INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9108167

Endopeptidase 24.15: A neuropeptide metabolizing enzyme. Isolation, localization, possible function

Reznik, Sandra Eve, Ph.D.

City University of New York, 1990

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

A

ENDOPEPTIDASE 24.15: A NEUROPEPTIDE METABOLIZING ENZYME. ISOLATION, LOCALIZATION, POSSIBLE FUNCTION.

by

SANDRA REZNIK

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.

1990

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

July 13, 1990
Date

Marian Orlowski
Marian Orlowski, M.D.
Chair of Examining Committee

July 16, 1990
Date

Terry A. Krulwich, Ph.D.
Executive Officer

Sherwin Wilk
Sherwin Wilk, Ph.D.

Dennis Healy
Dennis Healy, Ph.D.

Supervisory Committee

The City University of New York

ABSTRACT

Endopeptidase 24.15: A Neuropeptide Metabolizing Enzyme. Isolation, Localization, Possible Function.

by

Sandra Reznik

Adviser: Dr. Marian Orlowski

Endopeptidase 24.15, a metalloendopeptidase (EC 3.4.24.15) with an M_r of about 70,000 that rapidly converts dynorphin(1-8), α-neo-endorphin, β-neo-endorphin, Met-enkephalin-Arg-Gly-Leu, and metorphamide into the respective enkephalins, was purified to homogeneity from rat testes. The isolated enzyme is inhibited by metal chelators and by thiols. Loss of enzymic activity after dialysis against EDTA can be restored by low concentrations of Zn^{2+} and Co^{2+} ions. These results are consistent with the classification of the enzyme as a metalloendopeptidase. The testis enzyme is catalytically and immunologically closely related to the previously identified brain enzyme.

The enzyme was purified from rat brain by immunoaffinity chromatography. Both forms of the brain enzyme, soluble and

membrane-bound, were obtained; the soluble form was purified to homogeneity. As previous efforts to isolate the brain enzyme had failed to remove an inactive component from the preparation, this is the first time the brain enzyme has been so highly purified. The brain enzyme was inhibited by EDTA and EGTA in accordance with its classification as a metalloenzyme. The brain and testis enzymes appeared to be closely related catalytically and immunologically. The testis enzyme migrated somewhat more slowly in SDS PAGE than the brain enzyme, suggesting a higher molecular weight than the brain enzyme. The membrane-bound brain enzyme was found to be catalytically and immunologically similar to the soluble brain enzyme, but of less mobility in SDS PAGE. The slower migration suggests that the membrane-bound enzyme may contain an additional hydrophobic sequence for anchoring in the plasma membrane.

Endopeptidase 24.15 is most highly concentrated in testis, brain, anterior pituitary, and spinal cord. The high relative activity in testis prompted us to examine the localization of the enzyme within the testis immunocytochemically. Two cell populations containing the enzyme were identified immunocytochemically: Leydig cells and spermatazoa. Preincubation of the immune serum with the enzyme resulted in dramatically diminished or abolished staining. The enzyme may act on the peptide precursors of Met- and Leu-enkephalin to release these enkephalins in the testis and/or modulate the paracrine or autocrine function of LHRH in the testis.

ACKNOWLEDGEMENTS

I wish to express my thanks to:

Dr. Marian Orlowski, for his wisdom, patience, and guidance in every phase of my graduate career,

Dr. Dennis Healy, for his teaching and assistance in immunocytochemical studies,

Dr. Sherwin Wilk, for his advice on my thesis work,

Charlene Michaud, for her expert technical assistance and teaching,

Julia Ayala, for her interesting ideas and technical assistance,

Elizabeth Wilk, for her technical assistance in phlebotomy,

Dr. Terry A. Krulwich, for her enthusiasm for my work and for providing financial support of my studies through the Medical Scientist Training Program,

and to my dear husband, Eddie, for his constant love and for making the sacrifices that made this thesis project possible.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

EDTA, ethylenediaminetetraacetic acid

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

t-Boc-FAAF-pAB, tertiary butoxycarbonyl-Phe-Ala-Ala-Phe-para-aminobenzoate

cFP-AAF-pAB, N-[(1RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-para-aminobenzoate

cFP-AAY-pAB, N-[(1RS)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-para-aminobenzoate

cFE-AAF-pAB, N-[(1RS)-carboxy-2-phenylethyl]-Ala-Ala-para-aminobenzoate

SDS, sodium dodecyl sulfate

PAGE, polyacrylamide gel electrophoresis

LIST OF TABLES

Title	Page

LIST OF FIGURES

Title **Page**

BACKGROUND

Proteolytic enzymes function in physiological processes ranging from the digestion of proteins to amino acids to post-translational modifications of polypeptides. Localization of proteolytic enzymes has shown that they occur in association with cells and extracellularly. Although some extracellular proteolytic enzymes have been well studied, for many cellular proteases, the physiological substrates, mechanisms of regulating proteolysis, and physiological roles have not yet been identified. As the subject of this dissertation is a cellular endopeptidase, the following discussion will focus on cellular proteolytic enzymes.

Cellular proteases range in molecular weight from 20 to 800 kDa. Some, such as the lysosomal proteases, are monomeric, but oligomeric proteases containing subunits of 50 to 100 kDa occur. Non-proteinaceous components, such as sugar moieties, lipids, and divalent cations make up parts of cellular proteases. Cellular proteases are found in both the membrane fraction and the cytosolic fraction of cells.

Membrane-bound proteases identified so far have been classified as serine or metalloproteases. A distinction should be made between membrane-associated enzymes that can be dissociated from membranes by relatively mild treatments, such as washing with high ionic strength buffers, and truly intrinsic enzymes that must be extracted by harsh treatments, generally solubilization by detergents. Intrinsic membrane-bound enzymes can have very narrow to very broad specificities. Enteropeptidase,

for example, acts specifically on trypsinogen to release trypsin (Liepnieks & Light, 1979); procollagen C-terminal proteinase specifically hydrolyzes C-terminal peptide bonds in procollagen (Hojima et al., 1985). The membrane-bound enzyme meprin, on the other hand, acts on many different substrates (Beynon et al., 1981; Bond & Beynon, 1986). The intrinsic plasma membrane-bound proteases include enzymes such as endopeptidase 24.11 (EC 3.4.24.11), that preferentially act on small peptides (Turner et al., 1985), as well as enzymes that act on large proteins, such as meprin (Beynon et al., 1981; Bond & Beynon, 1986). The kidney brush border has proven particularly rich in membrane-bound proteases. Kidney brush border exopeptidases include aminopeptidase N and angiotensin converting enzyme (Kenny, 1986). Endopeptidases include meprin and .24.11. Ultrastructural immunocytochemistry indicates that both angiotensin converting enzyme and endopeptidase 24.11 are localized on the outside of the brush-border plasma membrane of epithelial cells in the proximal tubule (Schulz et al., 1988).

The possibility of a cellular protease occurring in both membrane-bound and soluble forms that may differ slightly in molecular weight, because of the absence or presence of a membrane anchoring sequence, should be addressed. Carboxypeptidase E (EC 3.4.17.10, also known as enkephalin convertase and as carboxypeptidase H, has been found in both soluble and membrane fractions of the secretory granules or tissue homogenates (Hook & Loh, 1984; Supattapone et al., 1984). After solubilization with detergent the membrane-bound form of carboxypeptidase E was

found to have similar properties to the soluble form of the enzyme, but to be of slightly higher molecular weight. The membrane-bound form of carboxypeptidase E was calculated to be 52-53 kD, while the soluble form of the enzyme had an M_r of 50 kD. This slight difference in molecular weight between the two forms occurred in enzymes isolated from bovine adrenal medulla, brain, and pituitary.

Endopeptidase 24.15 (EC 3.4.24.15), a metalloendopeptidase discussed in the following chapters, is predominantly associated with the soluble fraction of tissue homogenates (Orlowski et al., 1983), but also occurs in a membrane-bound form. Approximately 20% of enzyme activity is found in the membrane fraction in rat brain homogenates (Acker et al., 1983), while less than 10% of the activity is accounted for by a membrane-bound form of the enzyme in rat testis (Orlowski et al., 1989). Whether the two forms of the enzyme differ by a hydrophobic membrane-spanning region is not known.

One role of cellular proteases that has been identified is the post-translational processing of proteins. The expression of proteins in the form of pre- and proproteins is preserved across species and probably evolved before the appearance of vertebrates (Hobart et al., 1980; Chan et al., 1981). According to the Signal Hypothesis of Blobel and Dobberstein (Blobel & Dobberstein, 1974), bioactive peptides first occur within preproteins or preproproteins that include N-terminal signal peptides. The signal peptides play a role in the initial segregation of the peptides in the endoplasmic reticulum. The signal peptides are removed by signal peptidases, which are cellular proteases in the metalloenzyme class that

hydrolyze peptide bonds adjacent to small amino acid residues, such as Ala, Cys, Gly, and Ser. After the removal of the signal peptides, enzymes then process the proproteins until the active proteins or peptides are released.

With the exception of a few hormones, such as growth hormone and prolactin (Mittra, 1980a; Mittra, 1980b), processing is carried out by cellular endopeptidases. Two families of processing proteases in the serine protease class have been well characterized. One is a family of arginine residue specific serine proteases. Included in this family are epidermal growth factor (EGF) binding protein and nerve growth factor (NGF) endopeptidases (Ashley & MacDonald, 1985; Bothwell et al., 1979; Schenkein et al., 1981; Taylor et al., 1974; Thomas & Bradshaw, 1981; Frey et al., 1979). The NGF endopeptidases consist of multisubunit complexes containing two α, one β, and two γ subunits and one or two zinc atoms per molecule (Thomas et al., 1981). The γ subunits are processing endopeptidases highly specific for NGF proprotein. The tissue kallikreins make up another family of processing serine proteases. These enzymes release kinins from kininogens such as lysyl-bradykinin and may also be involved in the processing of insulin from proinsulin (Ole-Moi Yoi et al., 1979).

The processing endopeptidases generally have neutral to acidic pH optima. They are found in secretory granules, which are believed to arise from clathrin-coated vesicles. As the secretory granules mature, they become more acidic, inhibiting the activity of the processing enzymes. The processing takes place in the clathrin-

coated vesicles and secretory granules before release of the active peptides at the sites of action.

In addition to the peptide hormones and neuropeptides, many proteins must be activated by processing enzymes, including proalbumin (Russell & Geller, 1975) and prorenin (Panthier et al., 1982). Processing enzymes play a role in maturation for a class of bioactive peptides that has emerged in the past 15 years, the enkephalins.

The discovery of Met- and Leu-enkephalin (Hughes et al., 1975) was followed by the identification of several more peptides with opioid activities, some more potent than the pentapeptides. A series of peptides consisting of Met-enkephalin extended at the C-terminus by one to three amino acids emerged, including Tyr-Gly-Gly-Phe-Met-Arg, Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu, and Tyr-Gly-Gly-Phe-Met-Arg-Phe (Stern et al., 1979), the latter having eight times the antinociceptive activity of Met-enkephalin when administered into the cerebral ventricles of mice (Inturrisi et al., 1980). Dynorphin$_{1-13}$, a pituitary and guinea pig ileum muscle peptide with approximately 700 times the opioid activity of Leu-enkephalin (Goldstein et al., 1979) was identified at the same time (Huang et al., 1979), where opioid activity is measured by inhibition of the electrically stimulated twitch of guinea pig ileum longitudinal muscle.

The opioid peptides have been implicated in several physiological functions besides analgesia, including the regulation of body temperature (Gunne, 1960), learning (Stein & Belluzzi, 1979), sexual activity (Meyerson & Terenius, 1977; Meyerson & Berg,

1977), motor activity (Bhargava, 1978; Frigeni et al., 1978; Amir et al., 1979), feeding (Gambert et al., 1980; Grandison & Guidotti, 1977; Garthwaite et al., 1980; Aou et al., 1988), and drinking (DeCaro et al., 1979). The variety of functions served by the many identified opioid peptides in the central nervous system is reflected by the occurrence of multiple receptor types, selective for different peptides. Briefly, μ receptors have high affinity for β-endorphin, metorphamide (Weber et al., 1983), and the enkephalins; κ receptors bind preferentially to Leu-enkephalin and its C-terminally extended derivatives (Chavkin & Goldstein, 1981); and δ receptors interact mainly with the enkephalins (Paterson et al., 1983). The opioid peptides also occur peripherally and have local effects on intestinal motility (Rozé, 1989), cardiac function (Vargish & Beamer, 1989), and testicular function (Engelhardt, 1989).

The structural similarities among the identified opioid peptides suggested that they were derived from common parent molecules (Lewis et al., 1979). In 1982 the complete sequence of bovine proenkephalin was described simultaneously by two groups (Gubler et al, 1982; Noda et al, 1982) and sequencing of human proenkephalin soon followed (Comb et al., 1982). Proenkephalin contains one copy of Leu-enkephalin, four copies of Met-enkephalin and one copy each of Met-enkephalin-Arg^6-Phe^7 and Met-enkephalin-Arg^6-Gly^7-Leu^8 (Kakidani et al., 1982; Coomb et al, 1982; Noda et al., 1982, Rossier 1982; Gubler et al., 1982). In the same year prodynorphin was sequenced and found to contain dynorphin A_{1-17}, dynorphin A_{1-13}, dynorphin A_{1-8}, α-neoendorphin, β-neoendorphin, and dynorphin B, or rimorphin, each of which contains a Leu-

enkephalin sequence in the molecule (Kakidani et al., 1982). Proopiomelanocortin (POMC) had been sequenced previously and shown to be the common precursor of adrenocorticotrophic hormone (ACTH), the melanocyte stimulating hormones (MSH's), and β-endorphin (Nakanishi et al., 1979).

Homology among the three parent molecules has led to speculation that they evolved from a common ancestor molecule. All three precursors contain at least one enkephalin and/or enkephalin containing peptide in the C-terminal region, a cysteine rich N-terminal region, and an N-terminal signal peptide. Furthermore, sulfhydryl group containing amino acid residues, marking points of potential disulfide bridges, occur at the same sites in the N-terminal regions of the three molecules.

The opioid peptides released from the precursors discussed above, as well as many other bioactive peptides, are flanked by pairs of basic amino acids. This pattern was first described for proinsulin (Steiner et al., 1975). The importance of these framing amino acids is reflected by the fact that in their absence bioactive peptides are not released. For example, Robbins, et al. (1981) described a family in which several members with diabetes mellitus carried an autosomal dominant gene for hyperproinsulinemia. The proinsulin-like material in these patients' blood was identified as a biosynthetic intermediate of proinsulin conversion, in which the C peptide remains joined to the insulin A chain. Further analysis of the structure of this peptide showed that the Arg^{65} residue, at the normal site of cleavage between the A chain and the C peptide, was substituted. Similarly, seven patients with proalbuminemia

belonging to one family were found to have high blood levels of a proalbumin-like peptide. The terminal amino acid residue of the N-terminal segment normally removed in proalbumin conversion was changed in this peptide from Arg^6 to Gln^6. Pairs of basic amino acids at the N-terminus of β-MSH and at the C-terminus of γ_1-MSH do not occur in the rat precursors of these peptides, but do occur in the bovine precursors. Accordingly, the peptides are released in cow pituitaries but not in rat pituitaries.

All possible sequence combinations of Lys and Arg flank bioactive peptides; Lys-Arg has been found most often. Occasionally proteolytic cleavage to release bioactive peptides occurs adjacent to single basic amino acids, especially Arg. Such cleavages occur at the C-terminal end of neurophysin II and at the N-terminal ends of somatostatin-28 and cholecystokinin-8 (Docherty & Steiner, 1982; Goodman et al., 1982). In one third of such post-Arg cleavages a Pro residue is adjacent to the Arg residue.

In light of the flanking dibasic amino acid pairs, it was believed that the release of bioactive peptides such as insulin, gastrin, somatostatin, glucagon, ACTH, α-MSH, β-MSH, β-lipotropin, β-endorphin, Leu-enkephalin, α-neoendorphin, β-neoendorphin, Met-enkephalin, parathyroid hormone, and albumin (Steiner et al., 1980 for review) proceeded by the sequential actions of two enzymes (Steiner et al., 1980; Fricker & Snyder, 1984; Lindberg et al., 1984; Evangelista et al., 1982; Troy & Musacchio, 1982). According to the hypothesis, first a trypsin-like enzyme cleaved the peptide bond on the C-terminal side of the pair of basic amino acids. A carboxypeptidase B-like enzyme would then have to catalyze the

removal of the exposed Lys and/or Arg residues. The identification of a carboxypeptidase B-like metalloenzyme in various endocrine organs (Hook & Loh, 1984; Fricker & Snyder, 1982) supported this hypothesis. Furthermore, an enzyme that removes C-terminal Arg residues from enkephalins was identified and called "enkephalin convertase," although the same enzyme processes ACTH and vasopressin (Hook & Loh, 1984).

The finding that all pairs of basic amino acids are not hydrolytically cleaved off suggests that the release of bioactive peptides occurs not just by trypsin and carboxypeptidase B-like activity, but by enzymes that are affected by amino acids surrounding the flanking pairs. Furthermore, the finding that some bioactive peptides are released in some tissues but not in others suggests that processing enzymes may be tissue specific. For example, somatostatin-14 is the main somatostatin precursor derived peptide in the pancreas, whereas little somatostatin-14 but much somatostatin-28 is found in the stomach (Trent & Weir, 1981; Patel et al., 1981). Likewise, ACTH is found in the anterior lobe of the pituitary, but not the intermediary lobe, while MSH is present in the intermediary lobe and absent from the anterior lobe of the pituitary.

Further evidence of tissue specificity of the processing enzymes, and cellular specificity of these enzymes in heterogeneous tissues such as brain, is provided by the variation of concentrations of opioid peptides throughout the central nervous system. Both ratios of prodynorphin derived peptides and ratios of proenkephalin derived peptides vary, although ratios of prodynorphin derived

peptides vary more (Sonders & Weber, 1987). In the neurointermediate lobe of bovine pituitary, neoendorphins are five times as abundant as dynorphin A or B, but in the caudate-putamen, both dynorphin A and dynorphin B are twice as highly concentrated as the neoendorphins. Met-enkephalin-Arg-Phe is 1.4 times as abundant as Met-enkephalin-Arg-Gly-Leu in brain and pituitary, but half as abundant in spinal cord. The ratio of α–neoendorphin to β–neoendorphin is 13 in the bovine neurointermediary pituitary and 0.25 in the bovine hypothalamus. In most brain regions the ratio is much greater than 1 (Cone et al., 1983; Weber et al., 1982), a finding that suggests the presence of an enzyme that preferentially cleaves β-neoendorphin. In fact, the specificity constant of β–neoendorphin for endopeptidase 24.15 is much higher than that of α-neoendorphin (Chu & Orlowski, 1985).

Enzymes in the metalloprotease class such as endopeptidase 24.11 and endopeptidase 24.15 may each act on multiple substrates and play multiple roles in the organism, depending on the specific biochemical requirements of the cell or tissue containing the enzyme. Endopeptidase 24.11, first isolated from kidney, in addition to acting on angiotensin I, angiotensin II, and bradykinin in this tissue, may be involved in the digestion of filtered peptides to amino acids (Gafford et al., 1983). The enzyme is also known as "enkephalinase" because of its role in inactivation of enkephalinergic signals in the brain, where it cleaves Met- and Leu-enkephalin. (Schwartz et al., 1981). The same enzyme may play a role in the maturation of airway epithelium by acting on vasoactive peptides, such as bradykinin, in fetal lung (Johnson et al., 1985).

