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A SOLUBLE METALLOENDOPEPTIDASE FROM RAT BRAIN: POSSIBLE FUNCTION IN NEUROPEPTIDE PROCESSING AND DEGRADATION

by

THOMAS G. CHU

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

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ABSTRACT

A SOLUBLE METALLOENDOPEPTIDASE FROM RAT BRAIN:
POSSIBLE FUNCTION IN
NEUROPEPTIDE PROCESSING AND DEGRADATION

by

Thomas G. Chu

Advisor: M. Orlowski, M.D.

A metalloendopeptidase, optimally active at a neutral pH, was purified from the soluble fraction of rat brain homogenates. The enzyme (M_r about 67,000) is strongly inhibited by metal chelators such as EDTA and o-phenanthroline. An EDTA-treated enzyme can be reactivated by several divalent metal ions. The enzyme preferentially cleaves peptides having hydrophobic amino acid residues in the P'_3 position and also in the P_1 and P_2 position. Substitution of a D-amino acid residue in either position P_1 or P'_2 renders the substrate

resistant to hydrolysis. Specificity studies suggest the presence of an extended active site, binding a minimum of five amino acid residues. Bioactive peptides are hydrolyzed by the enzyme at sites consistent with the specificity deduced from the studies with synthetic substrates. Dynorphin(1-8), α-neo-endorphin, β-neo-endorphin and BAM-12P are hydrolyzed to form enkephalins. Kinetic studies indicate a high affinity of the enzyme towards dynorphin(1-8), β-neo-endorphin, bradykinin and neurotensin. The enzyme exhibits high activity in brain, testes, anterior pituitary and posterior pituitary. Other tissues show activity 10 to 28% of that in the brain.

A series of N-carboxymethyl peptide derivatives structurally related to model substrates and containing a carboxylate group capable of coordinating with the active site zinc atom were synthesized. One of these, N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-p-aminobenzoate, was found to be a potent competitive inhibitor of the enzyme with a K_i of 1.94 uM. Of the two diastereomers, the more potent diastereomer had a K_i of 0.81 uM. Effective inhibition requires the presence in the inhibitor of a group binding to the substrate recognition site of the enzyme and a group capable of coordinating with the active site metal atom. A hydrophobic residue in the inhibitor binding to the S_1 subsite of the enzyme greatly increases inhibitory potency. The high

activity of this enzyme in brain and pituitary, its preference for oligopeptides as substrates, its ability to generate enkephalins from several opioid peptides as well as its ability to degrade several neuropeptides, and its absence of activity towards large proteins and peptides suggests that the enzyme functions in the metabolism of neuropeptides.

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ABBREVIATIONS USED

ACTH .... adrenocorticotropic hormone

BAM-12P .... bovine adrenal medulla dodecapeptide

BAM-22P .... bovine adrenal medulla docosapeptide

BOC .... tert-butoxycarbonyl

Bz .... α-N-benzoyl

CH_3CN .... acetonitrile

DFP .... diisopropylfluorophosphate

DMF .... dimethylformamide

DTT .... dithiothreitol

EDTA .... ethylenediaminetetraacetic acid

EGTA .... ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid

HEPES .... (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)

HPLC .... high pressure liquid chromatography

LHRH .... luteinizing hormone-releasing hormone

βLPH .... β-lipotropin

βMSH .... β-melanotropin

2NA 2-naphthylamine

NaAc .... sodium acetate

$NaDodSO_4$ .... sodium dodecylsulfate

OPA .... o-pthalaldehyde

pAB .... p-aminobenzoate

PCMB .... p-chloromercuribenzoate

pNA .... p-nitroaniline

TCA .... trichloroacetic acid

TFA .... trifluoroacetic acid

THF .... tetrahydrofuran

TPCK .... L-1-tosylamide-2-phenylethyl chloromethyl ketone

Tris .... tri(hydroxymethyl)aminomethane

TRH .... thyrotropin releasing hormone

<u>Enzymes</u>. Membrane-bound neutral metalloendopeptidase (EC 3.4.24.11); angiotensin-converting enzyme (EC 3.4.15.1); aminopeptidase (EC 3.4.11.2); prolyl endopeptidase (EC 3.4.21.26); cathepsin B (EC 3.4.22.1).

<u>Nomenclature</u>. The nomenclature of Schechter and Berger (1967) is used to describe the interaction between enzyme and substrate. The amino acid residues in the substrate are designated as P_1, P_2, and P_3 etc. in the N-terminal direction and P_1', P_2', and P_3' etc. in the C-terminal direction from the bond undergoing cleavage. The corresponding subsites in the enzyme are identified by the letter S.

PUBLICATIONS RESULTING FROM THIS WORK

1. Chu, T. G. and Orlowski, M. (1983) Design of Potent Inhibitors of a Soluble Metalloendopeptidase from Rat Brain and Bovine Pituitary. Fed. Proc. 42, 1782.

2. Chu, T. G. and Orlowski, M. (1984) Active Site Directed N-Carboxymethyl Peptide Inhibitors of a Soluble Metalloendopeptidase from Rat Brain. Biochemistry 23, 3598-3603.

3. Orlowski, M., Chu, T. G. and Michaud, C. (1983) A Zinc-Metalloendopeptidase from Rat Brain: Interaction with Neuropeptides, Synthetic Substrates and Inhibitors. J. Neurochem. 41, S04.

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INTRODUCTION

Many physiological processes are controlled by peptide hormones and other bioactive peptides. It has recently been appreciated that peptides constitute a new class of molecules believed to play key roles as modulators or transmitters in the central and peripheral nervous system. Over 20 different peptides have been identified within neurons (Hokfelt et al., 1980). Formation of such bioactive peptides from high molecular weight precursors requires the specific hydrolysis of discrete peptide bonds; this non-random hydrolysis of peptide precursors has been termed "limited proteolysis" (Neurath and Walsh, 1976). Unlike some classical neurotransmitters such as norepinephrine, no reuptake mechanisms for neuropeptides have been elucidated. Once released, their inactivation necessitates the involvement of either membrane-bound proteases or intracellular degrading enzymes following internalization of the neuropeptide. Therefore, proteolytic enzymes appear to function in both the formation of peptide hormones and bioactive peptides, as well as their degradation to biologically inert fragments. Such enzymes would play key roles in the modulation of physiological systems. Unfortunately, the molecular mechanisms

involved in these reactions are poorly understood. Insight into the biochemical pathways controlling peptide hormones and neuropeptides can only come from careful study of the biochemistry and function of the enzymes involved.

In general, several biochemical criteria must be fulfilled in the identification of a proteolytic enzyme involved in the processing and degradation of peptide hormones and neuropeptides. The protease must correctly cleave the precursors or bioactive peptide to generate all known products. It must be resolved from contaminating proteases before characterization. Biochemical characterization should include studies of cleavage specificity and mechanism, pH characteristics, and susceptibility to known protease inhibitors. Cellular and subcellular distribution must be appropriate for its putative role. A number of studies have dealt with the enzymatic degradation of biologically active peptides and efforts have also been made to identify those enzymes responsible for the formation of peptide hormones and neuropeptides from inactive precursors (McDermott et al., 1983; McKelvy et al., 1982; Fletcher et al., 1981). The majority of these studies were carried out using crude enzyme preparations, with little regard to the contamination by additional proteases or careful identification of the reaction products. Proof of functional involve-

ment of a protease in bioactive peptide metabolism requires the demonstration that inhibition of activity results in the inhibition of formation or degradation of the respective peptide in intact cells or *in vivo*. Such proof necessitates the use of specific inhibitors, which could be used as probes in studies of the physiological function of the enzyme. The rational design and synthesis of such specific inhibitors, however, first requires the knowledge of the binding and specificity requirements of the active site of the involved enzymes. By affecting those functions which control the concentration of peptide hormones and neuropeptides, such specific enzyme inhibitors can also constitute a potentially new class of pharmacologically active agents. For example, an inhibitor of angiotensin-converting enzyme (EC 3.4.15.1), Captopril [D-3-mercapto-2-methylpropanoyl-L-proline], is used in the treatment of hypertension (Ondetti et al., 1977); its mechanism of action is believed to be the prevention of formation of angiotensin II, a vasospastic peptide, and the prevention of degradation of bradykinin, a vasodilating peptide.

An understanding of the enzymatic reactions involved in the formation and degradation of neuropeptides and peptide hormones requires knowledge of the amino acid sequences of the peptides and their precursors (see Figure 1). Met-enke-

Figure 1. Amino Acid Sequence of Opioid Peptide Precursors and Opioid Peptides

PROENKEPHALIN A (Human)

Met-Ala-Arg-Phe-Leu-Thr-Leu-Cys-Thr-Trp-Leu-Leu-Leu-Leu-Gly-Pro-Gly-Leu-Leu-Ala-Thr-Val-Arg-Ala-Glu-Cys-Ser-Gln-Asp-Cys-Ala-Thr-Cys-Ser-Tyr-Arg-Leu-Val-Arg-Pro-Ala-Asp-Ile-Asn-Phe-Leu-Ala-Cys-Val-Met-Glu-Cys-Glu-Gly-Lys-Leu-Pro-Ser-Leu-Lys-Ile-Trp-Glu-Thr-Cys-Lys-Glu-Leu-Leu-Gln-Leu-Ser-Lys-Pro-Glu-Leu-Pro-Gln-Asp-Gly-Thr-Ser-Thr-Leu-Arg-Glu-Asn-Ser-Lys-Pro-Glu-Glu-Ser-His-Leu-Leu-Ala-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Met-Glu-Pro-Glu-Glu-Glu-Ala-Asn-Gly-Ser-Glu-Ile-Leu-Ala-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Lys-Lys-Asp-Ala-Glu-Glu-Asp-Asp-Ser-Leu-Ala-Asn-Ser-Ser-Asp-Leu-Leu-Lys-Glu-Leu-Leu-Glu-Thr-Gly-Asp-Asn-Arg-Glu-Arg-Ser-His-His-Gln-Asp-Gly-Ser-Asp-Asn-Glu-Glu-Glu-Val-Ser-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu-Lys-Arg-Ser-Pro-Gln-Leu-Glu-Asp-Glu-Ala-Lys-Glu-Leu-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-Glu-Val-Pro-Glu-Met-Glu-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-Phe

PRODYNORPHIN (Porcine)

Met-Ala-Trp-Gln-Gly-Leu-Leu-Leu-Ala-Ala-Cys-Leu-Leu-Val-Leu-Pro-Ser-Thr-Met-Ala-Asp-Cys-Leu-Ser-Gly-Cys-Ser-Leu-Cys-Ala-Val-Lys-Thr-Gln-Asp-Gly-Pro-Lys-Pro-Ile-Asn-Pro-Leu-Ile-Cys-Ser-Leu-Glu-Cys-Gln-Ala-Ala-Leu-Gln-Pro-Ala-Glu-Glu-Trp-Glu-Arg-Cys-Gln-Gly-Leu-Leu-Ser-Phe-Leu-Ala-Pro-Leu-Ser-Leu-Gly-Leu-Glu-Gly-Lys-Glu-Asp-Leu-Glu-Ser-Lys-Ala-Ala-Leu-Glu-Glu-Pro-Ser-Ser-Glu-Leu-Val-Lys-Tyr-Met-Gly-Pro-Phe-Leu-Lys-Glu-Leu-Glu-Lys-Asn-Arg-Phe-Leu-Leu-Ser-Thr-Pro-Ala-Glu-Glu-Thr-Ser-Leu-Ser-Arg-Ser-Leu-Val-Glu-Lys-Leu-Arg-Ser-Leu-Pro-Gly-Arg-Leu-Gly-Glu-Glu-Thr-Glu-Ser-Glu-Leu-Met-Gly-Asp-Ala-Gln-Gln-Asp-GLy-Ala-Met-Glu-Ala-Ala-Ala-Ala-Leu-Asp-Ser-Ser-Val-Glu-Asp-Pro-Lys-Glu-Gln-Val-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-Arg-Ser-Ser-Glu-Val-Ala-Gly-Glu-Gly-Asp-Gly-Asp-Arg-Asp-Lys-Val-Gly-His-Glu-Asp-Leu-Tyr-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Gly-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val

Figure 1. continued

PROOPIOMELANOCORTIN (Bovine)

Met-Pro-Arg-Leu-Cys-Ser-Ser-Arg-Ser-Gly-Ala-Leu-Leu-Leu-Ala-Leu-Leu-Leu-
Gln-Ala-Ser-Met-Glu-Val-Arg-Gly-Trp-Cys-Leu-Glu-Ser-Ser-Gln-Cys-Gln-Asp-
Leu-Thr-Thr-Glu-Ser-Asn-Leu-Leu-Ala-Cys-Ile-Arg-Ala-Cys-Lys-Pro-Asp-Leu-
Ser-Ala-Glu-Thr-Pro-Val-Phe-Pro-Gly-Asn-Gly-Asp-Glu-Gln-Pro-Leu-Thr-Glu-
Asn-Pro-Arg-Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-
Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-Lys-Arg-Glu-Glu-Glu-
Val-Ala-Val-Gly-Glu-Gly-Pro-Gly-Pro-Arg-Gly-Asp-Asp-Ala-Glu-Thr-Gly-Pro-
Arg-Glu-Asp-Lys-Arg-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-
Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-
Ala-Gln-Ala-Phe-Pro-Leu-Glu-Phe-Lys-Arg-Glu-Leu-Thr-Gly-Glu-Arg-Leu-Glu-
Gln-Ala-Arg-Gly-Pro-Glu-Ala-Gln-Ala-Glu-Ser-Ala-Ala-Ala-Arg-Ala-Glu-Leu-
Glu-Tyr-Gly-Leu-Val-Ala-Glu-Ala-Glu-Ala-Glu-Ala-Ala-Glu-Lys-Lys-Asp-Ser-
Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-Lys-Arg-
<u>Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-
Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln</u>

BAM-12P Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu

BAM-22P Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-
Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly

Dynorphin$_{1-8}$ Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile

Dynorphin B Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr

α-Neo-Endorphin Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys

β-Neo-Endorphin Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro

Met-enkephalin Tyr-Gly-Gly-Phe-Met

Leu-enkephalin Tyr-Gly-Gly-Phe-Leu

β-Endorphin Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-
Thr-Leu-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln

phalin and Leu-enkephalin were the first endogenous opioid-like peptides to be isolated and sequenced (Hughes et al., 1975). It was observed that the sequence of Met-enkephalin is contained as residues 61-65 of the pituitary peptide β-lipotropin (β-LPH; Li et al., 1965). A fragment of β-LPH, β-endorphin, was later isolated and shown to have opioid-like activity (Li and Chung, 1976). Since the initial isolation and characterization of the enkephalins by Hughes and coworkers, a family of Met- and Leu-enkephalin-containing peptides in brain, pituitary and adrenal medulla have now been documented (Goldstein et al., 1979; Mizuno et al., 1980a; Mizuno et al., 1980b; Kangawa and Matsuo, 1979; Kangawa et al., 1981); the enkephalin sequence in these peptides is followed frequently by a pair of basic amino acid residues or by a single basic amino acid residue. With the advent of molecular genetic techniques, it has become possible to elucidate the structure of many of the precursor proteins of these opioid peptides, as well as other bioactive peptides. Using recombinant DNA technology, the amino acid sequences of three different precursors of opioid peptides have been determined. One of these precursors, proenkephalin A, contains four copies of Met-enkephalin and one copy each of Met-enkephalin-Arg^6-Phe^7, Met-enkephalin-Arg^6-Gly^7-Leu^8 and Leu-enkephalin (Coomb et al., 1982; Noda

et al., 1982). Another precursor, proopiomelanocortin (POMC), contains in its sequence β-endorphin and its fragments α- and γ-endorphin (Ling, 1977). It also contains several other bioactive fragments including adrenocorticotropic hormone (ACTH), β-melanocyte stimulating hormone (β-MSH; Geschwind et al., 1957) and βLPH (Nakanishi et al., 1979). The third and most recently characterized is the neoendorphin/dynorphin precursor (Kakidani et al., 1982), sometimes referred to as proenkephalin B. This precursor contains three copies of Leu-enkephalin included in the sequence of dynorphin (Goldstein et al., 1979), the neoendorphins (Kangawa and Matsuo, 1979; Kangawa et al., 1981), and in a peptide composed of the C-terminal 29 amino acid residues (residues 228-256) having in its sequence a peptide referred to as dynorphin B or rimorphin (Rossier, 1982). In addition to the opioid peptides, the amino acid sequence of several other neuropeptide precursors including somatostatin (Hobart et al., 1980) and vasopressin (Land et al., 1982) has been established.

It is apparent that release of bioactive peptides from precursors is dependent upon limited proteolysis of selected bonds. As first observed for proinsulin (Steiner, 1967) and later shown for other prohormones (Hobart et al., 1980; Land et al., 1982), most active peptide fragments contained

within larger precursors are frequently flanked on both the N-terminal and C-terminal sides by pairs of basic amino acid residues. This phenomenon also occurs in the opioid peptide precursors (Nakanishi et al., 1979; Coomb et al., 1982; Noda et al., 1982; Kakidani et al., 1982). It is widely regarded that peptide hormones and bioactive peptides, including the opioid peptides, are processed from their precursors by the combined action of a trypsin-like enzyme and a carboxypeptidase B-like enzyme (Steiner et al., 1980; Noda et al., 1982; Coomb et al., 1982). The conversion process is believed to proceed in two steps. Initially, a trypsin-like activity hydrolyzes bonds on the carboxyl side of basic amino acid residues; unlike trypsin, however, this processing activity must be highly specific. A carboxypeptidase B-like enzyme is then presumed to remove the exposed C-terminal basic residues. Such a process would be necessary for the generation of Leu-enkephalin from prodynorphin and both Met-enkephalin and Leu-enkephalin from proenkephalin A. The identification of the enzymes involved in these reactions are therefore an important objective. Several attempts have been made to purify the trypsin-like enzyme responsible for generating enkephalin molecules from precursors. A number of laboratories have reported partial purification of a trypsin-like enzyme from bovine adrenal chromaffin granules

(Troy and Musacchio, 1982; Evangelista et al., 1982; Lindberg et al., 1982; Lindberg et al., 1984). These reported enzyme activities cleave at the carboxyl side of basic amino acid residues. Lindberg and coworkers reported an enzyme which has a pH optimum of 8.0, is inhibited by DFP and has a molecular weight of about 20,000. The enzyme activity reported by Evangelista et al. (1982) has a pH optimun of 5.0 and is not inhibited by serine protease inhibitors. A pH optimum of 5.7 was obtained by Troy and Musacchio (1982) for what appears to be a sulfhydryl enzyme. Another study identified two enzyme activities, one soluble and one membrane-bound, from rat brains which specifically converts an enkephalin-containing substrate to enkephalin (Knight and Klee, 1979). The pH optimum of both these rat brain enzymes is about 7.0, with an intact sulfhydryl group appearing to be necessary for activity. All the enzymes described above exhibit low activity. Also, none of these studies have purified the activities to a sufficient degree to allow a comprehensive determination of specificity and biochemical properties.

