatives of Phe-pAB and Ala-pAB toward the brain enzyme. They are also expressed in the slightly higher sensitivity to inhibition by antibodies of the kidney enzyme than of the brain enzyme (Figure 4). Indeed, Ouchterlony immunodiffusion experiments gave results suggestive of the presence of antigenic determinants on the kidney enzyme that are not present on the brain enzyme (Figures 5-7). The underlying reasons for these differences are unknown. The kidney enzyme seems to be associated with the brush border (Kerr and Kenny, 1974a), a structure not present in brain tissue. Like other brush border enzymes, the metalloendopeptidase is a glycoprotein and thus the carbohydrate part of the molecule may be quantitatively and even qualitatively quite different from that of the brain enzyme. These differences might well account for the small immunological differences between the two enzymes. Although not changing the specificity of the enzyme, such differences could induce small changes in the conformation of the protein and its active site, thereby affecting the binding characteristics of the inhibitors. While this explanation seems attractive, the possibility cannot be excluded that the amino acid sequences of the two

enzymes may not be completely identical. Future studies of the structure of the two proteins should establish the exact nature of the observed differences.

The characteristics of the kidney metalloendopeptidase described here indicate that it is apparently identical with that purified by Kerr and Kenny (1974a), from rabbit kidney brush border preparations, and shown to cleave the B chain of insulin, by hydrolyzing bonds involving the amino group of hydrophobic residues. The enzyme also appears to have properties similar to those of a neutral metalloendopeptidase isolated from bovine pituitaries (Orlowski and Wilk, 1981) and also detected in pancreatic membrane preparations (Mumford et al., 1980), and in the rabbit and rat brain preparations described here. All of these enzymes are membrane-bound endopeptidases exhibiting a thermolysin-like specificity. All share the common property of being sensitive to inhibition by thiols and by metal chelators and being resistant to inhibition by thiol blocking agents, DFP, and several thiol, serine, and carboxyl protease inhibitors of bacterial origin, such as leupeptin, chymostatin, antipain, and pepstatin. Furthermore, the pituitary, brain and kidney enzymes have the same molecular weight of about 90,000-95,000 (Kerr and Kenny, 1974b) and seem to be composed of a single polypeptide chain.

Kerr and Kenny (1974a) have reported that the kidney enzyme was firmly bound to the microvillus membranes and

resisted solubilization by treatment with papain, Triton X-100, various concentrations of $NaDodSO_4$, and several proteolytic enzymes. Successful solubilization was only obtained after treatment with toluene and a rather prolonged subsequent exposure to trypsin. The procedure of deoxycholate extraction and papain digestion (Orlowski and Wilk, 1981) used here provides a rapid and effective method for solubilizing both the brain and kidney enzymes.

The role of this enzyme in peptide metabolism is unknown. Its localization in the kidney brush border suggests that in this organ, the enzyme may play a role in the degradation of peptides present in the glomerular filtrate. It was shown here that in addition to enkephalins, the enzyme hydrolyzes other biologically active peptides including bradykinin, oxytocin, angiotensin I, neurotensin, and dynorphin. It is therefore possible that the metalloendopeptidase present in brain and pituitary participates in the metabolism of neuropeptides, and could thus profoundly affect the function of the central nervous system and also of the pituitary. The activity of the enzyme hydrolyzing the Gly-Phe bond in enkephalins is of particular interest, because it is identical with an activity associated with brain membrane fractions and designated as enkephalinase (Roques et al., 1980; Sullivan et al., 1978). It was proposed that brain enkephalinase functions as a specific brain enkephalin inactivator in a

manner similar to the inactivation of acetylcholine by acetylcholinesterase. The main arguments advanced in support of this hypothesis were that the distribution of the enzyme in brain parallels the distribution of opiate receptors and that thiorphan, DL-3-mercapto-2-benzyl-propanoylglycine, a synthetic inhibitor of enkephalinase, potentiated the morphinomimetic effect of an enkephalin analogue, D-Ala^2-Met-enkephalin, and had itself some antinociceptive activity (Roques et al., 1980). A flaw in this work, however, is that while thiorphan is a potent enzyme inhibitor with antinociceptive activity, less potent metalloendopeptidase inhibitors with structures similar to thiorphan have not been tested. Thus, the antinociceptive properties of thiorphan cannot be correlated with its inhibitory potency for the metalloendopeptidase.