The enzyme occurs in placental microvilli and may be involved in the control of uterine stimulation by acting on oxytocin in that tissue (Johnson et al., 1984). Endopeptidase 24.11, along with endopeptidase 24.15 (Orlowski et al., 1989), angiotensin converting enzyme, and leucine-aminopeptidase (Heder et al., 1989), occurs in the male reproductive tract and may have a role in proacrosin activation and sperm maturation (Erdös et al., 1985). Endopeptidase 24.11 also occurs in cells involved in the immune response. Specifically, the enzyme has been found in neutrophils, where it may regulate chemotaxis and inflammation by acting on the chemotactic peptide formyl-Met-Leu-Phe and on bradykinin (Connelly et al., 1985; Painter et al., 1988).

In addition to having all of the potential physiological functions stated above, endopeptidase 24.11 has turned out to be identical with the CD (cluster differentiation) cell surface antigen CD10 or CALLA (common acute lymphoblastic leukemia antigen), a well-known protein occurring on lymphoid precursor and myoepithelial cells. (Malfroy et al., 1987; Letarte et al., 1988; Shipp et al., 1989). The same protein studied from different approaches was characterized in parallel as a malignant cell marker and as an endopeptidase. Shortly after the revelation that endopeptidase 24.11 and CALLA are identical, another specific hemopoietic marker, CD13, was shown to be identical to aminopeptidase N (Look et al., 1989). Dipeptidyl peptidase IV was soon after found to be identical to CD26 (Schön et al., 1989). Cell surface peptidases may function to regulate the concentrations of active peptides in the hemopoietic or myoepithelial cell milieu. The local concentrations of these

active peptides, in turn, may modulate cell growth and differentiation.

Endopeptidase 24.15 may also emerge as a metalloendopeptidase with multiple roles. Originally purified from brain, the enzyme may convert dynorphin $A_{(1-8)}$, α-neo-endorphin, and β-neo-endorphin to Leu-enkephalin, and Met-enkephalin-Arg-Gly-Leu to Met-enkephalin in the central nervous system. The enzyme is the primary factor that degrades luteinizing hormone releasing hormone in hypothalamic and pituitary particulate fractions (Molineaux et al., 1988) and may regulate this peptide hormone *in vivo* . Endopeptidase 24.15 activity is abundant in rat testes (Orlowski et al., 1989), where it may act on LHRH and opioid peptides. A common thread among the diversity of functions in various tissues of metalloendopeptidases such as endopeptidase 24.11 and endopeptidase 24.15 has yet to be identified.

INTRODUCTION

Previous work in this laboratory led to the identification and partial purification from rat brain of a metalloendopeptidase predominantly associated with the soluble fraction of homogenates (Orlowski et al., 1983). A membrane-bound form of the enzyme constituting approximately 20% of the total activity, and having properties similar to those of the soluble form, was found associated with brain particulate fractions, including synaptosomes (Acker et al., 1983). The finding that endopeptidase 24.15 is highly active in brain, pituitary and testis, and that it does not apparently cleave proteins and large peptides containing more than 20 amino acid residues, suggests that the enzyme is involved in the metabolism of bioactive peptides.

Endopeptidase 24.15 together with endopeptidase 24.11 (Kerr & Kenny, 1974; Almenoff et al., 1981; Matsas et al., 1984; Littlewood et al., 1988) seem to constitute the main two brain metalloendopeptidases. Unlike endopeptidase 24.11, also misleadingly called "enkephalinase" because of its cleavage of the Gly-Phe bond in both Leu- and Met-enkephalin (Malfroy et al., 1978), endopeptidase 24.15 does not attack the two enkephalins. Indeed, experiments with purified preparations of the enzyme from rat brain and also experiments with synaptosomal membranes have shown that the enzyme converts some larger opioid peptides such as dynorphin A_{1-8}, β-neoendorphin and Met-enkephalin-Arg^6-Gly7-Leu^8 into Leu- and Met-enkephalin respectively in reactions inhibited by

cFE-AAF-pAB, a substrate-related specific inhibitor of the enzyme (Acker et al., 1987; Chu & Orlowski, 1985).

Isolation of a homogeneous preparation of endopeptidase 24.15 from rat brain by conventional enzyme purification methods presented difficulties (Orlowski et al, 1983). Preparations contained an inactive protein contaminant of almost the same isoelectric point and molecular mass, that could not be removed by ion exchange chromatography, preparative gel electrophoresis or chromatofocusing. Since rat testes are rich in endopeptidase 24.15, efforts were directed towards isolation of the enzyme from this source. Most of the enzyme activity in rat testes is associated with the soluble protein fraction of homogenates, whereas the membrane-bound form of the enzyme constitutes only about 8% of the total activity. The enzyme was purified to homogeneity and its interaction with synthetic substrates and with substrate related active site directed inhibitors was studied.

Preparations of pure testicular enzyme were used for immunization to generate a polyclonal antiserum. A purification procedure using an immunoaffinity column constructed with the polyclonal antiserum was developed. The method eliminates several steps in the conventional purification procedure of the enzyme, including Sephadex G-100 chromatography, DEAE-Sephacel chromatography, and preparative PAGE. The immunoaffinity purification procedure yielded highly purified endopeptidase 24.15 in only a few days, allowing comparison studies of the enzyme obtained from different tissues as well as of the two forms of the enzyme found in a single tissue to be carried out. Accordingly, the soluble

testis enzyme is compared to the soluble brain enzyme, and the soluble brain enzyme to the membrane-bound brain enzyme, kinetically and immunologically. Evidence of differences in migration in SDS PAGE, suggesting slight differences in molecular weight among the three enzymes, is presented. Since potential substrates for the enzyme, including pro-dynorphin-derived peptides (Cox et al., 1987) and an LHRH-like peptide (Sharpe & Fraser, 1980a), have been identified in the rat testis and since endopeptidase 24.15 activity is highest in this tissue, we were interested in using the antiserum to localize the enzyme immunocytochemically in the rat testis.

Endopeptidase 24.15 is the primary LHRH-degrading enzyme in pituitary and hypothalamic membrane preparations (Acker et al., 1987) and appears to be the dominant factor regulating LHRH degradation in vivo (Lasdun et al., 1989). The main cleavage site of LHRH in vitro is at the Tyr^5-Gly^6 bond (Molineaux et al., 1988). This cleavage pattern places Tyr^5 and Arg^8 residues in the S_1 and S_3' subsites[1] of the enzyme, respectively. The fact that endopeptidase 24.15 acts preferentially on substrates that have hydrophobic or bulky residues in those subsites (Chu & Orlowski, 1985) suggests that LHRH may serve as a substrate in vivo.

Exogenous LHRH affects rat Leydig cell steroidogenic function directly by binding to specific receptors (Clayton et al., 1980). This finding reflects the paracrine action of an endogenous LHRH-like peptide, secreted from Sertoli cells, on Leydig cell function (Sharpe et al., 1981; Tähkä 1986). Endopeptidase 24.15 may modulate this paracrine system by regulating the degradation of a rat testicular

LHRH-like peptide. We present evidence here for the localization of endopeptidase 24.15 in rat testicular interstitial cells. The cells are abundant in the interstitium, occur in groups, are pyramidal in shape, and have regularly shaped nuclei containing several nucleoli. These characteristics are all consistent with the identification of these cells as Leydig cells. Immunocytochemical studies also showed that the enzyme is localized to germ cells that are near maturity, suggesting that the enzyme may be involved in the maturation of spermatazoa and/or in fertilization. The localization of endopeptidase 24.15 in spermatids, is in accordance with the finding that several other metalloenzymes, including angiotensin converting enzyme, endopeptidase 24.11, and leucine aminopeptidase, occur in bull ejaculates and that the activities of these enzymes are in close correlation with fertility parameters (Heder et al., 1989).

MATERIALS AND METHODS

Reagents

Endopeptidase 24.15 substrates and inhibitors were synthesized as previously described (Chu & Orlowski,1984; Orlowski et al., 1983; Orlowski et al., 1988). Sprague Dawley rat brains and testes were obtained frozen from Pel Freeze Inc. (Rogers, AR) or were dissected from sacrificed animals and then frozen. All other reagents were purchased from Fisher Scientific Co. (Pittsburgh, PA), Sigma Chemical Co. (St. Louis, MO), or Bio-Rad (Richmond, CA).

Isolation of Rat Testicular Endopeptidase 24.15

All steps were performed at 4°C. Deionized water was used for the preparation of all buffers. A group of 100 frozen rat testes from Pel Freeze Inc. was thawed and homogenized in 4 volumes of ice cold 0.01 M Tris-HCl buffer (pH 7.6) containing 0.32 M sucrose and 0.5 mM 2-mercaptoethanol. The homogenate was centrifuged at 30,000g for 2 h. The supernatant was collected and applied to the top of a DEAE-cellulose column (DE-52, 200 ml bed volume) equilibrated with 0.01 M Tris-HCl, pH 7.6 containing 0.5 mM 2-mercaptoethanol. The column was washed with 1 l of the equilibrating buffer. A linear gradient established between 400 ml of equilibrating buffer and 400 ml of the same buffer containing 0.3

M NaCl was then attached to start elution. Fractions of 8 ml were collected and monitored for protein concentration by measuring the absorbance at 280 nm. Enzymatic activity was followed by assaying with Bz-Gly-Ala-Ala-Phe-pAB as the substrate. The enzyme emerged after 360 ml of the eluting buffer had passed through the column. Fractions within the central portion of the activity peak were pooled. The pooled fractions were assayed for protein (Lowry et al., 1951) and for activity as described above and then concentrated to about 40 ml by ultrafiltration in an Amicon concentrator. The concentrate was applied to the top of a 2 l Sephadex G100 column equilibrated with 0.05 M Tris-HCl, pH 8.0. Elution was accomplished by washing the column with one bed volume of the equilibrating buffer. Fractions of 20 ml were collected and tested for protein and activity as described above. The enzyme emerged following a large inactive protein peak, after about 780 ml of the eluting buffer had passed through the column. Fractions in the central portion of the active peak were pooled and applied to the top of a 15 ml DEAE-Sephacel column equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.5 mM 2-mercaptoethanol. The column was washed with 220 ml of the equilibrating buffer and then eluted with a linear gradient established between 150 ml of the same buffer and 150 ml of the buffer containing 0.3 M NaCl. Fractions of 3 ml were collected and the enzyme emerged from the column after about 100 ml of the eluting buffer had passed through the column. A large amount of inactive protein was eluted before and after the main activity peak. Fractions containing enzyme with the highest specific activity were

combined and desalted by passing through a 150 ml Sephadex G-25 column equilibrated with 0.01 M Tris-acetate buffer (pH 7.5).

Samples of the enzyme after step 4 of the purification procedure were subjected to non-dissociating disc PAGE as described above. Staining with Coomassie Brilliant Blue revealed the presence of two protein bands (Figure 1). Unstained disc gels were run in parallel and cut into 2 mm slices. The distribution of activity was examined by homogenizing the slices in 0.25 ml of Tris-HCl buffer (0.05 M, pH 7.0), and determining the activity in the gel extracts as described above. Enzyme activity was associated only with the slower migrating protein band. For isolation of the homogeneous enzyme, aliquots of the enzyme after step 4 of the purification procedure containing about 1 mg protein were concentrated to about 0.5 ml and subjected to preparative slab PAGE. The location of protein bands was visualized by staining gel strips from the edges and the center of the slab gel with Coomassie Brilliant Blue. Good separation of the two protein bands was consistently obtained. The slab gel was sliced into 2 mm strips and the gel strips containing activity were placed in dialysis bags filled with 1.5 ml of Tris-HCl buffer (0.05 M, pH 8.3). The enzyme was electroeluted from the gel by placing the dialysis bags in a flat slab gel electrophoresis apparatus containing the same buffer and applying a current of 140 V for 5 h. The buffer containing the enzyme was recovered from the dialysis bags (1.35 ml from each bag) and the solutions were tested for activity and assayed for protein by the method of Peterson (1977). Enzyme purity was determined by subjecting the recovered enzyme to PAGE under non-

dissociating and denaturing and dissociating conditions. Immunoreactivity toward anti-endopeptidase 24.15 antiplasma was tested by Western blotting (see below). Enzyme samples obtained by this procedure (usually 35-40 µg protein) were homogeneous by several criteria (see below).

Determination of Enzyme Activity

Enzyme activity was determined in a coupled enzyme assay in the presence of excess aminopeptidase N (EC 3.4.11.2) using a-N-Bz-Gly-Ala-Ala-Phe-pAB (0.8 mM) or t-Boc-Phe-Ala-Ala-Phe-pAB (0.4 mM) as the substrate (Chu and Orlowski, 1985). In the case of the first substrate, endopeptidase 24.15 cleaves the Gly-Ala bond, releasing Bz-Gly and Ala-Ala-Phe-pAB. Aminopeptidase N catalyzes the release of pAB from the latter product and the chromogen is then quantified after diazotization.

To account for the possible degradation of the substrate by other enzyme(s) in crude tissue homogenates enzyme activity was determined in the absence and presence of 25 µM N-[1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB, a specific inhibitor of endopeptidase 24.15 (Orlowski et al., 1988). The difference in the rate of substrate degradation between incubation mixtures with and without the inhibitor was taken as a measure of the enzyme activity. Activity is expressed in units, one unit being defined as the amount of enzyme that catalyzes the release of 1 µmol of product per hour. Specific activity is expressed as units per mg of protein and protein

concentration was determined by the method of Lowry et al. (1951) or Peterson (1977) with bovine serum albumin as the standard.

In anticatalytic immunoinhibition assays, the enzyme was preincubated in the absence or presence of a varying amount of anti-endopeptidase 24.15 antiserum for 30 min at 37°C. The reaction was then begun by the addition of the substrate.

For endopeptidase 24.15 tissue distribution studies, rats were sacrificed by decapitation and tissues were immediately excised and homogenized in 4 ice cold vols of 0.01 M Tris-HCl, pH 7.6 with an ice-cooled Potter-Elvehjem homogenizer and motor-driven Teflon pestle. Homogenates were centrifuged for 1 h at 20,000g and enzyme activity in supernatants was determined.

Leydig cells were purified from rat testes by collagenase dispersion and Percoll density gradient centrifugation as described (Eskeland et al., 1989). At least 60% of the cells in Leydig cell-enriched preparations following this method immunostained with the monoclonal antibodies LC-1C6 and LC-6H6 to Leydig cell surface antigens (Hedger & Eddy, 1986, kindly donated by Dr. Beth Schachter) and more than 90% of the cells were viable by trypan blue exclusion.

Determination of Kinetic Constants

The steady state parameters, K_m and k_{cat} (=V/e, where e=total enzyme concentration) were determined from initial velocity measurements at various substrate concentrations. Michaelis-Menten kinetics were observed. K_m values were calculated from

double reciprocal plots by a linear regression program. Correlation coefficients were generally 0.99 or better. In the calculation of data a M_r of 67,000 was assumed for the brain enzymes and a M_r of 70,000 was assumed for the testis enzyme, with one catalytic site per enzyme molecule.

K_i measurements were carried out by the method of Dixon (1972) at three different substrate concentrations and at 5 different inhibitor concentrations (plots of 1/V versus [I]) using a computer program. Coefficients of determination (r^2) of better than 0.99 were generally obtained.

Polyacrylamide Gel Electrophoresis

Disc PAGE was carried out under nondissociating conditions in a 0.05 M Tris-HCl buffer (pH 8.3). A sample of 1 to 10 µg of enzyme protein was layered on the top of each gel and a current of 4 mA per tube was applied for a time period necessary for the tracking dye to reach the bottom of the gel. Preparative slab PAGE was performed under similar conditions in gels of 3 mm thickness using a slab gel apparatus (model 100, Aquebougue Machine Shop; Box 205 Aquebougue, New York 11931). Electrophoresis under dissociating conditions and molecular weight determinations were carried out in the same apparatus using a discontinuous buffer system (Laemmli, 1970). Gels (8%) contained 0.1% SDS and electrophoresis was run after the enzyme had been heated to 70°C for 10 min in a solution of 1% SDS and 5% 2-mercaptoethanol. Molecular weight standards were

2.5 µg each of phosphorylase b, bovine serum albumin, ovalbumin, and trypsinogen.

Production of Antibodies

Aliquots of homogeneous endopeptidase 24.15, obtained after preparative slab PAGE, containing about 20 µg of protein, were emulsified in equal volumes of Freund's complete adjuvant and injected intradermally at multiple sites into the back of a White New Zealand rabbit. Booster injections were administered at least two to three times after 14 to 20 day intervals. Blood was collected into heparinized tubes from the ear vein 10 days after the last injection, at which time a rather high titer of anticatalytic antibodies was present. Aliquots (0.2 ml) of plasma recovered by centrifugation were stored at -20°C.

Preparation of the IgG Fraction

Anti-endopeptidase 24.15 antiplasma was obtained from a New Zealand white rabbit immunized with aliquots of the homogeneous testis enzyme as described (Orlowski et al., 1989). Portions of three separate bleeds were pooled and an IgG fraction was prepared from a total volume of 35 ml of plasma having a rather high titer of anticatalytic antibodies. The plasma was diluted in an equal volume of 0.15 M NaCl and subjected to ammonium sulfate fractionation at

50% saturation. The redissolved precipitate was dialyzed extensively against 10 mM Tris-HCl, pH 7.8, loaded onto a DEAE-cellulose column equilibrated in the same buffer, and eluted by a 0 to 0.2 M NaCl gradient in the equilibration buffer. Fractions of 4 ml were collected and tested for anticatalytic immunoinhibitory activity. The active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 0.1 M MOPS, pH 7.5. After dialysis the IgG fraction contained 122 mg of protein in 35 ml of buffer.

Western Blotting

Electrophoretic transfer of proteins from SDS polyacrylamide slab gels was performed essentially as described (Towbin et al., 1979; Burnette, 1981) using a Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond CA). A 5% bovine serum albumin solution (20 mM Tris, 500 mM NaCl, pH 7.5) was used as the blocking solution. The primary antibody was diluted 1:500 to 1:1000 in a buffer containing 1% bovine serum albumin, 0.05% Tween 20 in 20 mM Tris-HCl-0.5 M NaCl (pH 7.5). An affinity purified goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad) was used as the secondary antibody and visualization was accomplished according to the manufacturer's instructions.

Preparation of Immunoaffinity Column

A slurry containing 20 ml of Affi-Gel 10 (Bio-Rad) was placed on a Buchner funnel and the gel was washed with cold water under a gentle vacuum. The washed gel was added to the IgG fraction and gently rocked overnight at 4°C. The next day 2.5 ml of 1 M ethanolamine-HCl, pH 8.0 were added to convert unreacted N-hydroxysuccinimide ester sites of the resin into amides and the mixture was again gently agitated overnight at 4°C. The gel was packed and washed with several column volumes of 0.1 M MOPS, pH 7.5, the coupling buffer. The column was then equilibrated with the same buffer containing 0.5 M NaCl, washed with 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 10.6, the elution buffer, and re-equilibrated with the coupling buffer. For immunoaffinity purifications of the enzyme, the column was equilibrated with 0.05 M Tris-HCl, pH 8.0 containing 0.5 M NaCl. After an enzyme purification the column was regenerated with 0.2 M Glycine/HCl, pH 2.3 containing 0.5 M NaCl and 0.1% Triton X-100. The column was stored in 0.05 M Tris-HCl, pH 8.0 containing 0.5 M NaCl and 0.02% Na_3N between runs.

Immunoaffinity Purification of the Soluble Brain Enzyme

A group of 74 frozen rat brains were allowed to thaw and homogenized as described (Orlowski et al., 1983). The homogenate was centrifuged and the supernatant was subjected to DEAE-cellulose chromatography, according to Chu and Orlowski (Orlowski

et al., 1983). To remove albumin from the preparation, the active fractions (50 ml) were pooled and pumped onto a 20 ml Cibacron Blue 3GA-agarose column (Sigma), previously equilibrated with 0.05 M Tris-HCl, pH 8.0. The column was washed with the equilibrating buffer and 5 ml fractions were collected. The enzyme emerged in the void volume. The active fractions were pooled and NaCl was added to a final concentration 0.375 M.