Two carboxypeptidase B-like activities have been described (Hook et al., 1982; Fricker and Snyder, 1982). One of these activities designated as "enkephalin convertase" has been purified from the soluble and membrane frac-

tions of bovine pituitary homogenates and adrenal chromaffin graule membranes, with a molecular weight of about 52,000 (Supattapone et al., 1984). Like carboxypeptidase B, this enzyme appears to require a divalent metal for catalytic activity. It is capable of generating enkephalin from both Met- and Leu-enkephalin-Arg^6 and -Lys^6.

The amino acid sequences of some opioid peptides indicates that their formation is due to the action of enzymes other than those responsible for cleavage at pairs of basic amino acid residues. This is evident from the comparison of the amino acid sequence of the C-terminal portion of such opioid peptides as β-endorphin, dynorphin B, BAM-12P, and BAM-22P with their respective parent protein. Another unanswered question is whether Met- and Leu-enkephalin are processed directly from proenkephalin A and prodynorphin, or whether the oligopeptides dynorphins, neoendorphins and bovine adrenal medulla fragments (BAM-12P and BAM-22P), peptides which have intrinsic biological activity, are first generated from these precursors before being selectively processed to enkephalins by an as yet unidentified enzyme. If these peptides are indeed the precursors of Leu-enkephalin, then inhibition of their conversion should result in a decrease in Leu-enkephalin concentration and a corresponding increase in the dynorphin and neoendorphin levels. Some

recent evidence indicates that Leu-enkephalin production in rat substantia nigra is attributable to a dynorphin containing pathway (Zamir et al., 1984). The isolation and determination of such opioid peptides as dynorphins, neoendorphins and bovine adrenal medulla fragments from brain and pituitary, even though these peptides contain pairs of basic residues following enkephalin sequences, seems to indicate that processing of precursors is selective; not all regions of pairs of basic residues are subject to cleavage by the trypsin-like processing enzymes. In fact, examination of the concentrations of dynorphins and neoendorphins in various brain regions suggests that processing of prodynorphin is not uniform in all brain regions (Weber et al., 1982; Seizinger et al., 1984). An analogous situation appears to exist in the processing of POMC in brain (Zakarian and Smyth, 1982). These discrepencies in opioid peptide concentrations in brain point to nonuniformity in enzymatic processing and serves as the impetus to identify all the enzymes involved.

As with efforts to characterize enzymes responsible for formation of bioactive peptides, attempts have been made to identify enzymes involved in neuropeptide degradation. A number of peptidases capable of hydrolyzing neuropeptides have been described in the central nervous system, however,

it is not yet known which, if any, of these are responsible for the regulation of peptide levels at nerve terminals. Again, many of these studies suffer from the use of crude synaptosomal preparations.

Several of these neuropeptide degrading activities have been isolated and characterized. A cation-sensitive high molecular weight (M_r 700,000) multicatalytic protease complex has been isolated from the soluble fraction of bovine pituitary homogenates (Wilk and Orlowski, 1980); this complex has chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide bond hydrolyzing activities. In addition to generating Met-enkephalin from α-endorphin (Orlowski et al., 1980), a peptide containing the amino acid sequence 61-76 of βLPH, this complex is also capable of hydrolyzing bradykinin, neurotensin, LHRH, substance P and angiotensin II.

Another peptidase isolated from rabbit brain with a potential role in neuropeptide regulation is prolyl endopeptidase (Orlowski et al., 1979), also referred to as post-proline cleaving enzyme (Koida and Walter, 1976) or "Kininase B" (Carvalho and Camargo, 1981). This enzyme has been isolated from other tissues including lamb brain and kidney (Yoshimoto et al., 1981), bovine brain (Tate, 1981) and rat brain (Knisatschek and Bauer, 1979). Prolyl endopeptidase

cleaves on the carboxyl side of proline residues and hydrolyzes such peptides as TRH, LHRH, substance P, bradykinin and neurotensin. It is a serine protease, rapidly inhibited by DFP and PCMB, however, resistant to inhibition by phenylmethylsulfonyl fluoride. It is activated by DTT, indicating the presence of a sulfhydryl group. The reported molecular weight of the enzyme varies from 66,000 to 77,000 (for review, see Wilk, 1983).

A substance P-degrading activity, isolated from human brain membranes, cleaves the peptide at the Gln^6-Phe^7, Phe^7-Phe^8 and Phe^8-Gly^9 bonds (Lee et al., 1981). This activity has a pH optimum in the the neutral range. It is strongly inhibited by EDTA and o-phenathroline, but not by the thiol blocking agents iodoacetate, N-ethylmaleimide and PCMB, or by pepstatin or DFP. This neutral metalloendopeptidase has a molecular weight of about 40,000 to 50,000. While substance P is a preferred substrate, it also appears to hydrolyze somatostatin, LHRH, angiotensin I and bradykinin.

The opioid peptide Met-enkephalin is hydrolyzed at the Gly^3-Phe^4 bond by rat brain membranes (Graf et al., 1982). Two membrane-bound enzymes capable of hydrolyzing enkephalins at this locus are angiotensin-converting enzyme and an

enzyme originally designated as "enkephalinase" (Malfroy et al., 1978). Angiotensin-converting enzyme, a dipeptidyl carboxypeptidase, has been purified to homogeneity from lung (Das and Soffer, 1975). As mentioned previously, it has a broad specificity capable of hydrolyzing several bioactive peptides, notably angiotensin I and bradykinin. "Enkephalinase", an enzyme suggested to function in the degradation of enkephalins (Schwartz et al., 1981), has been shown to be identical to a membrane bound metalloendopeptidase (Almenoff et al., 1981) present in bovine pituitary (Orlowski and Wilk, 1981) and rabbit kidney (Kerr and Kenny, 1974; Almenoff and Orlowski, 1983). This enzyme has a molecular weight of about 95,000 and exhibits a thermolysin-like specificity (Matsubara et al., 1966), cleaving peptide bonds on the amino side of hydrophobic amino acid residues.

This thesis involves studies of a metalloendopeptidase (Mr 67,000) identified and isolated from the soluble protein fraction of rat brain homogenates and optimally active at a neutral pH. The enzyme, with high activity in brain and pituitary, cleaves several biologically active peptides into inactive fragments that have been identified *in vivo*, and has considerable activity towards synthetic substrates. Specificity studies with synthetic and natural substrates indicate the presence of an extended substrate binding site

with a preference towards peptides having hydrophobic residues in the P_1', or both the P_1 and P_2 position. Furthermore, a hydrophobic residue at the P_3' position, at some distance from the hydrolyzed bond, greatly contributes to the enzyme specificity. Among its other properties, this enzyme is able to generate Met- and Leu-enkephalin from several precursors, as well as being able to hydrolyze several other peptides including bradykinin, neurotensin, substance P and LHRH. Using information derived from specificity studies, a series of active site directed inhibitors were synthesized, and are expected to be used as probes in studies of the *in vivo* function of the enzyme.

MATERIALS

Hippuric acid, dithiothreitol, bradykinin, neurotensin, angiotensin I, angiotensin II, substance P, LHRH, o-pthalaldehyde, urethane, 1-hydroxybenzotriazole, heparin, trifluoroacetic acid, N-hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide, and tert-butoxycarbonyl derivatives of amino acids were obtained from Sigma Chemical Co. (St. Louis, MO). Dynorphin (1-8), α-Neo-Endorphin, β-Neo-Endorphin, BAM-12P, and BAM-22P were obtained from Peninsula Labs (Belmont, CA). p-Aminobenzoate was obtained from Aldrich Chemical Co. (Milwaukee, WI). Somatostatin was obtained from Bachem (Torrance, CA). PE-50 polyethylene tubing was obtained from Becton, Dickinson and Co. (Parsippany, NJ). Frozen rat brains were obtained from Pel Freeze Inc. (Rogers, AR). Z-Gly-Pro-SM was graciously provided by S. Wilk. N-[1(R,S)-carboxy-2-phenylethyl]-Phe-pAB was synthesized as described previously (Almenoff and Orlowski, 1983).

METHODS

I. DESIGN OF MODEL SUBSTRATES

Model synthetic peptides are frequently used as substrates for the rapid determination of activity of proteolytic enzymes. The use of such peptides is often preferred over larger protein substrates. Since synthetic substrates have well defined chemical structures, primary enzyme specificity can be determined from knowledge of the site of peptide bond hydrolysis in such peptides. Also, proteases have extended binding sites, whose interaction with peptide substrates is a multipoint cooperative effect. By changing the amino acid residues at discrete postions in the peptide chain and studying the effect of such changes on kinetic parameters (for example K_m and k_{cat}), an enhanced understanding of the primary and secondary binding characteristics of the enzyme can be obtained. In many cases this can lead to the design of a substrate whose amino acid sequence strictly conforms with the binding requirements of the

active site of a protease. Such substrates are often specific for the detection and determination of a single protease. With crude tissue preparations it is convenient to block the carboxyl and amino terminus of the peptide in order to eliminate the action of exopeptidases on the substrate.

With few exceptions (for example proteases involved with blood coagulation), proteins are unsuitable as substrates for kinetic studies due to the presence of multiple sites of cleavage. In assays using crude homogenates, such substrates are susceptible to the action of many enzymes. The peptide products of such a reaction are probably a constantly changing mixture of secondary substrates and inhibitors. In contrast, model peptide substrates can be designed to offer only one potential site of peptide bond cleavage, thereby simplifying the kinetics of hydrolysis.

Just as the defined size and structure of peptide substrates can be advantageous, these properties can also prove to be limiting when attempting to characterize proteinases; not all endopeptidases attack large proteins. The secondary and tertiary interactions in a large protein may limit the accessibility of potentially sensitive peptide bonds in the substrate to the active site of a proteinase, whose primary

specificity permits cleavage of such units. Model substrates, due to their limited size and conformational restrictions, may prove to be misleading when characterizing a proteinase. For initial purification, kinetic studies and active site characterization, however, small model substrates are superior to their larger protein counterparts. Once enzyme purification and characterization with peptides is achieved, the interaction of an enzyme with larger proteins can be explored.

II. SYNTHESIS OF SUBSTRATES AND IDENTIFICATION OF PEPTIDE PRODUCTS

Peptides were synthesized in solution by the stepwise addition of amino acid residues starting at the carboxyl terminus. Model substrates were blocked at both the amino and carboxyl terminus by the presence of benzoyl (Bz) and p-aminobenzoate (pAB) groups respectively. The pAB group within these substrates functioned as the chromogen. The presence of the free carboxyl group of pAB also enhanced the solubility of peptides at neutral pH . Peptides blocked at the C terminus by pAB offered greater solubility over analo-

gous peptides blocked by the chromogens 2-naphthylamine or p-nitroanaline.

Peptide synthesis was accomplished by the stepwise addition of amino acid residues using the N-hydroxysuccinimide ester method of amino acid coupling (Anderson et al., 1963). Tertiary butyloxycarbonyl (Boc) derivatives of amino acids were obtained commercially and their N-hydroxysuccinimide esters were prepared using the dicyclohexylcarbodiimide method of coupling (Anderson et al., 1963).

Initially, the C-terminal amino acid residue was coupled to pAB using the active ester method. The N-hydroxysuccinimide ester of the Boc derivative of the C-terminal amino acid, in a 5% molar excess, was reacted with pAB in tetrahydrofuran (THF). One molar equivalent of 1-hydroxybenzotriazole was used as a catalyst. The Boc-amino acid-pAB derivative could frequently be crystallized from chloroform after the removal of THF _in vacuo_. Deprotection of the N-terminus was accomplished by addition of a 20 to 30 fold molar excess of trifluoroacetic acid (TFA), followed by removal of the acid 10 to 15 min later _in vacuo_. The TFA salt of the amino acid arylamide was precipitated by the addition of ether; the salt was then washed with ether and dried. The TFA salt was dissolved in ethanol and the

free amino form of the amino acid arylamide was crystallized by addition of one equivalent of triethylamine. The amino acid-pAB fragment was then washed with ethanol and dried.

Elongation of the peptide was typically carried out using the following reaction scheme. A 5% molar excess of the N-hydroxysuccinimide ester of a Boc-amino acid was reacted with an amino acid or peptide arylamide of pAB in THF, or in a mixture of THF and dimethylformamide (DMF). One molar equivalent of triethylamine was added to the reaction if the TFA salt was used instead of the free amino form of the peptide. Using high pressure liquid chromatography (HPLC), the progress of the reaction was monitored by following the consumption of starting material and the appearance of a new product. Upon completion of the reaction, the solvent was removed _in vacuo_. The reaction mixture was then dissolved in chloroform, washed twice with water then $NaHSO_4$ (0.02 M; pH 2.6), and dried over anhydrous sodium sulfate. Chloroform was removed under reduced pressure and the product was reacted with a 20 to 30 fold molar excess of TFA for 10 to 15 min. After removal of TFA _in vacuo_, the corresponding acid salt of the peptide was precipitated by addition of ether. The TFA salt was dissolved in THF and the free amino form of the peptide arylamide was crystallized by addition of one equivalent of triethylamine. The peptide-pAB frag-

ment was washed with THF and dried.

This cycle of stepwise addition of single amino acid residues was repeated to synthesize desired peptides. Intermediate products (Boc-peptide-pAB) having more than four amino acid residues could usually be purified by crystallization from either chloroform or ethyl acetate. Typically, the last amino acid residue added to the peptide fragment was blocked at the N-terminus by a benzoyl group. This was done by reacting the appropriate peptide fragment with the N-hydroxysuccinimide ester of hippuric acid (Bz-Gly).

Purity of peptide substrates were verified by : (1) amino acid analysis, (2) HPLC, and (3) elemental analysis. For amino acid analysis, 0.1 uM of substrate was hydrolyzed in evacuated tubes with 6 N HCl at 105° for 24 h. HCl was then removed and the residue was dissolved in 0.1 M sodium citrate buffer (pH 2.0); samples were then subjected to the ninhydrin method of amino acid analysis (Moore and Stein, 1951) in a Technicon-TSM amino acid autoanalyzer with norleucine used as an internal standard.

HPLC analysis of peptides were performed on a Waters Associates liquid chromatograph equipped with a variable wavelength detector; peptides were chromatographed on a C_{18}

reverse phase uBondapak column (30 X 10 um) were eluted by running a linear gradient established between 0.1% H_3PO_4 and acetonitrile (CH_3CN). Initial concentration of CH_3CN was usually 10%, which was then increased to 60% over a period of 20 min, at a flow rate of 1.0 ml/min. Emerging peaks were monitored at 210 nm.

Analysis of carbon, nitrogen, and hydrogen content of the peptides were performed by either the microanalytical service of the Rockefeller University (N.Y., N.Y.) or the Schwartzkopf Microanalytical Laboratory (Woodside, N.Y.). Table 1 summarizes the analytical data for the peptides.