Although Malfroy et al. (1978) originally reported that enkephalinase has a K_m of 9.0×10^{-8} M for Met-enkephalin, a K_m of 4.0×10^{-5} M was reported here for the pituitary metalloendopeptidase. The latter value, which is almost three orders of magnitude less than that of Malfroy et al., suggests that the enzyme is not a high affinity enkephalin degrading peptidase. Subsequent reports on the brain enzyme by Malfroy and Schwartz (1982) and by other investigators (Altstein et al., 1981; Benuck and Marks, 1980; Mumford et al., 1981) are in agreement with the K_m value presented here.

Mumford et al. (1981) have confirmed the findings reported here, that enkephalinase is a thermolysin-like endopeptidase rather than a dipeptidyl carboxypeptidase, and that it is similar to the kidney neutral metalloendopeptidase [EC 3.4.24.11] originally described by Kerr and Kenny (1974a). In addition, they have shown that the affinity of the kidney metalloendopeptidase for bradykinin, neurotensin, and for angiotensin I and II is similar to its affinity for Met- and Leu-enkephalin, thereby suggesting that the enkephalins are not preferred substrates. These observations are consistent with the findings presented here (Table 2), that both the rate of hydrolysis and the specificity constant of the pituitary enzyme are lower for Met-enkephalin than for the peptide naphthylamide substrates Hip-Arg-Arg-Leu-2NA and glutaryl-Ala-Ala-Phe-2NA.

In contrast to acetylcholinesterase, which is highly specific for the hydrolysis of acetylcholine, the metalloendopeptidase has a broad substrate specificity and does not preferentially hydrolyze enkephalins. In addition, acetylcholinesterase has been localized at the motor end plate of skeletal muscle (Davis and Koelle, 1967) and is in the vicinity of nicotinic receptors. Immunocytochemical or histochemical localization of the metalloendopeptidase has not yet been reported, thus its topographical relationship to the opiate receptors cannot be described. In conclusion, the available evidence does not suggest that

the metalloendopeptidase is a specific enkephalin inactivator or enkephalinase.

It is noteworthy that since the publication of the results described here (Almenoff et al., 1981; see p. vii), Malfroy and Schwartz (1982), who originally described the enzyme as a dipeptidyl carboxypeptidase, showed that their preparations of enkephalinase cleave Hip-Arg-Arg-Leu-2NA at a rate 10 times that of Hip-Arg-Arg-Ala-2NA, and that Hip-Arg-Arg-Gly-2NA was negligibly hydrolyzed. These results are similar to the data presented here (Table 2), and they confirm that enkephalinase is indeed a thermolysin-like endopeptidase.

The data indicate that the enzyme can act as a dipeptidyl carboxypeptidase on peptides having a hydrophobic residue in the penultimate position but that a free carboxyl group is not required for activity. It was observed however, that Phe-Leu is a more potent inhibitor of the metalloendopeptidase than Phe-Leu-NH_2. Similar observations were presented by Malfroy and Schwartz (1982), who showed that the K_m for enkephalinamide is 25 times greater than for enkephalin; likewise, the K_i for amidated thiorphan is 10-fold greater than for thiorphan. Further studies by this group showed that treatment of the metalloendopeptidase with butanedione, a reagent which has been reported to selectively and irreversibly block guanidinium groups, decreased the hydrolysis rate of enkephalin by 80% and of enkephalinamide by 50%. Based on this result, Malfroy and

Schwartz (1982) suggested that there is a "critical" arginine residue in the active site of the metalloendopeptidase as has been shown for pancreatic carboxypeptidase A (Quicho and Lipscomb, 1971). They speculated that this residue facilitates interaction of the metalloendopeptidase with peptides having free carboxyl groups on the dipeptide C-terminal to the hydrolyzed bond.