The enzyme was applied to the immunoaffinity column by hand and the effluent recycled twice. The column was washed with 7 column volumes of 0.05 M Tris-HCl, pH 8.0 containing 0.5 M NaCl and then eluted with 0.1 M $NaHCO_3$, pH 10.6 containing 0.5 M NaCl while 5 ml fractions were collected. The fractions were neutralized immediately with 1 M HAc/NaOH pH 3.5. The enzyme was eluted promptly when one column volume of elution buffer had passed through and the active fractions were pooled, concentrated, and dialyzed against 0.05 M Tris-HCl, pH 8.0. The purified enzyme was frozen in 200 µl aliquots.

Immunoaffinity Purification of the Membrane-Bound Brain Enzyme

Pellets from the centrifugation of 20% homogenates of 210 rat brains prepared as described (Orlowski et al., 1983) were washed twice in 0.01 M Tris-HCl, pH 7.6 containing 0.3 M NaCl and then extracted in 0.01 M Tris-HCl, pH 7.6 containing 1% Triton X-100. The extract was pumped onto a 150 ml DEAE-cellulose column equilibrated with 0.01 M Tris-HCl, pH 7.6 containing 0.5 mM 2-

mercaptoethanol and 0.1% Triton X-100 at 40 ml/h. The column was washed with 1 l of equilibrating buffer and a gradient of 300 ml of equilibrating buffer/300 ml equilibrating buffer containing 0.3 M NaCl was attached and pumped on at 60 ml/h. Fractions of 6 ml were collected and tested for enzyme activity. The enzyme emerged after 456 ml of the gradient had passed through. The active fractions were pooled and NaCl was added to a final concentration of 0.375 M.

The enzyme was applied to the immunoaffinity column, which was previously equilibrated with 0.01 M Tris-HCl, pH 8.0 containing 0.5 M NaCl and 0.1% Triton X-100, in two batches. The enzyme was applied by hand and the effluent was recycled. The column was washed with 15 column volumes of equilibrating buffer and eluted with 0.1 M $NaHCO_3$, pH 10.6 containing 0.5 M NaCl. Fractions of 5 ml were collected and immediately neutralized with 1 M HAc/NaOH pH 3.5. Fractions were assayed for enzyme activity and the active fractions were pooled, concentrated, and dialyzed against 0.05 M Tris-HCl containing 0.1% Triton X-100. The dialyzed enzyme was frozen in 200 µl aliquots.

Tissue Preparation for Immunocytochemistry

Male Sprague-Dawley rats purchased from Charles River Breeding Laboratory (Wilmington, MA), weighing 150-200 g, were heparinized and injected intraperitoneally (ip) with 0.5 ml of 50 mg/ml pentobarbital. Once anesthetized, rats were perfused with

approximately 100 ml of 0.13 M $NaH_2PO_4 \cdot H_2O$-0.14 M NaOH-73 mM NaCl pH 7.4 (PBS) and 500 ml of Zamboni's fixative, prepared as follows, for Leydig cell staining. A 20 g amount of paraformaldehyde was dissolved in 150 ml of heated picric acid with the dropwise addition of 10 N NaOH. The clear solution was diluted in 850 ml of 24 mM $NaH_2PO_4 \cdot H_2O$-126 mM $Na_2HPO_4 \cdot 7H_2O$ and the pH was adjusted to 7.4. The animal was fixed in 5% paraformaldehyde in 0.3 M $NaH_2PO_4 \cdot H_2O$, pH 7.4 for seminiferous tubule staining.

After perfusion with fixative the testes were removed and incubated in the fixing solution. As Leydig cell immunostaining decreased with increasing fixation times and with perforation of the testicular capsule, the testes were post-fixed for only 1 h with the capsules intact, for interstitial staining. Post-fixation was carried out for three to four hours, however, for optimal tubular staining. The tissue was then transferred sequentially into PBS containing 12, 16, and 18% sucrose every two hours. The testes were stored in the 18% sucrose PBS at 4°C for short periods or frozen at -70°C.

In preparation for tissue slicing the testicular capsule was removed from one testis and the organ was cut horizontally. The tissue was mounted onto a microtome chuck with OCT compound (Miles Laboratories) with the cut surface exposed, and frozen with powdered dry ice. After equilibration at -18°C the tissue was sliced into 20 μm sections, which were mounted directly onto acid washed glycerin coated microscopic slides.

Avidin-Biotin Complex Method of Immunostaining

The mounted tissue slices were allowed to warm to room temperature and washed in PBS. The slides were incubated for 30 minutes in PBS containing 0.1% Triton X-100 and 2% normal goat serum to block non-specific binding sites. The normal serum was removed.

Rabbit anti-endopeptidase 24.15 antiserum was added at a dilution of 1/5000 to 1/10,000 in PBS-Triton X-100 containing 1 mg/ml bovine serum albumin (BSA) and the slides were stored at 4°C for 48 h in a humidified chamber. For negative controls preimmune serum was used at the same dilution. To determine whether preabsorbtion with the antigen diminishes staining the antiserum was incubated in the absence or presence of homogeneous enzyme (1 μg antigen/0.025 μl antiserum) overnight at 4°C prior to application. After incubation with the primary antiserum the tissues were allowed to warm to room temperature and washed in PBS. Biotinylated goat anti-rabbit IgG secondary antibody from Vector Laboratories, diluted 1/222 in PBS-Triton X-100-BSA, was added and left on the slices for 45 min.

The slices were thoroughly washed in PBS to remove the secondary antibody. Avidin DH and biotinylated horseradish peroxidase H (Vector Laboratories) were reacted for 15 minutes at room temperature in PBS-Triton X-100-BSA and the tissues were incubated with the complex for 45 min. Unbound complex was then washed off with PBS. A substrate solution of 0.003% hydrogen peroxide and 0.5 mg/ml diaminobenzidine in phosphate buffer (37 mM

NaH_2PO_4-41 mM NaOH) was added and the reaction was allowed to proceed for 4-10 min. The slices were washed in water, air dried, dehydrated, and coverslipped with permount.

RESULTS

Isolation of Endopeptidase 24.15 From Rat Testes

A summary of the purification of the enzyme from the rat testis is presented in Table I. A 260-fold purification was necessary to obtain homogeneous enzyme. Disc PAGE after step 4 revealed the presence of two protein components (Figure 1). Activity was associated with the slower moving band only. Attempts to separate the two proteins by chromatography on various ion exchange columns, chromatofocusing or chromatography on a hydroxyapatite column were not successful. The enzyme was therefore isolated in small batches by preparative slab PAGE. Good separation of the two bands was consistently obtained when the gel was run for twice as long as it took for the tracking dye to reach the bottom. Previous attempts to isolate the brain enzyme by preparative PAGE had not been successful. The isolated testicular enzyme was purified to apparent homogeneity (Figure 1). PAGE under denaturing and dissociating conditions (Figure 2) also revealed the presence of a single protein band. The molecular mass of the enzyme calculated from its relative mobility with respect to marker proteins was approximately 70,000. This value is rather close to that obtained for the brain enzyme (67,000) on the basis of measurements of the elution volume from calibrated columns of Sephadex G-100 (Orlowski et al., 1983). Immunoblots also showed the presence of a single immunoreactive component in the isolated enzyme. These data taken together indicate the presence of a single

Table I. Summary of purification of the enzyme from rat testes.

Purification step	Vol (ml)	Protein (mg/ml)	Activity (units/ml)	Total (units)	Specific Activity (units/mg)	Purification (fold)	Recovery (%)
Supernatant	610	10.7	49	29,890	4.58	--	100
DE-52 Chromatography (pH 7.6)	77	6.6	194	14,940	29.4	6.4	50
Sephadex G-100	130	0.96	83	10,790	86.5	18.9	36
DEAE-Sephacel Chromatography (pH 8.0)	35	0.38	129	4,515	339	74	15
Prepara-tive PAGE	1.35	0.025	30.4	41	1220	266	0.14

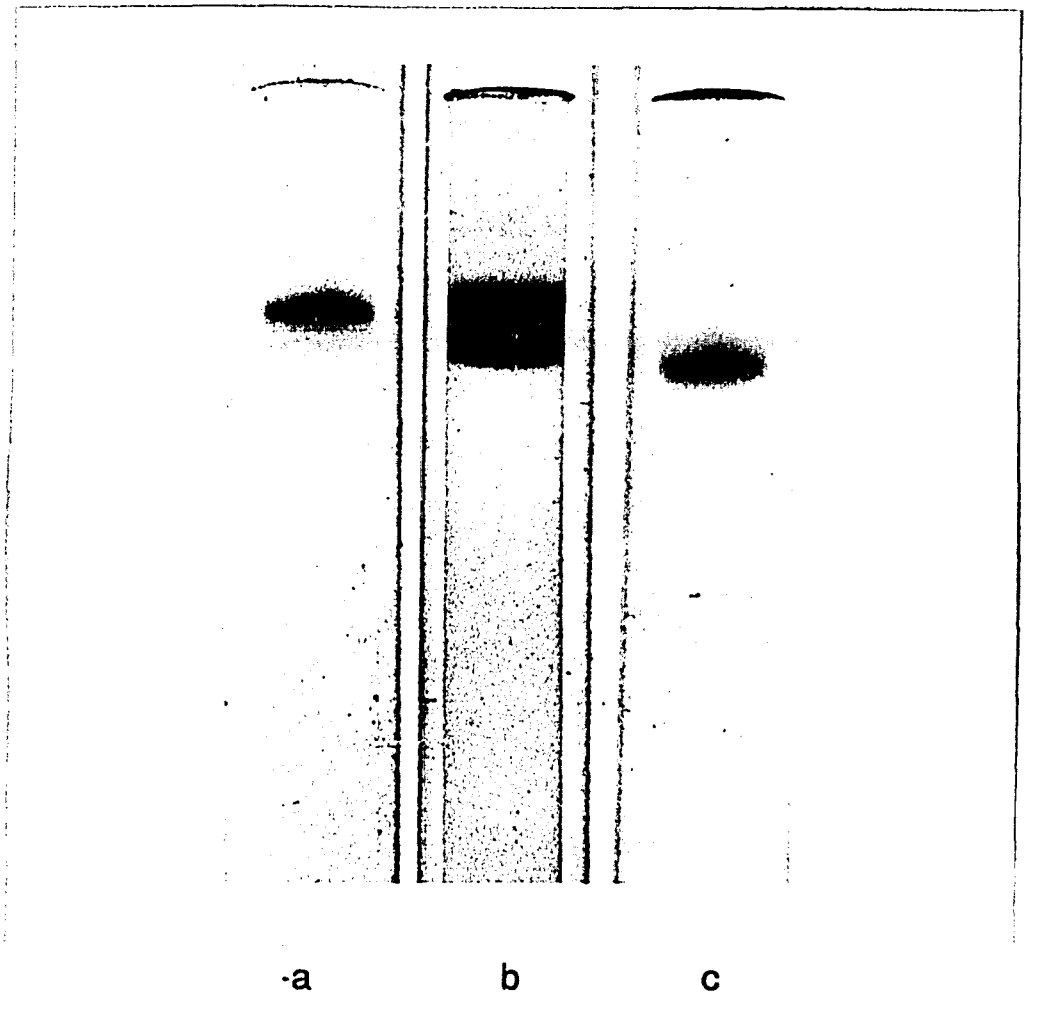

Figure 1. PAGE of the enzyme from testis. Gel (b) (10 μg of protein) represents the pattern obtained after step 4 of the purification procedure. Gel (a) and (c) (each containing abut 5 μg of protein) represent respectively the homogeneous enzyme and the impurity each isolated after preparative PAGE.

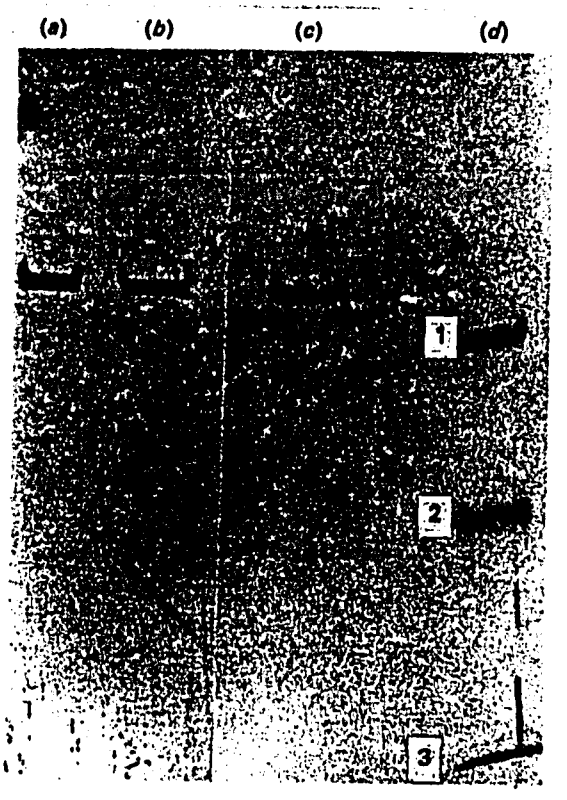

Figure 2. PAGE and immunoblots of the enzyme after step 5 of the purification. Electrophoresis was carried out under denaturing and dissociating conditions as described in the Materials and Methods section using 2.5 μg of protein. Lanes (a) and (b) are immunoblots of the enzyme. Lane (c) contains the isolated enzyme stained with Coomassie Brilliant Blue and lane (d) contains the standard marker proteins: 1, bovine serum albumin; 2, ovalbumin; 3, trypsinogen.

polypeptide chain. Crude testis supernatants contained an additional, somewhaf faster moving, immunoreactive band, that on further investigation proved to be serum albumin.

Double immunodiffusion experiments (Figure 3) (Ouchterlony, 1958) gave single precipitation lines with the testis enzyme, suggesting the presence of a single antigenic component. The antiserum was also tested by the same method for its reactivity toward the enzyme in a crude rat brain extract. As shown in Figure 3, patterns of identity were obtained for both the purified testis enzyme and the enzyme present in rat brain supernatant.

The effect of general protease inhibitors on the activity of the enzyme is summarized in Table II. High concentrations of leupeptin weakly inhibited the enzyme. No inhibition was obtained with pepstatin, antipain and chymostatin. Indeed, some increase in enzyme activity was observed in the presence of pepstatin and chymostatin. The detailed mechanism of this activation has not been further studied. DFP and PMSF, irreversible inhibitors of serine proteases, had no effect on activity. Among several thiol blocking agents, PCMB and N-ethylmaleimide partially inhibited the enzyme, while iodoacetamide and iodoacetic acid at rather high (mM) concentrations had no effect. Consistent inhibition was obtained with the metal chelators such as EDTA and EGTA. Unlike crude enzyme preparations which were moderately activated by addition of DTT (0.4-1.0 mM), the isolated homogeneous enzyme was inhibited by DTT.

The profile of inhibition indicated that the enzyme belongs to the class of metalloendopeptidases. Consistent with this conclusion

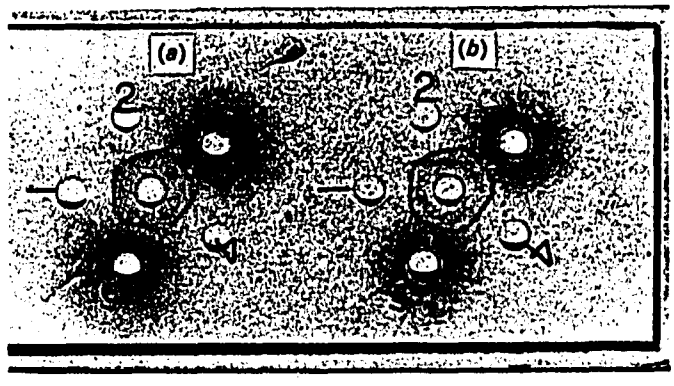

Figure 3. Ouchterlony double-immunodiffusion patterns of the enzyme from testis and brain. The rabbit antiserum was placed in the center well and allowed to diffuse toward the purified testis enzyme (wells 1, 2, and 4). Wells 3 and 5 contained a pH 5.0 supernatant of the soluble fraction of a rat brain homogenate after adjustment of pH to 7.6. The amounts of active enzyme are 0.14 unit in (a) and 0.07 unit in (b).

Table II. Effect of inhibitors on testis enzyme activity. Activity was determined with Bz-Gly-Ala-Ala-Phe-pAB (0.8 mM) as substrate. Activity in the presence of pepstatin was determined at 0.4 mM substrate concentration. Inhibitors were preincubated with the enzyme for 12 min and the reaction was then started by addition of substrate. Data are mean values obtained from two to four determinations.

Inhibitor	Final concentration (mM)	Relative Activity (%)
None		100
Leupeptin	0.04	90
	0.12	68
Pepstatin	0.008	142
Antipain	0.013	99
Chymostatin	0.013	108
DFP	1.0	102
PMSF	0.2	100
PCMB	1.0	35
Iodoacetamide	1.0	107
N-Ethylmaleimide	1.0	55
Iodoacetic acid	1.0	100
EDTA	1.0	40
EGTA	1.0	41
DTT	0.5	71
	1.0	48

was the finding that dialysis of the enzyme against EDTA (Table III) led to loss of activity. Activity could be restored by addition of some divalent metal ions. Complete reactivation was obtained after addition of zinc ions. The highest activity in reactivation experiments was obtained at Zn^{2+} concentrations as low as 1 μM. Indeed, at this concentration enzyme activity is somewhat higher than that of the undialysed enzyme, suggesting that some of the enzyme was stripped of its metal content during purification. Higher Zn^{2+} concentrations led to a progressive decrease of activity. The extent of reactivation was greater after addition of Co^{2+} than of Zn^{2+}; although the concentration of Co^{2+} required for maximal activation was 100 times greater (100 μM) than that of zinc. These data indicate that the Co^{2+} enzyme is more active than the Zn^{2+} enzyme toward Bz-Gly-Ala-Ala-Phe-pAB as the substrate. At an optimal concentration of each of the two metals, the Co^{2+} enzyme cleaved the substrate 2.5 times faster than the Zn^{2+} enzyme. The enzyme could also be reactivated by addition of Mn^{+2}, albeit at much higher concentrations than with cobalt or zinc (1.0-10 mM), while incomplete reactivation was obtained with calcium ions (0.05-2.0 mM). These data suggest that the enzyme is apparently a zinc-containing endopeptidase.

Table III. Effect of metal ions on enzyme activity after dialysis against EDTA. The enzyme after step 5 of the purification procedure was dialysed against three changes (1000 ml each) of a 1 mM solution of EDTA (pH 7.0) in deionized water, and then against three changes of a 1 mM solution of Tris/HCl (pH 7.0). Activity was then determined with Bz-Gly-Ala-Ala-Phe-pAB as the substrate. Activity was measured without preincubation with the metal ions.

Enzyme	Additions	Concentration (μM)	Activity (units/ml)	Relative Activity (%)
Not dialysed	None	--	71	100
Dialysed	None	--	22.8	32
	$ZnCl_2$	0.5	45.6	64
		1	91.6	129
		5	48.3	68
		50	42.8	60
		500	16.1	23
	$CoCl_2$	1	80.9	114
		5	130.0	183
		50	212.2	299
		100	232.1	327
		500	192.7	271
		1000	151.2	213
		2000	98.5	139

Immunoaffinity Chromatography of Endopeptidase 24.15

Coupling of the anti-endopeptidase 24.15 antiplasma IgG fraction to the Affi-Gel 10 resin was greater than 99%, as no detectable protein or anticatalytic immunoinhibitory activity was found in the effluent and washes of the packed column. Based on the number of units of enzyme activity inhibited by the IgG fraction before coupling, as determined by an anticatalytic immunoinhibition assay, the capacity of the immunoaffinity column is 1700 units of activity. As the homogeneous enzyme has a specific activity of approximately 1000, this represents a capacity of approximately 1.7 mg of enzyme.