Table 1. Analytical Data on Synthetic Substrates used for Specificity Studies of the Enzyme

Peptide	MW	Calcd			Found		
		C	H	N	C	H	N
1. Bz-Gly-Ala-Ala-Phe-pAB	605.65	61.48	5.82	11.56	61.40	5.50	11.32
2. Bz-Gly-Ala-Ala-Leu-pAB	589.64	57.04	6.67	11.88	57.00	6.49	11.12
3. Bz-Gly-Ala-Ala-Gly-pAB	515.52	55.92	5.67	13.58	55.95	5.67	13.30
4. Bz-Gly-Phe-Ala-Phe-pAB	663.71	66.96	5.62	10.55	66.15	5.58	10.28
5. Bz-Gly-Gly-Ala-Phe-pAB	591.62	60.91	5.62	11.84	60.87	5.66	12.14
6. Boc-Phe-Ala-Ala-Phe-pAB	691.78	62.50	6.56	10.12	62.47	6.35	10.17
7. Bz-Gly-Phe-Ala-Ala-Phe-pAB	752.82	63.82	5.89	11.16	64.07	5.67	11.20
8. Bz-Gly-Phe-Ala-Pro-Phe-pAB	756.86				NOT DETERMINED		
9. Bz-Gly-Phe-Ser-Pro-Phe-pAB	772.86				NOT DETERMINED		
10. Bz-Gly-DPhe-Ala-Ala-Phe-pAB	734.81	65.38	5.76	11.44	64.93	5.68	11.33
11. Bz-Gly-Phe-DAla-Ala-Phe-pAB	752.82	63.82	5.89	11.44	63.86	5.75	11.19
12. Bz-Gly-Arg-Ala-Ala-Phe-pAB	821.89	56.99	6.25	15.34	56.71	6.26	15.82
13. Bz-Gly-Asp-Ala-Ala-Phe-pAB	738.75	56.90	5.73	11.38	56.79	5.73	11.22
14. Bz-Gly-Phe-Phe-Ala-Ala-Phe-pAB	900.00	65.39	5.94	10.89	65.13	5.81	10.84
15. Bz-Gly-Lys-Arg-Ala-Ala-Phe-pAB	992.10	56.90	6.60	15.53	57.38	6.61	15.95
16. Bz-Gly-Ala-Phe-pAB	534.57	62.91	5.66	10.48	62.05	5.98	9.65

Table 1. continued

Peptide	HPLC Retention Time	m.p.	Amino Acid Analysis			
			Gly	Ala	Phe	others
	min	°C				
1. Bz-Gly-Ala-Ala-Phe-pAB	18.1(a)	270 dec	1.07	1.97	1.00	-------
2. Bz-Gly-Ala-Ala-Leu-pAB	23.9(b)	215 dec	1.03	2.02	----	1.00 Leu
3. Bz-Gly-Ala-Ala-Gly-pAB	9.4(a)	268 dec	1.00	0.97	----	-------
4. Bz-Gly-Phe-Ala-Phe-pAB	15.5(a)	260-261	1.05	0.99	2.00	-------
5. Bz-Gly-Gly-Ala-Phe-pAB	13.4(a)	281 dec	1.95	1.03	1.00	-------
6. Boc-Phe-Ala-Ala-Phe-pAB	28.4(b)	210-212	----	2.10	2.00	-------
7. Bz-Gly-Phe-Ala-Ala-Phe-pAB	25.9(b)	248-250	1.00	1.99	2.00	-------
8. Bz-Gly-Phe-Ala-Pro-Phe-pAB	27.0(b)	165-167	0.88	0.82	2.00	0.96 Pro
9. Bz-Gly-Phe-Ser-Pro-Phe-pAB	26.6(b)	175-177	0.81	----	2.00	0.91 Pro 0.71 Ser
10. Bz-Gly-DPhe-Ala-Ala-Phe-pAB	25.9(b)	278 dec	0.94	2.0	2.00	-------
11. Bz-Gly-Phe-DAla-Ala-Phe-pAB	26.0(b)	238-240	0.94	1.87	2.00	-------
12. Bz-Gly-Arg-Ala-Ala-Phe-pAB	11.2(a)	225 dec	1.09	1.96	1.00	1.04 Arg
13. Bz-Gly-Asp-Ala-Ala-Phe-pAB	23.4(b)	201-203	1.06	1.94	1.00	0.97 Asp
14. Bz-Gly-Phe-Phe-Ala-Ala-Phe-pAB	29.4(b)	271 dec	0.97	2.07	2.90	-------
15. Bz-Gly-Lys-Arg-Ala-Ala-Phe-pAB	9.1(a)	228 dec	1.07	2.07	1.00	1.08 Arg 0.9 Lys
16. Bz-Gly-Ala-Phe-pAB	24.6(b)	199-201	1.04	1.00	1.00	-------

Waters of hydration are included in the molecular weights. HPLC was performed with either methanol (a) or acetonitrile (b) as the organic phase as described under Methods.

III. SYNTHESIS OF INHIBITORS

Inhibitors were synthesized by reductive amination of aldehydes or α-keto acids with amino acid or peptide amides of pAB. Purity of these peptide derivatives was verified by HPLC, amino acid analysis, elemental analysis, and measurement of pAB content after enzymatic release of chromogen by incubation of the inhibitors with excess chymotrypsin.

The purity of inhibitors was analyzed by HPLC on a Waters Associates liquid chromatograph equipped with a variable wavelength detector. Emerging peaks were monitored at 210 nm. Samples were injected on a C_{18} reverse phase uBondapak column (30 cm X 0.4 cm; 10 um); elution was carried out with a gradient established between 0.1% phosphoric acid in water and CH_3CN. The initial concentration of CH_3CN was 10%, and its concentration was increased linearly to 40% over a 40 min period at a flow rate of 1.0 ml/min (gradient system A). Under other gradient conditions, the initial concentration of CH_3CN was 5%, and its concentration was linearly increased to either 40% over a 60 min period (gradient system B) or to 35% over a 60 min period (gradient system C). Both gradient system B and C used a flow rate of 1.0 ml/min.

Inhibitors containing impurities (Table 12; compounds III and V) were also purified by HPLC, using the same Waters Associates apparatus except for replacement of the analytic column with a preparative C_{18} reverse phase uBondapak column (30 cm X 0.8 cm; 10um). A linear gradient between 0.1% TFA in water and CH_3CN was used. Gradient conditions were identical with those used to analyze the inhibitors. Elemental analysis of peptide derivatives was performed by the microanalytical service of Rockefeller University (NY,NY). Melting points are uncorrected.

N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (I). TFA-Ala-Ala-Phe-pAB (0.54 g; 1.0 mmol), synthesized as described under Synthesis of Substrates and Identification of Peptide Products, and sodium phenylpyruvate (1.86 g; 10.0 mmol) were dissolved in water (15 ml). The solution was adjusted to pH 6.5 to 7.0 with 1 M NaOH. 10 ml of a 0.8 M solution of sodium cyanoborohydride was then added over a period of 3 h using a syringe pump. After stirring for 24 h, the reaction mixture was acidified to about pH 2 with 1 M HCl; the resulting precipitate was filtered and washed with water. The remaining solid was dried and then suspended in ethyl acetate and stirred for 3 h. The insoluble white solid was filtered and washed extensively with ethyl acetate: HPLC, double peak with retention times of 35.8 and 37.2 min.

(gradient system A); Anal. Calcd for $C_{31}H_{34}O_7N_4 \cdot H_2O$: C, 62.83; H, 6.12; N, 9.45. Found: C, 62.94; H, 5.90; N, 9.26; mp, 187-188°.

The diastereomers of (I) were separated by HPLC; 50 ul aliquots of a 10 mM mixture of the diastereomers were injected on a C_{18} reverse phase uBondapak column (30 X 0.4 cm; 10 um); elution was carried out with a linear gradient established between 0.1% TFA in water and CH_3CN, as described above. The emerging peaks were monitored at 210 nm and separately collected. After removal of solvent under reduced pressure, the residue from each peak was dissolved in 1.0 ml of 0.2 M Tris-HCl buffer pH 7.0 and 0.01 ml of 1.0 M NaOH. Upon reinjection on HPLC, each dissolved fraction eluted as a single peak indicating resolution of the diastereomers. The concentration of the inhibitor solution was determined by subjecting aliquots to chymotrypsin digestion and quantitating the amount of chromogen (pAB) released. 0.025 ml of an inhibitor solution, 0.175 ml of Tris-HCl buffer (0.1 M; pH 7.8) and 0.05 ml of chymotrypsin (400 units/ml: one unit being defined as the amount of enzyme required to hydrolyze 1 umol Bz-Tyr-OMe/min) were incubated for 2 h at 37°. After addition of 0.25 ml of 10% TCA, the amount of pAB released was quantified as described in "Determination of Enzyme Activity". Chymotrypsin digestion

resulted in total release of pAB, as followed by HPLC.

N-[1(R,S)-carboxybutyl]-Ala-Ala-Phe-pAB (II). (II) was prepared by reductive amination of α-ketovaleric acid with Ala-Ala-Phe-pAB as described for (I). HPLC of the product gave a double peak with retention times of 31.4 and 31.8 min. (gradient system A); Anal. Calcd. for $C_{27}H_{34}O_7N_4$ 0.5 H_2O : C, 60.55; H, 6.59; N, 10.46. Found: C, 60.31; H, 6.41; N, 10.26; mp, 218-219°.

N-[1(R,S)-carboxyethyl]-Ala-Ala-Phe-pAB (III). (III) was prepared by reductive amination of sodium pyruvate with Ala-Ala-Phe-pAB as described for (I). Following completion of the reaction, the solution was acidified to pH 3 by the addition of 1 M HCl and then applied to a 100 ml column of Dowex 50 (H^+, 100-200 mesh) previously equilibrated with a 0.1 M pyridine-formate buffer, pH 3.1. Removal of starting material was accomplished by washing the column with the equilibrating buffer. The product was eluted by washing the column with 2% pyridine. A white solid was obtained upon removal of solvent under reduced pressure. The compound was suspended in isopropanol, then filtered and washed with the same solvent: HPLC exhibited an asymmetric peak indicating the presence of two unresolved diastereomers. The retention time was 53.6 min. (gradient system C); Anal. Calcd for $C_{25}H_{30}O_7N_4 \cdot H_2O$: C, 58.13; H, 6.24. Found: C, 58.02; H,

5.89; mp, 290-295° dec.

N-[1-carboxymethyl]-Ala-Ala-Phe-pAB (IV). (IV) was prepared by reductive amination of glyoxylic acid with Ala-Ala-Phe-pAB as described for (I). The product was then isolated as in (III); HPLC, single peak with a retention time of 27.8 min. (gradient system A); Anal. Calcd for $C_{24}H_{28}O_7N_4$ H_2O : C, 57.36; H, 6.02; N, 11.15. Found: C, 57.02; H, 5.94; N, 11.09; mp, 176-178°.

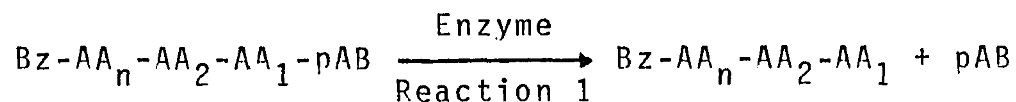
N-[1-phenylethyl]-Ala-Ala-Phe-pAB (V). Ala-Ala-Phe-pAB (0.434 g; 1.0 mmol) and 20 ml of phenylacetaldehyde (1:1 in isopropanol; 100 mmol) were dissolved in 15 ml of methanol:water (1:1). The pH was adjusted to 7.0 with 1 M NaOH. 10 ml of a 1.0 M solution of sodium cyanoborohydride was then added over a 3 h period using a syringe pump. After stirring for 24 h, the reaction mixture was transferred to a separatory funnel and washed twice with ethyl ether. The aqueous layer was collected and excess ether was removed under reduced pressure. The reaction mixture was then acidified to pH 3 with 1 M HCl; the resulting precipitate was filtered and extensively washed with water. The remaining solid was dried before suspension in ethyl acetate for 3 h. The insoluble white solid was filtered and thoroughly washed with ethyl acetate: HPLC, single peak with a retention time of 39.6 min. (gradient system A); Anal. Calcd. for

$C_{30}H_{34}O_7N_4$ 0.5 H_2O: C, 66.77; H,6.54. Found: C, 66.87; H, 6.47; mp, 198-200°.

N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Phe-pAB (VI). (VI) was prepared by reductive amination of sodium pyruvate with Ala-Phe-pAB as described for (I). The product was isolated as in (I) and recrystallized from ethyl acetate as the TFA salt: HPLC exhibited an asymmetric peak indicating the presence of two unresolved diastereomers. The retention time was 64.0 min. (gradient system B); Anal. Calcd. for $C_{28}H_{29}O_6N_3$·TFA: C, 58.35; H, 4.9. Found: C, 58.70; H, 5.10; mp, 191-192°.

IV. DETERMINATION OF ENZYME ACTIVITY

A general scheme for the detection of proteolytic activity is as follows:

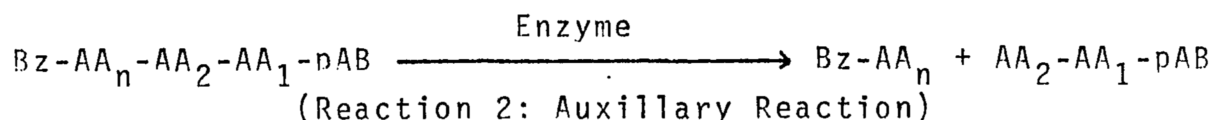


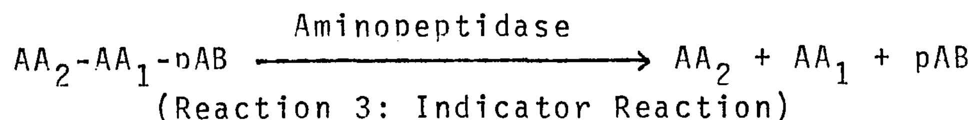
$$\text{Bz-AA}_n\text{-AA}_2\text{-AA}_1\text{-pAB} \xrightarrow[\text{Reaction 1}]{\text{Enzyme}} \text{Bz-AA}_n\text{-AA}_2\text{-AA}_1 + \text{pAB}$$

where AA represents an amino acid residue and n represents the nth amino acid residue of the substrate. In this reaction, an enzyme that cleaves the bond between the first

amino acid residue and pAB will release the chromogenic group directly. pAB can then be quantified by a diazotization procedure and conversion to a diazo dye.

An enzyme that attacks a peptide bond other than the AA -pAB bond can be determined in a coupled reaction. For example, an enzyme that cleaves the AA_n-AA_2 bond of a substrate can be determined as follows:

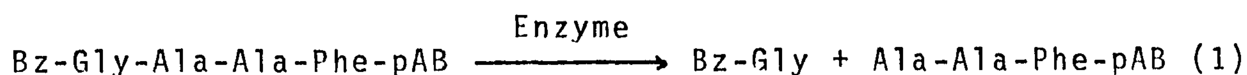


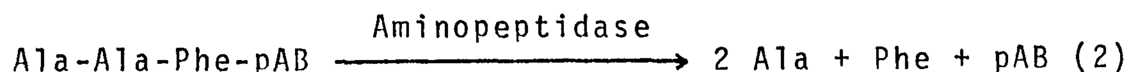


In the above scheme, the enzymatic hydrolysis of a peptide bond in the substrate results in the appearance of a free amino terminus. Aminopeptidase M, included in the incubation mixture, will then catalyze the release of pAB (Indicator Reaction). Since this is a coupled reaction, reaction 2 must be rate limiting to be correctly determined within the assay conditions. A large catalytic excess of aminopepti-

dase, therefore, must be present in the incubation mixture. A two stage reaction for the measurement of proteolytic activity can be used in those cases where more than one amino acid residue must be cleaved off by aminopeptidase before release of the chromogen, pAB. Enzyme is first incubated with substrate, resulting in the hydrolysis of a peptide bond and the appearance of a free amino terminus (Reaction 2). The reaction is stopped by placement of the incubation mixture in boiling water for 2 min. Aminopeptidase M is then added to the incubation mixture and Reaction 3 is allowed to procede to completion. This two stage reaction scheme enables the conservation of aminopeptidase and insures that the rate of reaction 2 is accurately determined, irrespective of the catalytic activity of the coupling enzyme.

The activity of the soluble metalloendopeptidase was assayed using Bz-Gly-Ala-Ala-Phe-pAB as substrate in a two stage reaction as follows:





In the first stage, the enzyme is incubated with substrate, resulting in hydrolysis of the Gly-Ala bond. The reaction is then terminated by placement of the incubation mixture in boiling water for 2 min. After cooling in ice and addition of the aminopeptidase, reaction (2) is allowed to proceed to completion at 37°. The amount of pAB released is determined by a diazotization procedure.

Incubation mixtures contained substrate (0.5 to 1.0 mM), dithiothreitol (0.25 mM), enzyme (5 to 25 ul), and Tris-HCl buffer (0.2 M; pH 7.0) in a final volume of 0.2 ml. Incubations were carried out at 37° for 15 to 60 min, and terminated by placement of reaction tubes in boiling water for 2 min, followed by cooling in ice. Aminopeptidase M (10 ug) and Tris-HCl buffer (0.2 M; pH 7.0) were added to a final volume of 0.25 ml. The tubes were then incubated for 120 min at 37°. This time interval was sufficient to catalyze the total release of pAB. The amount of pAB released was determined by a modification (Goldbarg and Rutenberg, 1958) of the diazotization procedure described by Bratton and Marshall (1939). The absorbance of the samples was determined at 555 nm and the amount of pAB present was calculated from a standard curve derived from known amounts of pAB. Controls in which the enzyme or substrate were separately omitted were carried through the procedure. In experiments with

inhibitors, various inhibitor concentrations were included in the incubation mixture and the reaction was initiated by addition of enzyme. In other experiments, varying amounts of inhibitor were preincubated with the enzyme for 10 min at 37° and the reaction was initiated by the addition of substrate. In experiments on the distribution of enzyme activity in rat tissue and rat brain, activity was determined in the presence of N-[1(R,S)-carboxy-2-phenylethyl]-Phe-pAB, an inhibitor of membrane-bound metalloendopeptidase (Almenoff and Orlowski, 1983), and in the presence of the above inhibitor and N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB, a specific inhibitor of the soluble metalloendopeptidase (discussed later in thesis). A membrane-bound metalloendopeptidase inhibitor was included in the incubations to prevent hydrolysis of the synthetic substrate used to determine enzyme activity, Bz-Gly-Ala-Ala-Phe-pAB, as well as to prevent hydrolysis of the soluble enzyme inhibitor. The difference in activity between these two incubations was taken as a measure of specific soluble metalloendopeptidase activity.

In all experiments, hydrolysis was linear with time for up to 60 min. Less than 10% of the total substrate was hydrolyzed in all experiments. Enzyme activity is expressed in units, one unit being equal to the amount of enzyme

required to release one umol product/h, under the conditions described above. Specific activity is in units/mg protein. Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

V. PURIFICATION OF THE ENZYME

All procedures were carried out at 4° C. Buffers were prepared in deionized water. Frozen rat brains were defrosted and homogenized with 4 volumes of an ice-cold solution of 0.32 M sucrose in 0.01 M Tris-HCl buffer, pH 7.6 (step 1, Table 2). An ice-cooled Potter-Elvehjem homogenizer with motor-driven Teflon pestle was used. The homogenate was centrifuged for 60 min at 33,000 X g and the supernatant was collected (step 2). The pH of the supernatant was adjusted to pH 5.0 by dropwise addition of 1 M acetic acid. The suspension was centrifuged and the precipitate was discarded. The supernatant was readjusted to pH 7.6 by the dropwise addition of a 1 M solution of Tris base (step 3) before being applied to the top of a DEAE-cellulose column (DE-52; 1.5 X 17.5 cm) equilibrated with a 0.01 M Tris-HCl buffer (pH 7.6) containing 0.5 mM 2-mercaptoethanol.

