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Processing of the oxytocin precursor by carboxypeptidase E

Bleakman, Alexandra

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Processing of the oxytocin precursor by carboxypeptidase E

Submitted by Alexandra Bleakman

for the degree of Ph.D. of

the University of Bath

1990

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CONTENTS

		Page
Title page		i
Contents		ii
List of Figures, Plates and Tables		iii
Acknowledgements		vi
Abstract		vii
Chapter 1.	Production of Oxytocin and Vasopressin	1
Chapter 2.	Preparation and Method Development	53
Chapter 3.	The Effects of Neurophysin and Protein Concentration on Carboxypeptidase E Activity	71
Chapter 4.	Comparison of Soluble and Membrane-Associated Forms of Carboxypeptidase E	92
Chapter 5.	Cleavage of Carboxy Terminal Lysine by Membrane-Associated Carboxypeptidase E	107
Chapter 6.	Discussion and Conclusions	115
Appendix		121
References		123

List of Figures, Plates and Tables

		Page
Fig.1.1.	Comparison of the structures of oxytocin and vasopressin	44
Fig.1.2.	The hypothalamo-neurohypophyseal system	45
Fig.1.3.	Structural organization of the genes coding for preprovasopressin and preprooxytocin	46
Fig.1.4.	Amino acid sequences of neurophysins from various species	47
Fig.1.5.	Disulphide pairing in bovine neurophysins forming separate domains	48
Fig.1.6.	A putative evolutionary pathway of neurohypophyseal hormones	49
Fig.1.7.	Processing of prooxytocin and provasopressin to generate active hormones, neurophysins and glycopeptide	50
Fig.2.1.	Separation of peptides by RP-hplc	65
Fig.2.2	Gel filtration of bovine neurosecretory granule soluble fraction	66
Fig.2.3.	pH activity profile of the soluble enzyme preparation	67
Fig.2.4.	Interaction of cyclic and oxidised forms of OT-GKR with agarose-neurophysin	68
Fig.2.5.	Cleavage of oxidised OT-GKR by the soluble enzyme preparation	69
Fig.3.1.	The effect of neurophysin concentration on cleavage of OT-GKR	82

Fig.3.2.	The effect of neurophysin concentration on cleavage of oxidised OT-GKR	83
Fig.3.3.	The effect of neurophysin in phosphate buffer	84
Fig.3.4.	The effect of BSA concentration on CPE activity	85
Fig.3.5.	The effect of various proteins on CPE activity	86
Fig.3.6.	The effect of 3-butanol on CPE activity	87
Fig.3.7a.	The effect of high neurophysin concentration on CPE activity	88
Fig.3.7b.	The effect of high neurophysin concentration on CPE activity against oxidised substrate	89
Fig.3.7c.	The effect of high neurophysin concentration on CPE activity with high substrate concentration	90
Fig.4.1a.	pH activity profile of the membrane enzyme preparation	99
Fig.4.1b.	pH activity profile of the soluble enzyme preparation	100
Fig.4.2.	Effect of high protein concentration on the membrane enzyme preparation	101
Fig.4.3a.	Time course of cleavage of cyclic and oxidised OT-GKR by the soluble enzyme preparation	102
Fig.4.3b.	Time course of cleavage of cyclic and oxidised OT-GKR by the membrane enzyme preparation	103
Fig.4.4a.	Substrate specificity of the membrane enzyme preparation with pH	104
Fig.4.4b.	Substrate specificity of the soluble enzyme preparation with pH	105

Fig.5.1. pH activity profiles of carboxy terminal arginine and lysine cleavages by the membrane enzyme preparation 113

Fig.5.2. The effect of oxidised OT-GK on the cleavage of cyclic OT-GK by the membrane enzyme preparation 114

Plate 1. Analysis of the affinity purification of neurophysin by polyacrylamide gel electrophoresis 91

Table 1.1. Sequences of the neurohypophyseal hormones 51

Table 1.2. The presence of neurohypophyseal hormones in vertebrates 52

Table 2.1. Characterization of carboxypeptidase E activity of the soluble enzyme preparation 70

Table 4.1. Characterization of carboxypeptidase E activity of the membrane enzyme preparation 106

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Abstract

Most peptide hormones are initially synthesized within a larger precursor protein and processed by a series of enzymes to the active molecule. Although much work has been undertaken in this field, many details of these reactions are not fully understood.

The biosynthesis of oxytocin and vasopressin involve at least three distinct processing activities which occur sequentially within the environment of the neurosecretory granule. Studies of granular extracts have identified the presence of all the individual activities; however, diverse conditions such as pH and cofactors have so far prevented the *in vitro* observation of complete processing from prohormone to active hormone in a single preparation.

The aim of this study, therefore, is to identify environmental factors which may operate *in vivo* to allow activity of all the processing enzymes under a common set of conditions.

I. Synthetic oxytocin-Gly-Lys-Arg was used as a substrate for a preparation of bovine neurosecretory granules containing carboxypeptidase E activity. The cleavage rate of the carboxy terminal arginine residue was affected by the presence of a range of proteins, including neurophysin, and this affect was shown

to be unrelated either to the presence of sulphydryl groups or to neurophysin-substrate binding. Carboxypeptidase E activity increased by fifty to one hundred percent in the presence of proteins at low concentrations (up to 100μM), while at 500μM protein this stimulation was lessened and under some conditions activity was inhibited. Treatment of the substrate to prevent it from binding to neurophysin was used to show that neurophysin can stimulate enzyme activity equally well whether or not it is binding the substrate.

II. Differences were shown between soluble and membrane-associated carboxypeptidase E activities in terms of both substrate specificity and response to changing pH. Although the membrane preparation cleaved carboxy terminal arginine more slowly than did the soluble enzyme, it was capable of cleaving the remaining lysine residue, a reaction that was not observed with the soluble preparation.

III. This study has shown that the activity of a widespread prohormone processing enzyme, carboxypeptidase E, is indeed influenced by environmental factors, including protein concentration and proximity to the granule membrane, which are likely to be operating *in vivo*. There is evidence that the soluble and membrane-bound forms of the enzyme are capable of fulfilling different roles. The presence of these two forms in the neurosecretory granule could be explained

by their different relative activities with the two substrate molecules. The soluble form may react more quickly with the arginyl substrate, while the membrane-bound enzyme is more efficient at cleaving the lysyl peptide.

These results should be considered in future studies of hormone biosynthesis if an integrated approach to the study of complete processing is to be followed.

CHAPTER 1:
PRODUCTION OF OXYTOCIN AND VASOPRESSIN

1.1. The Neurohypophyseal Hormones

1.1.1. Introduction

The mammalian neurohypophysis was shown early this century to possess four biological activities, namely uterotonic, milk-ejecting, pressor and antidiuretic properties (for review see Heller, 1974). There followed much controversy as to the number of peptides or proteins responsible for these properties until du Vigneaud and colleagues, in the 1950's, isolated oxytocin and vasopressin, determined their structures and established their activities using synthetic peptides (see du Vigneaud, 1957). Oxytocin possesses uterotonic and milk-ejecting properties, while vasopressin is responsible for pressor and antidiuretic functions, although the similarity in structures accounts for some overlap in activities. Both peptides consist of nine amino acids, with a ring formed between the two cysteine residues at positions one and six; the carboxy terminal amino acid (glycine) has an α-amino group. The two hormones differ at only two positions, in the ring at position three, and in the tail at position eight (figure 1.1).

Neurohypophyseal oxytocin and vasopressin are in fact synthesized by different cells in the paraventricular and supraoptic nuclei (cell groups) of the hypothalamus

(Mohr *et al*, 1988b). Both hormones are produced by magnocellular neurones in these areas; vasopressin is also made by parvicellular neurones in the suprachiasmatic nuclei, and axons from all these sites project to the posterior pituitary via the median eminence (figure 1.2). Some axons from the paraventricular nucleus also terminate in the median eminence itself and in extrahypothalamic areas of the brain and spinal cord.

The hormones are stored within the neurohypophysis inside granules that accumulate in axonal swellings (Herring bodies); the axons are supported by the surrounding pituitary cells (pituicytes) and account for about 40% of the total volume of the neurohypophysis (Nordmann, 1977). Action potentials passing down the axons from the hypothalamus lead to an increase in the concentration of calcium ions in the axonal cytoplasm; this causes exocytosis of the granules and release of oxytocin and vasopressin into the extracellular space (for review see Poulain & Wakerley, 1982); there is a preferential release of newly-arrived granules over older ones (Sachs & Haller, 1968, Norström & Sjöstrand, 1972 and Wong & Pickering, 1976).

Hormone from the neurohypophysis is released into the systemic circulation to fulfill endocrine roles. Vasopressin liberated in the median eminence enters the portal blood supply to the anterior pituitary, and is thought to act there as a releasing hormone of adrenocorticotropin (ACTH) (Carlson *et al*, 1982). Both hormones have been identified in diverse areas of the brain and spinal cord where their functions are uncertain, but may include effects on memory and learning behaviour (de Wied, 1980).

1.1.2. Endocrine Functions

For a review of hormone activities, see Baylis & Padfield (1985). Oxytocin is released from the pituitary in episodic secretion, and due to a fairly short half life (3-17 min), plasma levels are constantly fluctuating. Release is stimulated in the female by stretching of the lower genital tract (Ferguson's reflex) and tactile stimulation of the nipple. These physical stimuli are modulated by factors such as the presence of oestrogens, which stimulate oxytocin release (Robinson *et al*, 1977), neurotransmitters (see Richard *et al*, 1985) and the emotional status of the animal.

Oxytocin can cause contraction of smooth muscle in the uterus and the breast, but its effectiveness depends directly on the numbers of oxytocin receptors in these tissues, and this is in turn dependent on the reproductive state of the animal. Again, oestrogens play a role by stimulating the expression of oxytocin receptors in uterine tissue in late pregnancy (Solof *et al*, 1979). An increase in receptor numbers occurs in the breast, but this is not thought to be initiated by increased oestrogen concentrations (Solof *et al*, 1979).