Shown in Table IV is a summary of the immunoaffinity purification procedure of the soluble rat brain enzyme. Of the 1476 units applied to the immunoaffinity column 1138 bound to the column (1.1 mg enzyme protein). Elution resulted in the recovery of 256 units, a yield of 22%. The capacity of the column was consistent for applications of approximately this number of units and did not decrease over the course of several months and purifications. The specific activity of the final preparation is slightly lower than that reported for the isolated testis enzyme (Orlowski et al., 1989). A 1710-fold purification was needed to isolate the brain enzyme from the supernatant, whereas an only 266-fold purification resulted in isolation of the testis enzyme, as testis is much richer in endopeptidase 24.15 than brain.
Shown in Figure 4A is an SDS polyacrylamide gel in which the immunoaffinity purified soluble brain enzyme was run alongside the

Table IV. Summary of purification of rat brain soluble endopeptidase 24.15 using immunoaffinity chromatography.

Purification Step	Volume (ml)	Protein (mg/ml)	Activity: Concentration (units/ml)	Activity: Total (units)	Activity: Specific Activity (units/mg)	Recovery %	Purification -fold
1. Homogenate	570	24.8	5.34	3072	0.22	100	-
2. Supernatant	390	10.5	5.95	2322	0.57	76	2.59
3. DEAE-Cellulose Chromatography	50	7.43	32.9	1646	4.43	54	20.1
4. Affi-Gel Blue Chromatography	68	1.88	21.7	1476	11.5	48	52
5. Immunoaffinity	51	0.00515	5.02	256	975	8.3	4432

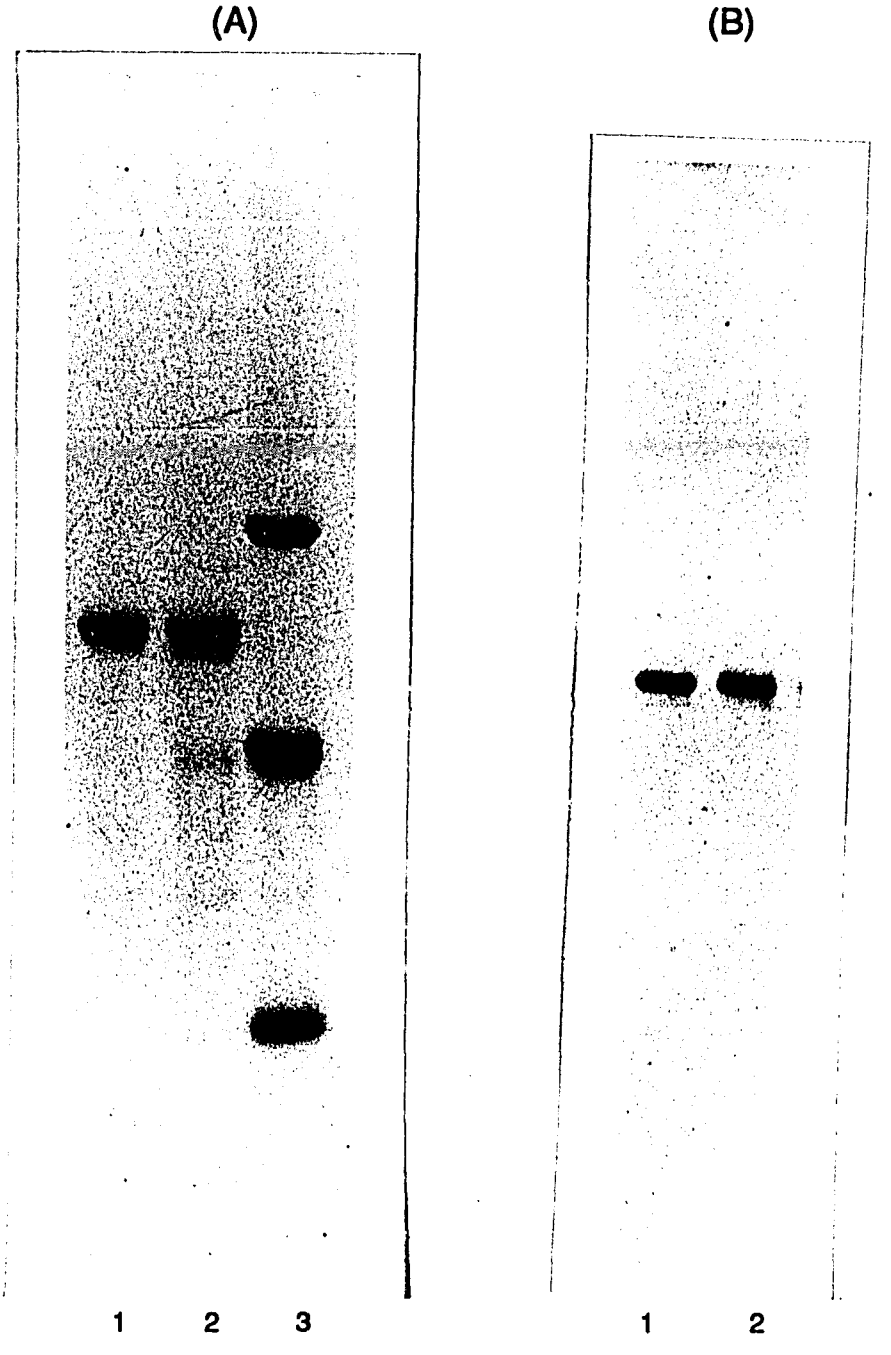

Figure 4. SDS PAGE and immunoblots of immunoaffinity purified soluble brain enzyme and conventionally purified soluble testis enzyme. (A) Electrophoresis was carried out under denaturing and dissociating conditions as described in the Materials and Methods section using 1 μg of protein for the enzymes and 2.5 μg for molecular weight standards. Lane 1, testis enzyme; lane 2 brain enzyme; lane 3, standard marker proteins phosphorylase b, bovine serum albumin, and ovalbumin. (B) Immunoblots were carried out with 0.2 μg of each enzyme. Lane 1, brain enzyme; lane 2, testis enzyme.

soluble testis enzyme isolated as described (Orlowski et al., 1989). A single protein band is present in both enzyme preparations. The testis enzyme appears to migrate somewhat more slowly in the SDS gel, suggesting that the brain enzyme may be of slightly lower molecular weight than the enzyme from testis. The apparent molecular weights of both enzymes are approximately 70,000, consistent with the molecular weight of the testis enzyme of 70,000 calculated from its relative mobility with respect to marker proteins (Orlowski et al., 1989) and the molecular weight of the brain enzyme of 67,000 calculated from measurements of the elution volume from calibrated columns of Sephadex G-100 (Orlowski et al., 1983). Immunoblots also showed the presence of a single band in each enzyme preparation, with the testis enzyme migrating more slowly.

The effects of general protease inhibitors on the activities of the brain and testis enzymes are summarized in Table V. An increase in both activities was observed in the presence of leupeptin, pepstatin, and chymostatin; the mechanism of this activation has not been further studied. DFP and PMSF, irreversible inhibitors of serine proteases, had no effect on either activity. The thiol blocking agents iodoacetamide and iodoacetic acid did not inhibit either enzyme at rather high concentrations; indeed, iodoacetamide caused slight activation of both enzymes. N-ethylmaleimide partially inhibited the brain enzyme at a high concentration. Consistent inhibition of both enzymes was obtained with metal chelators such as EDTA and EGTA. Nearly complete

Table V. Effects of protease inhibitors on brain and testis enzyme activities. [a]Enzyme activity was determined as described in Materials and Methods, using α-N-Bz-Gly-Ala-Ala-Phe-pAB as the substrate. Activities are relative to specific activities of enzymes in the absence of inhibitors, set arbitrarily to 100. Mean values±standard error are listed.

Inhibitor	Final Concentration (mM)	Relative Activity[a] Brain	Testis
None	-	100	100
Leupeptin	0.02	115±0	103±5
Pepstatin	0.16	132±14.5	115±6
Chymostatin	0.008	117	116
Antipain	0.008	93	92
DFP	0.1	107±4	105±1
PMSF	0.2	103	105
Iodoacetamide	1.0	119±3	110±2.5
Iodoacetic Acid	1.0	91±4	97±1.5
N-ethlymaleimide	1.0	72±1	99±1
EDTA	1.0	53±2.5	59±1.5
EGTA	1.0	48	54
o-Phenanthroline	0.04	97	84
	0.16	29	24
	0.24	7	5

inhibition of both enzyme activities was obtained by concentrations of o-phenanthroline in the μM range.

The immunological properties of the brain and testis enzymes were compared in an anticatalytic immunoinhibition assay (Figure 5). Approximately equal numbers of units of both activities were preincubated in the absence or presence of varying amounts of antiserum. Incubation with preimmune serum caused no inhibition of brain or testis enzyme activity. The fact that the two inhibition curves are nearly superimposable indicates that the two forms of the enzyme are similar immunologically. This conclusion is consistent with the pattern of identity obtained in double immunodiffusion experiments with these two enzymes (Orlowski et al., 1989).

Shown in Table VI are the apparent K_m's, turnover rate constants (k_{cat}'s), and specificity constants (k_{cat}/K_m's) of the testis and brain enzymes for t-Boc-Phe-Ala-Ala-Phe-pAB, a synthetic substrate of the enzyme (Orlowski et al., 1983). The K_m's for the two enzymes are similar. The difference in the k_{cat}'s and hence in the specificity constants is a reflection of the lower specific activity of the brain enzyme and may be due to partial inactivation of the enzyme during the immunoaffinity purification. Inhibition by two active-site directed specific inhibitors of endopeptidase 24.15, cFP-Ala-Ala-Phe-pAB and cFP-Ala-Ala-Tyr-pAB (Chu & Orlowski, 1984; Orlowski et al., 1988), is shown in Table VII. The substitution of the phenylalanyl residue with a tyrosyl residue results in significantly lower K_i's with both enzymes. The tyrosyl residue may better fit the S_3' enzyme subsite[1], which is

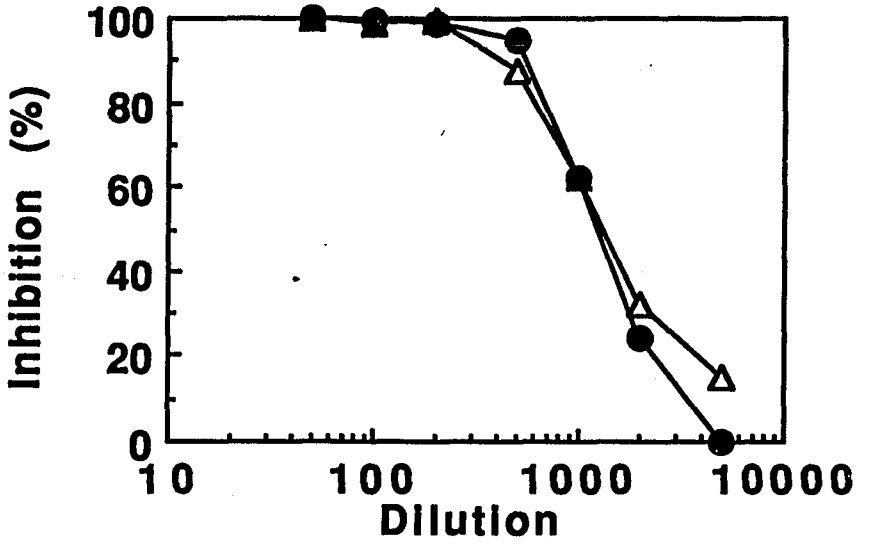

Figure 5. Anticatalytic immunoinhibition assays with anti-endopeptidase 24.15 antiserum and soluble brain and soluble testis enzymes. Enzyme was preincubated in the presence of varying dilutions of antiserum and reactions were begun by addition of substrate as described in Materials and Methods. (●) brain enzyme; (Δ) testis enzyme.

Table VI. Kinetic parameters of soluble testis, soluble brain, and membrane-bound brain endopeptidase 24.15-catalyzed hydrolysis of t-Boc-Phe-Ala-Ala-Phe-pAB. Data are mean values±S.E.M. obtained from two to four determinations.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
Soluble Testis	0.10±0.01	94.1	9.4×10^5
Soluble Brain	0.17±0.03	24.4	1.4×10^5
Membrane-Bound Brain	0.26±0.03	9.65	3.7×10^4

Table VII. Inhibition of soluble testis, soluble brain, and membrane-bound brain endopeptidase 24.15 by two active site-directed inhibitors. Enzyme activity was determined with the Bz-Gly-Ala-Ala-Phe-pAB substrate as described in Materials and Methods and K_i's were determined by the method of Dixon (1972) at three different substrate concentrations and five different inhibitor concentrations. Data are means ±S.E.M. of at least three determinations.

Enzyme	Inhibitor	
	cFP-Ala-Ala-Phe-pAB	cFP-Ala-Ala-Tyr-pAB
	K_i (nM)	
Soluble Testis	70.5±15	27.3±0.88
Soluble Brain	40.5±2.5	17.5±0.50
Membrane-Bound Brain	34.0±2.0	16.0±1.0

believed to accommodate hydrophobic or bulky residues in substrates (Orlowski et al., 1983; Orlowski et al., 1989). Similar K_i's of the inhibitors for the enzymes suggest similar active sites, although a statistically significant difference ($p<0.05$, student's t test) was noted for the better inhibitor.

The immunoaffinity column was used for the purification of the membrane-bound form of brain endopeptidase 24.15 (Table VIII). In a typical preparation, of 850 units of enzyme applied to the column, in two separate runs, 785 bound. After elution 82 units were recovered and the specific activity determined to be 63 units/mg, which is much lower than the specific activity calculated for the soluble brain enzyme isolated by immunoaffinity chromatography (975). SDS PAGE revealed the presence of one major polypeptide band and a few significantly more minor bands (Figure 6). The identity of the major band was verified to be the enzyme by Western blotting. As the enzyme appears to constitute most of the protein in the preparation, the low specific activity may be accounted for by inactivation of the enzyme during the preparation. We have found that storing the enzyme in buffer containing Triton X-100 leads to loss of enzyme activity.

The membrane-bound form of the brain enzyme apparently migrates slightly more slowly than the soluble form. In light of this difference between the soluble and membrane-bound forms of the brain enzyme, the possibility of immunological differences was considered. Immunoinhibition curves generated in an anticatalytic assay with anti-endopeptidase 24.15 antiserum and the brain enzymes were essentially superimposable (Figure 7). No inhibition

Table VIII. Summary of purification of rat brain membrane-bound endopeptidase 24.15 using immunoaffinity chromatography.

Purification Step	Vol	Protein Concentration	Activity Total	Specific Activity	Purification	Recovery
	(ml)	(mg/ml)	(units/ml)	(units)	(units/mg)	-fold	
Homogenate	1670	22.9	8.47	14,138	0.37	-	100
Washed Pellets	1000	8.33	2.73	2,730	0.33	0.89	19
Triton X-100 Extract	678	9.09	2.31	1,563	0.25	0.68	11
DE-52 Chromatography	130	4.51	6.54	850	1.45	3.9	6.0
Immunoaffinity Chromatography	106	0.0096	0.6	82	63.2	171	0.6

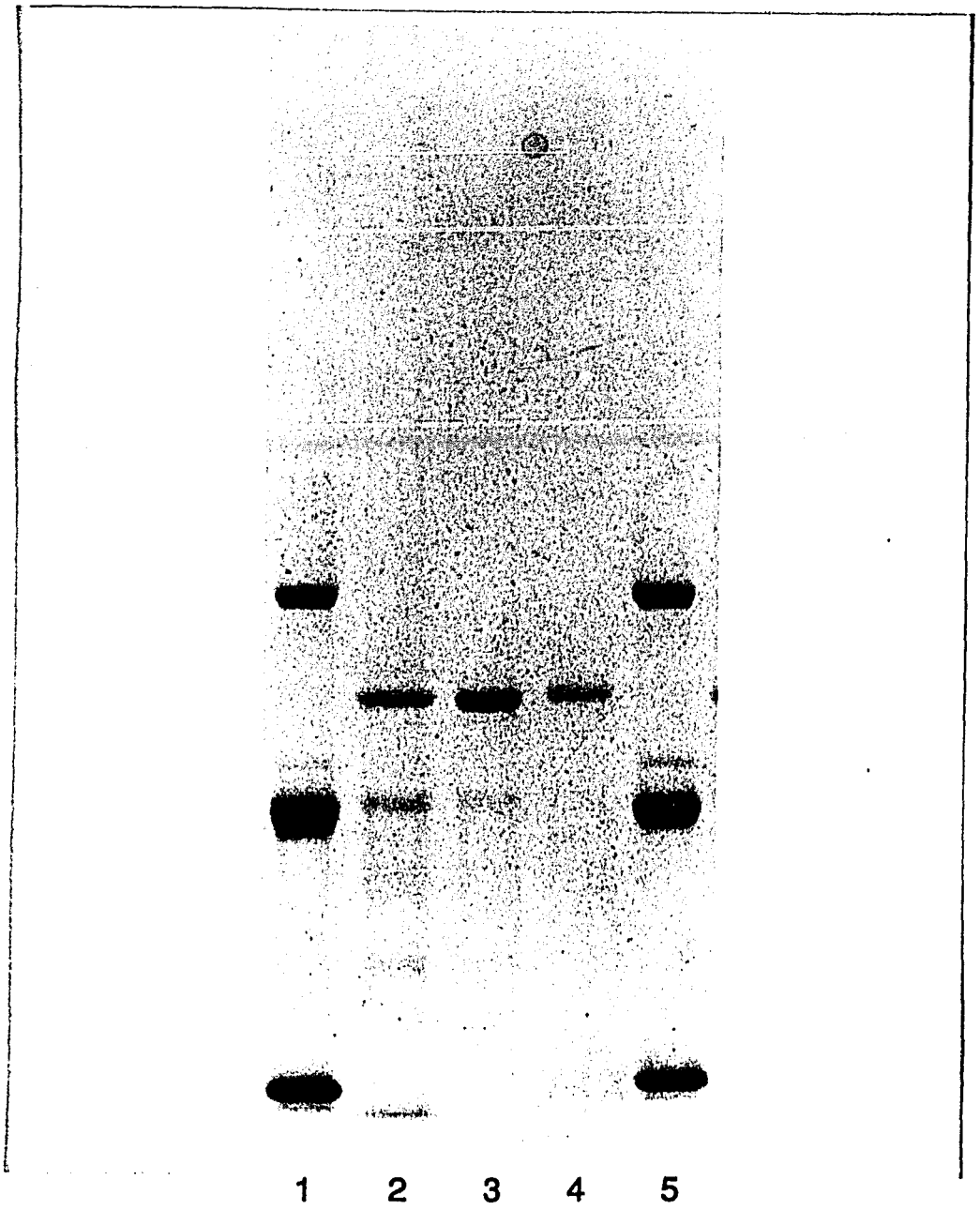

Figure 6. SDS PAGE of immunoaffinity purified membrane-bound brain enzyme, immunoaffinity purified soluble brain enzyme, and conventionally purified testis enzyme. Electrophoresis was carried out under denaturing and dissociating conditions as described in the Materials and Methods section using 0.8 μg of membrane-bound enzyme protein (lane 2), 1 μg of soluble brain enzyme protein (lane 3), and 0.3 μg of testis enzyme protein (lane 4). Molecular weight standards were 2.5 μg each of phosphorylase b, bovine serum albumin, and ovalbumin (lanes 1 and 5).