The column was washed with 100 ml of the same buffer and then eluted with a linear gradient established between 150 ml of 0.01 M Tris-HCl buffer (pH 7.6) containing 0.5 mM 2-mercaptoethanol and 150 ml of the same buffer containing 0.3 M NaCl. Fractions of 3 ml were collected. The concentration of protein was monitored by measuring the absorbance at 280 nm and the activity of the enzyme was determined as described above. The enzyme emerged as a single peak of activity after about 180 ml of the eluting buffer had passed through the column (step 4, Figure 1). Active fractions were pooled and concentrated by ultrafiltration (Amicon) to a volume of about 3 ml before being applied to the top of a Sephadex G-100 column (2.5 X 60 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. The column was eluted with the same buffer at a flow rate of about 20 ml/h and fractions of about 4 ml were collected and monitored for protein and enzyme activity (step 5, Figure 2). The enzyme emerged from the column as a single peak of activity coinciding with a distinct protein peak. Active fractions were pooled and concentrated to a volume of about 7 ml. A saturated ammonium sulfate solution was added to achieve a final saturation with respect to ammonium sulfate of 20%. The enzyme was applied to a phenyl-Sepharose CL4B column (0.9 X 7.0 cm) equilibrated with 30 ml of 0.05 M Tris-HCl buffer (pH 8.0)

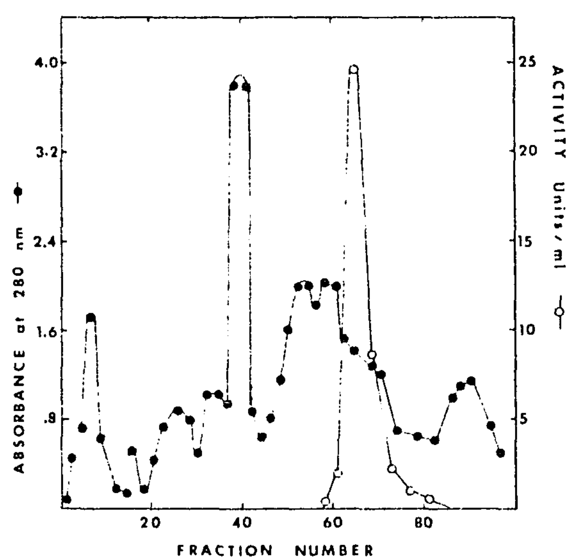


Figure 2. Chromatography of the Enzyme on DEAE-cellulose. This is step 4 of the purification procedure.

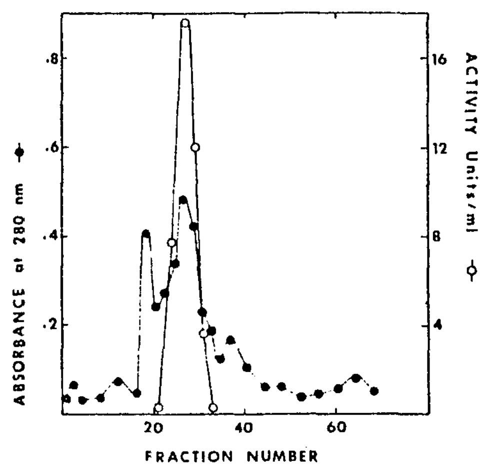


Figure 3. Chromatography of the Enzyme on Sephadex G-100. This is step 5 of the purification procedure.

containing 20% ammonium sulfate by saturation; the column was washed with 30 ml of the same buffer. Enzyme elution was carried out with a linear gradient established between 100 ml of 0.05 M Tris-HCl buffer (pH 8.0) containing ammonium sulfate (20% by saturation), and 100 ml of 0.05 M Tris-HCl buffer of the same pH. Fractions of about 3 ml were collected, examined for protein by absorbance at 280 nm, and assayed for activity (step 6). The enzyme emerged from the column as a single peak of activity in Fractions 60-78. Fractions containing the highest activity were pooled and used in subsequent studies.

VI. SUBSTRATE CLEAVAGE SITE DETERMINATION

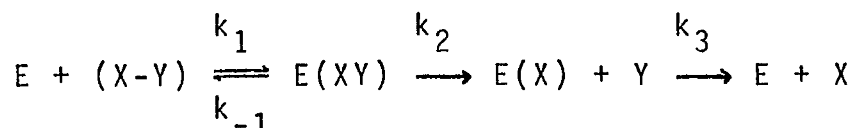
The site of cleavage of the peptide bond in bioactive and synthetic substrates was determined by a combination of HPLC and amino acid analysis. Since the synthetic and bioactive substrates studied are small oligopeptides, it is easy to deduce the site of cleavage from the amino acid content of the reaction products. 0.1 umole of substrate was incubated at 37° with enzyme, 0.4 mM DTT and Tris-HCl buffer (0.2 M; pH 7.0) in a final volume of 0.25 ml. The reaction was fol-

lowed by withdrawing at various time intervals 10 ul aliquots of the reaction mixture, which were then subjected to HPLC. After the reaction had proceeded to about 50% completion, the entire incubation mixture was chromatographed, and the products and starting material were collected. After removal of solvent, the residues were hydrolyzed in 6 N HCl for 24 h at 105° and their amino acid content determined. The conditions for HPLC and amino acid analysis are described under "Synthesis of Substrates". Studies on the site of hydrolysis of peptides containing enkephalin sequences were conducted using only micromolar amounts of substrates; therefore, a more sensitive method of amino acid analysis was required. Hydrolysates containing picomoles of free amino acid were analyzed fluorometrically by reaction of amino acids with o-phthalaldehyde (OPA). OPA-amino acids were separated by reverse phase HPLC. Excess HCl was removed from the hydrolysis tubes under reduced pressure. The amino acids were then dissolved in 0.5 M sodium borate buffer, pH 10.4. OPA derivatizing agent (5 mg OPA in 0.4 ml methanol, 0.025 ml 2-mercaptoethanol and 0.575 ml of 0.5 M sodium borate buffer, pH 10.4) was added in a two-fold excess to amino acid hydrolysates and allowed to react for 1 min. The reaction mixtures were then injected into a Waters Associates liquid chromatograph equipped with a 5 u spheri-

cal C_{18} column (RESOLVE; 0.4 X 15 cm). Elution of OPA-amino acids was accomplished by a linear gradient established between (A) 0.05 M NaAc, 0.05 M Na_2HPO_4 pH 7.0, 2% methanol, 2% THF and (B) 65:35 methanol:H_2O. Initial concentration of (B) was 0% and was increased to 100% over a 40 min period at a flow rate of 1.5 ml/min. Fluorescence was monitored on a Waters Associates Fluorescence Detector equipped with a 338 nm excitation filter and a 425 nm long pass emission filter.

VII. KINETIC CONSIDERATIONS

A minimum scheme for the action of a protease, designated E, on an oligopeptide substrate, designated X-Y, may be written as follows:



$$E + (X\text{-}Y) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(XY) \xrightarrow{k_2} E(X) + Y \xrightarrow{k_3} E + X$$

The bond between X and Y is hydrolyzed and the products derived from X and Y are released sequentially. For example, in the interaction of chymotrypsin with ester substrates, E(X) represents an acyl enzyme intermediate. Two kinetic parameters are frequently determined in the study of

enzymes, k_{cat} and K_m. K_m represents the substrate concentration at half maximal initial velocity and is frequently cited as a measure of binding affinity; k_{cat}, or turnover rate constant, is a measure of catalytic efficiency and represents the maximal initial velocity per unit enzyme concentration (V_{max}/e). Where $k_{-1} >> k_2$, K_m and k_{cat} are related to the various rate constants of equation 1 as follows:

$$K_m = \frac{K_s k_3}{k_2 + k_3} \qquad k_{cat} = \frac{k_2 k_3}{k_2 + k_3}$$

where K_s is the dissociation constant of the enzyme substrate complex (k_{-1}/k_1). When k_2 is rate limiting ($k_3 >> k_2$), K_m is equal to K_s and k_{cat} is equal to k_2. Under these conditions, the dissociation constant of the enzyme-substrate complex can be regarded as a measure of the binding specificity of the substrate. When the release of X from the enzyme is rate limiting ($k_2 >> k_3$), k_{cat} is equal to k_3.

Comparisons of k_{cat} and K_m values for different substrates may give misleading information in those cases in which alternate modes of enzyme-substrate interaction exist; a limiting case is the one in which a peptide substrate has been bound at the active site in such a manner that the catalytic groups are positioned near a peptide bond resistant to such an enzymatic attack. This nonproductive mode of

binding influences specificity studies when the dissociation constant of the nonproductive complex is much lower (of higher affinity) than for the productive mode. K_m and k_{cat} values are both changed by an equivalent factor in such a case; therefore, the ratio k_{cat}/K_m is independent of nonproductive binding. This ratio is the preferred parameter when characterizing the specificity of proteases with several substrates. (Bender and Kezdy, 1965).

VIII. KINETIC STUDIES

The steady state parameters, K_m and k_{cat}, were determined for the model synthetic substrates from initial velocity measurements at various substrate concentrations. Each value represented an average of at least four separate kinetic measurements. Data were analyzed either by double reciprocal plots obtained by linear regression analysis, or by a calculator linear regression program utilizing an Eadie-Hofstee transformation (Zivin and Waud, 1982). For the double reciprocal plots, correlation coefficients of 0.99 or better were obtained for the data utilized; the coefficient of variation of the data used in the calculator

program was typically less than 8%. In the calculation of k_{cat}, a molecular weight of 67,000 was used. The specific activity of the enzyme was taken as 172 units/mg, using Bz-Gly-Ala-Ala-Phe-pAB as substrate at a concentration of 1.0 mM.

Kinetic analyses of bioactive peptides were performed by measuring the rate of disappearance of substrate and the rate of appearance of product by HPLC. Various initial concentrations of bioactive peptides were incubated with enzyme and aliquots were withdrawn after 3 to 10 min. These samples were subjected to HPLC on a Waters Associates liquid chromatograph equipped with a variable wavelength detector. Emerging peaks were monitored at 210 nm. Samples were injected on a C_{18} reverse phase uBondapak column (30 cm X 0.4 cm; 10 um); elution was carried out with a gradient established between 0.1% phosphoric acid in water and CH_3CN. The initial concentration of CH_3CN was 10%, and its concentration was increased linearly to 45% over a 30 min period at a flow rate of 1.0 ml/min.

Since substrate consumption was greater that 5%, velocity measurements were not at initial conditions; therefore, the steady state parameters K_m and k_{cat} (=V/e where e is equal to total enzyme concentration) were derived by an averaging

method (Lee and Wilson, 1971). Velocity measurements at substrate concentrations determined by taking an arithmetic mean of the initial and final substrate concentration were used in the calculator linear regression program utilizing an Eadie-Hofstee data transformation (Zivin and Waud, 1982). The coefficient of variation of the data was typically less than 8%. Again in the calculations, the molecular weight of the enzyme was taken as 67,000.

The type of inhibition for all N-carboxymethyl-peptides was determined from double reciprocal plots obtained in the presence and absence of inhibitor. K_i values for all synthetic inhibitors were determined by the method of Dixon (1953; plot 1/v vs. [I] at several different substrate concentrations).

The inhibitory constants (K_i values) of bioactive peptides were determined by several methods. The K_i values of bradykinin, neurotensin, angiotensin I and angiotensin II were determined from Dixon plots obtained at two different concentrations using Bz-Gly-Ala-Ala-Phe-pAB as substrate. At each concentration of substrate, rates were determined with five diffent peptide concentrations. For the enkephalin containing peptides, K_i values were determined from a single concentration of peptide, using the equation:

$$\frac{v_i}{v_0} = \frac{K_m + S}{K_m(1 + I/K_i) + S}$$

where v_i and v_0 were initial velocities in the presence and absence of peptide. Bz-Gly-Phe-Ser-Pro-Phe-pAB was used as substrate. The inhibitory constants of LHRH and substance P were calculated from IC_{50} values, using Bz-Gly-Phe-Ser-Pro-Phe-pAB as substrate. K_i values were calculated from the equation :

$$IC_{50} = (1 + S/K_m)K_i$$

IX. POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis under nondissociating conditions was performed as described by Davis and Ornstein (Ornstein, 1964; Davis 1964) on eight cm polyacrylamide gels (8%) in 0.05 M Tris-HCl buffer, pH 8.3. Enzyme samples containing 3 to 10 ug of protein were layered on top of the gels and a current of four milliamps per tube was applied. Electrophoresis was continued until a tracking dye included with the sample (Bromophenol Blue) reached the base of the gel. A 0.05% solution of Coomassie Brilliant Blue in 10% acetic acid - 25% isopropanol was used to stain for protein.

For enzyme localization within gels, matched pairs of gels were run. While one of the gels was stained for protein, the other was sliced into two mm segments by a gel slicer. The segments were homogenized in 0.2 M Tris-HCl buffer, pH 7.0, using small glass homogenizers equipped with a Teflon pestle. Aliquots of the gel suspension were then used to determine enzyme activity, as described above.

Electrophoresis under dissociating conditions was run under identical conditions to those described above, in gels containing 0.1% $NaDodSO_4$. Enzyme samples were pretreated with 1.0% $NaDodSO_4$ and 1.0% 2-mercaptoethanol at 60° for 15 min before layering on gels.

X. MOLECULAR WEIGHT DETERMINATION

Molecular weight was determined by both gel electrophoresis and gel filtration. Enzyme molecular weight determination, using gel electrophoresis, was carried out in 8% polyacrylamide gels under dissociating conditions. The mobility of the enzyme relative to that of standard marker proteins of known molecular weight (Weber and Osborn, 1969) was used to calculate the molecular weight. Molecular

weight of the enzyme was also determined by gel filtration on calibrated Sephadex G-100 columns with bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome C as marker proteins (Andrews, 1965).

XI. ANIMAL STUDIES

Animal studies were performed on male Sprague-Dawley rats weighing 225-300 g. The rats were anesthetized with urethane (1.2 g/kg) injected intraperitoneally. Arterial and venous cannules, using PE-50 polyethylene tubing, were introduced into the femoral artery and vein, respectively. The arterial line contained heparinized normal saline (10 Units heparin/ml saline); the venous line contained normal saline. The rats were allowed to stabilize for 30 min postoperatively before further manipulation. The animal temperature was maintained at 37° by using a heat lamp equipped with a rectal probe.

For experiments involving blood sugar measurements, the rats were injected into the venous line with a 10 mM solution of N-{1(R,S)-carboxy-2-phenylethyl}-Ala-Ala-Phe-pAB to reach a final concentration of 40 umol/kg. 5 min after

injection of this inhibitor, neurotensin (3 nmol/kg) was injected into the venous line. Arterial blood samples were withdrawn at various times and immediately centrifuged in a Beckman desktop centrifuge at 9000 X g for 5 min. The plasma was then collected and frozen at -20° until further use. Plasma glucose was determined by a glucose oxidase and peroxidase method (Raabo and Terkildsen, 1960), using a Sigma kit.

RESULTS

I. PURIFICATION OF THE ENZYME

A representative summary of the enzyme purification is given in Table 2. A large proportion (45%) of activity towards Bz-Gly-Ala-Ala-Phe-pAB was associated with the particulate fraction of homogenates of rat brains. As shown in Table 3, about 40% of the activity in the particulate fraction of homogenates is similar to the predominant (78%) activity in the supernatant; about 20% of the metalloendopeptidase activity in homogenates is found in the pellet. Most of the remaining activity in the particulate fraction can be attributed to a membrane-bound metalloendopeptidase similar to that purified by Orlowski and coworkers (Orlowski and Wilk, 1981; Almenoff et al., 1983) from the membrane fractions of bovine pituitary and rabbit kidney. This membrane-bound enzyme cleaves Bz-Gly-Ala-Ala-Phe-pAB between the Ala-Phe bond as opposed to the soluble enzyme which cleaves between the Gly-Ala bond. During DEAE-cellulose and Sepha-

Table 2. Summary of Purification of the Enzyme from Rat Brain

Purification Step	Vol (ml)	Protein (mg/ml)	Activity conc (units/ml)	Activity total (units)	Spec Act (units/mg)	Recovery (%)	Purification (-fold)
1. Homogenate	112.0	24.0	14	1570	0.58	100	1
2. Supernatant	87	7.1	10	870	1.41	55	2.4
3. pH 5 Supernatant	82	2.93	8.4	689	2.87	44	5.9
4. DEAE-cellulose chromatography	37	0.76	9.85	364	13.0	23	22.4
5. Sephadex G-100 chromatography	29	0.39	14.0	406	35.9	26	62.0
6. Phenyl-sepharose chromatography	14.5	0.056	9.62	139	172.0	9	296.0

The enzyme was isolated from 22.5 g of brain tissue. Activity was determined with Bz-Gly-Ala-Ala-Phe-pAB as substrate. One unit of activity is defined as the amount of enzyme that liberates 1 umol of product/h.

Table 3. Distribution of Soluble Metalloendopeptidase Activity Between the Supernatant and Particulate Fraction of Rat Brain Homogenates

Fraction	Vol (ml)	Activity (umol/ml/h) Total	Activity (umol/ml/h) SMEP	Protein (mg/ml)	Spec Act (umol/mg) Total	Spec Act (umol/mg) SMEP	Units (umol/h) Total	Units (umol/h) SMEP	%SMEP Act in Fract	% Total SMEP Act
1. Homogenate	10.0	8.319	4.841	26.54	0.313	0.182	83.19	48.41	52	100
2. Supernatant	8.85	5.429	4.254	8.49	0.639	0.501	46.05	37.65	78	78
3. Wash	8.85	0.696	0.432	1.33	0.523	0.325	6.16	3.82	62	8
4. Pellet	10.0	2.576	1.072	15.68	0.164	0.068	25.76	10.71	42	22

The homogenate and supernatant fractions were obtained as in Step 1 and 2 of the purification procedure. After removing the supernatant, the pellet was washed once with an equivalent volume of homogenizing buffer (0.2 M Tris-HCl, pH 7.6, 0.32 M sucrose). The pellet was resuspended in another equivalent volume of this buffer. Enzyme activity was determined with Bz-Gly-Ala-Ala-Phe-pAB as substrate. Specific soluble metalloendopeptidase activity was determined as described in "Determination of Enzyme Activity".

dex G-100 chromatography, the enzyme emerged as a single peak of activity. An overall purification of about 300 fold was achieved.