Plasma levels of oxytocin rise towards the end of pregnancy, and exhibit a maximum during the expulsive phase of labour. Receptors in the uterus appear only in late pregnancy and disappear rapidly after parturition; a single injection of oxytocin at this stage will cause a series of muscular contractions which continue after the hormone has been cleared from the circulation. Receptor numbers change less in the mammary gland, and although there is some increase after parturition, a similar injection of oxytocin will only cause a single contraction in the myoepithelium of the breast (Lincoln & Paisley, 1982).

The physiological function of oxytocin in labour is uncertain; it can be used to artificially induce parturition in late pregnancy, but is not thought to be needed for normal initiation of labour. It may be involved in retraction of the uterus after delivery or constriction of the uterine and umbilical vessels, where it is a more powerful vasoconstrictor than vasopressin, to prevent excessive bleeding after expulsion of the placenta (see Chard, 1985). In lactation, oxytocin causes milk ejection, but again the importance of the hormone in this activity is uncertain; milk let-down often occurs before suckling begins, when oxytocin levels are low. The increases in receptor numbers in these tissues during late pregnancy, however, may cause them to become unusually responsive to "basal" levels of circulating hormone (Solof *et al*, 1979), so oxytocin could indeed trigger initiation of labour and milk ejection. Observations of substantial oxytocin gene activity in the male hypothalamus suggest an endocrine role for oxytocin in the male (Richter, 1985).

Release of vasopressin is stimulated by osmotic stress and blood volume depletion (for review see Robinson & Verbalis, 1987). The osmotic response is exquisitely sensitive and secretion is triggered by as little as a 1% change in plasma osmolality. Vasopressin acts to combat osmotic stress by causing increased water resorption in the distal tubules of the kidney; this water dilutes the plasma, increasing its volume and reducing its osmolality. Secretion of vasopressin can also be induced by a drop in blood volume, but a 5-10% decrease in volume is needed to activate this pathway (see Robertson, 1977). Although vasopressin release is much less sensitive to this trigger, when a response occurs it can result in much higher levels of vasopressin in the circulation; up to ten times those occurring due to a hyperosmotic stimulus (Robertson, 1977). These levels are sufficient to cause vasoconstriction and

so provide a rapid correction of the pressure loss experienced by a sudden decrease in blood volume. While vasopressin acts as a constant sensitive adjustment mechanism for plasma osmolality, its function in maintaining blood volume is probably not operating under normal physiologic circumstances but is a fast response to an acute drop in volume, such as in haemorrhage. Vasopressin is also released in response to a decrease in blood pressure. A drop of 5% in the human, which is within normal daily fluctuations in a healthy adult, can result in a significant increase in plasma vasopressin (Robertson, 1977).

1.1.3. Gene Structure and Translation Products

The genes which code for oxytocin and vasopressin have been isolated and sequenced from rat (Schmale *et al*, 1983 and Ivell & Richter, 1984), cow (Ruppert *et al*, 1984) and human (Sausville *et al*, 1985). Complementary DNA (cDNA) has also been cloned from the mRNA for bovine vasopressin (Land *et al*, 1982) and oxytocin (Land *et al*, 1983), and comparison of the genomic DNA and cDNA reveal a common structure for the two genes, suggesting they have evolved from a single ancestral gene.

All the genes sequenced to date are less than 2.5kb in size, with the vasopressin gene typically about 1kb larger than the oxytocin gene. The structure comprises three exons, A, B and C, and two introns (figure 1.3). The resulting (bovine) translation products are proteins of 166 amino acids for vasopressin and 125 amino acids for oxytocin (Land *et al*, 1982 and Land *et al*, 1983). The sizes of these proteins are obviously far in excess of the hormones themselves which are each only 9 amino acids long. Like many other peptide hormones and neuropeptides, oxytocin and

vasopressin are synthesized as parts of larger precursor proteins. The gene organization that leads to this synthesis is shown in figure 1.3.

The resulting translation product consists of a signal peptide, the 9 amino acids of the hormone, and the sequence of a small protein called neurophysin. The vasopressin precursor has an additional carboxy terminal glycoprotein domain of 39 amino acids. In both cases the hormone and neurophysin sequences are separated by 3 amino acids, Gly-Lys-Arg, and the neurophysin sequence is followed by a single residue of either Arg or His. In this form the protein is known as a preprohormone, and after removal of the signal peptide simply as a prohormone. The processing of these precursor molecules to the active hormones is detailed in section 1.2.

1.1.4. Genetic Control and Conservation

Where studied, the two genes occur close together on opposite strands of the same chromosome. In the human, they are both on chromosome 20 (Riddell *et al*, 1985), 12kb apart (Sausville *et al*, 1985), and in the rat 11kbp apart (Mohr *et al*, 1988a). Although the levels of transcription of both genes appear to respond to the same stimuli, for example osmotic stress (Mohr *et al*, 1988b) and lactation (Zingg & Lebebvre, 1988), the two genes are never active in the same cell (Mohr *et al*, 1988b). This would suggest that while some factors are able to act on control regions of both genes, there must be important differences at these sites to prevent transcription of both genes in the same nucleus.

Studies in the rat show homology at the 5'-flanking regions of both genes (the putative promoter regions), including some potential control sites; this area of the oxytocin gene for instance includes sequences that may be responsive to

glucocorticoids and some to oestrogens, as well as a possible interferon-responsive enhancer sequence (Mohr *et al*, 1988a). Some similarity is seen in the vasopressin gene, which also contains a consensus sequence for a glucocorticoid response element, but this gene possesses a region resembling a cAMP response element and four putative AP2 (transcription factor) binding sites (Mohr & Richter, 1990).

Protein sequencing studies on the neurophysins revealed that within each species the two types (from prooxytocin and provasopressin) differ by about 20%; however, between species the substitution rate is much lower, only 1-5% in neurophysins from provasopressin and 6% in the proteins from prooxytocin (for review see Acher, 1980). This apparently high level of conservation between various mammalian species was confirmed when the gene sequences became available. Not only is there a high degree of homology in the sequences for the corresponding rat, bovine and human genes, there also emerged extensive nucleotide homology between the two different genes of each species, with over 99% homology in each case for the sequence encoded by exon B. This level of homology is far higher than would be expected even for two genes coming from a common ancestor, particularly because of the absence of silent mutations, which are not prevented by conservation of amino acid sequence. The likely explanation, therefore, is of gene conversion events involving the second exon of both genes and part of the first intron, extending upstream of exon B (where homology is also seen). The ancestral gene is thought to have undergone duplication and divergence to give the oxytocin and vasopressin lineages before the appearance of fishes 450 million years ago (Acher, 1980). Calculations using the mutation rate for silent substitutions suggest that conversion of

the central regions of the genes occurred about 10 million years ago in the rat, and less than 1 million years ago in the bovine gene (Ruppert *et al*, 1984).

While gene conversion can account for conservation of the central sequence of the two neurophysins within a given species, the high level of homology seen between species cannot be explained in this way, and is therefore perhaps due to an important function played by these proteins that is dependent upon their sequence.

1.1.5. Neurophysins

Neurophysins are stable, cysteine-rich proteins of about 10kd, encoded by the oxytocin and vasopressin genes. They were first isolated by Acher and Fromageot (1957) from a noncovalent complex with the two hormones (the "van Dyke protein"). Further work revealed that neurophysin specifically binds both hormones with high affinity and that at least two types of neurophysin could be isolated from most species studied (for review see Hope, 1975). When neurophysin was found to be synthesized as part of the hormone precursor, it was clear that the two forms of the protein correlated with the two different precursors. Nomenclature of the neurophysins has become confusing, based on relative electrophoretic mobilities or amino acids at certain positions, as proteins from different species do not give a consistent classification; it is easier to refer to them, therefore, by their origin from prooxytocin as oxytocin-neurophysin (OT-Np), or from provasopressin as vasopressin-neurophysin (VP-Np).

Although neurophysins also occur with neurohypophyseal hormones in non-mammalian species, studies into structure and hormone binding have been conducted using only mammalian neurophysins (for reviews see Breslow, 1979 and Breslow &

Burman, 1990). The amino acid sequences of neurophysins from several mammalian and non-mammalian species are shown in figure 1.4. As already stated, these proteins show a very high degree of conservation both within and between species, particularly in the central region that is encoded by exon B. Other features of the primary sequence include a high cysteine content, 14 out of 93-95 residues, and internally duplicated segments at residues 12-31 and 60-77 showing about 60% homology (Capra *et al*, 1972).

Predictions of secondary structure suggest a low content of α-helix (5-20%) and about 40% β-pleated sheet (Breslow, 1979). The tertiary structure is highly stable due to the involvement of all 14 cysteine residues in disulphide bonds; all of the pairs have been assigned and reveal the formation of two separate domains with similar pairing patterns within each domain (Burman *et al*, 1989) (see figure 1.5).

Neurophysin binds both hormones in a 1:1 molar ratio, and extensive studies have been undertaken using both hormones and smaller peptide analogues in order to identify the residues involved in binding, both in the hormone and neurophysin sequences (for review see Breslow, 1979). The hormone residues important for binding are relatively easy to study using synthetic analogues; this has shown the major involvement of the α-amino group and the side chains of the first three amino acids in the hormone sequences. The protonated α-amino group of the hormone forms a salt-bridge with an ionized carboxyl on the side-chain of a neurophysin residue which has not yet been identified; this bond accounts for the pH dependence of the hormone-neurophysin interaction, which is maximal at pH 5-6 and declines rapidly below pH 5.0 and above pH 6.5. The hormone side chains of Cys^1, Tyr^2 and Phe^3 or Ile^3 contribute to the binding with hydrophobic interactions with the protein;

the presence of a hydrophobic aromatic residue at position 2 (either tyrosine or phenylalanine) is essential for hormone-neurophysin binding (Breslow, 1975). Although a synthetic tripeptide based on these three residues will bind to neurophysin, it does so with lower affinity suggesting that other residues in the hormone sequence or the conformation of the hormone molecule are involved in stabilizing the binding (Breslow, 1975). In solution, neurophysin exists in an equilibrium between monomeric and dimeric forms, and dimerisation potentiates hormone binding; neurophysin dimers are stabilized by the addition of phosphate ions, although this is not believed to be significant under normal physiological conditions (Tellam & Winzor, 1980).