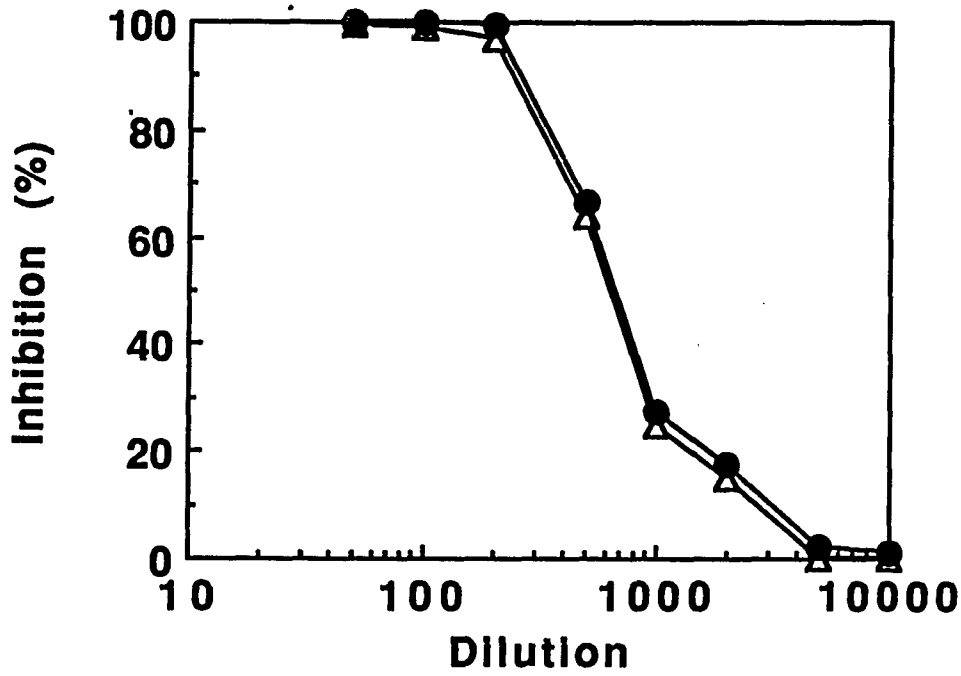

Figure 7. Anticatalytic immunoinhibition assays with anti-endopeptidase 24.15 antiserum and soluble and membrane bound forms of the brain enzyme Enzyme was preincubated in the presence of varying dilutions of antiserum and reactions were begun by addition of substrate as described in Materials and Methods. (●) soluble enzyme; (Δ) membrane-bound enzyme.

of either enzyme was detected with preimmune serum. Ouchterlony immunodiffusion experiments yielded patterns of identity with the two forms of the enzyme (Figure 8). These results indicate that each epitope on one enzyme recognized by the antiserum was shared by the other.

No significant difference between the K_m's of the two enzymes for the synthetic substrate t-Boc-Phe-Ala-Ala-Phe-pAB was detected (Table VI). An apparent difference in specificity constants may have resulted from an underestimation of the k_{cat} of the membrane-bound enzyme as the membrane-bound enzyme was not purified to homogeneity. As expected, the K_i of cFP-Ala-Ala-Tyr-pAB for the membrane-bound enzyme was determined to be lower than that of cFP-Ala-Ala-Phe-pAB. No significant difference in K_i's of cFP-Ala-Ala-Phe-pAB for the two enzymes was detected (Table VII). Likewise, the K_i's of cFP-Ala-Ala-Tyr-pAB for the two forms were essentially the same. These results are consistent with identical active sites in the soluble and membrane-bound forms of the brain enzyme.

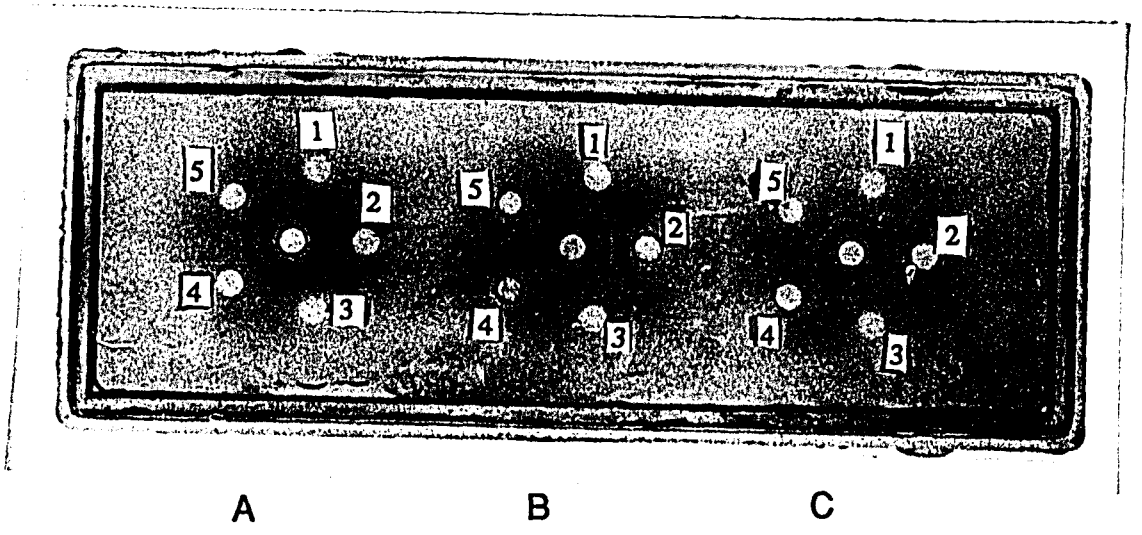

Figure 8. Ouchterlony double immunodiffusion patterns of the soluble and membrane-bound forms of the enzyme from brain. Anti-endopeptidase 24.15 antiserum was placed in the center well and allowed to diffuse toward aliquots of 0.08 (A), 0.3 (B), and 0.15 (C) unit of active enzyme. Wells 1 and 3, soluble enzyme; wells 2, 4, and 5, membrane-bound enzyme.

Immunocytochemical Localization of Rat Testicular Endopeptidase 24.15

The distribution of endopeptidase 24.15 activity in the soluble protein fractions of rat tissues is shown in Table IX. All tissues examined had detectable activity. Specific activity in the testis is over 4 times as high as in the brain, which has the second highest activity. These results differ from those reported previously (Chu & Orlowski, 1985), where brain supernatants were found to be slightly more active than testis supernatants. The inconsistency may be due to the fact that much older rats were used in the experiments here and rat testicular endopeptidase 24.15 activity increases with age (Molineaux et al., 1989, unpublished work). Relatively high activities in brain, anterior pituitary, and spinal cord are consistent with the neuropeptide metabolizing properties of the enzyme. The abundance of activity in the testis prompted us to examine its localization in that tissue.

Since approximately 7% of endopeptidase 24.15 activity is associated with the membrane fraction in rat testes (Orlowski et al., 1989), it was of interest to determine whether the antiserum raised against soluble enzyme would react with the membrane-bound form and whether the antiserum would distinguish between the two forms. The results of a typical anticatalytic immunoinhibition assay with both forms of the testicular enzyme are shown in Figure 9. Approximately equal amounts of both activities in units were

Table IX. Rat tissue distribution of endopeptidase 24.15 activity. Enzyme activities and protein concentrations were determined in tissue homogenate supernatants as described in Materials and Methods. The substrate was α-N-benzoyl-Gly-Ala-Ala-Phe-p-aminobenzoate (0.8 mM). Relative activities are in comparison to the highest specific activity, set arbitrarily to 100.

Tissue	Specific Activity (μmol/mg/h)	Relative Activity (%)
Testis	3.75	100
Brain	0.865	23
Spinal Cord	0.652	17
Spleen	0.631	17
Anterior Pituitary	0.521	14
Pancreas	0.332	9
Kidney Cortex	0.273	7
Stomach	0.270	7
Kidney Medulla	0.260	7
Lung	0.252	7
Adrenals	0.250	7
Heart	0.244	7
Skeletal Muscle	0.239	6
Vas Deferens	0.239	6
Posterior Pituitary	0.231	6
Liver	0.216	6
Small Intestine	0.165	4

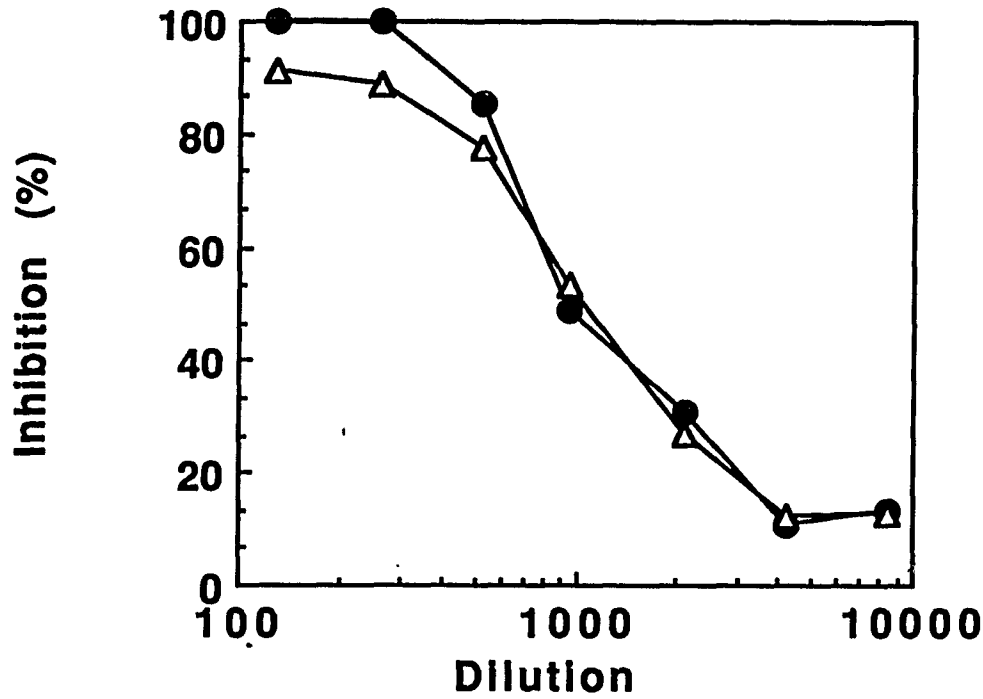

Figure 9. Anticatalytic immunoinhibition assay with anti-endopeptidase 24.15 antiserum and soluble and membrane-bound forms of the testis enzyme. Approximately equal amounts of both soluble and membrane-bound activities were preincubated in the absence or presence of varying dilutions of antiserum in 0.1 M Tris-HCl, pH 7.6 containing 0.2 mg/ml BSA. The reaction was begun by addition of a cocktail containing α-N-benzoyl-Gly-Phe-Ala-Ala-Phe-pAB as the substrate (final concentration 0.8 mM), dithiothreitol (final concentration 0.4 mM) and an excess of aminopeptidase N in the same buffer. (●) membrane-bound enzyme; (Δ) soluble enzyme.

preincubated in the absence or presence of varying amounts of antiserum. Half of the activity was inhibited at dilutions of the antiserum of 1/1000 in both the case of the soluble and the membrane-bound enzyme. Incubation with preimmune serum caused no inhibition of soluble or membrane-bound activity. The fact that the two inhibition curves are nearly superimposable indicates that the two forms of the enzyme are similar immunologically. These results suggested that we would be able to detect the membrane-bound form of the enzyme as readily as the soluble form immunocytochemically with this antiserum.

Immunocytochemical staining of rat testis slices indicated that the enzyme was localized in two cell populations: spermatazoa and Leydig cells. Staining of the germ cells improved with increasing tissue fixation times; Leydig cell staining, on the other hand, was improved as the tissue fixation time was decreased.

Seminiferous tubules throughout the slices stained consistently with anti-endopeptidase 24.15 antiserum diluted 1/5000 (Figure 10.) Selected tubules contained cells bordering the lumen filled with peroxidase reaction product. The cells are clearly spermatazoa, oriented with the tails pointing toward the tubule lumen and the enzyme-filled heads embedded in the cell layer of the tubule wall apposing the lumen (Figure 10). Testis slices incubated with immune serum preincubated with the enzyme, had no evidence of immunostaining in the tubules as compared to immune serum preincubated in buffer (Figure 11.) Immunocytochemical staining of rat testis slices yielded dark granular deposits when antiserum was diluted 1/10,000. As shown in Figure 12A, an abundance of

immunostaining occurred in the testicular interstitium. All interstitial areas were consistently stained to the extent shown. A subpopulation of the interstitial cells contains well-delimited punctate granules, arranged along the periphery of the cells. In some of the endopeptidase 24.15 positive cells the dark granules lie in the cytoplasm superimposed over areas of lighter staining. Indeed, the only part of the cell unstained is the nucleus in some cases. Any staining apparent in slices treated with preabsorbed serum is dramatically diminished (Figure 12B).

The localization of the immunostained cells in the interstitium, the arrangement of the cells in small groups, and the spherical and pyramidal shapes of the cells are consistent with their identity as Leydig cells. The possibility that the enzyme was concentrated in a single cell type in the testis led us to purify Leydig cells and test for concentration of enzymatic activity in those cells.

Figure 13 shows relative specific activities in rat seminiferous tubules, Leydig cells, and interstitial cells minus Leydig cells. Seminiferous tubules, Leydig cell enriched fractions of approximately 10^6 cells, and interstitial cells separated from the Leydig cell fractions (approximately 10^7 cells per purification) were homogenized and assayed for endopeptidase 24.15 activity in the absence and presence of 25 μM cFP-Ala-Ala-Phe-pAB, a specific inhibitor of the enzyme (Chu & Orlowski, 1984; Orlowski et al., 1988), as described in Materials and Methods. The seminiferous tubules contained the highest enzyme specific activity, 1.7-fold higher than Leydig cells. The Leydig cell enriched fractions

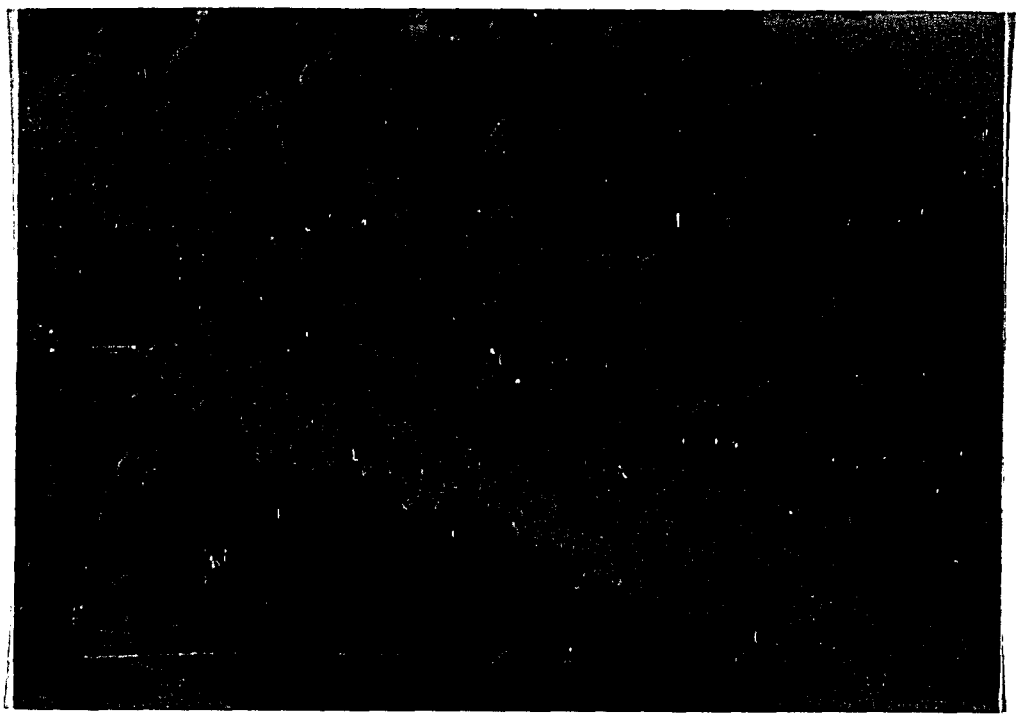

Figure 10. Endopeptidase 24.15 immunostaining in rat testis seminiferous tubules. Antiserum was diluted 1/5000. Immunocytochemistry was performed using an ABC kit from Vector Laboratories, following the manufacturer's instructions.

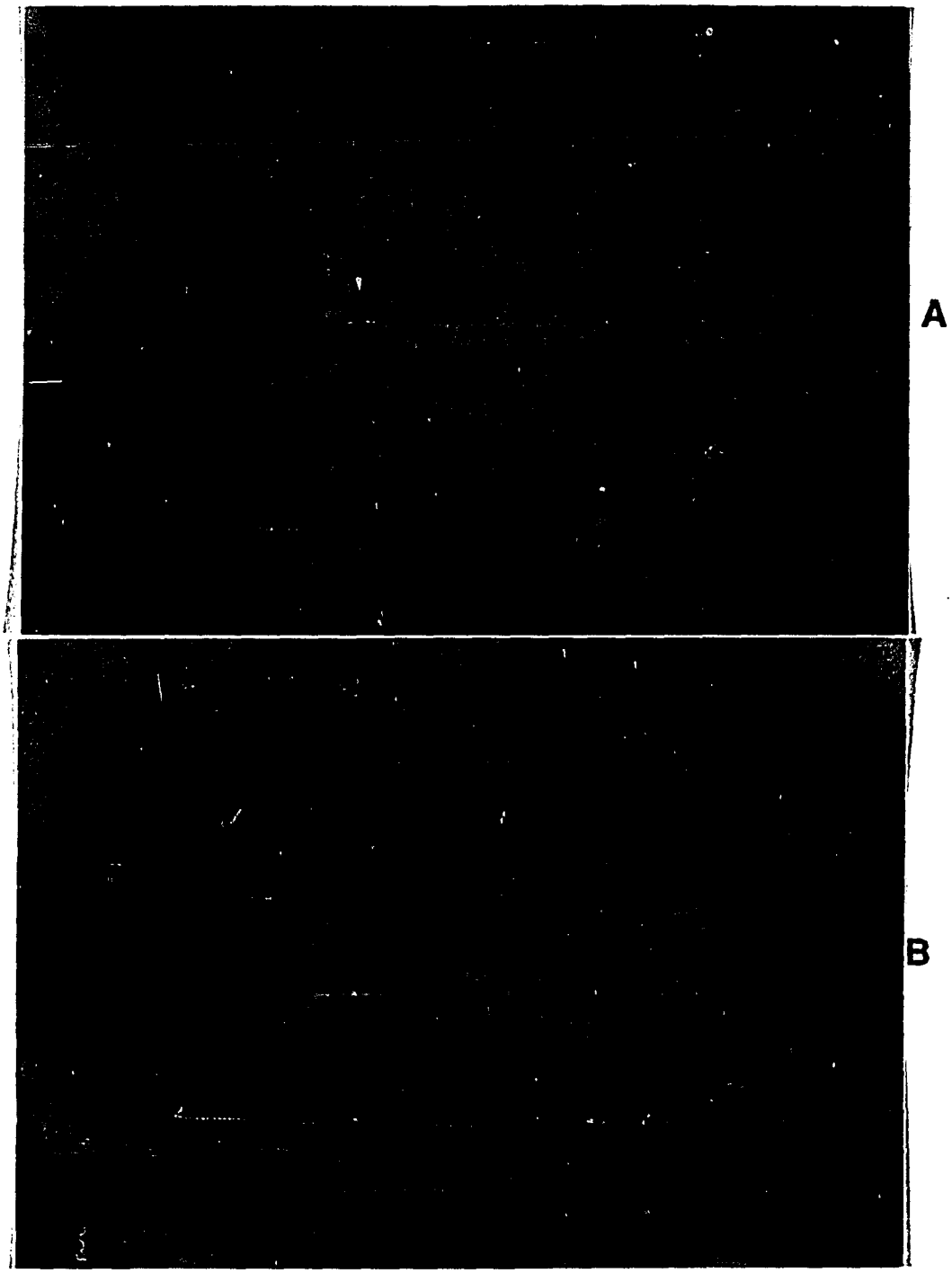

Figure 11. Blocking of tubular endopeptidase 24.15 immunostaining with the enzyme. Immunocytochemistry was performed with antiserum incubated in the absence (A) or presence (B) of the enzyme as described in Materials and Methods.