Enzyme obtained after chromatography on Phenyl Sepharose CL4B was subjected to polyacrylamide gel electrophoresis. A minor slower moving component comprizing 10-20% of the total protein, and a major protein component constituting the remainder was found. Analysis of the enzyme activity within unstained gels sliced into 2 mm segments, in comparison with duplicate protein stained gels, revealed that the slower moving component was responsible for enzyme activity. The major protein band nad no enzymatic activity. Attempts were made to further purify this enzyme using the following techniques: DEAE-cellulose and carboxymethyl-cellulose chromatography, chromatography on hydroxyapatite columns, preparative gel electrophoresis, chromatofocusing, affinity chromatography using Blue Sepharose or Concanavelin A, and affinity chromatography using a specific inhibitor of the enzyme, N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (discussed later in thesis), as a ligand. All these attempts, however, were unsuccessful. The enzyme was found to be free of aminopeptidase activity, cathepsin B and prolyl endopeptidase (Orlowski et al, 1979).

II. MOLECULAR WEIGHT DETERMINATION

A molecular weight of 67,000 was found in experiments with gel filtration on calibrated Sephadex G-100 columns. A similar molecular weight was determined from polyacrylamide gel electrophoresis under dissociating conditions, using albumin, ovalbumin, chymotrypsinogen and cytochrome C as marker proteins. The single protein band found under dissociating conditions suggests a single polypeptide chain with a molecular weight of 67,000.

III. PH OPTIMUM

The pH optimum was determined in 0.2 M Tris-HCl, Hepes, imidazole and potassium phosphate buffers in the pH range of 6.0 to 8.8. In each of the buffers, activity was maximal at pH 7.0; however, the nature of the buffer greatly influenced the degree of activity. At pH 7.0, the activity in phosphate, Hepes and imidazole buffers was respectively 84%, 48% and 14% of that in Tris-HCl buffer. The low activity in imidazole buffer can be attributed to its metal-chelating

properties.

IV. EFFECT OF PROTEASE INHIBITORS

The effect of various protease inhibitors on soluble metalloendopeptidase activity is summarized in Table 4. No inhibition of enzyme activity was observed with leupeptin, pepstatin, antipain or chymostatin, inhibitors of bacterial origin of several serine, thiol and carboxy proteases. Similarly, the serine protease inhibitor DFP had no effect on activity. A weak and variable degree of inhibition was observed with relatively high concentrations of thiol blocking agents. Dithiothreitol, a reducing agent, activated the enzyme at concentrations below 1.5 mM; however, at higher concentrations it had a pronounced inhibitory effect. Metal chelators such as EDTA, EGTA and o-phenanthroline consistently inhibited the enzyme. On the basis of these findings, it was concluded that the enzyme could be classified as a metalloendopeptidase. Accordingly, while the activating effect of DTT at low concentrations may be due to reduction of a thiol group important for activity, the inhibition at higher concentrations could result from the metal coordi-

Table 4. Effect of Protease Inhibitors on Enzyme Activity

Inhibitor	Final Conc (mM)	Inhibition (%)
Leupeptin	0.022	0
Pepstatin	0.168	0
Antipain	0.013	0
Chymostatin	0.013	0
DFP	0.10	0
PCMB	0.20	33
Iodoacetamide	1.00	14
N-ethylmaleimide	1.00	45
Iodoacetic Acid	1.00	6
EDTA	1.00	64
EGTA	1.00	62
o-phenanthroline	0.042	23
	0.166	82
	0.25	93

Activity was determined with Bz-Gly-Ala-Ala-Phe-pAB as substrate in Tris-HCl buffer (0.2 M; pH 7.0). The enzyme was incubated with the inhibitor for 12 min at 37° before addition of substrate. Controls in which the enzyme was preincubated in the absence of inhibitor were included in each experiment. Data are mean values of two to four determinations.

nating properties of this thiol.

V. REACTIVATION OF ENZYME ACTIVITY BY METAL IONS

Further evidence that the enzyme is a metalloendopeptidase was obtained from experiments in which enzyme dialysis against EDTA (1.0 mM), followed by removal of the chelating agent by dialysis against 1.0 mM Tris-HCl buffer, pH 7.0, resulted in almost complete inactivation of the enzyme (Table 5). Full reactivation of enzyme activity was obtained with several different metal ions. Reactivation with Zn^{2+} occurred at quite low concentrations. By analogy with other metalloendopeptidases, it can be assumed that the enzyme is a Zn^{2+}-metalloprotease. Remarkably, the Co^{2+}-endopeptidase (Table 5) degraded Bz-Gly-Ala-Ala-Phe-pAB more efficiently than the native (undialyzed) enzyme. Furthermore, addition of Zn^{2+} to the apoenzyme resulted in an almost twofold increase in activity over the undialyzed holoenzyme. This finding suggests that part of the isolated enzyme was either stripped of its metal content resulting in incomplete saturation of the enzyme catalytic site with metal ions, or that dialysis against EDTA removed enzyme-

Table 5. Reactivation of Metalloendopeptidase by Metal Ions after Dialysis Against EDTA

Enzyme	Additions	Conc (mM)	Activity (units/ml)	Proportion of initial act. (%)
Not Dialyzed	None	-	0.454	100
Dialyzed	None	-	0.055	12
	$CoCl_2$	0.10	1.69	372
		0.25	1.65	363
		0.50	0.39	86
	$ZnCl_2$	0.01	0.19	42
		0.025	0.83	183
		0.05	0.69	152
	$MnCl_2$	1.00	0.89	196
		5.00	0.26	57
		10.00	0.17	37
	$CaCl_2$	1.00	0.56	123
		5.00	0.22	48
		10.00	0.20	44

The enzyme was dialyzed against 1.0 mM EDTA (pH 7.0) and then against 1.0 mM Tris-HCl buffer (pH 7.0). Activity was determined with Bz-Gly-Ala-Ala-Phe-pAB (1.0 mM) as substrate.

bound metal ions that have an inhibitory effect on its activity.

VI. SPECIFICITY STUDIES: SYNTHETIC SUBSTRATES

The specificity of the enzyme was studied with a series of model synthetic substrates. The results, summarized in Table 6, indicate that both the nature of the amino acid residues that participate in the formation of the hydrolyzed bond, as well as amino acid residues situated at some distance from the bond undergoing hydrolysis, greatly influence the interaction between the substrate and the active site of the metalloprotease. A distinct feature of the specificity is that a phenylalanine residue in position P_3' (substrate 1; Table 6) greatly contributes to the specificity constant. Replacement of phenylalanine by either a leucine or a glycine residue (substrate 2 and 3; Table 6) markedly increases the K_m and decreases the turnover rate constant, resulting in a pronounced decrease in the specificity constant. Specificity is also greatly influenced by the nature of the residue in the P_1 position. The substrates demonstrating the highest specificity constants (substrates 6-9, and 14)

Table 6. Kinetic Parameters of Soluble Metalloendopeptidase-Catalyzed Hydrolysis of Synthetic Substrates

	$P_4 - P_3 - P_2 - P_1 \downarrow P_1' - P_2' - P_3' - P_4'$	K_m(mM)	$k_{cat}(s^{-1})$	$k_{cat}/K_m(s^{-1}/M^{-1})$
1.	Bz - Gly- Ala- Ala- Phe- pAB	0.49 ± 0.03	4.77	9.73×10^3
2.	Bz - Gly- Ala- Ala- Leu- pAB	2.50 ± 0.23	3.10	1.24×10^3
3.	Bz - Gly- Ala- Ala- Gly- pAB	3.10 ± 0.10	1.15	0.37×10^3
4.	Bz - Gly- Phe- Ala- Phe- pAB	0.51 ± 0.07	2.75	5.39×10^3
5.	Bz - Gly- Gly- Ala- Phe- pAB	2.00 ± 0.42	1.45	0.73×10^3
6.	BOC- Phe- Ala- Ala- Phe- pAB	0.22 ± 0.02	21.50	9.77×10^4
7.	Bz - Gly- Phe- Ala- Ala- Phe- pAB	0.16 ± 0.05	8.60	5.38×10^4
8.	Bz - Gly- Phe- Ala- Pro- Phe- pAB	0.071 ± 0.012	24.39	3.44×10^5
9.	Bz - Gly- Phe- Ser- Pro- Phe- pAB	0.16 ± 0.05	21.14	1.32×10^5
10.	Bz - Gly- DPhe-Ala- Ala- Phe- pAB	INACTIVE		
11.	Bz - Gly- Phe- DAla-Ala- Phe- pAB	INACTIVE		
12.	Bz - Gly- Arg- Ala- Ala- Phe- pAB	0.37 ± 0.05	5.25	1.42×10^4
13.	Bz - Gly- Asp- Ala- Ala- Phe- pAB	0.72 ± 0.03	2.20	3.10×10^3
14.	Bz - Gly- Phe- Phe- Ala- Ala- Phe- pAB	0.031 ± 0.004	6.20	2.00×10^5
15.	Bz - Gly- Lys- Arg- Ala- Ala- Phe- pAB	0.24 ± 0.02	10.10	4.20×10^4

Data are mean values ± S. E. obtained from 4 to 8 determinations. The site of cleavage is indicated by an arrow. k_{cat} is expressed in moles of product formed per mole of enzyme per second.

all contain a phenylalanine residue in this position. A further increase in the specificity constant is obtained with the introduction of an additional phenylalanine residue in the P_2 position. Basic residues in position P_1 and P_2 (substrates 12 and 15) decrease the specificity constant; however, these substrates show higher specificity than those having either an aspartyl (substrate 13) or a glycine residue (substrate 1-5) in this position.

Substrates 7-9 all have phenylalanine residues in both the P_1 and P_3' position. Unlike substrate 7, however, substrates 8 and 9 have a proline residue instead of an alanine residue in the P_2' position. This single amino acid substitution appears to greatly increase the specificity constant; a comparison of substrate 7 and 8 indicates that replacement of a position P_2' alanine residue with a proline residue increases the specificity constant over six-fold. In contrast, a comparison of substrate 8 and 9 shows that replacement of an alanine residue in position P_2' with a serine residue results in a decrease in substrate specificity; however, the specificity constant of substrate 9 is still considerably greater than the majority of substrates listed in Table 6.

The endopeptidase exhibits stereospecificity toward the

amino acids forming the hydrolyzed bond. Peptides having D-amino acid residues in either position P_1 or P_1' (substrates 10 and 11) are completely resistant to hydrolysis. Furthermore, these peptides did not inhibit, even at high concentrations, the hydrolysis of susceptible substrates suggesting their inability to bind to the active site of the enzyme. In the series of substrates studied, peptides containing four amino acid residues or less were not hydrolyzed. At least five amino acid residues, three on the C-terminal side and two on the N-terminal side of the scissle bond, have a clear effect on the kinetic parameters of the reaction. This indicates the presence of an extended binding site capable of accomodating a minimum of five amino acid residues.

VII. SPECIFICITY STUDIES: NATURAL SUBSTRATES

A. SITE OF CLEAVAGE

The pattern of specificity observed with model synthetic substrates was also clearly apparent when the cleavage of several biologically active peptides was examined (Table 7).

Table 7. Hydrolysis of Biologically Active Peptides by Soluble Metalloendopeptidase

Peptide	Structure and Site of Hydrolysis
1. Dynorphin$_{1-8}$	Tyr-Gly-Gly-Phe-Leu↓Arg-Arg-Ile
2. α-Neo-Endorphin	Tyr-Gly-Gly-Phe-Leu↓Arg-Lys-Tyr-Pro-Lys
3. β-Neo-Endorphin	Tyr-Gly-Gly-Phe-Leu↓Arg-Lys-Tyr-Pro
4. BAM-12P	Tyr-Gly-Gly-Phe-Met↓Arg-Arg-Val-Gly-Arg-Pro-Glu
5. Bradykinin	Arg-Pro-Pro-Gly-Phe↓Ser-Pro-Phe-Arg
6. Neurotensin	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg↓Arg-Pro-Tyr-Ile-Leu
7. Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro↓Phe-His-Leu
8. Angiotensin II	Asp-Arg-Val-Tyr↓Ile-His-Pro-Phe
9. LHRH	pGlu-His↓Trp-Ser-Tyr↓Gly-Leu-Arg-Pro-Gly-NH_2
10. Substance P	Arg-Pro-Lys-Pro↓Gln-Gln-Phe↓Phe↓Gly-Leu-Met-NH_2
11. Somatostatin	Ala-Gly-Cys-Lys↓Asn-Phe-Phe-Trp↓Lys-Thr-Phe-Thr-Ser-Cys (Cys–Cys bridged)
12. Met-enkephalin	Tyr-Gly-Gly-Phe-Met Not Hydrolyzed
13. Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu Not Hydrolyzed

The conditions of the experiments are described under "Substrate Cleavage Site Determination".

Leu-enkephalin was generated from dynorphin(1-8) by the hydrolysis of the Leu^5-Arg^6 bond. This cleavage is consistent with the relatively high specificity of the enzyme towards substrates having hydrophobic amino acid residues in positions P_1, P_2 and P_3'. Binding of dynorphin(1-8) to the enzyme active site appears to be facilitated by binding of Phe, Leu and Ile to the S_1, S_2 and S_3' subsites of the enzyme respectively. In a similar fashion, Leu-enkephalin was also generated from α-neo-endorphin and β-neo-endorphin. In both these cases, a tyrosine residue is situated in the P_3' position. Likewise, cleavage of the Met^5-Arg^6 bond in BAM-12P resulted in generation of Met-enkephalin. In this instance, hydrophobic amino acid residues occupy the P_1, P_2 and P_3' positions within the substrate (Phe, Met and Val residues respectively). A larger Met-enkephalin containing opioid peptide, BAM-22P, was not hydrolyzed. Unlike BAM-12P, the failure of this similar 22 amino acid peptide to be cleaved by soluble metalloendopeptidase indicates its substrate size limitations. In fact, several other large peptides were also not hydrolyzed, including insulin B chain (30 amino acid residues) and cortrosyn, a peptide containing the sequence 1-24 of human ACTH.

Several other non-opioid peptides were also hydrolyzed by soluble metalloendopeptidase. Bradykinin was hydrolyzed

between the Phe^5-Ser^6 bond. This cleavage site is consistent with the high specificity of the enzyme towards substrates having aromatic residues in position P_1 and P'_3, and a proline residue in position P'_2. Indeed, substrate 8 of Table 6 was synthesized after it was determined that bradykinin was hydrolyzed between the Phe^5-Ser^6 bond; therefore, this model synthetic substrate was designed to mimck the primary structure of bradykinin. The cleavage of the His^2-Trp^3 bond in LHRH is apparently facilitated by the binding of the Tyr^5 residue to the S'_3 subsite, while the binding of the same Tyr residue to the S_1 subsite renders the Tyr^5-Gly^6 bond susceptible to hydrolysis. In a similar fashion, Tyr^4 of angiotensin II appears to bind to the S_1 subsite of the enzyme, resulting in hydrolysis of the Tyr^4-Ile^5 bond. As with model synthetic substrates, the activity of the enzyme towards biologically active peptides is not restricted to hydrolysis of bonds between neutral amino acid residues. In neurotensin, the only bond hydrolyzed was that between arginine residues (Arg^8-Arg^9). It should be noted that in the process of hydrolysis of this bond, the peptide must bind to the enzyme in such a manner that Tyr^{11} becomes aligned with the S'_3 subsite. The importance of a hydrophobic residue in the P'_3 position is further exemplified in the cleavage of angiotensin I, substance P

and somatostatin. Angiotensin I was hydrolyzed between the Pro^7-Phe^8 bond; in this instance, a hydrophobic amino acid residue (Leu) is located in position P_3'. In fact, the enzyme was unable to cleave the same Pro-Phe bond in angiotensin II demonstrating the contribution to hydrolysis rendered by binding of leucine to the S_3' subsite. In the case of substance P, the hydrolysis of bonds on the carboxyl side of the two phenylalanine residues occurs apparently after binding of these two residues to the S_1 and S_2 subsites of the enzyme while hydrolysis of the Pro-Gln bond requires Phe^7 to align again with the S_3' subsite. It is interesting to note that neither angiotensin I nor angiotensin II was cleaved between the Ile^5-His^6 bond, even though positioning of this bond in the catalytic site of the enzyme would also favorably position a Tyr and Phe residue in the S_2 and S_3' subsites respectively, while also allowing a proline residue to be situated in the S_2' subsite. Despite all these desireable interactions, the enzyme failed to cleave this bond. It therefore appears that a histidine residue in the P_1' position results in a highly unfavorable configuration. Even though the enzyme was able to cleave on the carboxyl side of proline residues in both angiotensin I and substance P, the enzyme exhibited no prolyl endopeptidase activity (Orlowski et al., 1979) when assayed with Z-Gly-Pro-SM, a

chromogenic substrate specific for this enzyme. Somatostatin was cleaved between the Lys^4-Asn^5 and Trp^8-Lys^9 bonds. Binding of Phe^7 to the hydrophobic S_3' subsite enables hydrolysis of the Lys-Asn bond. Alternately, binding of Phe^{11} to the S_3' subsite and Trp^8 to the S_1 subsite facilitates cleavage of the Trp-Lys bond. It should be noted that the enzyme was able to cleave a cyclic peptide since somatostatin was not reduced under the conditions of the experiment. Furthermore, neither Met-enkephalin nor Leu-enkephalin was cleaved by soluble metalloendopeptidase. Apparently the presence of a free amino group prevents the binding of the tyrosine residue to the S_1 subsite, in spite of the potential interaction of the Phe^4 residue in the enkephalin molecule with the S_3' subsite.