Little is so far know about the hormone binding site on the neurophysin molecule (see Breslow & Burman, 1990). Chemical cross-linking suggests the contribution of the carboxyl group for the salt bridge by Glu^{31} (Walter & Hoffman, 1973), and neurophysin Arg^{8} has been shown by circular dichroism studies to stabilize the bound state (Breslow *et al*, 1989). Much work has also been conducted into the role of Tyr^{49} in hormone binding, with evidence of involvement of this residue from studies using, among other techniques, photoaffinity labelling (Abercrombie *et al*, 1982), nuclear magnetic resonance spectroscopy (Peyton & Breslow, 1985) and chemical cross-linking (Sardana *et al*, 1987). All three of these residues occur in the "amino domain" of neurophysin as deduced by Burman *et al* (1989) from disulphide pairing (see figure 1.5), and they are also conserved throughout the mammalian neurophysins so far studied (see figure 1.4). However, sequences of non-mammalian neurophysins reveal that although Arg^{8} appears in all of these sequences, in frog and toad VP-Np the Asp^{30}-Glu^{31} pair is reversed (Michel

et al, 1987 and Nojiri *et al*, 1987); in one fish neurophysin the glutamine is again moved to position 30, and in another it is absent (Heierhorst *et al*, 1989). Tyr^{49}, about which there is most evidence of binding involvement, does not occur in one of the neurophysins from both ostrich (Chauvet *et al*, 1986) and toad, but these molecules both have a tyrosine at position 35 which is not seen in mammalian neurophysins. Two neurophysins of the white sucker fish *Catostomus commersoni* (which has at least three neurophysin species due to duplication of the vasotocin gene) also lack Tyr^{49}: one has a tyrosine at position 22 and the other at position 63; thus the second fish neurophysin is the only one that does not have a tyrosine residue in the amino domain of the protein (Heierhorst *et al*, 1989).

The high degree of evolutionary conservation of the neurophysins suggests an important role for these proteins. Evidence has been presented that neurophysin can act as a growth factor for the non-neuronal glial cells found at the site of neurophysin synthesis in the hypothalamus (Worley & Pickering, 1984). Several studies looking for physiological effects of neurophysin outside the hypothalamo-pituitary system revealed apparent lipolytic, hypocalcaemic, hyperglycaemic and melanotropic actions, but these have since been shown to have resulted from contamination of the neurophysin preparations (for review see Pickering & Jones, 1978). The only activities of neurophysin so far confirmed, then, are the potential to promote cell growth in the hypothalamus and the ability to bind hormone at acid pH; the dissociation of the complex at a neutral pH discounts neurophysin from acting as a carrier for the hormone in the circulation. Its importance would therefore appear to lie in the formation of the hormone-neurophysin complex within the neurosecretory granule, which has an acidic nature (Russell & Holz, 1981 and Sherman &

Nordmann, 1982). The purpose of this binding has been variously suggested as being to prevent leakage of hormone out of the granule, to prevent disulphide interchange between hormone molecules, or to protect the hormones from proteolysis (Breslow, 1979). The finding that most of the hormone-neurophysin complex is present in the granule in a crystalline form (Nordmann and Morris, 1984) has also led to the suggestions that the binding acts to reduce the osmotic potential within the granule (Breslow, 1979), or to assist in driving packaging of the precursor molecules into granules at the Golgi apparatus (Rose *et al*, 1988 and Breslow & Burman, 1990). Aggregation of individual molecules into larger complexes would reduce the apparent concentration gradient caused by packaging and thus reduce the energy requirement. Evidence against these ideas comes mainly from the existence of other small peptide hormones which are successfully packaged and stored in similar granules and do not appear to suffer from leakage, proteolysis or osmotic problems, although they are not known to be bound by any protein within the granule. Thus the confirmation of a role for these apparently important proteins is still lacking.

1.1.6. Extrahypothalamic Synthesis

Oxytocin and vasopressin have commonly been identified by immunohistochemistry, radioimmunoassay or high performance liquid chromatography (hplc) in areas other than the hypothalamus and pituitary, such as the adrenal, placenta and ovary (for review see Ivell, 1987). Initially it was not known whether the hormone found in these organs derived from the hypothalamus or was in fact being made in these areas. Ovarian synthesis was confirmed by incorporation of [^{35}S]-cysteine into oxytocin by cells of the corpus luteum (Swann *et al*, 1984) and

discovery of preprooxytocin mRNA in luteal extracts (Ivell & Richter, 1984). Knowledge of the mRNA sequences for the preprohormones makes it possible to determine the exact sites of precursor synthesis using cDNA or cRNA probes for precursor mRNA, and this technique has located preprooxytocin mRNA in the cell bodies of magnocellular neurones in the supraoptic, suprachiasmatic and paraventricular nuclei of the rat hypothalamus (Mohr *et al*, 1988b), as well as the bovine corpus luteum (Ivell & Richter, 1984), rat cerebellum and testis (Ivell *et al*, 1986), and human thymus (Geenan *et al*, 1987). The functions of oxytocin and vasopressin synthesized outside the hypothalamus are uncertain, but ovarian oxytocin is believed to play a role in luteolysis (Flint & Sheldrick, 1985), and the hormone is seen to cause increased contractility of rat seminiferous tubules *in vitro* (Worley *et al*, 1985).

1.1.7. Evolution of Neurohypophyseal Hormones

After the initial identification of oxytocin and vasopressin in the 1950's, du Vigneaud's group synthesized various analogues of the hormones including arginine vasotocin, which has the ring of oxytocin and the tail of vasopressin, and showed similar pharmacological characteristics to chicken pituitary extracts. Meanwhile, Heller in Bristol was looking for the "water balance principle" in fish, amphibians and birds, known to be a molecule with pressor and oxytocic activities, but not vasopressin or oxytocin. The two groups met and the unnamed molecule was identified as vasotocin (see Pickering, 1985).

Chemical studies of hormones related to oxytocin and vasopressin have been carried out so far almost exclusively in vertebrates resulting in the identification of

ten peptides (table 1.1), although extensive immunocytochemical studies have been conducted in insects and coelenterates with positive results (Mizuno & Takeda, 1988a and Mizuno & Takeda, 1988b) and a molecule related to vasotocin has been isolated from locusts (Cupo & Proux, 1983 and Proux *et al*, 1987). Most of the fifty or so vertebrate species studied have two neurohypophyseal hormones, one related to oxytocin and one to vasopressin. The relationship to the mammalian hormones is decided by the nature of the amino acid at position eight; in oxytocin-like peptides this is neutral while in vasopressin-like peptides it is a basic amino acid. This family of hormones exhibits strong evolutionary stability; they share a common basic structure, with the same pairs of peptides being present in related species and amino acid substitutions usually only involving one or two amino acids.

The evolutionary pathway of this hormone family is not entirely clear (for review see Acher & Chauvet, 1988); their presence in vertebrate species is shown in table 1.2. Cyclostomes, the most ancient surviving vertebrates, appear to have only vasotocin. A peptide has been isolated from locusts which has the same basic structure as these hormones (nine amino acids, 1-6 forming a ring, and a carboxy terminal amide), and is most closely related in sequence to vasotocin (Ile^3 and Arg^8). Vasotocin is also thought to be present in all non-mammalian vertebrates, so this seems the most likely of the hormones to have been present at the beginning of vertebrate evolution. In attempting to follow the possible development of the hormones by gene duplication and single base changes as the vertebrates evolved, it must be remembered firstly that only a limited number of present day species have been studied, so there may be more hormones as yet undiscovered, and secondly that the species alive today have continued to evolve from their ancestral forms for

millions of years and do not represent an unaltered sequence of evolutionary types. With these facts in mind, a possible pathway of evolution has been suggested for the neurohypophyseal hormones (figure 1.6).

The evolution of the vasopressin line appears straightforward. Vasotocin persisted in the non-mammalian vertebrates, then was replaced by arginine vasopressin in early mammals, where it is seen now in the primitive prototherian echidnas. The change from vasotocin to arginine vasopressin, Ile^3 to Phe^3, requires only a single base substitution. Arginine vasopressin remains present in eutherian mammals, except where another single base change has given rise to lysine vasopressin, identified in the pig (Popenoe *et al*, 1952) and Peruvian mouse (Stewart, 1968). In marsupials, an apparent duplication of the vasopressin gene has led to some species exhibiting two vasopressin-like peptides; in Australian *Macropodidae* and *Peramelidae*, both lysine vasopressin and phenypressin (which could again be produced from arginine vasopressin by a single base change) are seen (Chauvet *et al*, 1983), whereas in American opossums arginine and lysine vasopressins are found together (Chauvet *et al*, 1985).

The lineage of oxytocin is harder to follow. An initial duplication of the vasotocin gene before the appearance of the fishes would have allowed mutation of the duplicate gene to give rise to this new line of peptides, but it is not possible to follow it through vertebrate groups with only single base alterations as can be done with the vasopressin line. The major problem is that three of the four oxytocin-like peptides found in fishes, namely isotocin, glumitocin and aspargtocin, have residues at position 4 that cannot be reached by a single base change from Gln^4 in vasotocin. Similarly, Ser^4 in isotocin would have to go through Pro to become Gln^4 again in

mesotocin, which is the presumed path of development (from bony fishes to tetrapods). It may be that proline has appeared in position 4 and that this hormone has either been lost in evolution or not yet identified. A synthetic analogue of vasopressin, Pro^4-Arg^8-vasopressin, exhibits some antidiuretic activity (Buku *et al*, 1987), so Pro^4-vasotocin may be a viable hormone. Conversely, an early gene duplication could have given rise to the fish peptides after the separation of fishes from the tetrapod lineage, where another gene duplication event would allow a single base change to produce mesotocin from vasotocin (Arg^8 to Ile^8). Another point mutation in mesotocin will produce oxytocin. As with the vasopressin peptides, some marsupials appear to display duplication of the oxytocin-like hormones; both oxytocin and mesotocin have been found in the North American opossum (Chauvet *et al*, 1984) and the Australian Northern bandicoot (Rouillé *et al*, 1988).