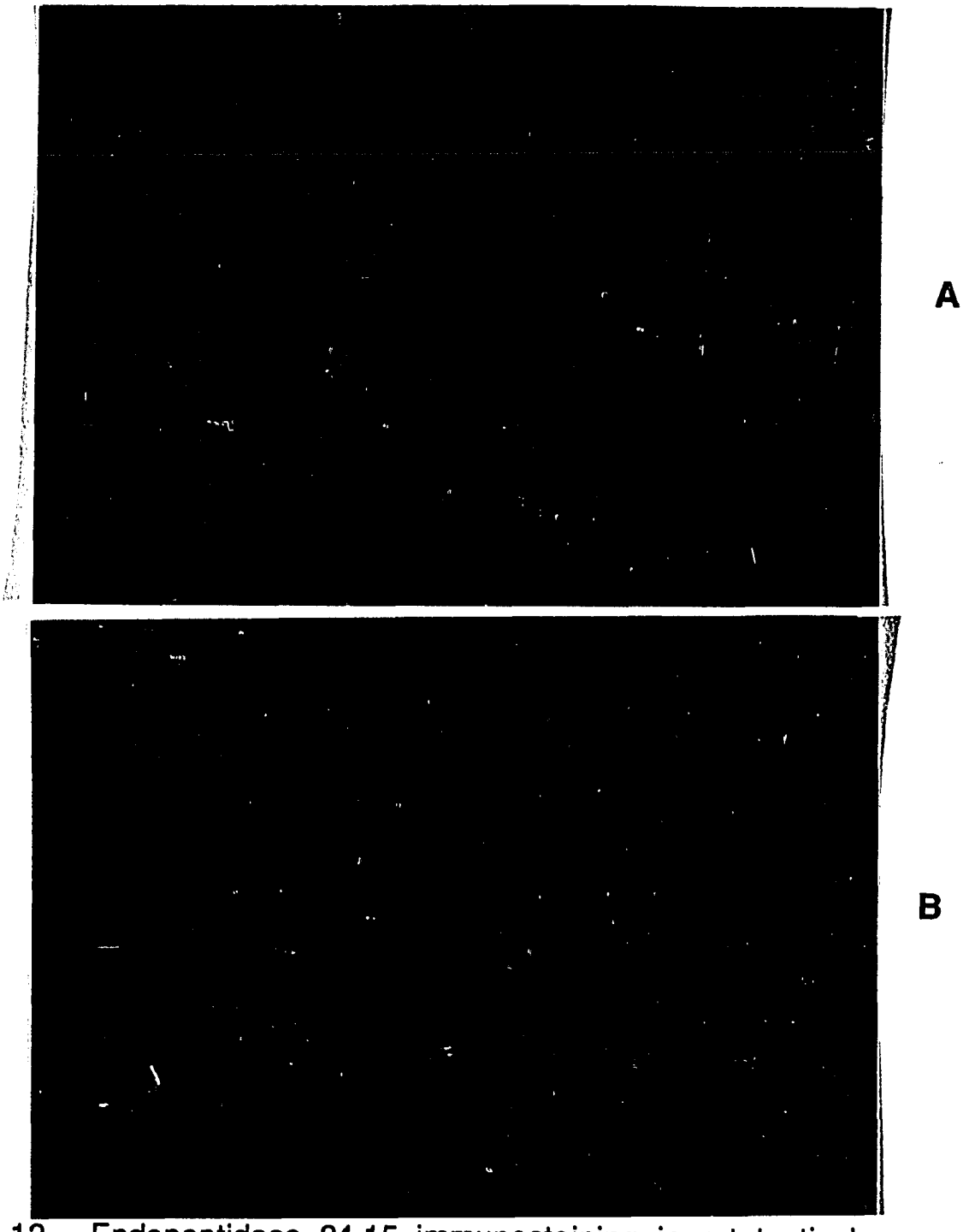

Figure 12. Endopeptidase 24.15 immunostaining in rat testicular interstitium. Antiserum was incubated overnight at 4°C in the absence (A) or presence (B) of the enzyme as described in Materials and Methods.

contained approximately 150% as much endopeptidase 24.15 activity as the fraction containing residual interstitial cells. This increase of endopeptidase 24.15 specific activity in Leydig cells as compared to other interstitial cells is an underestimation of the enrichment of the enzyme in Leydig cells for two reasons. Broken Leydig cells, which may contain enzyme activity, are not separated from residual interstitial cells during Percoll density centrifugation. Furthermore, macrophages may be copurified with Leydig cells in the Percoll density centrifugation, lowering the calculated enzyme specific activity.

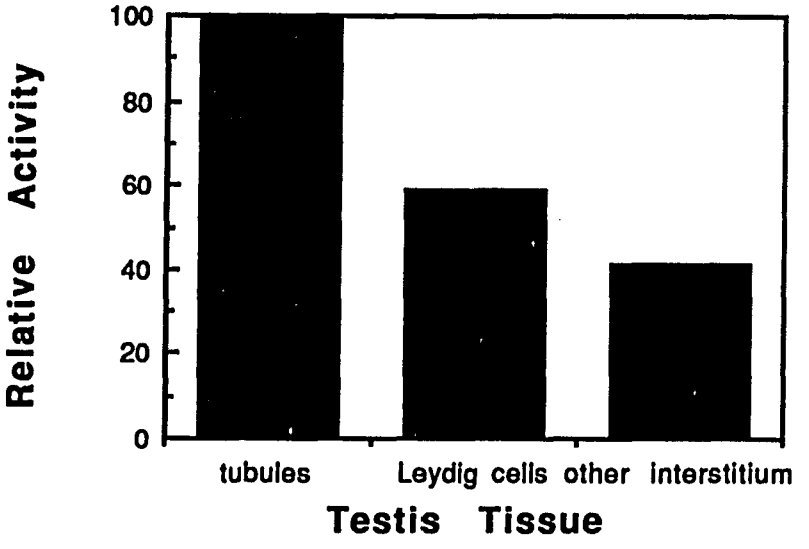

Figure 13. Distribution of endopeptidase 24.15 activity in rat testis. Interstitial cells were separated from tubules and Leydig cells were purified by collagenase dispersion followed by Percoll density centrifugation. The tubules, Leydig cell-enriched fraction, and the residual interstitial cell fraction were homogenized and assayed for enzyme activity in the absence and presence of a specific endopeptidase 24.15 inhibitor as described in Materials and Methods. Results are averages of duplicate determinations from two separate preparations. The differences in relative activities between the tubules and the other fractions and between Leydig cells and other interstitial cells are significant (Student's t test, $p<0.01$). Relative specific activity of the tubular fraction is arbitrarily set to 100.

DISCUSSION

Previous efforts to isolate a homogeneous preparation of endopeptidase 24.15 from rat brain failed because of the presence of an inactive contaminating protein having virtually the same molecular mass and isoelectric point. Efforts were therefore directed toward the isolation of the enzyme from rat testes, a tissue rich in endopeptidase 24.15 activity. The purification was modified from that reported previously (Orlowski et al., 1983) by eliminating the pH 5.0 precipitation step and hydrophobic chromatography and introducing two ion-exchange chromatography steps at two different pH values. An additional purification step consisting of preparative PAGE followed by electroelution of the enzyme was needed to obtain a homogeneous preparation of the enzyme. While the yield in the last step was rather low, it could be used repeatedly on fractions from a single purification run to obtain small batches of a homogeneous enzyme each containing about 40 μg of protein. The isolated enzyme gave a single band in PAGE under non-dissociating and denaturing and dissociating conditions, and homogeneity was also suggested by Ouchterlony immunodiffusion experiments and Western blots using a polyclonal antiserum raised in rabbits. The electrophoretic pattern of the isolated enzyme indicated the presence of a single polypeptide chain with a molecular mass of about 70 kDa, similar to that estimated for the brain enzyme (67 kDa). The immunological properties of the brain and testis enzyme are very similar if not identical.

Although the metal content of the enzyme has not yet been determined, several lines of evidence indicate that it is a metalloendopeptidase. This conclusion is based on the finding that metal chelators (o-phenanthroline, EDTA, EGTA) consistently inhibit the enzyme, that dialysis against chelators leads to loss of activity which can be restored by Zn^{2+} and Co^{2+} ions, that the Co^{2+} enzyme is more active than the Zn^{2+} enzyme, and finally that substrate-related N-carboxymethyl peptides act as specific and potent enzyme inhibitors (Chu & Orlowski, 1984; Orlowski et al., 1988). All these findings are characteristic of Zn^{2+} peptidases. An additional argument for classification of the enzyme as a metalloendopeptidase is inhibition of the isolated enzyme by low concentrations of DTT (0.4 mM). Crude enzyme preparations (Orlowski et al., 1983) from brain and testis are weakly activated by low concentrations of DTT (0.4-1 mM), but inhibited at higher concentrations (over 1.5 mM), whereas the isolated enzyme is inhibited even at low DTT concentrations. While the causes of this behavior are not clear, we have observed that enzyme preparations after step 4 of the purification procedure have a tendency to form heterodimers with the contaminating impurity. Accordingly the activating effect of DTT in crude preparations may result from preventing formation of such heterodimers.

A highly purified preparation of endopeptidase 24.15 from brain was isolated by immunoaffinity chromatography. The specific activity of the isolated enzyme (975 units/mg) was several fold higher than that of the preparations obtained by conventional purification methods (172 units/mg) (Orlowski et al., 1983). The

immunoaffinity column eliminates the need for preparative gel electrophoresis, a purification step in which 90% of enzyme activity is lost (Orlowski et al, 1989) and allows preparations of highly purified enzyme to be obtained in a few days.

A drawback of the procedure is that the recovery of enzyme from the immunoaffinity column is relatively low, generally 20-25%. These yields are in the same range as those obtained by Gee et al., (1983) and Fulcher and Kenny (1983) for the isolation of endopeptidase 24.11 by immunoaffinity chromatography. The low yield may be partly accounted for by a wide range of affinities of the polyclonal antibodies. Some high affinity antibodies may have only released bound enzyme when the column was regenerated with pH 2.3 buffer, a process that would inactivate any enzyme then stripped off, while other antibodies with low affinity for the enzyme may have allowed the elution of dilute enzyme during the washing cycles.

SDS PAGE revealed that the preparation of the soluble brain enzyme obtained by immunoaffinity chromatography is essentially homogeneous. A small difference in migration in SDS PAGE, possibly accounted for by tissue specific post-translational modifications, was consistently observed. The modifications do not seem to include the addition or deletion of an epitope, as the anti-endopeptidase 24.15 antiserum, raised against the testis enzyme, did not distinguish between the enzymes in Ouchterlony immunodiffusion experiments or in anticatalytic immunoinhibition assays. The latter experiments are consistent with an active site preserved in the two tissues, although the possibility that the

antibodies are anticatalytic because of interactions with the enzyme that cause inhibition indirectly can not be ruled out. The similar K_m's and K_i's determined with the two enzymes are further evidence for very similar if not identical active sites. On the other hand, the K_i's for cFP-Ala-Ala-Tyr-pAB, the more potent inhibitor, were statistically significantly different. The potency of the specific inhibitors and affinity of the synthetic substrate t-Boc-Phe-Ala-Ala-Phe-pAB for the enzymes are consistent with the presence of an extended binding site with major determinants of specificity being a hydrophobic residue in the P_1 position and a hydrophobic or bulky residue in the P_3' position (Orlowski et al, 1983; Orlowski et al., 1989).

SDS PAGE revealed that preparations of the membrane-bound brain endopeptidase 24.15 were not homogeneous, but contained two or three minor lower molecular weight components. Of interest is the finding that the membrane-bound form migrates slightly more slowly than the soluble form of the enzyme in brain. The addition of an amino acid sequence containing a hydrophobic side chain for anchoring in the plasma membrane would be consistent with this observation. Such a modification of the enzyme would be similar to the addition of a hydrophobic sequence found in a form of cytochrome b_5 (Spatz & Strittmatter, 1973a) or the addition of a hydrophobic membrane-binding segment found in a form of reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase (Spatz & Strittmatter, 1973b). A common mode of membrane attachment for several membrane-bound enzymes is a glycosyl-phosphoinositol anchor attached at the C-terminus (Ferguson & Williams, 1988).

Such proteins from mammalian cells, however, are poorly soluble in nonionic detergents, including Triton X-100.

Immunologically and catalytically the soluble and membrane-bound brain enzymes are very similar if not identical. Ouchterlony immunodiffusion experiments with anti-endopeptidase 24.15 antiserum and the two forms of the enzyme yielded patterns of identity. Immunoinhibition curves generated in anticatalytic assays were superimposable. K_m's with a synthetic substrate and K_i's with two substrate-related inhibitors did not differ for the two forms of the enzyme.

As the enzyme is most highly concentrated in the testis, the distribution of endopeptidase 24.15 was examined in this tissue. The data in the preceding chapter indicate that rat testicular endopeptidase 24.15 immunoreactivity is localized in two cell populations: Spermatids and Leydig cells. Furthermore, the seminiferous tubules are enriched with the enzyme biochemically, relative to the interstitium, and Leydig cells are endopeptidase 24.15 enriched relative to other interstitial cells.

Immunostaining of spermatids was consistently observed and the staining was absent when antiserum preabsorbed with the enzyme was used. Various seminiferous tubules throughout the tissue slices, but not every tubule, contained staining. This finding is consistent with the heterogeneity of the tubules. Rat seminiferous tubules pass through 14 different stages, or cell associations, as the germ cells mature (Clermont & Bustos-Obregon,1968). The immunostained tubules appeared to be at approximately stage XII.

Leydig cells contained dark well-delimited granules consistently observed with high dilutions of anti-endopeptidase 24.15 antiserum (1/10,000). The identity of the immunostaining with the enzyme was confirmed by the dramatic reduction in staining with antiserum preabsorbed with the enzyme. The location of immunostained cells in the interstitium, the grouping of the cells in small clusters, and the shapes of the cells are in accordance with their identity as Leydig cells. As immunostained granules occurred both along the periphery of cells and in the cytoplasm superimposed over diffuse staining, both the soluble and membrane-bound forms of the enzyme were probably detected in Leydig cells.

The biochemical studies involving separation of the seminiferous tubules from the interstitium and purification of Leydig cells from other interstitial cells were undertaken to determine whether the distribution of the enzyme activity in the rat testis determined biochemically corresponds to the distribution indicated by immunocytochemistry. The enrichment in endopeptidase 24.15 activity of the Leydig cell fraction, as compared to the fraction of other interstitial cells, confirms that the stained cells are indeed Leydig cells. As immunostaining is difficult to quantify, the biochemical studies were necessary to determine whether the specific activity of the enzyme was higher in the tubules or in the interstitium. This question was particularly difficult to address with immunocytochemical studies, because the conditions for optimal staining differed for the two populations of cells that contain the enzyme. Spermatids are apparently several-fold richer in the enzyme biochemically than are Leydig cells.

Significance

The finding that catalytically and immunologically very similar forms of the enzyme occur in the brain and in the testis is not surprising in light of the similar potential substrates for the enzyme found in these tissues. Several lines of evidence point to the presence of opioid peptides in the testis, specifically in Leydig cells. The prodynorphin gene in the R2C Leydig tumor cell line is expressed and prodynorphin-derived peptides are synthesized in those cells (McMurray et al., 1989). Immunoreactivity for both Leu- and Met-enkephalin has been identified in the rat testis (Cox et al., 1987; Engelhardt et al., 1986). Indeed, immunostaining for both enkephalins occurs in the form of well-delimited granules, reminiscent of endopeptidase 24.15 immunocytochemical staining presented here, along the periphery of Leydig cells (Engelhardt et al, 1986). The opioid peptides may modulate Leydig cell steroidogenic function, as intratesticular administration of the opioid antagonists naloxone or nalmefene to rats results in significantly decreased testosterone production in vitro and in vivo. (Gerendai et al., 1984).

A potential substrate for the enzyme in Leydig cells is substance P, as endopeptidase 24.15 is known to act on this peptide, although the affinity of substance P for the enzyme is relatively low, (Chu & Orlowski, 1985; Orlowski et al, 1989) and as immunoreactivity for a substance P-like peptide has been detected

in Leydig cells (Schulze et al, 1987). Whereas the opioid peptides have been identified in a wide range of tissues (Zaigon et al., 1986), substance P-like immunoreactivity has been detected only in tissues believed to be developmentally related to the nervous system and in Leydig cells. The detection of substance P-like immunoreactivity in human Leydig cells prompted Schulze et al. (1987) to speculate that Leydig cells may be of neural crest origin. This speculation is in accordance with the concentration of the enzyme in brain, pituitary, spinal cord, and testes and the function of endopeptidase 24.15 as a neuropeptide metabolizing enzyme.

Production and secretion of testosterone by Leydig cells is regulated not only in an endocrine manner by LH but in a paracrine or autocrine manner by LHRH in the testis. LHRH affects Leydig cell function in hypophysectomized animals (Hsueh & Erickson, 1979) and in isolated Leydig cells (Hunter et al, 1982). Receptors for LHRH and LHRH agonists have been identified on Leydig cells (Hazum & Keinan, 1983; Sharpe & Fraser, 1980) and shown to be initially distributed uniformly on the cell surface and then form clusters and internalize upon binding LHRH (Sharpe & Fraser, 1980).

LHRH or an LHRH-like factor, distinct from hypothalamic LHRH, may be produced in the testis by Sertoli cells. It has been demonstrated that rat testicular immunoreactive LHRH differs from hypothalamic LHRH (Dutlow & Millar, 1981). LH-stimulated production of testosterone in a co-culture of Leydig and Sertoli cells was inhibited by a partially purified fraction of a testis extract. The inhibition was prevented by addition of an LHRH antagonist to the incubation medium (Bhasin & Swerdloff, 1984).

Testicular LHRH or LHRH-like factor may be involved in Sertoli-Leydig cell communication (Sharpe et al., 1981). Recent studies have shown that endopeptidase 24.15 is the primary enzyme responsible for cleavage of the Tyr^5-Gly^6 bond in LHRH in membrane preparations from hypothalamus and pituitary (Molineaux et al., 1988). Experiments have also shown that specific inhibitors of the enzyme cause a dramatic increase in the half-life of intravenously administered LHRH (Lasdun et al., 1989). Taken together, the evidence for endopeptidase 24.15 degradation of LHRH and for the presence of LHRH in the testis suggest that the enzyme may be involved in the regulation of testicular LHRH available to interact with Leydig cell receptors.

The Leydig cell response to chronically administered LHRH is mainly decreased production and release of testosterone, although an initial increase in steroidogenesis occurs (Jones & Hsueh, 1984). Regulation of the concentration of LHRH in the vicinity of the Leydig cells, then, is necessary for maintaining testosterone production. The presence of opioid peptides in the Leydig cell milieu apparently also stimulates testosterone production and release (Hsueh & Erickson, 1979). Endopeptidase 24.15, then, may play a double role in maintaining serum testosterone levels by local action in the testis on both LHRH and enkephalin precursors. This dual effect of the testicular enzyme may be analogous to that of the enzyme in the brain, where levels of hypothalamic LHRH may be regulated by the enzyme in two ways. The enzyme may degrade LHRH directly and may also release opioid peptides from peptide precursors, which affects production and secretion of hypothalamic LHRH (Rasmussen

et al., 1988). A change in hypothalamic LHRH levels would in turn affect release of pituitary LH and finally release of testosterone from Leydig cells. A highly speculative scheme of the central and peripheral effects of endopeptidase 24.15 on testosterone release from Leydig cells is diagrammed in Figure 14. Regulation of blood testosterone concentration at the level of the central nervous system and at the level of the gonads may be an example of enzyme mediated regulation of a single physiological function through activity in different tissues.