B. KINETIC PARAMETERS

The ability of several bioactive peptides to competitively inhibit enzyme activity when assayed with model chromogenic substrates was determined; the dissociation constants (K_i values) derived for these peptides, including the enkephalin containing peptides, are summarized in Table 8. Both β-neo-endorphin and dynorphin(1-8) exhibited high affinity for the enzyme, with K_i values of 18.4 and 42.4 uM respect-

Table 8. Inhibition Constants of Biologically Active Peptides

Peptide	$K_i \pm$ SEM (uM)
$Dynorphin_{1-8}$[@]	42.4 ± 5.6
α-Neo-Endorphin[@]	131.0 ± 24.8
β-Neo-Endorphin[@]	18.4 ± 4.7
BAM-12P[@]	393.7 ± 236.3
Leu-enkephalin[@]	245.0 ± 69.0
Met-enkephalin[@]	294.0 ± 66.0
Bradykinin[§]	51.2 ± 1.4
Neurotensin[§]	37.3 ± 5.4
Substance P[†]	974.0 ± 183.0
LHRH[†]	811.0 ± 112.0
Angiotensin I[§]	14.7 ± 8.9
Angiotensin II[§]	59.6 ± 23.7

[@] K_i values were determined from a single concentration of peptide, using the equation: $(v_i/v_o) = (K_m + S)/(K_m(1 + I/K_i) + S)$ where v_i and v_o are the initial velocities in the presence and absence of peptide.

[§] K_i values were determined from Dixon plots obtained at two different substrate concentrations using either Bz-Gly-Ala-Ala-Phe-pAB or Bz-Gly-Phe-Ser-Pro-Phe-pAB as substrate. At each concentration of substrate, rates were determined with five different inhibitor concentrations.

[†] K_i values were determined from IC_{50} values.

Data are mean values of two to five determinations ± SEM.

ively. Moreover, β-neo-endorphin had over a seven-fold greater affinity for the enzyme than α-neo-endorphin. The Met-enkephalin containing peptide BAM-12P had the lowest affinity of all the opioid peptides tested (K_i value of 393.7 uM). Among the other bioactive peptides, angiotensin I exhibited the strongest inhibition of enzyme activity toward synthetic substrates, with a K_i of 14.7 uM. Angiotensin II, however, had a K_i four fold greater (K_i of 59.6) than angiotensin I. Bradykinin and neurotensin both strongly inhibited enzyme activity toward chromogenic substrates with K_i values of 51.2 uM and 37.3 uM respectively. Conversely, LHRH and substance P were poor competitive inhibitors with K_i values about sixty fold greater than angiotensin I. Further kinetic studies were performed to determine the specificity of the enzyme towards the enkephalin containing peptides as well as bradykinin and neurotensin (Table 9). Among the opioid peptides, dynorphin(1-8) and β-neo-endorphin were found to be highly efficient substrates as exemplified by their large specificity constants (k_{cat}/K_m ratio). Interestingly, the enzyme was over five times more selective for β-neo-endorphin than α-neo-endorphin. In comparison with dynorphin(1-8) and β-neo-endorphin, BAM-12P had a higher turnover rate constant (k_{cat}), however, due to a relatively high K_m its specificity

Table 9. Kinetic Parameters of Rat Brain Metalloendopeptidase Catalyzed Hydrolysis of Biologically Active Peptides

Peptide	K_m (uM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/M^{-1})
1. Dynorphin$_{1-8}$	60.4	15.55	2.58×10^5
2. β-Neo-Endorphin	37.9	9.13	2.41×10^5
3. α-Neo-Endorphin	92.9	5.72	4.36×10^4
4. BAM-12P	393.7[b]	18.19	4.62×10^4
5. Bradykinin	67.1	19.88	2.96×10^5
6. Neurotensin	37.3[b]	11.27	3.02×10^5

[a]The kinetic parameters K_m and k_{cat} were derived by measuring the rate of disappearance of substrate and the rate of appearance of product by HPLC, as described under "Kinetic Studies".

[b]K_i values (see Table 8) were used.

constant was lower. The kinetic studies with bradykinin and neurotensin indicate that both these peptides are highly efficient substrates, with specificity constants of the same order of magnitude as those of dynorphin(1-8) and β-neo-endorphin. The K_m values of these peptides determined in the specificity studies are in good agreement with the K_i values derived from the inhibition studies (Table 8). A further comparison of the specificity constants of the natural peptides in Table 9 with those determined with the model synthetic substrates (Table 6) indicate that the specificity constants for all the bioactive peptides are comparable to those obtained with the best synthetic substrates. In particular, dynorphin(1-8), β-neo-endorphin, bradykinin and neurotensin had the highest specificity constants of any substrates tested, whether synthetic or natural.

VIII. REGIONAL DISTRIBUTION OF ENZYME ACTIVITY

The distribution of soluble metalloendopeptidase activity in various organs of the rat (Table 10), as well as the regional distribution of enzyme activity in rat brain (Table 11) was determined. The highest soluble metalloendopepti-

Table 10. Distribution of Soluble Metalloendopeptidase Activity in Rat Tissue

Tissue	Specific Activity (um/ml/h)	Relative Activity (%)
Brain	1.15	100
Testis	1.10	92
Anterior Pituitary	0.72	63
Posterior Pituitary	0.75	50
Ovary	0.31	28
Spleen	0.23	18
Adrenal	0.19	16
Liver	0.14	11
Kidney	0.13	10
Lung	0.12	10
Thyroid	0.11	9
Heart		<5

Tissue homogenates were prepared as in Step 1 of the purification procedure. Activity was determined in supernatants using Bz-Gly-Ala-Ala-Phe-pAB as substrate. Specific enzyme activity was determined by taking the difference in activity in the presence of N-[1(R,S)-carboxy-2-phenylethyl]-Phe-pAB and in the presence of N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB and N-[1(R,S)-carboxy-2-phenylethyl]-Phe-pAB, as described in "Determination of Enzyme Activity". Data are mean values of two to three determinations. The deviations of individual results from the mean did not exceed 10%. Relative activities are expressed relative to that of the brain arbitrarily set as 100%.

Table 11. Regional Distribution of Soluble Metalloendopeptidase Activity in Rat Brain

Region	Specific Activity (um/mg/h)	Relative Activity (%)
Cerebellum	0.213	100
Hippocampus	0.194	91
Substantia Nigra	0.190	89
Cortex	0.181	85
Striatum	0.175	82
Hypothalamus	0.144	68
Midbrain	0.133	62
Thalamus	0.122	57
Medulla/Pons	0.108	51

Tissue homogenates were prepared as in Step 1 of the purification procedure. Activity was determined using Bz-Gly-Ala-Ala-Phe-pAB as substrate under the same conditions given in Table 10. Data are mean values of two to three determinations. The deviations of individual results from the mean did not exceed 10%. Relative activities are expressed relative to that of the cerebellum arbitrarily set as 100%.

dase activity was found in brain, testes, anterior pituitary and posterior pituitary. Enzyme activity in other organs was considerably lower when compared with brain and testes. For example, activity in liver, lung, and kidney is only respectively 11, 10 and 10% of that of the brain. As shown in Table 11, enzyme activity in rat brain is highest in cerebellum, with activity in cerebellum > hippocampus > substantia nigra > cortex > striatum > hypothalamus > midbrain > thalamus > medulla/pons. The activity in medulla/pons is 51% of that in cerebellum.

IX. DESIGN OF SPECIFIC SOLUBLE METALLOENDOPEPTIDASE INHIBITORS

A series of N-carboxymethyl peptides were synthesized by reductive amination of various α-keto acids and aldehydes with Ala-Ala-Phe-pAB. This peptide was used because we have shown in previous studies (Orlowski et al., 1983) that the phenylalanyl residue is important for substrate binding by apparently interacting with a hydrophobic pocket in the S_3' subsite of the enzyne, and because the two Ala residues are needed for binding to the S_1' and S_2' subsites. Furthermore,

by introducing this fragment into all the inhibitors, it became possible to evaluate the importance of interactions at the S_1 subsite for inhibition and also to evaluate the contribution of the carboxylate anion as a zinc-coordinating group.

Table 12 summarizes the inhibition constants obtained with all the inhibitors. Reductive amination of α-ketoacids generates a new asymmetric carbon, and accordingly two diastereomers are formed in about equimolar amounts as determined by HPLC. With the exception of N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB, which was resolved into its diastereomers by HPLC, mixtures containing both diastereomers were used in studies of the other derivatives. As expected, reductive amination of glyoxylic acid (inhibitor IV; Table 12) and phenylacetaldehyde (inhibitor V; Table 12) yielded single products.

N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB produced the strongest inhibition of the soluble metalloendopeptidase. A mixture of this inhibitor had a K_i of 1.94 uM. The more potent diastereomer had a K_i of 0.81 uM, while the less active diastereomer had a K_i of 7.4 uM. This almost ten fold difference in inhibitory potency suggests considerable stereoselectivity of inhibitor binding. Decreasing the hy-

Table 12. Inhibitors of Rat Brain Soluble Metalloendopeptidase

Compound	$K_i \pm$ SEM (uM)
N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (I)	1.94 ± 0.65
Diastereomer I	0.81 ± 0.16
Diastereomer II	7.39 ± 1.77
N-[1(R,S)-carboxybutyl]-Ala-Ala-Phe-pAB (II)	5.17 ± 0.67
N-[1(R,S)-carboxyethyl]-Ala-Ala-Phe-pAB (III)	20.90 ± 2.30
N-carboxymethyl-Ala-Ala-Phe-pAB (IV)	69.40 ± 7.72
N-phenylethyl-Ala-Ala-Phe-pAB (V)	2360.0 ± 700.0
N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Phe-pAB (VI)	2640.0 ± 720.0
N-[1(R,S)-carboxy-2-phenylethyl]-Ala-pAB (VII)	No Inhibition*
N-[1(R,S)-carboxy-2-phenylethyl]-Phe-pAB (VIII)	No Inhibition*
Phosphoramidon (IX)	No Inhibition*

K_i values were determined from Dixon Plots obtained at two different substrate concentrations, using Bz-Gly-Ala-Ala-Phe-pAB as substrate. At each concentration of substrate, rates were determined with five different inhibitor concentrations. Each K_i value represents the average of three to five determinations.

*The final concentration of compound VII, VIII and IX were 1.25 mM, 0.5 mM and 0.125 mM respectively. These concentrations are respectively 250, 7,000 and 36,000 times greater than the K_i values of these inhibitors towards the membrane-bound metalloendopeptidase.

drophobicity of the residue binding to the S_1 subsite of the enzyme greatly decreased the inhibitory potency of the N-carboxymethyl peptides. For example, while the N-carboxybutyl derivative was only 2.5 times less potent than the N-carboxy-2-phenylethyl derivative, the N-carboxyethyl was 10 fold less potent. The N-carboxymethyl peptide was even less potent, being 35 fold less inhibitory compared with the mixture of N-[1(R,S)-carboxy- 2-phenylethyl]-Ala-Ala-Phe-pAB and 86 fold less inhibitory compared with the more potent isolated diastereomer.

N-phenylethyl-Ala-Ala-Phe-pAB, a compound similar to N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB but lacking the Zn^{2+} coordinating carboxylate group was only weakly inhibitory. This derivative had a K_i almost 1200 fold higher than the corresponding carboxylate containing compound. A similar dramatic decrease in inhibitory potency was caused by replacement of the Ala-Ala-Phe-pAB with Ala-Phe-pAB. Inhibitors of membrane-bound metalloendopeptidase ("enkephalinase"; EC 3.4.24.11) such as N-[1(R,S)-carboxy-2-phenylethyl]-Ala-pAB, N-[1(R,S)-carboxy-2-phenylethyl]-Phe-pAB and phosporamidon had no effect on the soluble metalloendopeptidase activity.

The inhibition of the soluble metalloendopeptidase by the

inhibitors listed in Table 12 was competitive in nature since it could be surmounted by increasing substrate concentrations. Double reciprocal plots (1/v versus 1/S) in the presence and absence of inhibitor gave straight lines intersecting at the y axis. The reversibility of this inhibition was also apparent in dialysis experiments. Enzyme activity, which had been inhibited by a 6.25 uM solution of N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB, could be completely restored upon dialysis against Tris-HCl buffer (0.1 M; pH 7.6). Experiments in which the enzyme was preincubated with inhibitor for 10 min at 37° before the reaction was started by addition of substrate, showed the same degree of inhibition as those in which the enzyme was not preincubated, and the reaction was initiated by adding the enzyme to mixtures containing substrate and buffer. This indicates that formation and dissociation of the enzyme-inhibitor complex is a rapid equilibrium process. No change in the degree of inhibition was also noted in experiments in which 0.05 mM $ZnCl_2$ was added to incubation mixtures containing enzyme and N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (2.5 uM). This indicates that inhibition was not due to an EDTA-like effect, whereby the metalloendopeptidase is stripped of its catalytically essential metal ion by the inhibitor.

The soluble metalloendopeptidase showed considerable sensitivity to heat denaturation in the absence of substrate. More than 90% of activity was lost by heating the enzyme at 57° for 60 min (Figure 4). Even at 37°, more than 50% of activity was lost after 60 min. Considerable protection against heat denaturation was, however, provided by addition of inhibitor. Thus, in the presence of 0.1 M N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB only about 30% of activity was lost by heating at 57° for 1 h, suggesting that inhibitor binding increases the heat stability of the enzyme.

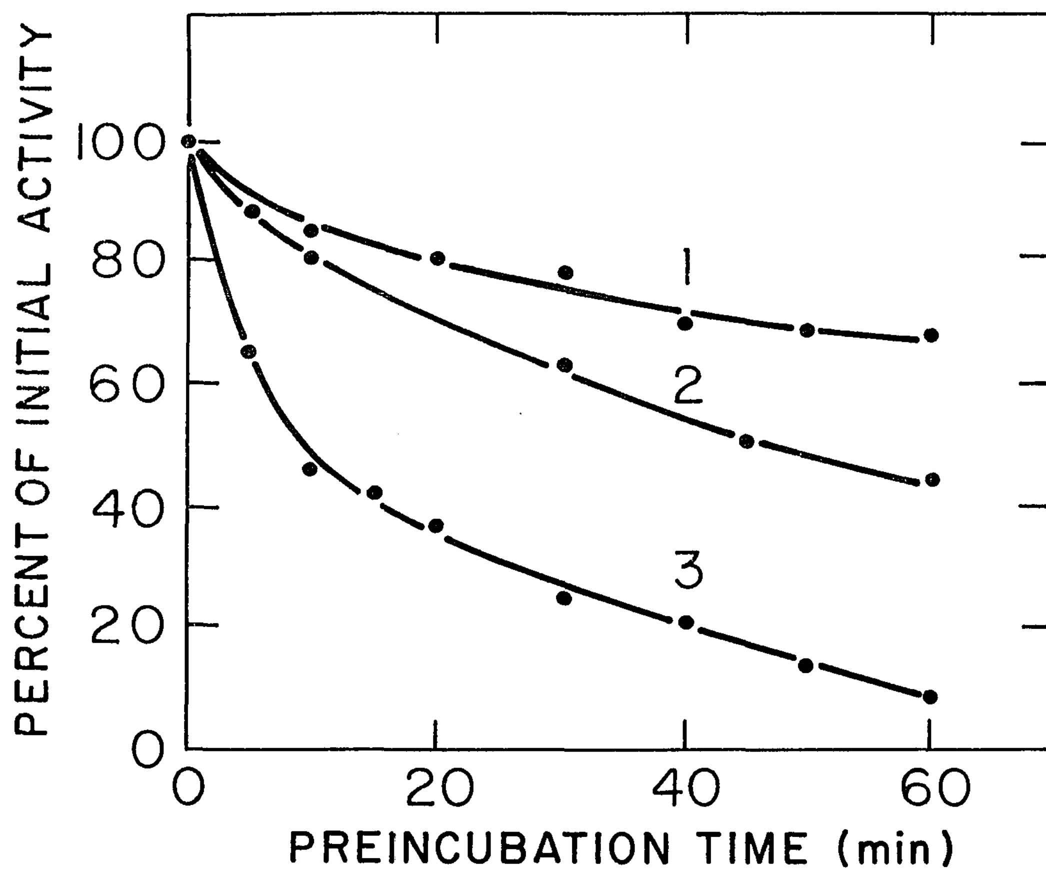


Figure 4. Protection of the Enzyme Against Heat Denaturation by N- 1(R,S)-carboxy-2-phenylethyl -Ala-Ala-Phe-pAB.

The enzyme (after step 3 of the purification procedure) was preincubated in Tris-HCl buffer (0.2 M, pH 7.0) at 57° C (curve 3) and 37° C (curve 2) in the absence of inhibitor and at 57° C (curve 1) in the presence of 0.1 M inhibitor. Aliquots were withdrawn at various times for determination of activity as described under Methods.

DISCUSSION

A series of model pAB-containing substrates were synthesized during the course of this present study, making possible the identification, purification, and determination of specificity of an endopeptidase predominantly associated with the soluble cytoplasmic protein fraction of homogenates of rat brain. These pAB-containing substrates offer a considerable advantage over analogous 2NA-containing and pNA-containing substrates. The pAB-containing substrates are much more water soluble than their 2NA or pNA counterparts; this increased solubility allowed the testing of substrates containing hydrophobic amino acid residues. These substrates were essential in mapping out the substrate recognition site of the enzyme.

On the basis of available data, the enzyme has been classified as a metalloendopeptidase. This classification is supported by the finding that metal chelators, such as EDTA, EGTA and o-phenanthroline strongly inhibit enzyme activity. Futhermore, activity lost upon dialysis against EDTA can be restored by addition of one of several divalent cations. By analogy with other metalloproteases, such as carboxypepti-

dase A, angiotensin converting enzyme, and membrane-bound metalloendopeptidase, it can be assumed that the enzyme is a zinc-containing protein; however, greater amounts of enzyme need to be isolated in order to determine its metal content.