The possible evolutionary connections between vasotocin and arginine vasopressin, as well as mesotocin and oxytocin are strongly supported by evidence of precursor structure from cDNA sequencing. Studies using the bufo toad show that provasotocin has a structure similar to provasopressin (hormone, neurophysin and glycopeptide moieties) while promesotocin, like prooxytocin, lacks the glycopeptide domain (Nojiri *et al*, 1987). The same investigation also provided evidence of a gene conversion event in the toad similar to those suggested for mammals, involving the central region of the neurophysins. This has also been suggested from neurophysin sequence studies in birds (Lévy *et al*, 1987). The recent cDNA sequencing of vasotocin and isotocin precursors from the teleost fish *Catostomus commersoni* has provided more information about the possible evolutionary pathways of the hormones; both precursors contain a carboxy terminal sequence after neurophysin

which exhibit some homology with the glycopeptide moiety of provasotocin from the toad (Heierhorst *et al*, 1989). This strengthens the connection between the vasotocin genes in the fish and the toad, but does not support the evolution of promesotocin from proisotocin as the two prohormones have different structures.

It should be noted that the theory of vasotocin being the sole original vertebrate peptide is brought into doubt by some immunohistochemical studies suggesting the presence of both oxytocin-like and vasotocin/vasopressin-like immunoreactivities in a wide range of invertebrates (eg. Mizuno & Takeda, 1988a and Mizuno & Takeda, 1988b). These results support a gene duplication at a much earlier stage of evolution, although this could have occurred after the ancient divergence of the invertebrate and vertebrate lines. However, duplication of both genes in some marsupials, as well as the discoveries of duplicate vasotocin genes in the sucker fish (Heierhorst *et al*, 1989) and chum salmon, *Oncorhynchus keta* (Heierhorst *et al*, 1990), may demonstrate particular susceptibility of the genes to duplication. There is also evidence for duplication of the vasopressin gene in pig, which appears to express both arginine and lysine vasopressins in the testis (Nicholson *et al*, 1988). In the Brattleboro rat, which is deficient in vasopressin,some neurones in the magnocellular system have been reported to be immunoreactive for both vasopressin and the associated glycopeptide (Richards *et al*, 1985). The deficiency is caused by a base deletion in exon B (Schmale & Richter, 1984), so the region of the gene which normally codes for the glycopeptide domain is subject to a frame-shift and consequent loss of sequence, glycosylation site and stop codon (with translation continuing into the poly-A region). The apparent presence of immunoreactive glycopeptide in a particular cell group of these animals is therefore unexpected and could be explained by the expression of a

duplicate, unaffected vasopressin gene in these cells. However, the apparent difficulty that these cells encounter in packaging and transporting the immunoreactive material (Richards *et al*, 1985) may indicate that alternative splicing of the mutant transcript is occuring, joining exon A (which codes for the hormone sequence) directly to exon C (which includes the glycopeptide region), so overcoming the frame-shift and resulting in a product containing both of the immunoreactive regions, but which cannot be processed normally by the cell.

Despite the unlikelihood of a second vasopressin gene in the Brattleboro rat, there is much evidence of several gene duplication events in the evolution of the neurohypophyseal hormones. This may allow the possibility of a single vasotocin gene entering the vertebrate line; the first duplication after divergence of the fishes producing isotocin, and the second during early tetrapod evolution, where the duplicated gene this time lost its carboxy terminal extension, giving rise to mesotocin. The oxytocin family would then have evolved at least twice (i.e. parallel evolution), once in fish, and again in higher vertebrates.

1.2. Prohormone Processing

1.2.1. Introduction

Many functional proteins are made as integral parts of longer precursor molecules; for example albumin, catalase, melittin and zymogens, as well as peptide hormones, are initially synthesized within polyprotein sequences (for review see Zimmerman *et al*, 1980). The purpose behind synthesizing proteins in this way is not

always clear, although it is obviously important to store certain enzymes in an inactive state to prevent cell damage, and this can be achieved by synthesis of an inactive precursor, or zymogen. In other cases it may be necessary to ensure correct folding of the mature peptide, as in the case of insulin (Steiner, 1974), or to provide the minimum size of protein that can be translocated and packaged by the cell, or perhaps to coordinate synthesis of different active molecules contained within the same precursor sequence (Docherty & Steiner, 1982).

The enzyme activities involved in the processing of peptide prohormones generally include specific endoproteolysis to release the hormone domain from the bulk of the precursor, followed by trimming of excess residues at one or both ends by amino- or carboxypeptidase activities. The hormone sequence is then commonly modified by such reactions as amino terminal acetylation or, as with oxytocin and vasopressin, carboxy terminal amidation (Loh *et al*, 1984).

The action of selective enzymes on precursor molecules is thus widespread in nature and can serve diverse purposes (Zimmerman *et al*, 1980). A single polyprotein can be cleaved to release several peptides with related functions. For example in viral reproduction the enzymes and structural proteins required for complete replication and assembly of the new virion can be contained within a single precursor molecule; this allows both coordination of synthesis of all the components needed for the replicative process and efficient use of genetic material (Koch & Richter, 1980). Identical precursor proteins can also be differentially processed to give rise to appropriate products in different tissues and/or at varying stages of development. An example of this tissue specific processing is seen in the proteolysis of pro-opiomelanocortin (POMC) in the mammalian pituitary; in the anterior lobe the

primary products are adrenocorticotropin (ACTH) and β-lipotropin, while the neurointermediate lobe produces mainly α-melanocyte stimulating hormone, corticotrophin-like intermediate lobe peptide, χ-lipotropin and β-endorphin (Roberts *et al*, 1978). Differential processing of this kind could be achieved by several tissue-specific properties, including differences in processing enzymes, intracellular compartmentalization of precursor or enzymes, and accessibility of different processing sites within the precursor sequence by structural modification due to different environmental conditions (Thomas *et al*, 1988a).

Tissue-specific processing can also affect the activity of a peptide as well as its quantity in a given tissue. For example, differential processing of β-endorphin (a product of POMC) in the regions of the pituitary can result in widely differing activities in the anterior and intermediate lobes with little difference in quantity (Smyth & Zakarian, 1980).

During sexual development of the female rat, changes in the processing of β-endorphin in the hypothalamus and pituitary have been detected (Martensz, 1985). While processing of POMC to β-endorphin in the anterior lobe increases during this time, the levels of active β-endorphin in the hypothalamus are reduced in the mature animal by increased carboxy terminal proteolysis; this decrease in active β-endorphin may allow an increase in the secretion of lutenizing hormone and onset of reproductive activity (Martensz, 1985). Ontogenic differences in the processing of prooxytocin have also been described (Altstein & Gainer, 1986 and Altstein *et al*, 1988) which allow control of the level of oxytocin found in the hypothalamo-neurohypophyseal system of the rat at different stages of development.

Thus, precursor processing can be important in determining the type, quantity and potency of bioactive products from a single precursor. It is also necessary to understand the processes involved if such peptides are to be produced by gene cloning and fermentation methods, as the initial translation product needs to be processed correctly and efficiently in order to obtain the active peptide.

The primary sequences of many prohormones have been elucidated from cloned cDNA or genomic DNA fragments (for review see Douglass *et al*, 1984); similarities can be seen between all of these molecules. As the final products are destined for export from the cell, the precursors like all secretory proteins have an amino terminal signal peptide of 18-25 hydrophobic amino acids. This sequence facilitates co-translational translocation of the preprohormone into the lumen of the rER, where the signal peptide is removed by a specific endoprotease (Lingappa & Blobel, 1980). The remaining prohormone may contain a single active hormone sequence, as in probradykinin (Nawa *et al*, 1983), several different active sequences such as within POMC (Uhler & Herbert, 1983) multiple copies of similar peptides, as in proenkephalin, or of the same peptide, for example in yeast pro α-mating factor (Kurjan & Herskowitz, 1982). In all cases, there is a striking similarity in that all the active peptide domains are flanked by basic amino acids. These are usually arranged as pairs, or less often as singlets, triplets or quadruplets (Rholam *et al*, 1986), and do not appear in the final hormone sequence.

Several putative processing enzymes have been studied that will cleave prohormones *in vitro* at these basic pairs. Cleavage has been observed on the amino terminal side of the doublet in the case of prosomatostatin (Gluschankof *et al*, 1985), between the pair in proenkephalin B (Mizuno *et al*, 1985) and yeast α-mating factor

(Mizuno & Matsuo, 1984), and on the carboxyl side in POMC (Chang & Loh, 1983), prooxytocin (Clamagirand *et al*, 1986) and provasopressin (Parish *et al*, 1986). These basic amino acids appear to be the primary signal for endoproteolytic cleavage as there is no other consensus seen between prohormones at these regions. However, further examination of the structures of these peptides reveals additional basic amino acid pairs at sites that do not appear to be susceptible to proteolysis, for example within the sequences of growth hormone releasing factor (Gubler *et al*, 1983) and glucagon (Bell *et al*, 1983 and Lund *et al*, 1983). In a study using analogues of prooxytocin, it was demonstrated that cleavage could occur when the Lys^{11}-Arg^{12} doublet was reversed, but not when either was replaced by its D-isomer or by a neutral amino acid (Nle); analogues which contained the native basic pair, but which had substitutions affecting the predicted secondary structure upstream of the doublet also abolished cleavage susceptibility (Créminon *et al*, 1988).

It is therefore apparent that although the basic pair is an essential signal for endoprotease cleavage, there must be additional information within the precursor molecule that directs endoproteolysis only to appropriate basic pairs. Calculation of the predicted structures around such cleavage sites of twenty prohormones (using the prediction method of Chou & Fasman, 1978) revealed that such sites are always found within or immediately adjacent to regions with high β-turn formation probability; the sequences on either side of these regions have high probabilities for forming highly ordered structures, i.e. α-helix or β-sheet (Rholam *et al*, 1986). These properties are well illustrated by prooxytocin, which is cleaved on the carboxyl side of the dibasic pair Lys^{11}-Arg^{12}. Predictions suggest that residues 8 to 12 form a β-turn typical of a region subject to endoprotease attack, while the flanking sequences

are ordered: residues 1 to 7 forming a β-sheet and 13 to 21 an α-helix (Créminon *et al*, 1988).