Because of the proposed role of the metalloendopeptidase as an enkephalinase and the finding that thiorphan, a metalloendopeptidase inhibitor, has analgesic properties, it was of interest to test N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB and its analogues for antinociceptive activity. These compounds are resistant to enzymatic degradation by the metalloendopeptidase, aminopeptidase, and dipeptidyl carboxypeptidase, and are therefore excellent tools for *in vivo* studies.

When administered intraperitoneally, N-[1-(R,S)-carboxy-2-phenylethyl] derivatives of phenylalanyl, alanyl, and glycyl-pAB increased latency about two-fold in the rat tail-flick test. Significant effects were seen with all three compounds, with N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB being slightly more potent than the alanyl and glycyl derivatives. All compounds produced analgesia at 1-24 h but not at 48 h after administration. N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB was active at 3.12 umol/kg, which, assuming uniform tissue distribution in the rat, is

at a concentration about 40 times its K_i for the metalloendopeptidase. The N-[1-(R,S)-carboxy-2-phenylethyl] derivatives of alanyl-pAB and glycyl-pAB were equally efficacious at 25 umol/kg. Assuming uniform tissue distribution, this represents a concentration of about 5 times the K_i of the alanyl derivative and 0.16 times the K_i of the glycyl derivative. The data suggest, therefore, that the antinociceptive effect is apparently not correlated with the extent of metalloendopeptidase inhibition.

The mechanism of action for these compounds is not clear. Since their antinociceptive activity does not correlate with their ability to inhibit enzyme activity, it is difficult to explain the observed effects on this basis. Preliminary data indicate that naloxone (1 mg/kg) does not reverse the antinociceptive effects of N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB and thus suggests a mechanism of action unrelated to opiate binding or to accumulation of endogenous opiates such as enkephalins. Future studies of other behavioral and biological effects of these three compounds, and identification of systems which are selective for N-[1-(R,S)-carboxy-2-phenylethyl]-Phe-pAB over the less potent analogues, may provide insight into the in vivo role of the metalloendopeptidase.

While a report on the synthesis of N-(1-carboxy-2-phenylethyl) derivatives of phenylalanyl and alanyl-pAB was in press (Almenoff and Orlowski, 1983; see p. vii), several

similar inhibitors of the metalloendopeptidase were described. Roques et al. (1982) synthesized N-[(R,S)-2-carboxy-3-benzylpropanoyl]-L-Leucine, a competitive inhibitor with a K_i of 3.4×10^{-7} M. Mumford et al. (1982) synthesized similarly potent N-(1-carboxy-3-phenylethyl)-phenylalanyl derivatives of glycine, β-alanine, and γ-aminobutyric acid. Future studies on the in vivo properties of these compounds are of great interest.

B. SERUM METALLOENDOPEPTIDASE

Increased activities of angiotensin converting enzyme have been found in the serum of patients with active sarcoidosis (Fanburg et al., 1976; Lieberman, 1975; Rhogati and Ryan, 1980; Rohrbach and DeRemee, 1979). Since this enzyme is similar to the metalloendopeptidase in its mechanism of action and relative abundance in lung membranes, it was of interest to determine whether the metalloendopeptidase could be detected in human serum and whether it, too, is elevated in sarcoidosis. The studies presented here show that the enzyme is indeed present in serum. The data indicate that serum metalloendopeptidase activity can be reliably determined with the substrate glutaryl-Ala-Ala-Phe-2NA and the coupled enzyme assay used for determination of the brain and kidney enzymes. The biochemical properties of the serum enzyme and its activities in healthy subjects and sarcoidosis patients were studied.

The properties of the serum enzyme described here indicate that it is similar to the thermolysin-like neutral metalloendopeptidase found in kidney, pituitary, brain, and other tissues. All of these, like the serum enzyme, hydrolyze peptide bonds on the amino side of hydrophobic amino acids, are inhibited by metal chelators, and active site directed inhibitors that fulfill the binding requirement of the active site, and are capable of complexing with the active site zinc atom. The strong crossreactivity between an antiserum against the purified kidney enzyme and the enzyme in human serum shows that the two enzymes have common antigenic determinants.