To our knowledge, the isolated endopeptidase represents the first metalloendopeptidase characterized in the soluble protein fraction of brain homogenates. It distinctly differs from the membrane-bound metalloendopeptidase shown to be identical with "enkephalinase" (Almenoff and Orlowski, 1983; Almenoff et al., 1981; Fulcher et al., 1982), an enzyme suggested to function in the degradation of enkephalins (Schwartz et al., 1981). Membrane-bound metalloendopeptidase, a zinc-containing enzyme present in bovine pituitary and many peripheral tissues (Orlowski and Wilk, 1981; Kerr and Kenny, 1974), has a molecular weight (95,000) significantly greater than that of the soluble enzyme (67,000). It exhibits a thermolysin-like specificity (Matsubara et al., 1966), cleaving peptide bonds on the amino side of hydrophobic amino acid residues. In contrast, the soluble metalloendopeptidase can potentially cleave peptide bonds involving the carbonyl group contributed by neutral, basic and even acidic amino acid residues. In this sense the "primary specificity" of the enzyme, as represented by the two amino acids forming the scissle bond, can be regarded as

rather broad. Examination, however, of the kinetic parameters obtained with model synthetic substrates (Table 6) shows a great preference towards substrates having aromatic residues in position P_1 or both P_1 and P_2. Furthermore, an outstanding characteristic of the enzyme is that binding of an aromatic or hydrophobic residue to the S_3' subsite of the enzyme has considerable influence on the specificity constant (K_m/k_{cat} ratio) and the type of bond being hydrolyzed. The presence of a substituent on the α-amino group in the P_1 position of the substrate appears to be necessary, as evidenced by the failure of the enzyme to hydrolyze either Met- or Leu-enkephalin [Tyr-Gly-Gly-Phe-Met(Leu)]. Inclusion of a proline residue at position P_2' also appears to contribute greatly to the specificity constant. Indeed, the best substrate incorporates aromatic residues in positions P_2, P_1 and P_3' as well as a proline residue in position P_2' (substrate 8; Table 6) and shows a specificity constant almost 3 orders of magnitude greater than a substrate having glycine residues in position P_1 and P_3' (substrate 3). On the basis of the data derived from the specificity studies, the active site of the enzyme and its interaction with one of the best model synthetic substrates can be schematically represented as shown in Figure 5; The S_1, S_2 and S_3' subsites (probably hydrophobic pockets) accomodate the hydrophobic residues of

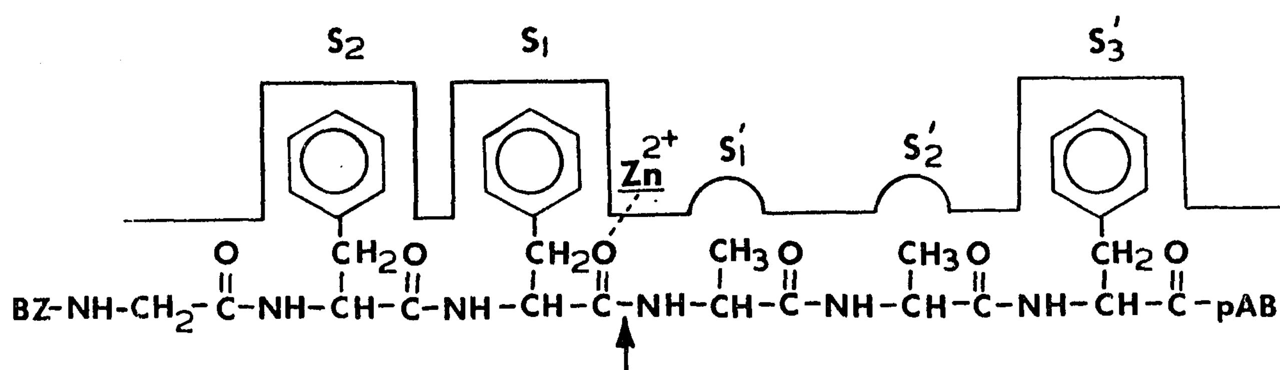


Figure 5. Schematic Representation of the Active Site of the Enzyme and its Binding of the Substrate Bz-Gly-Phe-Phe-Ala-Ala-Phe-pAB.

the substrate and the Zn^{2+} atom coordinates with the carbonyl oxygen of the hydrolyzed bond. The presence of an interaction between an aromatic or hydrophobic residue in position P_3' with the S_3' subsite of the enzyme has also a considerable influence on the pattern of hydrolysis of natural peptides. Interestingly, the importance for binding of an aromatic residue located at some distance from the bond undergoing hydrolysis is not a property unique to this metalloendopeptidase. In fact, a phenylalanine residue located at a distance from the attacked bond is also recognized as important for the action of thrombin (Magnusson, 1971; Marsh et al., 1982).

The enzyme described here is clearly different from the chymotrypsin-like activity of a high molecular weight (700,000) proteolytic complex characterized in bovine pituitaries and rabbit brain (Wilk and Orlowski, 1980; Orlowski and Wilk, 1981; Wilk and Orlowski, 1983). The substrate Cbz-Gly-Gly-Leu-pNA readily cleaved by the complex is resistant to hydrolysis by the soluble metalloendopeptidase. Furthermore, the chymotrypsin-like activity of the complex is inhibited by sodium and potassium ions, and unaffected by EDTA, properties which are at variance with those of the metalloendopeptidase.

Soluble fractions of rabbit brain homogenates contain a thiol-dependent activity, having a molecular weight of 74,000. This activity, first described by Camargo and his coworkers and designated as 'Kininase A', hydrolyzes the Phe^5-Ser^6 bond in bradykinin, and seems to be inactive toward LHRH (Oliveira et al., 1976; Camargo et al., 1982); however, the enzyme does cleave des-Gly-NH_2-LHRH between the Tyr^5-Gly^6 bond (Camargo et al., 1982). A closely related activity was purified from bovine pituitaries and shown to hydrolyze the Tyr^5-Ser^6 and His^2-Trp^3 bonds in LHRH (Horsthemke and Bauer, 1980). The reported molecular weight of this enzyme was 83,000, somewhat higher than that of Kininase A. While the sites of cleavage of bradykinin and LHRH by these enzymes are similar to those found with the metalloendopeptidase, a distinguishing feature of both these enzymes is their insensitivity to inhibition by EDTA (Horsthemke and Bauer, 1980; Camargo et al., 1973). In contrast, the brain metalloenzyme is strongly inhibited by EDTA and other metal chelators. Furthermore, while the pituitary enzyme is significantly inhibited by relatively low concentrations of chymostatin, pepstatin and leupeptin (Horsthemke and Bauer, 1982), these inhibitors have no effect on the enzyme described here. Kininase A activity is inhibited by PCMB and enhanced by DTT (Camargo et al., 1973); therefore,

Camargo and coworker suggest that the enzyme is a thiol protease, requiring free sulfhydryl groups for catalytic activity. Initial studies on Kininase A and the pituitary enzyme have been primarily limited to the degradation of bradykinin and LHRH. Recently, attempts have been made to define more broadly the specificity of these enzymes. For example, Camargo and coworkers (1983) have reported that Kininase A cleaves neurotensin between the Arg^{8}-Arg^{9} bond. D-Tyr^{11}-neurotensin, however, was resistant to hydrolysis by this enzyme. Horsthemke and coworkers (1981) also showed that modifications at positions remote from the scissle peptide bond also influenced the degrading ability of the bovine pituitary enzyme, using LHRH and other superactive analogs. In particular, analogs containing additional aromatic amino acid residues were more effective in inhibiting the degradation of [H^{3}]LHRH. Horsthemke et al. report that enzyme-ligand binding was also strongly influenced by hydrophobic interactions. Studies on the regional distribution of soluble metalloendopeptidase activity in rat tissue (Table 10) clearly indicates that the pituitary is a rich source of this enzyme. The possibility therefore arises that the activity described in bovine pituitaries is identical with the enzyme described in our laboratory. Until further data on Kininase A becomes available, one is compelled

to conclude that soluble metalloendopeptidase is different from this activity, unless the differences between these enzymes can be explained on the basis of species differences, or by a gross error on the part of the authors reporting on Kininase A in that they failed to recognize the metalloendopeptidase nature of the enzyme.

Several unpurified or partially purified enzyme activities have been reported to be involved in the processing of various peptide precursors. Proinsulin and proglucagon-converting activities have been partially characterized in isolated anglerfish islet secretory granules (Fletcher et al., 1981). These activities have a pH optimum of 4.5-5.5 and are strongly inhibited by antipain, leupeptin and PCMB; however, the enzyme activities are unaffected by EDTA. A lysed crude secretory granule fraction from rat islets was also shown to process endogenous proinsulin to insulin with a pH optimum of 5.0-6.0 (Docherty et al., 1982). Similarly, this converting enzyme activity is not inhibited by the metalloprotease inhibitors EDTA and o-phenanthroline, but is inhibited by the thiol protease reagents PCMB, antipain and leupeptin. Docherty and coworkers estimate that the molecular weight of this thiol protease to be 31,000. Another suggestively similar thiol-dependent protease activity was determined in purified rat neurointermediate lobe secretory gran-

ules (Loh and Gainer, 1982). This enzyme also had an acidic pH optimum of 5.0 and appears to have proopiomelanocortin-converting activity. These cathepsin B-like activities all cleave at pairs of basic amino acid residues in the precursor. Furthermore, they all appear to be present in both soluble and membrane fractions of granule lysates. Even though these enzyme activites have yet to be purified, they appear to be clearly distinct from soluble metalloendopeptidase in their specificity, pH optimum and sensitivity to protease inhibitors.

A number of laboratories have reported on the partial purification of a trypsin-like enzyme from bovine chromaffin granules (Troy and Musacchio, 1982; Evangelista et al., 1982; Lindberg, et al., 1982), responsible for generating enkephalin moieties from precursors. Again, all the enzyme activities cleave at the carboxyl side of basic amino acid residues. Lindberg and coworkers (1982) reported an enzyme activity, optimal at pH 8.0, which appears to be a serine protease. Troy and Musacchio (1982) and Evangelista et al. (1982) characterized the enkephalin generating activities as being optimal in the acidic range (pH optimum of 5.7 and 5.0 respectively) and not being sensitive to serine protease inhibitors such as DFP. Furthermore, Troy and Musacchio suggest that the processing enzyme is thiol-dependent. What

physiologic role, if any, these enzyme activities play in the processing of enkephalin precursors remains to be determined; however, it clearly appears that these enzymes are distinct from soluble metalloendopeptidase.

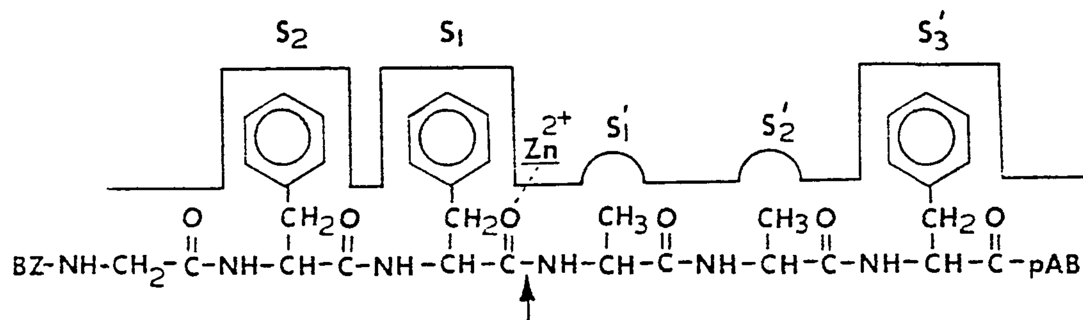
A recent report described a dynorphin converting enzyme with unusual specificity from rat brain (Devi and Goldstein, 1984). A rat brain membrane extract was shown to convert synthetic dynorphin B-29 ("leumorphin") to dynorphin B ("rimorphin"). This enzyme apparently cleaves at the amino side of single arginine residues, at a Thr-Arg bond at positions 13 and 14 of the substrate. The pH optimum for this enzyme is about 8.0. There is a marked increase in enzyme activity in Tris-HCl buffer as compared with sodium phosphate buffer. While these characteristics are in some way similar to those of soluble metalloendopeptidase, the sensitivity of this dynorphin converting activity to protease inhibitors distinctly differs from the soluble enzyme. The converting activity is not inhibited by either EDTA or o-phenanthroline; however, it is strongly inhibited by p-chloromercuribenzenesulfonic acid (PCMS). This inhibition by PCMS suggests that the activity is due to a thiol protease. Moreover, this activity does not appear to degrade dynorphin(1-8), a particularly specific substrate for soluble metalloendopeptidase.

Another enzyme activity with striking similarities to the metalloenzyme is able to generate Met-enkephalin by cleavage of the substrate [Homoarg-^{14}C]-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Homoarg (Knight and Klee, 1979). This activity is present in both soluble and membrane fractions of homogenates of rat brain. The soluble activity has a pH optimum of about 7.0, is competitively inhibited by angiotensin II and neurotensin, is uniformly distributed throughout the brain (cerebellum highest and medulla/pons lowest), and appears to require an intact sulfhydryl group for activity. This soluble activity has not been further purified nor characterized; however, its relationship to the metalloendopeptidase described here remains to be established.

Marks and coworkers have described a labile neutral endopeptidase activity in the supernatant fraction of rat brain homogenates (Marks and Pirotta, 1971). This activity, designated Cathepsin M, appears to cleave several biologically active peptides, as well as such proteins as hemoglobin. Bradykinin is cleaved between the Phe^5-Ser^6 bond (Marks and Pirotta, 1971), somatostatin primarily between the Trp^8-Lys^9 bond (Marks and Stern, 1975; Marks et al., 1976), LHRH between either the Tyr^5-Gly^6 or the Gly^6-Leu^7 bond (Marks and Stern, 1974) and substance P between either the Gln^6-Phe^7 or Phe^7-Phe^8 bond and the Gly^9-Leu^{10} bond (Benuck

and Marks, 1975). Until more information about the physical characteristics and the specificity requirements of Cathepsin M become available, the relationship between this activity and soluble metalloendopeptidase cannot be determined.

On the basis of the data derived from the specificity studies, it is apparent that the enzyme might play a role in hydrolyzing several biologically active peptides. Several Leu- and Met-enkephalin precursor peptides contain amino acid sequences that fulfill the hydrophobic requirements of the S_1 and S_3' subsites of the enzyme. Figure 6 is a schematic representation of the interaction of several opioid peptides with the active site of the enzyme. Indeed, dynorphin(1-8) (Goldstein et al., 1979), and α-neo-endorphin and β-neo-endorphin (Kangawa and Matsou, 1979) are specifically cleaved to generate Leu-enkephalin. Met-enkephalin is generated from cleavage of BAM-12P (Mizuno et al., 1980a). The failure of the enzyme to cleave BAM-22P (Mizuno et al., 1980b) indicated that the enzyme has substrate size restrictions, being capable of only hydrolyzing peptides of relatively small length. Furthermore, kinetic studies of these enkephalin containing peptides indicated that the enzyme has a high specificity for these substrates, particularly for dynorphin(1-8) and β-neo-endorphin. The K_i values and dissociation constants of these opioid peptides were found to



ENK$_{1-3}$ – PHE – LEU – ARG – ARG – ILE　　DYNORPHIN$_{1-8}$

ENK$_{1-3}$ – PHE – LEU – ARG – LYS – TYR – PRO – LYS　　α-NEO-ENDORPHI

ENK$_{1-3}$ – PHE – LEU – ARG – LYS – TYR – PRO　　β-NEO-ENDORPHI

ENK$_{1-3}$ – PHE – MET – ARG – ARG – VAL – GLY – ARG –　　BAM-12P

Figure 6. Schematic Representation of the Active Site of the Enzyme and its Binding of Enkephalin-containing Peptides. Enk$_{1-3}$ denotes Tyr-Gly-Gly.

be in the umolar range. β-neo-endorphin and dynorphin(1-8) have particularly high affinity, K_m values of 37.9 and 60.4 uM and K_i values of 18.4 and 42.4 uM respectively, for the metalloenzyme. These observations, in conjunction with the finding that soluble metalloendopeptidase is primarily found in brain and pituitary tissue, are highly suggestive of a role for this enzyme in the processing of certain enkephalin precursors. The Leu-enkephalin containing peptides α-neo-endorphin, β-neo-endorphin and dynorphin(1-8) are believed to arise from a single precursor protein, prodynorphin (Kakidani et al., 1982) and the Met-enkephalin containing peptides BAM-12P and BAM-22P arise from another distinct precursor molecule, proenkephalin A (Coomb et al., 1982; Noda et al., 1982). Since enkephalin moieties within these larger precursor proteins are flanked on both sides by pairs of basic amino acid residues, especially Lys-Arg, it is widely regarded that enkephalin molecules are processed from precursors by the combined action of a trypsin-like enzyme and a carboxypeptidase B-like activity (Steiner et al., 1980; Noda et al., 1982; Coomb et al., 1982). The conversion process is believed to proceed by a trypsin-like activity hydrolyzing bonds on the carboxyl side of basic residues, followed by a carboxypeptidase B-like activity removing the exposed C-terminal basic residues. The amino

acid sequences of some opioid peptides, however, indicates that their formation is not due to the action of such enzymes; therefore, processing of certain opioid peptides is selective or "atypical". For example, atypical processing regions in proenkephalin A (Noda et al., 1982; Coomb et al., 1982) have been observed in the generation of BAM-12P and BAM-22P (Mizuno et al., 1980a,b). It has recently been suggested that selective processing of prodynorphin into different opioid peptide forms occurs in various regions of rat brain and pituitary (Seizinger et al., 1984). Immunoreactive α-neo-endorphin was found in much higher concentration than β-neo-endorphin in several brain regions, suggesting either the absence of the carboxypeptidase necessary for the conversion of α-neo-endorphin to β-neo-endorphin, and or the presence of enzymes which selectively degrade β-neo-endorphin over α-neo-endorphin. It is interesting to note that soluble metalloendopeptidase had a much higher affinity for β-neo-endorphin than α-neo-endorphin and was more than five times more specific for β-neo-endorphin than α-neo-endorphin. Differential processing of opioid peptides is not limited to the enkephalin precursors. Zakarian and Smyth (1982) have reported that β-endorphin is processed differentially in specific regions of rat brain and pituitary. Thus there appears to be a clear distinction between

processing of high molecular weight precursors such as proenkephalin A and prodynorphin from that of the smaller opioid peptides. This concept is reinforced by examination of the amino acid sequences of several opioid peptides. As shown in Figure 1, the dynorphins, neoendorphins, and bovine adrenal medulla peptides all contain pairs of basic amino acid residues. The isolation of these peptides from brain, pituitary and adrenal medulla suggest that not all loci of pairs of basic residues are susceptible to processing by the trypsin-like activity. Whether enkephalins are generated directly from high molecular weight precursors or by the further cleavage of such peptides as dynorphin(1-8), the neoendorphins, BAM-12P, BAM-22P, Met-enkephalin-Arg^6-Phe^7, and Met-enkephalin-Arg^6-Gly^7-Leu^8 is also still unresolved. If, however, enkephalins are generated from these opioid peptides, then another as yet unidentified enzyme must be involved. Since soluble metalloendopeptidase is specific for only small oligopeptides but not higher molecular weight peptides and proteins, it might be a good candidate for the further processing of low molecular weight opioid peptides.