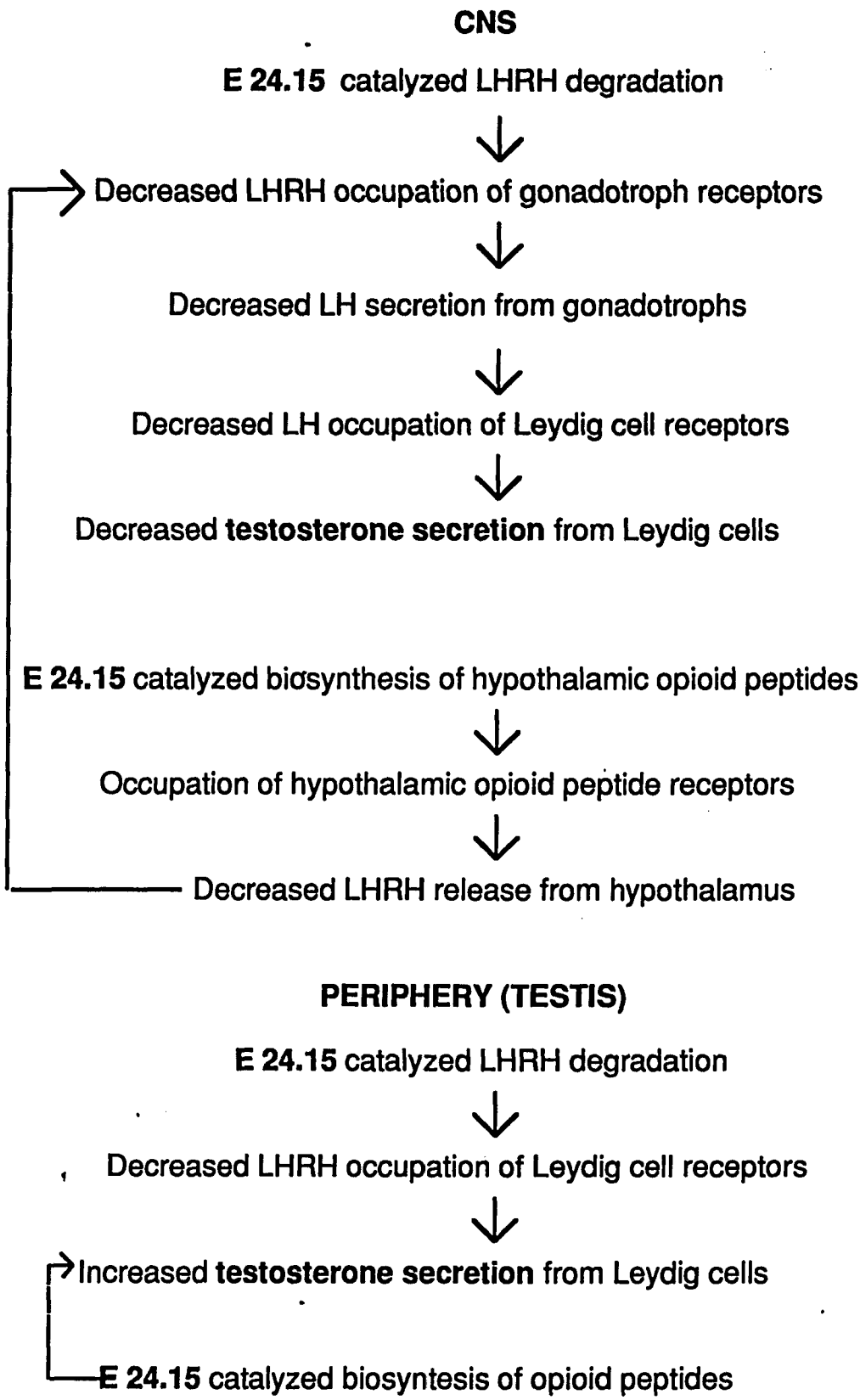

Figure 14. Central and peripheral endopeptidase 24.15 mediated regulation of Leydig cell testosterone secretion.

The immunostaining in the seminiferous tubules occurred in selected tubules at or around one of the 14 stages. Judging from the wide bundles of spermatids deeply embedded in the Sertoli cytoplasm, the tubules containing immunostaining appear to be at approximately Stage XII. Spermatids at this stage are fairly mature; they are in the twelfth of nineteen stages of spermatid development, the nineteenth stage just preceding spermatid release. The data do not indicate that endopeptidase 24.15 is absent from germ cells that are not close to Stage XII in development, but that enzyme activity peaks around this stage. Endopeptidase 24.15 may play a role in the part of the transformation of the spermatid into a spermatazoan that occurs during Stage XII, i.e., the flattening of the acrosome from a triangular shape along the dorsal edge of the nucleus.

Metalloendoproteases have been implicated in membrane fusion events and, interestingly, in the acrosome reaction in sea urchin sperm. Farach et al. (1986) found that metalloendoprotease inhibitors and a synthetic substrate blocked the acrosome reaction in sea urchin sperm. Metalloendoprotease-catalyzed proteolysis is required to generate a "fusogenic" peptide in paramyxovirus-host cell fusion (White et al., 1983: Scheid & Choppin, 1977). Alternatively, a metalloprotease might be involved in membrane fusion events by catalyzing the cleavage of the exposed domains of membrane proteins, removing charge or steric restraints to membrane apposition. The findings of Heder et al. (1989) that the activities of the metallopeptidases endopeptidase 24.11, angiotensin converting enzyme, and leucine aminopeptidase are well

correlated with fertility parameters of bull ejaculates suggest that metallopeptidases may have a role in fertilization.

The affinity of endopeptidase 24.15 for opioid peptide precursors suggests that the primary role of the enzyme, however, may be in the biosynthesis of opioid peptides. Although neuropeptides and peptide hormones have emerged as the modulators of central nervous system functions, only a few of the enzymes that catalyze their formation and degradation have been isolated and characterized. Endopeptidase 24.11, shown to cleave peptides on the amino side of hydrophobic amino acid residues in this laboratory (Orlowski & Wilk, 1981), effects the breakdown of Met- and Leu-enkephalin. The biosynthesis of these pentapeptides, on the other hand, involves the actions of other enzymes that catalyze their release from larger precursor molecules. The occurrence of intermediate size neuropeptides, such as dynorphin $A_{(1-8)}$, α-neoendorphin, and β-neoendorphin raises the question of whether Met-enkephalin and Leu-enkephalin are released from the high molecular weight precursors directly, or from the intermediate enkephalin-containing peptides.

The specificity of endopeptidase 24.15 was studied with a series of model synthetic substrates and it was determined that the enzyme preferentially cleaves peptides containing at least five amino acid residues, with substrates having hydrophobic residues at the P_1, P_2, and P_3' positions being preferred[1] (Chu and Orlowski, 1983). The enzyme was shown to catalyze the breakdown of several bioactive peptides at sites consistent with these specificity

criteria. Specifically, Chu and Orlowski (1985) showed that the enzyme catalyzes the release of Leu-enkephalin from dynorphin $A_{(1-8)}$, α-neoendorphin, and β-neoendorphin and the release of Met-enkephalin from bovine adrenal medulla dodecapeptide (BAM-12P), a Met-enkephalin-containing endogenous opioid peptide (Mizuno et al., 1980). Analysis of reaction products revealed that Leu or Met fills the S_1 subsite, Phe the S_2 subsite, and Ile, Tyr, or Val the S_3' subsite, an arrangement consistent with the scheme described.

In addition to the prodynorphin-derived peptides, several other bioactive peptides are subject to endopeptidase 24.15 catalyzed degradation *in vitro* (Table X). The affinity of the enzyme for bradykinin, neurotensin, and the angiotensins is similar to the affinity of the enzyme for prodynorphin derived opioid peptides (Chu & Orlowski, 1985). Endopeptidase 24.15 may be involved in the transformation and degradation of neuropeptides, peptide hormones, and bioactive peptides in general, modulating particular physiological functions at various tissue sites.

Table X. Hydrolysis of bioactive peptides by endopeptidase 24.15.

Peptide	Structure and site of hydrolysis
Dynorphin(1-8)	Tyr-Gly-Gly-Phe-Leu ↓ - Arg-Arg-Ile
α-Neo-Endorphin	Tyr-Gly-Gly-Phe-Leu ↓ - Arg-Lys-Try-Pro-Lys
β-Neo-Endorphin	Tyr-Gly-Gly-Phe-Leu ↓ - Arg-Lys-Tyr-Pro
Bradykinin	Arg-Pro-Pro-Gly-Phe ↓ - Ser-Pro-Phe-Arg
Neurotensin	<Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg ↓ Arg-Pro-Tyr-Ile-Leu
Substance P	Arg-Pro-Lys-Pro ↓ - Gln-Gln-Phe ↓ - Phe ↓ - Gly-Leu-Met-NH2
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro ↓ - Phe-His-Leu
Angiotensin II	Asp-Arg-Val-Tyr ↓ - Ile-His-Pro-Phe
Somatostatin	Ala-Gly-Cys-Lys ↓ - Asn-Phe-Phe-Trp ↓ - Lys-Thr-Phe-Thr-Ser-Cys (disulfide bridge)

FOOTNOTE

[1]The nomenclature proposed by Schecter and Berger (1967) is used to describe the position (P) of the residues in the substrate and the corresponding subsites (S) in the active site of the enzyme.

REFERENCES

Acker, G. R., Molineaux, C. J., & Orlowski, M. (1987) Synaptosomal Membrane-Bound Form of Endopeptidase-24.15 Generates Leu-enkephalin From Dynorphin(1-8), α- and β-Neoendorphin, and Met-enkephalin From Met-enkephalin-Arg-Gly-Leu. **J. Neurochem. 48**, 284.

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

Aou, S., Oomura, Y., Noshino, H., Nakano, Y., & Lénárd, L. (1988) Feeding-Related Activity of Opiate-Sensitive Neurons in the Monkey. In **Regulatory Roles of Opioid Peptides**. Illes, P. & Farsang, P. (eds.) VCH Publishers, Weinheim/Basel/Cambridge/New York.

Ashley, P. L. & MacDonald, R. J. (1985) Tissue-Specific Expression of Kallikrein-Related Genes in the Rat. **Biochemistry 24**, 4520.

Beynon, R. J., Shannon, J. D., & Bond, J. S. (1981) Purification and Characterization of a Metalloendoproteinase From Mouse Kidney. **Biochem. J. 199**, 591.

Bhargava, H. N. (1978) Effects of Methionine on Spontaneous Locomotor Activity: Antagonism By Naloxone. **Pharmacol. Biochem. Behav. 9**, 167.

Bhasin, S. & Swerdloff, R. S. (1984) Testicular GnRH-Like Factors: Characterization of Biologic Activity. **Biochem. Biophys. Res. Comm.122**, 1070.

Blobel, G. & Dobberstein, B. (1975) Transfer of Proteins Across Membranes. I. Presence of Proteolytically Processed and Unprocessed Nascent Immunoglobulin Light Chains on Membrane-Bound Ribosomes of Murine Myeloma. **J. Cell Biol. 67**, 835.

Bothwell, M. A., Wilson, W. H., & Shooter, E. M. (1979) The Relationship Between Glandular Kallikrein and Growth Factor-Processing Proteases of Mouse Submaxillary Gland. **J. Biol. Chem. 254**, 7287.

Bond, J. S., & Beynon, R. J. (1986) Meprin: A Membrane-Bound Metallo-endopeptidase. **Curr. Top. Cell. Regul. 28**, 263.

Brennan, S. O. & Carrell, R. W. (1978) A Circulating Variant of Human Proalbumin. **Nature 274**, 908.

Burnette, W. N. (1981) "Western Blotting": Electrophoretic Transfer of Proteins From Sodium Dodecyl Sulfate-Polyacrylamide Gels to Unmodified Nitrocellulose and Radiographic Detection with Antibody and Radioiodinated Protein A. **Anal. Biochem. 112**, 195.

Chan, S. J., Emdin, S. O., Kwok, S. C. M., Kramer, J. M., Falkmer, S., & Steiner, D. F. (1981) Messenger RNA Sequence and Primary Structure of Preproinsulin In a Primitive Vertebrate, the Atlantic Hagfish. **J. Biol. Chem. 256**, 7595.

Chavkin, C. & Goldstein, A. (1981) Specific Receptor for the Opioid Peptide Dynorphin: Structure-Activity Relationships. **Proc. Natl. Acad. Sci. U.S.A. 78**, 6543.

Chu, T. G. & Orlowski, M. (1984) Active-Site Directed N-Carboxymethyl Peptide Inhibitors of a Soluble Metalloendopeptidase From Rat Brain. **Biochemistry 23**, 3598.

Chu, T. G. & Orlowski, M. (1985) Soluble Metalloendopeptidase From Rat Brain: Action on Enkephalin-Containing Peptides and Other Bioactive Peptides. **Endocrinol. 116**, 141.

Clermont, Y. & Bustos-Obregon, E. (1968) Re-examination of Spermatogonial Renewal in the Rat by Means of Seminiferous Tubules Mounted "in toto". **Am. J. Anat. 122**, 237.

Clayton, R. N., Katikineni, M., Chan, V., Dufau, M. L., & Catt, K. J. (1980) Direct Inhibition of Testicular Function by Gonadotropin-Releasing Hormone: Mediation By Specific Gonadotropin-Releasing Hormone Receptors in Interstitial Cells. **Proc. Natl. Acad. Sci. (USA) 77**, 4459.

Coomb, M., Seeburg, P. H., Adelman, J., Eiden, L., & Herbert, E. (1982) Primary Structure of the Human Met- and Leu-enkephalin Precursor and Its mRNA. **Nature 295**, 663.

Cone, R. I., Weber, E., Barchas, J. D., & Goldstein, A. (1983) A Regional Distribution of Dynorphin and Neo-endorphin Peptides in Rat Brain, Spinal Cord, and Pituitary. **J. Neurosci. 3**, 2146.

Connelly, J. C., Skidgel, R. A., Schulz, W. W., Johnson, A. R., & Erdös, E. G. (1985) Neutral Endopeptidase 24.11 in Human Neutrophils: Cleavage of Chemotactic Peptide. **Proc. Natl. Acad. Sci. USA 82**, 8737.

Cox, B., Rosenberger, J. G., & Douglass, J. (1987) Chromatographic Characterization of Dynorphin and [Leu^5]enkephalin Immunoreactivity in Guinea Pig and Rat Testis. **Regul. Peptides19**, 1.

DeCaro, G., Micossi, L. G. & Venturi, F. (1979) Drinking Behaviour Induced by Intracerebroventricular Administration of Enkephalins to Rats. **Nature 277**, 51.

Dixon, M. (1972) The Graphical Determination of K_m and K_i. **Biochem. J. 129,** 197.

Docherty, K. & Steiner, D. F. (1982) Post-translational Proteolysis in Polypeptide Hormone Biosynthesis. **Annu. Rev. Physiol. 44**, 625.

Dutlow, C. M. & Millar, R. P. (1981) Rat Testis Immunoreactive LH-RH Differs Structurally From Hypothalamic LH-RH. **Biochem. Biophys. Res. Comm. 101**, 486.

Engelhardt, R. P., Saint-Pol, P., Tramu, G., & Leonardelli, J. (1986) Immunohistochemical Localization of Enkephalinlike Peptides During Testicular Development in Rats. **Arch. Androl. 17**, 49.

Engelhardt, R. P. (1989) Gonadal Opioids and Testicular Function. **Ann. Endocrinol. 50**, 64.

Erdös, E. G., Schulz, W. Gafford, J. T., & Defendini, R. (1985) Neutral Metalloendopeptidase in Human Male Genital Tract. Comparison to Angiotensin I-Converting Enzyme. **Lab. Invest. 52**, 437.

Eskeland, N. L., Lugo, D. I., Pintar, J. E., & Schachter, B. S. (1989) Stimulation of β-endorphin Secretion by Corticotropin-Releasing Factor in Primary Rat Leydig Cell Cultures. **Endocrinology 124**, 2914.

Evangelista, R., Purinima, R., & Lewis, R. V. (1982) A "Trypsin-Like" Enzyme in Adrenal Chromaffin Granules: A Proenkephalin Processing Enzyme. **Biochem. Biophys. Res. Commun. 106**, 895.

Farach, F. A. Jr., Mundy, D. I., Strittmatter, W. J., & Lennarz, W. J. (1986) Evidence for the Involvement of Metalloendoproteases in the Acrosome Reaction in Sea Urchin Sperm. **J. Biol. Chem. 262**, 5483.

Ferguson, M.A.J. & Williams, A.F. (1988) Cell-Surface Anchoring of Proteins Via Glycosyl-Phosphatidylinositol Structures. **Ann. Rev. Biochem. 57**, 285.

Frey, P., Forand, R., Maciag, T., & Shooter, E. M. (1979) The Biosynthetic Precursor of Epidermal Growth Factor and the Mechanism of Its Processing. **Proc. Natl. Acad. Sci. U.S.A. 76**, 6294.

Fricker, L. D. & Snyder, S. H. (1984) Enkephalin Convertase: Purification and Characterization of a Specific Enkephalin-Synthesizing Carboxypeptidase Localized to Adrenal Chromaffin Granules. **Proc. Natl. Acad. Sci. U.S.A. 79**, 3886.

Frigeni, V., Bruno F., Carenzi, A., Racagni, R., & Santini, V. (1978) Analgesia and Motor Activity Elicited By Morphine and Enkephalins in Two Inbred Strains of Mice. **J. Pharm. Pharmacol. 30**, 310.

Fulcher, I. S. & Kenny, A. J. (1983) Proteins of the Kidney Microvillar Membrane. The Amphipathic Forms of Endopeptidase Purified From Pig Kidneys. **Biochem. J. 211**, 743.

Gafford, J., Skidgel, R. A., Erdös, E. G., & Hersh, L. B. (1983) Human Kidney "Enkephalinase", a Neutral Metalloendopeptidase That Cleaves Active Peptides. **Biochemistry 22**, 3265.

Gambert, S. R., Garthwaite, T. L., Pontzer, C. H., & Hagen, T. C. (1980) Fasting Associated with Decrease in Hypothalamic β-Endorphin. **Science 210**, 1271.

Garthwaite, T. L., Martinson, D. R., Tseng, L. F., Hagen, T. C., & Menahan, L. A. (1980) A Longitudinal Hormonal Profile of the Genetically Obese Mouse. **Endocrinology 107**, 671.

Gee, N. S., Matsas, R., & Kenny, A. J. (1983) A Monoclonal Antibody to Kidney Endopeptidase 24.11. Its Application in Immunoabsorbent Purification of the Enzyme and Immunofluorescent Microscopy of Kidney and Intestine. **Biochem. J. 214**, 377.

Gerendai, I., Shaha, C., Thau, R., & Bardin, W. (1984) Do Testicular Opiates Regulate Leydig Cell Function? **Endocrinol. 84**, 1154.

Goldstein, A., Tachibana, S., Lowney, L. I., Hunkapiller, M., & Hood, L. (1979) Dynorphin-(1-13), an Extraordinarily Potent Opioid Peptide. **Proc. Natl. Acad. Sci. USA 76,** 6666.

Goodman, R. H., Jacobs, J. W., Dee, P. C., & Habener, J. F. (1982) Somatostatin-28 Encoded in a Cloned cDNA Obtained From a Rat Medullary Thyroid Carcinoma. **J. Biol. Chem. 257,** 1156.

Grandison, L. & Guidotti, A. (1977) Stimulation of Food Intake by Muscimol and Beta Endorphin. **Neuropharmacology 16**, 533.

Gubler, U., Seeburg, P., Hoffman, B. J., Gage, L. P., & Udenfriend, S. (1982) Molecular Cloning Established Proenkephalin as Precursor of Enkephalin-Containing Peptides. **Nature 295**, 206.