A more recent study of predicted prohormone structure near to endoproteolytic cleavage sites, which included the newly defined structural feature, the Ω loop, found that of the potential sites studied, 25% occur in β-turns, 32% in Ω loops and 42% in α-helices (Bek & Berry, 1990).

Following the action of an endoprotease cleaving on the carboxyl side of a dibasic pair, as for prooxytocin, the signalling amino acids are removed sequentially by an enzyme specific for carboxy terminal basic amino acids. Because of its specificity, this activity was initially termed "carboxypeptidase B-like", but it has since been shown to be more highly specific for basic residues than is carboxypeptidase B (CPB), and to differ in certain properties (Fricker, 1988a).

The presence of a carboxy terminal α-amide group is seen in about half of all neuropeptides and peptide hormones; in the majority of cases this amide group can be shown to be essential to activity (Eipper & Mains, 1988). An assay for the α-amide moiety has been used to identify novel bioactive peptides, and has also revealed that such amidated peptides are only present in neural and endocrine tissues (Tatemoto & Mutt, 1978 and Tatemoto *et al*, 1986). These peptides have also been discovered in relatively primitive animals such as the sea anemone (Grimmelikhuijzen & Graff, 1986) and locust (Proux *et al*, 1987), suggesting that they are an ancient phenomenon.

All carboxy terminally amidated peptides are initially synthesized within precursor molecules, and sequencing has revealed that the carboxy terminal amino acid of the final peptide sequence is always followed in the precursor by a glycine residue. This glycine is targeted by an enzyme and donates the nitrogen from its α-

amino group to form the carboxy terminal α-amide in the active peptide; thus the inactive sequence -X-Gly is transformed to the active -X-NH_2 and the residual glycine is released as glyoxylic acid (Bradbury *et al*, 1982). The enzyme activity that catalyses this reaction can only occur after both the endoprotease and the carboxypeptidase activities have released the peptide sequence from the precursor and removed the extra basic amino acids to leave glycine as the carboxy terminal residue (Bleakman & Smyth, 1987).

1.2.2. Processing Enzymes for Oxytocin and Vasopressin

When trying to identify an enzyme involved in the processing of a precursor such as prooxytocin, it is important to know the intracellular site of activity. The hypothalamic oxytocin precursor is transported along the axon of the secretory neurone down the pituitary stalk and into the neural lobe of the pituitary; processing occurs during this axonal transport and within the axon terminals where the products of processing are stored (Brownstein *et al*, 1980). As all of the protein transported from the hypothalamus to the neural lobe is contained within secretory granules, it was concluded that axonal processing was taking place within these neurosecretory granules (Brownstein *et al*, 1980). Pulse-chase labelling experiments, originally used by Palade in studies on the pathway of protein secretion (Palade, 1975), provided additional evidence that processing could only occur once the precursor had been translocated across the Golgi and packaged into neurosecretory granules (Gainer *et al*, 1982).

The overall picture of oxytocin and vasopressin biosynthesis in the hypothalamo-neurohypophyseal system is, therefore, as follows: gene transcription

and RNA splicing occur within the nuclei of the magnocellular neurons of the supraoptic, suprachiasmatic and paraventricular nuclei. Translation of the mRNA on the rough ER results in translocation of the nascent protein into the lumen of the ER and removal of the signal peptide. The prohormone then travels to the Golgi, and emerges from there packed inside a neurosecretory granule with the components necessary for its conversion to active products during axonal transport to the neural lobe (or any alternative destination). If axonal transport is artificially blocked by the use of colchicine, granules accumulate in the cell bodies but processing proceeds at a normal rate (Birkett *et al*, 1983); this demonstrates that the granule is acting as a discrete environment providing everything needed for complete processing of the precursors, independent of its location along the hypothalamo-neurohypophyseal tract.

In order to identify an enzyme studied *in vitro* as one involved in precursor processing *in vivo*, Docherty and Steiner (1982) proposed criteria specifically for proteases, but which can be adapted to include all the processing enzymes. As well as being present in the correct location, in this case the neurosecretory granules of the hypothalamo-neurohypophyseal system, the enzyme must be able to act on the natural substrate to produce all known products. The enzyme should be purified of all similar, contaminating activities and biochemically characterized to assess whether it would be active under the conditions found at the *in vivo* site of processing (e.g. pH optimum and cofactor requirements). Conclusive evidence of participation in prohormone maturation requires demonstration that specific inhibition of the enzyme by a suitable method can prevent normal processing from occurring *in vivo*.

There are three enzyme activities involved in processing prooxytocin or provasopressin within the secretory granule (see figure 1.7). Endoproteolytic cleavage occurs in both prohormones between Arg^{12} and Ala^{13} to release the amino terminal hormone domain with the Gly-Lys-Arg extension, and between Arg^{108} and Ala^{109} in provasopressin only to separate the neurophysin and glycopeptide domains. The first of these steps occurs after a typical basic pair, Lys^{11}-Arg^{12}, which as stated earlier is probably at a β-turn. The second site of endoproteolysis, however, has only the single basic Arg^{108}, and is not always observed; sequencing of a frog neurophysin revealed that cleavage had not occurred between the neurophysin and glycopeptide domains, and the molecule remained intact as "big" neurophysin (Michel *et al*, 1987); this has also been demonstrated in the green turtle (Licht *et al*, 1984).

The basic amino acids that signalled endoproteolysis are then trimmed by an enzyme or enzymes specific for carboxy terminal basic residues. This involves Arg^{12} and Lys^{11} of both extended hormone sequences, as well as the extra residue at the carboxy terminus of neurophysin from prooxytocin (His^{106} or Arg^{106}) or Arg^{108} from provasopressin. The neurophysins and glycopeptide are now in their mature forms, but the hormone sequences are still extended by Gly^{10}, which is acted upon by another enzyme to leave the active carboxy terminally amidated oxytocin or vasopressin. While this scheme of processing has been assumed for many years to be the correct one, there is now much evidence to support it in the form of identification of the various predicted intermediate forms in secretory granule preparations (Clamagirand *et al*, 1987a) and tissue sections (Altstein *et al*, 1988), as well as

isolation of appropriate enzyme activities from various tissue preparations (see below).

1.2.3. Endoprotease Activity

Early investigations of endoproteolytic enzymes in neurosecretory granules utilized *in vitro* cleavage of POMC to ACTH and β-endorphin (Chang *et al*, 1982); this study revealed the presence of an acid-thiol protease which was specific for pairs of basic amino acids, in granules from bovine neural lobes. Further studies with similar granule preparations used substrates of synthetic prooxytocin$^{1-18}NH_2$ (Clamagirand *et al*, 1986) and cancer cell-derived human provasopressin (Parish *et al*, 1986), and both identified proteolytic enzymes cleaving between Arg^{12} and Ala^{13}. The former report was of a 58kd molecule that was not a serine protease, while the latter described a 70kd glycoprotein with aspartyl protease activity that did not appear to cleave provasopressin at the single Arg residue between neurophysin and the glycopeptide. The 58kd enzyme has since been further characterized, using a prooxytocin$^{1-20}$ peptide substrate, as a thiol-metalloprotease with an optimum pH around 7.0; it is specific for the basic pair, but will also cleave when Lys^{11}-Arg^{12} is reversed to Arg^{11}-Lys^{12} (Clamagirand *et al*, 1987a). Although essential for cleavage, the dibasic pair alone is not a sufficient feature for recognition by this endoprotease; a minimum substrate length of nine residues, either 7-15 or 8-16, is necessary, and the structural nature of the regions immediately flanking the dibasic pair also influences enzyme activity (Brakch *et al*, 1989). A similar enzyme has also been found in secretory granules from bovine corpus luteum, with a size of 52-58kd and similar specificity and thiol-metalloprotease activity (Clamagirand *et al*, 1987b).

It differs, however, in its acidic nature (pI 4.9 compared with 6.9/7.1 for the neural enzyme) and kinetic properties towards the prooxytocin$^{1-20}$ substrate (Plevrakis *et al*, 1989)).

There are thus two proteases reported in bovine neurosecretory granules that both cleave suitable substrates *in vitro* to give the expected products, but they have different sizes (70kd and 58kd) and widely differing characteristics. Both enzymes are found at the correct intracellular site for processing, and both generate the predicted products *in vitro*. However, only the 70kd enzyme has a pH optimum appropriate for the acidic nature of the neurosecretory granule.

To date, no cleavage between neurophysin and glycopeptide has been demonstrated *in vitro*. The inabilities of the 58kd enzyme to cleave at single basic residues within a peptide (Clamagirand *et al*, 1986) and of the 70kd enzyme to cleave provasopressin at Arg^{108}, together with the absence of cleavage at this position in frog provasopressin (Michel *et al*, 1987), suggests that this activity is due to a different enzyme *in vivo*, which has not yet been isolated from neural lobe extracts. An enzyme thought to be involved in the processing of enkephalins has been found in chromaffin granules which cleaves a model substrate at a single Arg residue; it is a thiol metalloprotease of about 45kd, with a pH optimum of 7.8 but still active at pH 6.0 (Tezapsidis & Parish, 1989). A related enzyme may exist in neurosecretory granules to process provasopressin, however the pH profile does not support its function in acidic granules, and it would be expected to occur in granule preparations that exibit activities of other processing enzymes.

A different approach to the identification of a mammalian prohormone-processing endoprotease has been taken by searching for a gene sequence related to

that of a yeast enzyme with appropriate endoproteolytic activity. The enzyme Kex2 is a calcium-dependent, neutral serine protease which cleaves pro-peptides at pairs of basic amino acid residues; it is a product of the KEX2 gene in *Saccharomyces cerevisiae* (Fuller *et al*, 1989a). The enzyme is heavily glycosylated and is anchored into the membrane by a hydrophobic region near the carboxy terminus; the remaining part of the protein protrudes into the cytoplasm and is believed to be important in directing the enzyme to its functional location in late Golgi vesicles (Fuller *et al*, 1989b). Kex2 can correctly process human proinsulin that is expressed in yeast cells (Thim *et al*, 1986), will cleave POMC when KEX2 is expressed in processing-deficient mouse cells (Thomas *et al*, 1988b) and membrane preparations with Kex2 activity can process human proalbumin (Bathurst *et al*, 1987). It was therefore anticipated that the corresponding mammalian endoprotease(s) would have similar structural features to Kex2, and a database search revealed a human gene, *fur*, which showed partial sequence homology to KEX2 (Fuller *et al*, 1989b). The product of this gene, furin, displays 50% identity with the catalytic domain of Kex2; it also has a potential transmembrane region followed by a hydrophilic and highly charged tail, as does the yeast enzyme (Fuller *et al*, 1989b). It therefore appears likely that furin is a mammalian prohormone-processing endoprotease, although the ability of this protein to function as a protease has not yet been demonstrated.