The regional distribution of soluble metalloendopeptidase in rat brain is also suggestive of a possible role of this enzyme in processing of prodynorphin to Leu-enkephalin. Leu-enkephalin was found to be particularly rich in rat

striatum (Yang et al., 1977), an area of high enzymatic activity (Table 11). Furthermore, it was recently suggested that Leu-enkephalin production in rat substantia nigra, another region of high enzymatic activity, was the result of processing of the precursor prodynorphin (Zamir et al., 1984). It must be recognized, however, that generation of enkephalin moieties from precursor molecules is dependent upon the rate of synthesis of such precursors, the presence of enzymes capable of processing these large peptides and proteins, as well as the absence of enkephalin degrading enzymes.

In addition to hydrolyzing dynorphin(1-8), α-neo-endorphin, β-neo-endorphin and BAM-12P, soluble metalloendopeptidase also cleaved several other biologically active peptides (Table 7). Whether this enzyme is involved in degradation and termination of action of such neuropeptides must be determined. The dissociation constants derived from competition studies with chromogenic substrates indicate that bradykinin, neurotensin, angiotensin I and angiotensin II all have high affinity for the enzyme (Table 8). In addition, both bradykinin and neurotensin have high turnover rate constants as well as high specificity constants for the enzyme (Table 9).

LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), is responsible for stimulating the release of LH and FSH from the pituitary (Guillemin, 1978). This peptide is blocked at both the N- and C-terminals and is therefore susceptible only to the action of endopeptidases. This decapeptide amide is hydrolyzed by the soluble metalloendopeptidase at the Tyr^5-Gly^6 bond. This bond is reported to be the major site of hydrolysis of LHRH *in vivo* (Marks, 1977). In another *in vitro* study, a peptidase activity from rat brain synaptosomes catalyzed a single cleavage of LHRH at this same Tyr^5-Gly^6 bond (McKelvy et al., 1982). Preliminary characterization of this peptidase activity indicated that it is a metalloendopeptidase from the soluble fraction of brain synaptosomes which requires a thiol group for activity. This activity was not inhibited by the chymotrypsin inhibitor TPCK nor the serine protease inhibitor DFP. Furthermore, an activity described by McDermott et al. (1983), also from synaptosomes of rat hypothalamus and cortex, specifically cleaves LHRH at the Tyr^5-Gly^6 locus. This activity, found predominantly in the soluble protein fraction, has a pH optimum of 7.5; moreover, the activity is inhibited by o-phenanthroline and PCMB.

What physiological role these LHRH-degrading enzymes play in the regulation of LHRH levels is unknown; however, there

appears to be a strong correlation in the rat median eminence between the levels of a LHRH-degrading peptidase similar to soluble metalloendopeptidase (McKelvy et al., 1982), and the content of LHRH (Advis et al., 1982). In fact, LHRH-degrading activity appears to decrease significantly before the content of LHRH peaks in the median eminence during the first estrous cycle of puberty. This relationship suggests that hydrolysis of the Tyr^5-Gly^6 bond in LHRH may possibly be the mechanism by which the physiological actions of this peptide are terminated.

Several highly potent and stable analogs of LHRH have been synthesized (Coy et al., 1976). These peptides incorporate several modifications including the removal of the N-terminal glycine amide, N-ethylamidation of the penultimate Pro residue, and D-amino acid substitutions in specific positions. In particular, replacement of the Gly residue in position 6 with a D-Trp residue results in an analog with about a 13-fold increase in LHRH activity. It has been suggested that the higher potency of LHRH analogs, as well as other bioactive peptide analogs, is due in part to increased resistance of the peptide to degradation (Koch et al., 1977). In fact, a study in humans on the metabolic clearance and half-disappearance time of D-Trp^6-LHRH compared with exogenous LHRH indicate that LHRH is cleared about 3

times more rapidly from the plasma *in vivo* than is D-Trp[6]-LHRH (Barron et al., 1982). These findings are suggestive of a decreased rate of degradation of D-Trp[6]-LHRH. Furthermore, studies on the degradation of LHRH and its analogs by the nonchymotrypsin-like endopeptidase from bovine pituitary (Horsthemke et al., 1981), indicate that superactive analogs of LHRH modified in position 6 and 10 are degraded more slowly than the parent peptide. It is therefore interesting to note that replacement of Gly[6] by a D-Trp results in a peptide resistant to hydrolysis by soluble metalloendopeptidase. As shown in Table 6, this stereospecific enzyme will not cleave peptides containing D-amino acid residues in either the P_1 or P_1' position. Whether higher potency of LHRH analogs, or other bioactive peptide analogs, is due to higher receptor affinity, increased resistance to proteolytic enzymes or decreased clearance from the circulation, or a combination of all the above, is still open to debate. The observation, however, that the analog D-Leu[6] des-Gly-NH_2[10]-LHRH ethylamide is about 30 times more active than LHRH (Vilchez-Martinez et al., 1974) and yet appears to have the same affinity as LHRH for the receptor (Wagner et al., 1979) suggests that an increased resistance to degradation might be the more important factor.

Another peptide of interest, neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), a tridecapeptide first discovered in and isolated from bovine hypothalamic extracts (Carraway and Leeman, 1973) has also been characterized in calf (Kitabgi et al., 1976) and human small intestine (Hammer et al., 1980). This peptide induces many biological responses such as vasodilation, hypotension, increased vascular permeability, hypoglycemia, hypothermia, and increased secretion of ACTH, LH, FSH, GH, and prolactin (Leeman and Carraway, 1982). Soluble metalloendopeptidase hydrolyzes neurotensin between the Arg^8-Arg^9 bond. The enzyme cleaves this peptide extremely efficiently, with a specificity constant almost 30 times greater than the model synthetic substrate used in routine assays (Tables 9 and 10). Studies were conducted by Aronin et al. (1982) on the metabolic breakdown products formed after intravenous injection of synthetic neurotensin into rats. It was found that the major metabolite of neurotensin involved a trypsin-like split between Arg^8 and Arg^9. Neurotensin was rapidly metabolized with an apparent half-life of 0.55 min. An activity from the soluble fraction of rat brain synaptic membranes was found to inactivate neurotensin by cleavage of this Arg^8-Arg^9 bond (Checler et al., 1983a). Conversion of neurotensin to neurotensin(1-8) was the major route by which

this peptide was inactivated *in vitro*. This soluble degrading activity was inhibited by the metalloprotease inhibitor o-phenanthroline, but was not inhibited by DTT. Furthermore, the same LHRH-degrading activity described by McDermott and coworker (1983) also specifically cleaves neurotensin at this Arg^8-Arg^9 bond.

By analogy with the LHRH superagonists, it might be expected that highly potent analogs of neurotensin would incorporate D-amino acid substitutions at specific positions. Not suprisingly, the analogs D-Tyr^{11}-neurotensin and D-Phe^{11}-neurotensin were found to be resistant to brain peptidases *in vivo* and *in vitro* (Checler et al., 1983b). These analogs were not degraded by the synaptosomal metallo-endopeptidase suggestively similar to soluble metalloendopeptidase (Checler et al., 1983a). Soluble metalloendopeptidase apparently requires binding of the position 11 Tyr residue to the S_3' subsite of the enzyme for proper hydrolysis of neurotensin. Whether a D-amino acid residue in the P_3' position can bind to the S_3' subsite of the enzyme remains to be determined.

The high activity of the enzyme in brain and pituitary, and its possible function in neuropeptide processing and degradation prompted efforts aimed at designing specific

active site directed inhibitors. Such inhibitors could be used as probes to demonstrate that inhibition of the enzyme prevents the processing and degradation of specific neuropeptides within the intact organism, thereby proving the participation of the enzyme in peptide metabolism. It was expected that inhibitor studies would provide additional insight into the substrate recognition site of the enzyme

Metalloproteases are inhibited by simple anions and chelating agents, such as EDTA and o-phenanthroline. Data have been presented showing that metal coordinating substrate analogs, compounds which incorporate characteristics essential for binding to the substrate recognition site of the enzyme and having a group capable of coordinating with the active site zinc atom, act as highly specific inhibitors of such enzymes (Holmquist and Vallee, 1979). Indeed, peptides fulfilling the binding requirements of thermolysin, a bacterial metalloendopeptidase, and containing N-terminal hydroxamate, thiol, carboxyl, or phosphoramidate zinc-coordinating groups have been shown to act as potent competitive inhibitors of this enzyme (Kam et al., 1979; Nishino and Powers, 1978; Maycock et al., 1981). Similar highly specific inhibitors of angiotensin converting enzyme (Cushman et al., 1977; Patchett et al., 1980) and membrane-bound metalloendopeptidase (Fournie-Zaluski et al., 1982; Mumford

et al., 1982; Almenoff and Orlowski, 1983) have also been synthesized. We proceeded, therefore, to synthesize a series of N-carboxymethyl derivatives containing that part of the substrate which was postulated to bind to the S_1'-S_3' subsites of the enzyme (Figure 5). Indeed, as expected the synthesized derivatives (Table 12) act as strong inhibitors of the soluble metalloendopeptidase.

The assumption that the interaction of a hydrophobic residue with a hydrophobic pocket in the S_1 subsite of the enzyme is important for binding was tested by determining the inhibitory potency of a series of N-carboxymethyl derivatives with varying degrees of hydrophobicity in the P_1 position. The finding that the inhibitory constant of N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (Table 12) is 35 times lower than that of the corresponding N-carboxymethyl derivative (K_i 1.94 uM versus 69.4 uM) indeed supports this assumption.

The importance for inhibition of the carboxylate group as a moiety capable of coordinating with the active site zinc atom is shown by the finding that N-phenylethyl-Ala-Ala-Phe-pAB, a compound not having this group, is by three orders of magnitude less inhibitory than the analogous N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB. In addi-

tion to coordinating with the zinc atom, this group may also impose some conformational restrictions on the hydrophobic side chain of the inhibitor, thereby favoring its interaction with the S_1 subsite of the enzyme. In the absence of this group, the benzyl group of the phenylethyl derivative is free to assume many additional conformations which might not lead to interaction with the hydrophobic S_1 subsite.

The importance of interactions at the active site zinc and at both the S_1 and S_3' subsites of the enzyme for inhibition is also indicated by the finding that N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Phe-pAB, an analog of the most potent inhibitor missing a single Ala residue, is only weakly inhibitory (K_i 2640 uM versus 1.94 uM; Table 12). One can suggest that the binding of this inhibitor to the S_1 subsite and to the zinc atom prevents interaction between the Phe residue and the S_3' subsite and vice versa, binding to the S_3' subsite prevents interactions with the zinc atom and the S_1 subsite. Neither of the membrane-bound metalloendopeptidase inhibitors (compounds II and III, Table 12) inhibited the soluble brain enzyme.

Our data indicate that inhibition of the soluble metalloendopeptidase requires the presence of a group fulfilling the binding requirements of the active site and the presence

of a zinc coordinating group, as postulated by Holmquist and Vallee (1979) for metallopeptidases in general. In addition, a tetrahedral geometry is present in these inhibitors at the scissile CO-NH locus, through the R, H, COOH, and NH groups, probably mimicking the transition state for peptide hydrolysis. As suggested by Wolfenden (1972) and Leinhard (1973), compounds mimicking the transition state of a substrate would be expected to bind tightly to the active site of an enzyme and therefore act as potent enzyme inhibitors. The proposed mechanism by which metalloproteases catalyze the hydrolysis of peptide bonds is not totally resolved, however, there are some indications that substrates for these enzymes undergo a transient change into a tetrahedral geometry (Hartsuck and Lipscomb, 1971). Therefore, the inhibition exhibited by the N-carboxymethyl derivatives might be partially contributed by their resemblence to the transition state for peptide hydrolysis.

The endopeptidase purified in our laboratory exhibits all the characteristics indicative of a metalloenzyme. Since, however, only minute amounts of enzyme could be isolated from rat brain, we have not been able to determine its zinc content by metal analysis. The demonstration that the presence of a carboxylate group capable of interacting with the active site metal is required for inhibition in the series

of N-carboxymethyl peptides described here, represents additional proof that this enzyme is indeed a metalloendopeptidase. Furthermore, our inhibitor studies fully confirm the conclusions related to the topography of the active site as derived from studies with model synthetic substrates. A schematic representation of the interaction of the inhibitor with the active site is given in Figure 7. The phenylethyl group and the Phe residue of the inhibitor fit into the hydrophobic pockets of the active site in a similar fashion as the Phe residues of the substrate (Figure 5). Also, the zinc ion that polarizes the carbonyl group of the scissle bond of the substrate is coordinated by the carboxylate group of the inhibitor. While these studies verify the preference of the enzyme for hydrophobic residues in the P_1 position, information concerning the binding requirements of the S_1' and S_2' subsites and the importance of hydrogen bonding between groups of the enzyme and the carbonyl oxygens of the substrate is still lacking. Such information could provide a basis for the synthesis of even more potent inhibitors than those described here.

Using the specific active site directed inhibitor, N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB, preliminary studies were undertaken to determine what role the soluble metalloenzyme plays in neuropeptide processing and

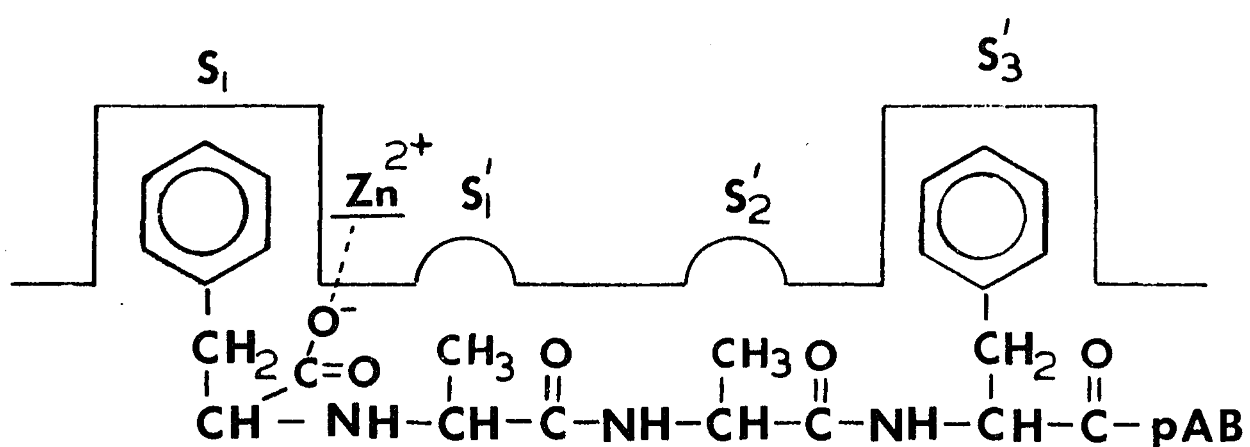


Figure 7. Schematic Representation of the Active Site of the Enzyme and its binding of the inhibitor N- 1-carboxy-2-phenylethyl -Ala-Ala-Phe-pAB.

degradation. Since the decapeptide neurotensin appears to be inactivated *in vivo* by a soluble metalloendopeptidase-like activity (Checler et al., 1983a; McDermott et al., 1983), initial efforts were aimed at attempting to potentiate the hyperglycemic response in rats to peripherally administered neurotensin (Carraway et al., 1976), by inhibition of this enzyme. Animals were first pretreated with N-[1(R,S)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB before injection of neurotensin. These experiments, however, failed to show a potentiation of neurotensin induced plasma glucose increase (data not shown). Inability of this inhibitor to potentiate the actions of this decapeptide does not necessarily preclude a role for soluble metalloendopeptidase in neurotensin metabolism. Several factors may have negatively effected the outcome of these preliminary studies. Animals were given a single bolus of inhibitor at a concentration of 40 umol/kg. Since the K_i of this inhibitor is only about 2 uM, the amount administered may not have been sufficient. Also, since the inhibitor contains a peptide sequence, it is susceptible to cleavage by other peptidases such as membrane-bound metalloendopeptidase. Metabolism of the inhibitor would therefore further diminish its effective concentration. Attempts at infusing larger amounts of inhibitor over the course of the experiments, however, suf-

fered from volume overloading of the rats. Furthermore, the possibility exists that the neurotensin-induced hyperglycemic response is partially regulated at the receptor level. Desensitization of these receptors would obscure a prolonged hyperglycemic response due to an increased half-life of neurotensin. Actual measurement of peptide levels in plasma would therefore give more accurate information than measurement of plasma glucose levels. The enzyme is found in the soluble fraction of rat brain homogenates; an important issue unanswered in these studies is whether the inhibitor actually reaches this target, as well as other organs containing this enzyme. All these points must be addressed before one can conclude that soluble metalloendopeptidase is not involved in the degradation of neurotensin.

The direction of further study of this enzyme is apparent. Of primary importance is the design of new inhibitors with substantially higher affinities. Ideally, irreversible or "suicide" inhibitors of this enzyme should be developed. Design of such inhibitors should also incorporate features to minimize degradation by other proteolytic enzymes. With proper targeting of these inhibitors, one should be able to probe the _in vivo_ function of the enzyme in neuropeptide processing and degradation.

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