The serum neutral metalloendopeptidase has a molecular weight in excess of 200,000, and therefore differs in this respect from the kidney metalloendopeptidase, which, when solubilized by deoxycholate treatment and papain digestion, has a molecular weight of 95,000. Several explanations can account for this difference. The serum enzyme may have an oligomeric structure, or it may be released from tissues as part of a high molecular weight membrane fragment. Alternatively, it may be associated as a complex with other serum proteins including α_2-Macroglobulin, which is known to be capable of binding endopeptidases without inhibiting their activity toward small peptides. It is noteworthy that Werb et al. (1974) have reported the binding of thermolysin to α_2-Macroglobulin. Exploration of these

and other possibilities is needed to account for the unusually high molecular weight of the serum enzyme.

The coupled enzyme assay introduced for the determination of serum metalloendopeptidase activity is sufficiently sensitive for measuring even low enzyme activities and gives reproducible results. Some interference with determination of 2-naphthylamine could, however, be expected from sulfonamides present in serum of patients treated with these agents. A hemolysate of red blood cells had very low enzyme activity (average of 2.6 nmoles product formed per ml of packed erythrocytes per min), and therefore suggests that hemolysis of samples should not interfere with determination of enzyme activity. It should be noted that Hip-Arg-Arg-Leu-2NA, a substrate used for determination of metalloendopeptidase activity in purified preparations, is not suitable for these studies because it can be hydrolyzed by trypsin-like enzymes that are present in serum.

The metalloendopeptidase and the angiotensin converting enzyme have similar mechanisms of action and they are both abundant in lung membranes. It was therefore of interest to study serum metalloendopeptidase activity in sarcoidosis, a disease in which elevated serum angiotensin converting enzyme activity has been described. The descriptive statistics in Table 18 indicate that metalloendopeptidase activities in sarcoidosis patients tend to be higher than

activities in the control group. The difference between the two groups is statistically significant ($p < 0.001$). Both distributions have similar shapes and are skewed toward higher values (Table 19). Although approximately 90% of the healthy controls had less than 5 units of enzyme activity, 5 of the 150 individuals studied had 10-20 units of activity. Since these 5 subjects had no known medical illnesses, it appears that a small percentage of asymptomatic, presumably healthy individuals have elevated activities of the metallo-endopeptidase. The significance of this finding is unclear. It is noteworthy, however, that a small percentage of healthy individuals have elevated serum angiotensin converting enzyme (Fanburg et al., 1976) and that elevated serum angiotensin converting enzyme has been found in diseases other than sarcoidosis (for a review see Rhogati, 1982).

The data in Table 20 show that the highest enzyme activities were seen in patients with stage II x-rays (hilar lymphadenopathy with parenchymal infiltrates). Although this suggests that serum enzyme elevations may be related to the extent of granulomatous involvement of the lung, it should be emphasized that only 60% of these patients have elevated activities. This suggests that there may be other factors such as disease activity, duration, and extrathoracic involvement, which may be related to enzyme elevation. It is noteworthy that most studies of serum angiotensin converting enzyme in

sarcoidosis have shown that highest enzyme activities are found in patients with pulmonary parenchymal involvement (stages II and III).

The source of the metalloendopeptidase in serum remains to be determined. Since the enzyme is membrane bound, it is possible that the serum activity is solubilized from the surface of the lung and of other tissues. Alternatively, the enzyme may be derived from the sarcoid granulomas themselves. This mechanism has been postulated for the production of serum angiotensin converting enzyme in sarcoidosis based on the finding of increased angiotensin converting enzyme in lung and lymph node biopsies of patients with sarcoidosis (Lieberman, 1976) and of immunoreactive angiotensin converting enzyme present in the epithelioid and giant cells of sarcoidosis granulomas (Silverstein et al., 1979).

The activity measurements in patients with sarcoidosis were not correlated with disease activity or duration. Since the population studied consisted of patients in remission as well as those with active disease, it is perhaps not surprising that a wide range of enzyme activities was found in the sarcoidosis group. Detailed analysis of the relationship between various criteria of disease activity and serum enzyme activity should provide insight into the clinical significance of the enzyme.

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