1.2.4. Carboxypeptidase Activity

Studies on various neural and endocrine tissues, using substrates related to many different neuropeptides and peptide hormones, have revealed the association of a single carboxypeptidase enzyme with the processing of this family of molecules

(for review see Fricker, 1988a); it has been designated EC 3.4.17.10 and is widely known as carboxypeptidase E (CPE), also as enkephalin convertase or carboxypeptidase H. Originally described as having CPB-like activity, it is in fact much more highly specific for basic amino acids than is CPB, which shows moderate affinity for some other residues.

As with the endoprotease, initial studies on carboxypeptidase activity were not carried out on neurohypophyseal precursors, but in this case using carboxy terminally extended enkephalins as substrates, and enzyme preparations from bovine adrenal chromaffin granules (Fricker & Snyder, 1982 and Hook *et al*, 1982). These and further studies characterized the enzyme as an acid thiol protease, maximally active at pH 5-6 and stimulated by cobalt ions, and strongly inhibited by guanidinoethylmercaptosuccinic acid (GEMSA), a site-directed inhibitor (Fricker *et al*, 1983, Fricker & Snyder, 1983 and Hook & Eiden, 1984). These properties can be used to distinguish CPE from the other known carboxypeptidases. Investigation of other tissues revealed the presence of similar activities in insulin secretory granules (Docherty & Hutton, 1983), hypothalamus, hippocampus, striatum, thalamus and mid brain, with highest activity in the pituitary (Fricker *et al*, 1982). The pituitary activity was further characterized (Hook & Loh, 1984), and found to be similar in all three lobes; moreover incubation of neural lobe granule lysate with vasopressin-Gly-Lys-Arg resulted in the appearance of the expected products, vasopressin-Gly-Lys and vasopressin-Gly (Hook & Loh, 1984). Extracts of bovine neurosecretory granules have also been shown to convert oxytocin-Gly-Lys-Arg to its corresponding products (Kanmera & Chaiken, 1985a and Clamagirand *et al*, 1986).

CPE is found in both membrane-bound and soluble forms in bovine adrenal medulla, brain and pituitary, with identical physical properties in all the tissues, including molecular weights of 52-53kd for the membrane-bound and 50kd for the soluble form (Fricker & Snyder, 1983 and Supattapone *et al*, 1984). Evidence that there is a single enzyme providing activities in all these tissues is provided by these common sizes, identical characteristics of pH optimum, stimulation and inhibition, as well as similarity of kinetic parameters to a range of peptide substrates (Fricker & Snyder, 1983).

Because of the apparently high affinity of GEMSA for CPE over other carboxypeptidases, [^{3}H]GEMSA has been widely used to locate CPE in tissue studies (for review see Fricker, 1988a); however this specificity has recently been disputed because carboxypeptidase M (CPM) is also inhibited significantly by GEMSA at low pH (Deddish *et al*, 1989), so CPM could give false results when probing for CPE with GEMSA at a low pH. CPE has been demonstrated using this technique in a wide range of tissues that are involved in production of neuropeptides and peptide hormones, including brain, pituitary, salivary gland, lung, ileum, colon, heart, pancreas, testis and spleen (Fricker, 1988a). In many of these tissues, CPE is shown to be localized in cells producing such peptides.

Dehydration and rehydration of an animal cause depletion and restoration, respectively, of the numbers of [^{3}H]GEMSA binding sites in the posterior pituitary; such changes are not seen in the other lobes of the pituitary nor in the hypothalamus (Strittmatter *et al*, 1985). This mirrors the depletion in pituitary levels of oxytocin and vasopressin caused by their release in response to dehydration, and strongly suggests colocalization of CPE with the hormones inside neurosecretory granules.

The unaltered levels of CPE in the hypothalamus, however, suggest that CPE synthesis is not stimulated by dehydration in the way that prohormone production is.

It was noticed in a study of CPE in cultured medullary chromaffin cells that the ratio of CPE enzymic activity to CPE immunoreactivity differed between cellular extracts and secreted material; the activity was much lower in the cellular material (Hook & Eiden, 1985). This could be explained by the presence of a precursor to CPE, which is immunoreactive but enzymically inactive, found only inside the cell and not secreted. This idea is supported by DNA studies. Although a gene coding for CPE has not yet been isolated, cDNA studies have revealed some useful information about the enzyme. The first cDNA clone from a bovine pituitary cDNA library was incomplete, but provided information on a partial sequence for the enzyme, including several pairs of basic amino acids (Fricker *et al*, 1986). Protein sequencing of both the soluble and membrane-bound forms of CPE revealed a common amino terminus, and the sequence deduced from the cDNA contained a pair of basic amino acids 22 residues from the carboxy terminus; cleavage of the enzyme at this site would reduce its size by about 3kd, which could account for the difference between the membrane bound (52-53kd) and soluble (50kd) types (Fricker *et al*, 1986). Later estimations of size using more rapid purification methods have re-defined these values at 53-55kDa for the soluble and 55-58kDa for the membrane-bound forms (Laslop *et al*, 1986 and Fricker *et al*, 1990). Using the prediction method of Chou and Fasman, these workers calculated that the predicted structure of this 22 residue region is an α-helix, and although most of the amino acids are charged, there are three pairs of hydrophobic residues which would protrude from one

side of the helix and possibly provide a means of interaction with the membrane of the secretory granule (Fricker *et al*, 1986).

Re-screening of the bovine cDNA library resulted in the isolation of a longer clone (Fricker, 1988b). Examination of this longer sequence suggested the presence of a signal peptide followed by a short "pro-peptide" that is not seen in the enzyme isolated from tissues. Immediately before the amino terminal sequence common to both forms of the enzyme are 5 adjacent Arg residues, which are a likely target for cleavage (Fricker, 1988b). As the intact "proenzyme" sequence has not been isolated, it is not known whether it has any enzyme activity. Southern blotting of bovine genomic DNA suggests the presence of only one gene for CPE, and this supports the evidence for a common enzyme in many different tissues (Fricker *et al*, 1986).

Recently, a full length cDNA clone has been isolated from cDNA libraries of both hippocampus and hypothalamus of rat (Fricker *et al*, 1989), and a shorter clone from libraries of rat insulinoma and thyroid carcinoma cell lines (Rodríguez *et al*, 1989); the protein sequences from these studies differ at only one residue. Northern blot analysis reveals corresponding mRNA of a constant size of about 2.1kb in rat cerebellum, cortex, hippocampus, hypothalamus, midbrain, brainstem and striatum, as well as heart, adrenal, testis and duodenum (Fricker *et al*, 1989); CPE mRNA could not be detected in rat liver, despite reported GEMSA binding in this tissue (Lynch *et al*, 1987). cDNA for human CPE has also been prepared and indicates an mRNA transcript of 2.5kb leading to a proenzyme product of similar size and structure to those of both rat and bovine origin (Manser *et al*, 1990). Comparison of the sequences of rat and bovine CPE displays a high level of conservation: 91.6% homology for the entire preprotein (proCPE plus signal peptide) and 94.3% for the

active enzyme sequences (Fricker *et al*, 1989). This is much higher than the level of homology between rat and bovine carboxypeptidase A (CPA) which is only 78% for the active region (Quinto *et al*, 1982).

The proteins with the highest homology with bovine CPE (discounting CPE from other species) are bovine CPB and bovine and rat CPA, and the residues thought to be important for activity in CPA and CPB are highly conserved in both bovine and rat CPE (Fricker *et al*, 1986 and Fricker *et al*, 1989). The major differences between the CPE sequences and those for CPA and CPB involve two internal regions of little homology and an additional 120 amino acids at the carboxy terminus of CPE; if the tertiary structure of CPE is similar to the other enzymes, the two additional internal regions in CPE would lie close to the substrate binding site, and so could account for the difference in substrate specificity of CPE (Fricker *et al*, 1986). The difference in pH optimum between CPE (5.6) and CPA/B (7.0) also has to be accounted for.

The sequences of CPA and CPB show 48% homology, compared with 20% for CPE and CPA, and 17% for CPE and CPB, suggesting that although CPE probably evolved originally from this digestive enzyme lineage, it was a considerable time before the divergence of CPA and CPB (Fricker *et al*, 1986). This is supported by the presence of very similar enzymes in frog, shark and *Aplysia* neural tissues; although the amino acid sequences are not yet known, most of the physical and enzymatic properties of CPE from these species are identical to the mammalian enzyme (Fricker & Herbert, 1988).

Further work on the membrane-bound form of CPE has shown that association with the membrane leads to reduced enzyme activity (at least against short, synthetic substrates) and that activity is increased when membranes are treated either with

detergent or high pH buffers to solubilize the enzyme (Fricker, 1988c). The dependence of CPE-membrane association on pH is interesting; all the CPE activity in preparations of bovine pituitary membranes remained bound at pH values below 6, but the majority was solubilized by extraction with buffers above pH 8 (Fricker, 1988c). Similar results have been presented on CPE from pancreatic islets of the anglerfish (Mackin & Noe, 1987). These findings suggest that at the intragranular pH at which *in vivo* processing occurs, much of the carboxypeptidase activity will be at the membrane.