Gunne, L. M. (1960) The Temperature Response in Rats During Acute and Chronic Morphine Administration, A Study of Morphine Tolerance. **Arch. Int. Pharmacodyn. Ther. 129**, 416.

Hazum, E. & Keinan, D. (1983) Testicular GnRH receptors: Photoaffinity Labeling and Fluorescence Distribution Studies. **Peptides 5**, 119.

Heder, G., Siems, W.-E., Nehring, H., Mueller, K., Hilse, H., & Jentzsch, K. D. (1989) Occurrence and Potential Importance of Selected Peptidases in Bull Ejaculates. **Andrologia 21**, 247.

Hedger, M. P. & Eddy, E. M. (1986) Monoclonal Antibodies Against Rat Leydig Cell Surface Antigen. **Biol. Reprod. 35**, 1309.

Hobart, P. M., Shen, L-P., Crawford, R., Pictet, R. L., & Rutter, W. J. (1980) Comparison of the Nucleic Acid Sequence of Anglerfish and Mammalian Insulin mRNA's From Cloned cDNA's. **Science 210**,1360.

Hojima, Y., van der Rest, M., & Prockop, D. J. (1985) Type I Procollagen Carboxyl-Terminal Proteinase From Chick Embryo Tendons. **J. Biol. Chem. 260**, 15996.

Hook, V. Y. H. & Loh, Y. P. (1984) Carboxypeptidase B-Like Converting Enzyme Activity in Secretory Granules of Rat Pituitary. **Proc. Natl. Acad. Sci. U.S.A. 81**, 2776.

Hsueh, A. J. W. & Erickson, G. F. (1979) Extra-Pituitary Inhibition of Testicular Function by Luteinizing Hormone Releasing Hormone. **Nature 281**, 66.

Huang, W-Y., Chang, R. C., Kastin, A. J., Coy, D. H., & Schally, A. V. (1979) Isolation and Structure of Pro-methionine-enkephalin: Potential Enkephalin Precursor From Porcine Hypothalamus. **Proc. Natl. Acad. Sci. U.S.A. 76,** 6177.

Hughes, J., Kosterlitz, H. W., & McKnight, A. T. (1978) The Incorporation of [^{3}H]-Tyrosine into the Enkephalins of Striatal Slices of Guinea-Pig Brain [Proceedings]. **Br. J. Pharmacol. Chemother. 63**, 396P.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A. & Morris, H. R. (1975) Identification of Two Related Pentapeptides From the Brain With Potent Opiate Agonist Activity. **Nature 258**, 577.

Hunter, M. G., Sullivan, M. H. F., Dix, C. J., Aldred, L. F., & Cooke, B. A. (1982) Stimulation and Inhibition by LHRH Analogues of Cultured Rat Leydig Cell Function and Lack of Effect on Mouse Leydig Cells. **Mol. Cell. Endocrinol. 27**, 31.

Inturrisi, C. E., Umans, J. G., Wolff, D. Stern, A. S., Lewis, R. V., Stein, S., & Udenfriend, S. (1980) Analgesic Activity of the Naturally Occurring Heptapeptide [Met]enkephalin-Arg^6-Phe^7. **Proc. Natl. Acad. Sci. U.S.A. 77**, 5512.

Johnson, A. R., Ashton, J., Schulz, W. W., & Erdös, E. G. (1985) Neutral Metalloendopeptidase in Human Lung Tissue and Cultured Cells. **Am. Rev. Respir. Dis. 132**, 564.

Johnson, A. R., Skidgel, R. A., Gaffard, J. T., & Erdös, E. G. (1984) Enzymes in Placental Microvilli: Angiotensin I Converting Enzyme, Angiotensinase A, Carboxypeptidase, and Neutral Endopeptidase ("Enkephalinase"). **Peptides 5**, 789.

Jones, P. B. C. & Hsueh, A. J. W. (1984) Direct Antigonadal Actions of LHRH. In: Vikery, B. H., Nestor, J. J. Jr., & Hafez, E. S. E. (eds.) **LHRH and Its Analogs: Contraceptive and Therapeutic Applications**. MTP Press Limited, Lancaster/Boston/The Hague/Dordrecht.

Kakidani, H., Furutani, Y., Takahashi, H., Noda, M., Morimoto, Y., Hiroe, T., Asai, M., Inayama, S., Nakanishi, S., & Numa, S. (1982) Cloning and Sequence Analysis of cDNA for Porcine α-Neo-endorphin/Dynorphin Precursor. **Nature 298**, 245.

Kerr, M. A. & Kenny, A. J. (1974) The Purification and Specificity of a Neutral Endopeptidase From Rabbit Kidney Brush Border. **Biochem. J. 137**, 447.

Laemmli, U. K. (1970) Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. **Nature 227**, 680.

Lasdun A., Reznik S., Molineaux C. G., & Orlowski M. (1989) Inhibition of Endopeptidase 24.15 Slows the in vivo Degradation of LHRH. **J. Pharm. Exper. Ther. 251**, 439.

Letarte, M., Vera, S., Tran, R., Addis, J. B., Onizuka, R. J., Quackenbush, E. J., Jongencel, C. V., McInnes, R. R. (1988) Common Acute Lymphocytic Leukemia Antigen Is Identical to Neutral Endopeptidase. **J. Exp. Med. 168**, 1247.

Lewis, R. V., Stern, A. S., Kimura, S., Rossier, J., Stein, S., & Udenfriend, S. (1979) An About 50,000-Dalton Protein in Adrenal Medulla: A Common Precursor of [Met]- and [Leu]enkephalin. **Science 208**, 1459.

Liepnieks, J. J. & Light A. (1979) The Preparation and Properties of Bovine Enterokinase. **J. Biol. Chem. 254**, 1677.

Lindberg, I., Yang, H. Y. T., & Costa, E. (1984) Further Characterization of an Enkephalin-Generating Enzyme From Adrenal Medullary Chromaffin Granules. **J. Neurochem. 42**, 1411.

Look, A. T., Ashmun, R. A., Shapiro, L. H., & Peiper, S. C. (1989) Human Myeloid Plasma Membrane Glycoprotein CD13 (gp150) Is Identical to Aminopeptidase N. **J. Clin. Invest. 83**, 1299.

Lowry, O. H., Rosebrough, A. L., Farr, A. L., & Randall, R. J. (1951) Protein Measurement with the Folin Phenol Reagent. **J. Biol. Chem. 193**, 265.

Malfroy, B., Swerts, J. P., Guyon, A., Roques, B. P. & Schwartz, J.-C. (1978) High Affinity Enkephalin Degrading Peptidase in Mouse Brain and Its Enhanced Activity Following Morphine. **Nature 276**, 523.

Malfroy, B., Schofield, P. R., Kuang, W.-J., Seeburg, P. H., Mason, A. J., & Henzel, W. J. (1987) Molecular Cloning and Amino Acid Sequence of Rat Enkephalinase. **Biochem. Biophys. Res. Commun. 144**, 59.

Matsas, R., Kenny, A. J. & Turner, A. J. (1984) The Metabolism of Neuropeptidases. The Hydrolysis of Peptides, Including Enkephalins, Tachykinins and Their Analogues, by Endopeptidase 24.11. **Biochem. J. 223**, 433.

McMurray, C. T., Lakshmi, D., Calavetta, L., & Douglass, J. O. (1989) Regulated Expression of the Prodynorphin Gene in the R2C Leydig Tumor Cell Line. **Endocrinology 124**, 49.

Meyerson, B. J. & Terenius, L. (1977) Beta-endorphin and Male Sexual Behavior. **Eur. J. Pharmacol. 42,** 191.

Mittra, I. (1980a) A Novel "Cleaved Prolactin" in the Rat Pituitary: Part I. Biosynthesis, Characterization, and Regulatory Control. **Biochem. Biophys. Res. Commun. 95**, 1750.

Mittra, I. (1980b) A Novel "Cleaved Prolactin" in the Rat Pituitary: Part II. In vivo Mammary Mitogenic Activity of Its N-Terminal 16K Moiety. **Biochem. Biophys. Res. Commun. 95**, 1760.

Mizuno, K., Minamino, N., Kanagawa, K., & Matsuo, H. (1980) A New Endogenous Opioid Peptide From Bovine Adrenal Medulla: Isolation and Amino Acid Sequence of a Dodecapeptide (BAM-12P). **Biochem. Biophys. Res. Commun. 95**, 1482.

Molineaux, C. J., Lasdun, A., Michaud, C., & Orlowski, M. (1988) Endopeptidase 24.15 Is the Primary Enzyme Which Degrades Luteinizing Hormone Releasing Hormone Both in vitro and in vivo. **J. Neurochem. 51**, 624.

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N., and Numa, S. (1979) Nucleotide Sequence of Cloned cDNA for Bovine Corticotropin-β-Lipotropin Precursor. **Nature 278**, 423.

Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Horose, T., Inayama, S., Nakanishi, S., & Numa, S. (1982) Cloning and Sequence Analysis of cDNA for Bovine Adrenal Preproenkephalin. **Nature 295**, 202.

Ole-Moi Yoi, O., Pinkus, G. S., Spragg, J., & Austen, K. F. (1979) Identification of Human Glandular Kallikrein in the Beta Cell of the Pancreas. **N. Engl. J. Med. 300**, 1289.

Orlowski, M. & Wilk, S. (1981) Purification and Specificity of a Membrane-Bound Metalloendopeptidase From Bovine Pituitaries. **Biochemistry 20**, 4942.

Orlowski, M., Michaud, C., & Chu, T. G. (1983) A Soluble Metallopeptidase From Rat Brain. Purification of the Enzyme and Determination of Specificity with Synthetic and Natural Peptides. **Eur. J. Biochem. 135**, 81.

Orlowski, M., Michaud, C., & Molineaux, C. (1988) Substrate-Related Potent Inhibitors of Endopeptidase 24.15 (EC 3.4.24.15). **Biochemistry 27**, 597.

Orlowski, M., Reznik, S., Ayala, J., & Perotti, A. R. (1989) Endopeptidase 24.15 From Rat Testes. Isolation of the Enzyme and Its Specificity Toward Synthetic and Natural Peptides, Including Enkephalin-Containing Peptides. **Biochem. J. 261**, 951.

Ouchterlony, O. (1958) Diffusion-In-Gel Methods for Immunological Analysis. **Prog. Allergy 5,** 1.

Painter, R. G., Dukes, R., Sullivan, J., Carter, R., Erdös, E. G., & Johnson, A. R. (1988) Function of Neutral Endopeptidase on the Cell Membrane of Human Neutrophils. **J. Biol. Chem. 263**, 9456.

Panthier, J. J., Foote, S., Chambraud, B., Strosberg, A. D., Corvol, P., & Rougeon, F. (1982) Complete Amino Acid Sequence and Maturation of the Mouse Submaxillary Gland Renin Precursor. **Nature 298**, 90.

Patel, Y. C., Wheatley, T., & Ning, C. (1981) Multiple Forms of Immunoreactive Somatostatin: Comparison of Distribution in Neural and Nonneural Tissues and Portal Plasma of the Rat. **Endocrinol. 109**, 1943.

Paterson, S. J., Robson, L. E. & Kosterlitz, H. W. (1983) Classification of Opioid Receptors. **Br. Med. Bull. 39**, 31.

Peterson, G. L. (1977) A Simplification of the Protein Assay Method of Lowry et al. Which is More Generally Applicable. **Anal. Biochem. 83**, 346.

Rasmussen, D. D., Kennedy, B. P., Ziegler, M. G., & Nett, T. M. (1988) Endogenous Opioid inhibition and Facilitation of Gonadotropin-Releasing Hormone Release From the Median Eminence in vitro: Potential Role of Catecholamines. **Endocrinol. 123**, 2916.

Robbins, D. C., Blix, P. M., Rubinstein, A. H., Kanazawa, Y., Kosaha, K., & Tager, H. S. (1981) A Human Proinsulin Variant at Arginine 65. **Nature 291**, 679.

Rossier, J. (1982) Opioid Peptides Have Found Their Roots. **Nature 298**, 221.

Rozé, C. (1989) Rôle des Peptides Opioïdes dans la Motricité Intestinale. **Presse Med. 18**, 269.

Russell, M. H. & Geller, D. M. (1975) The Structure of Rat Proalbumin. **J. Biol. Chem. 250**, 3409.

Schechter I. & Berger A. (1967) On the Size of the Active Site in Proteases. I. Papain. **Biochem. Biophys. Res. Commun. 27**, 157.

Scheid, A. & Choppin, P. W. (1977) Two Disulfide-Linked Polypeptide Chains Constitute the Active F Protein of Paramyxoviruses. **Virology 80**, 54.

Schenkein, I., Franklin, E. C., & Frangione, B. (1981) Proteolytic Enzymes From the Mouse Submaxillary Gland: A Partial Sequence and Demonstration of Spontaneous Cleavages. **Arch. Biochem. Biophys. 209**, 57.

Schön, E., Demuth, H. U., Eichmann, E., Horst, J. J., Körner, I. J., Kopp, J., Mattern, T., Neubert, K., Noll, F., Ulmer, A. J., et al. (1989) Dipeptidyl Peptidase IV in Human T Lymphocytes. Impaired Induction of Interleukin 2 and Gamma Interferon Due to Specific Inhibition of Dipeptidyl Peptidase IV. **Scand. J. Immunol. 29**, 127.

Schulz, W. W., Hagler, H. K., Buja, L. M., & Erdös, E. G. (1988) Ultrastructural Localization of Angiotensin I-Converting Enzyme (E.C. 3.4.15.1) and Neutral Metalloendopeptidase (E.C. 3.4.24.11) in the Proximal Tubule of the Human Kidney. **Lab. Invest. 59**, 789.

Schulze, W., Davidoff, M. S., & Holstein, A-F. (1987) Substance P-Like Immunoreactivity in Human Testicular Tissue. **Acta Endocrinol. (Copenh.) 155**, 373.

Schwartz, J. C., Malfroy, B., & De La Baume, S. (1981) Biological Inactivation of Enkephalins and the "Role of Enkephalin-Dipeptidyl Carboxypeptidase ("Enkephalinase") as Neuropeptidase. **Life Sci. 29**,1715.

Sharpe R. M. & Fraser, H. M. (1980a) HCG Stimulation of Testicular LHRH-Like Activity. **Nature 287**, 642.

Sharpe, R. M. & Fraser, H. M. (1980b) Leydig Cell Receptors for Luteinizing Hormone Releasing Hormone and its Agonists and Their Modulation by Administration or Deprivation of the Releasing Hormone. **Biochem. Biophys. Res. Comm. 95**, 256.

Sharpe, R. M., Fraser, H. M., Cooper, I., & Rommerts, F. F. G. (1981) Sertoli-Leydig Cell Communication Via an LHRH-Like Factor. **Nature 290**, 785.

Shipp, M. A., Vijayaraghavan, J., Schmidt, E. V., Masteller, E. L., D'Amio, L., Hersh, L. B., & Reinherz, E. L. (1989) Common Acute Lymphoblastic Leukemia Antigen (CALLA) Is Active Neutral Endopeptidase 24.11 ("Enkephalinase"): Direct Evidence By cDNA Transfection Analysis. **Proc. Natl. Acad. Sci. USA 86**, 297.

Sonders, M. & Weber, E. (1987) Distribution Pattern of Metorphamide Compared With Other Opioid Peptides From Proenkephalin and Prodynorphin in the Bovine Brain. **J. Neurochem. 49**, 671.

Spatz, L. & Strittmatter, P. (1973a) A Form of Cytochrome b5 That Contains an Additional Hydrophobic Sequence of 40 Amino Acid Residues. **Proc. Natl. Acad. Sci. (U.S.A.) 68**, 1042.

Spatz, L. & Strittmatter, P. (1973b) A Form of Reduced Nicotinamide Adenine Dinucleotide-Cytochrome b5 Reductase Containing Both the Catalytic Site and an Additional Hydrophobic Membrane-Binding Segment. **J. Biol. Chem. 248**, 79.

Stein, L. & Belluzzi, J. D. (1979) Brain Endorphins: Possible Role in Reward and Memory Formation. **Fed. Proc. 38**, 2468.

Steiner, D. F., Kemmler, W., Tager, H. S., Rubinstein, A. H., Lernmark, A., & Zühlke, H. (1975) Proteolytic Mechanisms in the Biosynthesis of Polypeptide Hormones. In **Proteases and Biological Control**. Reich, E., Rifkin, D., & Shaw, E. (eds.) Cold Spring Harbor NY: Cold Spring Harbor Lab.

Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J., Tager, H. S. (1980) Processing Mechanisms in the Biosynthesis of Proteins. **Ann. N. Y. Acad. Sci. 343**, 1.

Stern, A. S., Lewis, R. V., Kimura, S., Rossier, J., Gerber, L. D., Brink, L., Stein, S., & Udenfriend, S. (1979) Isolation of the Opioid Heptapeptide Met-enkephalin [Arg^6, Phe^7] From Bovine Adrenal Medullary Granules and Striatum. **Proc. Natl. Acad. Sci. U.S.A. 76**, 6680.

Supattapone, S., Fricker, L. D., Snyder, S. H. (1984) Purification and Characterization of a Membrane-Bound Enkephalin Forming Carboxypeptidase, "Enkephalin Convertase." **J. Neurochem. 42**,1017.

Tähkä, K. M. (1986) Current Aspects of Leydig Cell Function and Its Regulation. **J. Reprod. Fert. 78**, 367.

Taylor, J. M., Mitchell, W. M., & Cohen, S. (1974) Characterization of the Binding Protein for Epidermal Growth Factor. **J. Biol. Chem. 249**, 2188.

Thomas, K. A., Baglan, N. C., & Bradshaw, R. A. (1981) The Amino Acid Sequence of the γ-Subunit of Mouse Submaxillary Gland 7S Nerve Growth Factor. **J. Biol. Chem. 256**, 9156.

Towbin, H., Staehelin, T. & Gordon, J. (1979) Electroletic Transfer of Proteins From Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. **Proc. Natl. Acad. Sci. U.S.A. 76**, 4350.

Trent, D. F. & Weir, G. (1981) Heterogeneity of Somatostatin-like Peptides in Rat Brain, Pancreas, and Gastrointestinal Tract. **Endocrinol. 108**, 2033.

Troy, C. M. & Musacchio, J. M., (1982) Processing of Enkephalin Precursors by Chromaffin Granule Enzymes. **Life Sci. 31**, 1717.

Turner, A. J., Matsas, R., & Kenny, A. J. (1985) Endopeptidase-24.11 and Neuropeptide Metabolism. **Biochem. Soc. Transactions 13**, 39.

Vargish, T. & Beamer, K. C. (1989) Delta and Mu Receptor Agonists Correlate with Greater Depression of Cardiac Function Than Morphine Sulfate in Perfused Rat Hearts. **Circ. Shock 27**, 245.

Weber, E., Evans, S. J., Chang, J. K., Barchas, J. D. (1982) Brain Distribution of α-Neo-endorphin and β-Neo-endorphin: Evidence for Regional Processing Differences. **Biochem. Biophys. Res. Commun. 108**, 81.

Weber, E., Esch, R. S., Böhlen, P., Paterson, S., Corbett, A. D., McKnight, A. T., Kosterlitz, H. W., Barchas, J. D., & Evans, C. J. (1983) Metorphamide: Isolation, Structure, and Biologic Activity of an Amidated Opioid Octapeptide From Bovine Brain. **Proc. Natl. Acad. Sci. U.S.A. 80**, 7362.

White, J., Kielan, M., & Helenius, A. (1983) Membrane Fusion Proteins of Enveloped Animal Viruses. **Q. Rev. Biophys. 16**, 151.

Zagon I. S., Rhodes R. E., & McLaughlin P. G. (1986) Localization of Enkephalin Immunoreactivity in Diverse Tissues and Cells of the Developing and Adult Rat. **Cell Tissue Res. 246**, 561.