The mechanism by which CPE is held to the membrane has also been the subject of detailed study. Synthetic peptides 11-24 residues long and corresponding to the carboxy terminus of the enzyme have been examined for their abilities to bind to bovine pituitary membranes (Fricker *et al*, 1990). All of the peptides bound to membrane preparations and were released under similar conditions to the complete enzyme; the mechanisms involved included both hydrophobic and ionic interactions, supporting the theory that the extra carboxy terminal portion of the membrane-associated form of CPE is an amphiphilic α-helix (Fricker *et al*, 1990). However, an antisera raised to the 24-residue carboxy terminal peptide was seen to cross-react with one of the soluble forms of the enzyme (Fricker *et al*, 1990), so it would seem that cleavage of the membrane-bound to the soluble form could not take place at the Arg^{413}-Lys^{414} dibasic pair as previously suggested (Fricker *et al*, 1986), as this would result in removal of the entire 22-residue carboxy terminus.

The carboxypeptidase activity involved in producing oxytocin and vasopressin is thus almost certainly CPE. This enzyme acts on precursors of a wide range of hormones and neuropeptides to remove the basic amino acids left at the carboxy

terminus after signalling for endoproteolytic cleavage. It is probably encoded by a single gene and is synthesized as a precursor with an amino terminal extension; this extension may be removed by the action of the same endoprotease that acts on the prohormone, as it is joined to the active enzyme sequence by five adjacent Arg residues in both the rat and bovine forms. The gene that codes for the CPE precursor is related to those for CPA and CPB. Little is known about the regulation of CPE synthesis and activity *in vivo*.

Single Arg residues are also removed from the carboxy terminus of neurophysins from human and rat prooxytocin and from provasopressin; this is presumably achieved by CPE. However, removal of the His residue from the carboxy terminus of neurophysin from bovine prooxytocin may be by a different enzyme. CPE has been shown to remove carboxy terminal His from synthetic peptides, but this reaction is extremely slow - about 6,000 times slower than cleavage of Lys from a similar substrate under the experimental conditions chosen (Smyth *et al*, 1989). The ability to remove a carboxy terminal His residue is also required in the processing of β-endorphin to forms that have been isolated from the CNS (Smyth *et al*, 1981). An enzyme has been isolated from bovine neurosecretory granules that will cleave the carboxy terminal His from prooxytocin as well as from a synthetic hexapeptide (Nörenberg & Richter, 1988). It displays remarkably similar properties to CPE: a thiol protease, stimulated by Co^{2+} with a pH optimum of 5.0-5.5, but it differs from CPE in size, being only 45kd (Nörenberg & Richter, 1988).

1.2.5. Amidating Activity

Enzymes with the ability to convert a carboxy terminal Gly to a -$CONH_2$ group have been isolated from a wide range of tissues and display similar properties (for review see Eipper & Mains, 1988). Most of the studies have used the synthetic substrate [^{125}I]-D-Tyr-Val-Gly in a simple assay of amidation that was first used to demonstrate such activity in porcine pituitary secretory granules (Bradbury *et al*, 1982). The same method was then used to demonstrate activity in secretory granules of anterior, intermediate and neural lobes of rat pituitary, as well as bovine intermediate lobe (Eipper *et al*, 1983).

Amidating activity has now been well characterized in mammalian hypothalamus (Emeson, 1984), cerebrospinal fluid (Vaeröy *et al*, 1987), small intestine (Noguchi *et al*, 1988), spinal cord (Graham & Gallop, 1989), heart (Kojima *et al*, 1989) and medullary thyroid carcinoma cells (Gilligan *et al*, 1989), as well as pituitary and frog skin (see Eipper & Mains, 1988). The enzymes all exhibit common characteristics of stimulation by copper and ascorbate, a requirement for molecular oxygen, and a pH optimum usually in the range pH 6.5-8.0. The apparent molecular weights vary, however, between 26 and 92kd. The properties of the enzymes have led to designation of the collective name peptidyl-glycine α-amidating monooxygenase (PAM).

PAM is found in tissues associated with oxytocin and vasopressin synthesis, i.e. neural lobe (Eipper *et al*, 1983) and corpus luteum (Sheldrick & Flint, 1989), and in both cases the enzyme is co-localized with the hormones in secretory granules. However, the pH inside neurosecretory granules is 5.8 (Russell & Holz, 1981 and Sherman & Nordmann, 1982), and the activity of the pituitary enzyme *in vitro* is

maximal at pH 7.0, dropping sharply below pH 6.0 (Eipper *et al*, 1983). PAM has been shown to have the ability to convert oxytocin-Gly to mature oxytocin (Ando *et al*, 1988), but again only at a high pH, in this case pH 8.5.

1.2.6. Problems Remaining in Understanding Oxytocin and Vasopressin Production

When trying to identify and characterize a prohormone processing enzyme, the criteria of Docherty and Steiner, detailed earlier, must be kept in mind. The use of small, synthetic peptide substrates can be useful for the initial localization of a particular enzyme activity, but that enzyme must be shown to have sufficient activity on the natural substrate under conditions as close as possible to those found *in vivo*. Detailed studies of enzyme characteristics are often based on model substrates and conditions decided entirely by achieving maximal activity, rather than considering the environment in which the enzyme is acting in the cell. This is particularly apparent in the case of the amidating enzyme, where technical problems make it difficult to study amidation of natural substrates, and a wide range of cofactors are used often without consideration of their presence or otherwise at the *in vivo* site of activity. The only study using oxytocin-Gly as substrate still involved the use of a pH optimal for *in vitro* enzyme activity rather than the acidic pH of the neurosecretory granule (Ando *et al*, 1988), so the relevance of the findings of such studies to the *in vivo* situation must be questioned.

The initial consideration when studying the processing of prooxytocin, then, is the form of the substrate for each processing enzyme. Most evidence presented so far points to the scheme outlined in figure 1.7; the endoprotease enzymes act on

prooxytocin, the carboxypeptidase on oxytocin-Gly-Lys-Arg and oxytocin-Gly-Lys, and the amidating enzyme on oxytocin-Gly. However, the presence of the neurophysin sequence in the prohormone, and its affinity for oxytocin, must be considered. In a study using a semi-synthetic prohormone, the hormone sequence was shown to be already associated with the hormone binding site on the neurophysin moiety before endoproteolytic cleavage (Kanmera & Chaiken, 1985b), so this is likely to be the initial substrate for processing. Neurophysin has been shown by affinity chromatography studies to bind oxytocin-Gly-Lys-Arg in a similar manner to oxytocin at pH 5.7 (Kanmera & Chaiken, 1985a), so it can be assumed that after initial endoproteolytic cleavage, the extended hormone and the neurophysin sequences remain bound, and this complex is in fact the substrate seen by both carboxypeptidase and amidating enzymes. Working on these assumptions, studies have been carried out on the ability of enzymes from neurosecretory granules to process oxytocin-Gly-Lys-Arg and oxytocin-Gly in the presence of neurophysin (Kanmera & Chaiken, 1985a and Ando *et al*, 1988). Both of these studies reported slightly increased carboxypeptidase activity in the presence of neurophysin equimolar to the substrate, but decreased activity at higher neurophysin concentrations; the later study showed similar effects on amidation. The rate enhancing effect was also achieved with a protein of similar size to neurophysin, so the effect of neurophysin was described as being non-specific (Ando *et al*, 1988). The inhibition of activities by high concentrations of neurophysin was thought to be due to stearic constraints on processing due to association of neurophysin-hormone complexes with additional neurophysin. However, the situation *in vivo* will not allow any excess of neurophysin

over hormone as they are produced in equal amounts from the same precursor, so again the validity of comparing these results to the *in vivo* situation appears flawed.

The *in vivo* site of processing, the intra-granular environment, has been studied with respect to pH (Russell & Holz, 1981) protein concentration (Nordmann & Morris, 1984), and ascorbate and metal ion content (Thorn *et al*, 1986). These reports have built the picture of an acidic environment, with extremely high concentrations of hormone precursor and cleaved products (0.06M), such that a large proportion of the complex may be in crystalline form and there is very little volume remaining in the granule for accomodation of other molecules (Nordmann & Morris, 1984). The relatively high ascorbate level (45 to 60nmol/mg protein) provides a reducing environment, sustained by electron transport across the granule membrane (Russell *et al*, 1985). This transport is thought to be mediated by cytochrome b_{561} in both the neurosecretory granule (Russell *et al*, 1985) and the adrenal chomaffin granule (Njus *et al*, 1983), where the ascorbic acid may act as an electron donor to the amidating enzyme.

Measurement of metal ions in neurosecretory granules resulted in no detection of cobalt (<5pmol/mg protein), but fairly high levels of both copper and zinc (Thorn *et al*, 1986). If the total contents of these metals were in soluble forms, the concentrations would be considerably higher than in plasma, and the copper would be far in excess of the level required for maximal activity of the amidating enzyme; however, both metals are shown to be largely associated with the membrane fraction of granule preparations, presumably bound to proteins, perhaps copper to the amidating enzyme and zinc to CPE (Thorn *et al*, 1986).

Another environmental effect on the enzymes *in vivo* that has not been widely considered is the entrapment by, and therefore proximity to the granule membrane. All three types of processing activity necessary for maturation of oxytocin and vasopressin have been isolated as forms with either a membrane spanning region or part of the enzyme in close association with the membrane, yet nearly all enzyme studies are carried out in aqueous solution in the absence of any lipid component. Granules from rat anterior pituitary contain comparable amounts of soluble and membrane bound endoprotease activity (Chang & Loh, 1983); similar activity purified from bovine neurohypophysis and corpus luteum is found in two isoforms, possibly a membrane-bound enzyme and a smaller, soluble molecule formed by processing of the larger form (Plevrakis *et al*, 1989). As detailed in the above section on CPE, this enzyme has also been found to be associated with the granule membrane. Studies on the relative activities of the soluble and membrane-bound forms suggest that the soluble enzyme has a higher specific activity (Hook, 1985 and Fricker, 1988b); however these studies used unnatural substrate molecules: respectively iodinated Met-enkephalin and a short synthetic tripeptide. The amidating enzyme is commonly found in several different forms within the same tissue, including some on the membrane, and many of these are believed to be bound by a membrane spanning region; for example in frog skin (Ohsuye *et al*, 1988), mammalian heart (Kojima *et al*, 1989, Eipper *et al*, 1988 and Stoffers *et al*, 1989), and bovine pituitary (Eipper *et al*, 1987, and May *et al*, 1988). These enzyme studies, taken together with the finding of copper, a cofactor for the amidating enzyme, and zinc, a cofactor for CPE, mostly in the membrane fraction, strongly suggest that the inner surface of the granule membrane may be a site of processing activity.

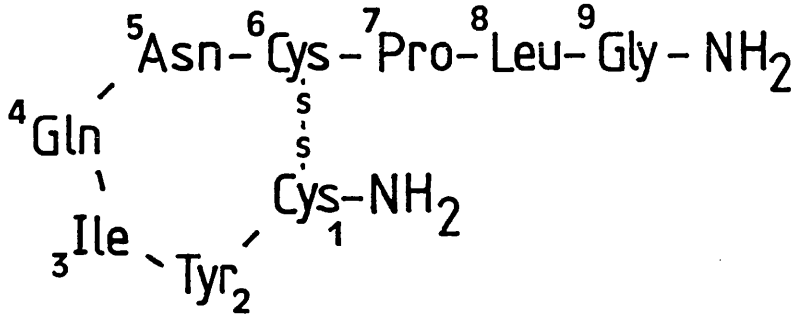
If it is accepted that all the processing enzymes may be active at the membrane surface, the problem remaining is the location of the peptide substrates. Newly synthesized POMC has been found to be largely associated with the granule membranes of both frog and mouse intermediate lobe cells (Loh & Tam, 1985). Neurophysin has also been shown to associate with membrane lipids (Audhya & Walter, 1979) and has been isolated from neurosecretory granule membrane preparations (Swann & Pickering, 1976); this introduces the possibility of a mechanism by which the immature hormone substrates, bound to neurophysin, could be held close to the granule membrane where the processing enzymes could effectively exist as a multi-enzyme complex with their metal ion cofactors. The chemical environment adjacent to the membrane will differ considerably from the typical aqueous solution used in most enzyme studies and could possibly allow for activity of all the required enzymes under identical conditions, which has never been achieved in an *in vitro* study.

Another problem which has not been addressed is the slow removal of carboxy terminal lysine residues from substrates by CPE. If the assumption that CPE is the only carboxypeptidase involved in prohormone processing (Fricker *et al*, 1989) is correct, it appears puzzling that the enzyme is far more efficient in cleavage of arginine than of lysine. Perhaps under different conditions, both residues could be cleaved with equal efficiency.

Full understanding of processing of the oxytocin precursor therefore appears to be restricted by the lack of consideration for the granular environment where the reactions are taking place. A more integrated approach involving the use of native substrates, all of the processing enzymes and conditions approaching those found *in*

vivo may serve to overcome some of the remaining gaps in the knowledge of this system - including the apparent incompatability of isolated enzymes (in terms of pH and cofactor requirements) and the low rates of cleavage observed with lysyl substrates. The aims of this study, therefore, are to examine the effects of some of the factors discussed here on the activities of the processing enzymes. Those factors include substrate form and chemical environment, including protein concentration and the presence of neurophysin, and the influence of membrane components, with the objective of working towards a single set of conditions that would allow complete processing to occur.

OXYTOCIN



ARGININE
VASOPRESSIN

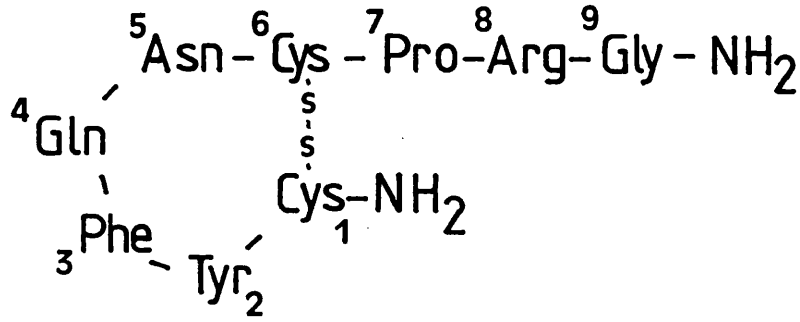


Figure 1.1. Comparison of the structures of oxytocin and vasopressin.

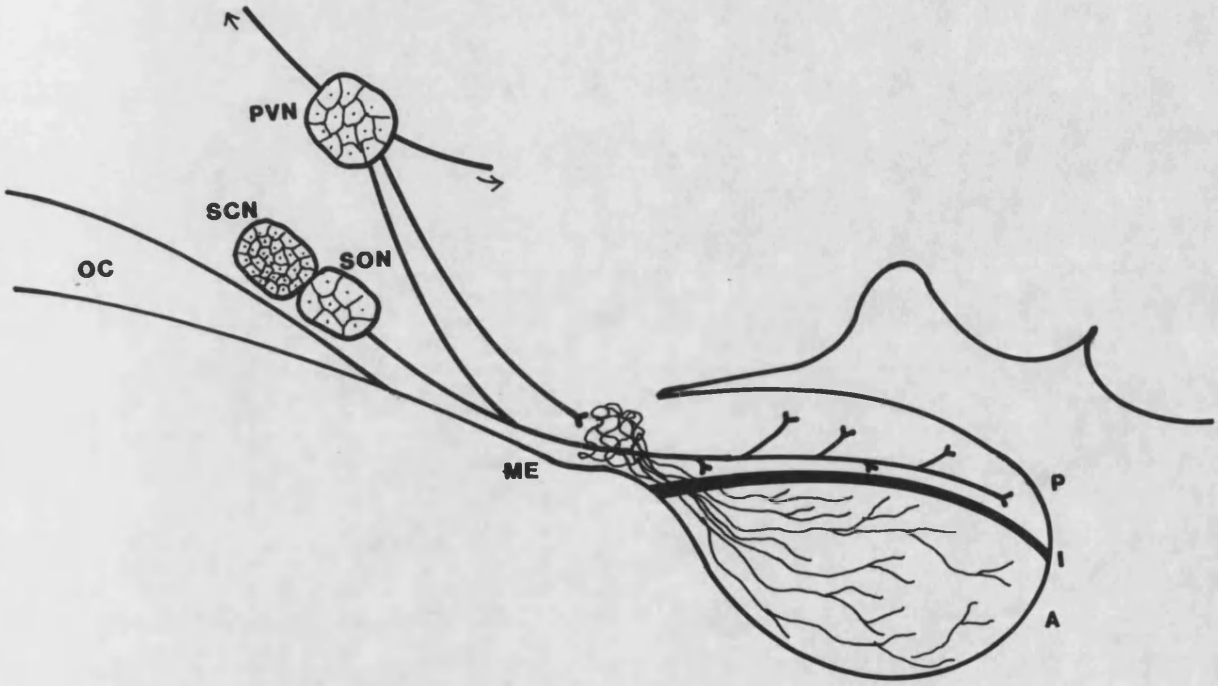


Figure 1.2. The hypothalamo-neurohypophyseal system. OC, optic chiasma, PVN, paraventricular nucleus, SCN, suprachiasmatic nucleus, SON, supraoptic nucleus, ME, median eminence, P, posterior pituitary, I, intermediate pituitary, A, anterior pituitary.

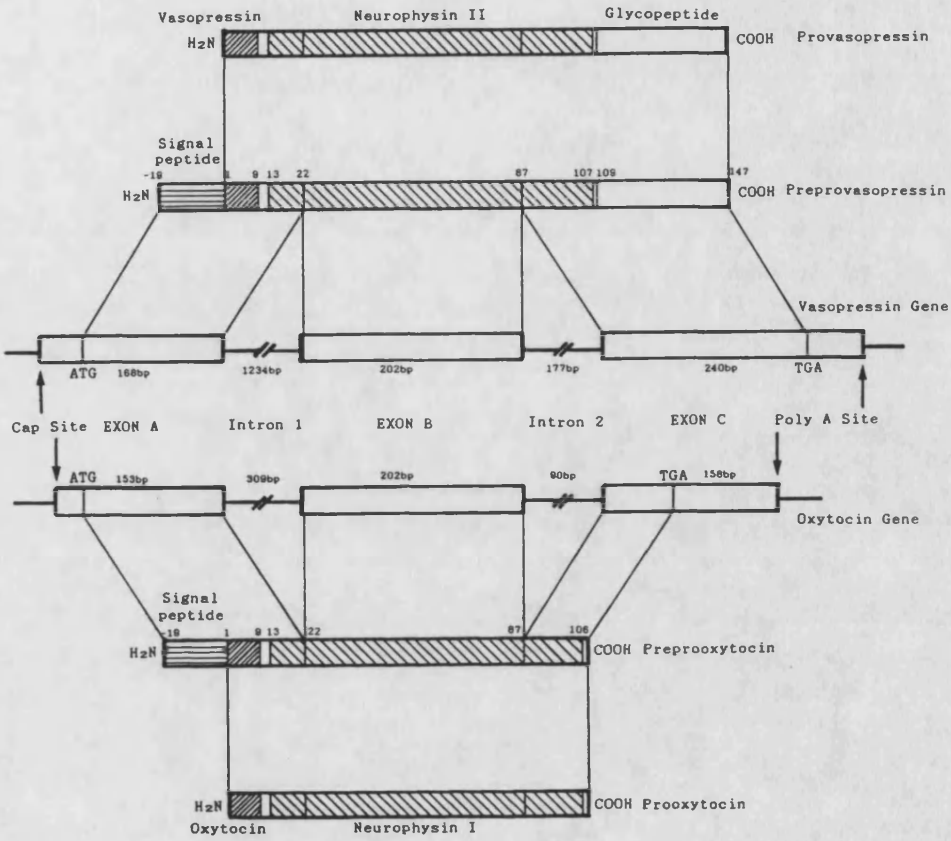


Figure 1.3. Structural organization of the genes coding for preprovasopressin and preprooxytocin. Exon A of both genes codes for 19 amino acids that comprise the signal peptide, as well as the hormone sequence itself, 3 separating amino acids and the 9 amino terminal residues of neurophysin. Exon B codes for the central part of neurophysin. Exon C codes for the carboxyl end of neurophysin plus a single amino acid in preprooxytocin, or a 39-residue glycoprotein in provasopressin.

```
                    1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16
Bovine (VP)        Ala-Met-Ser-Asp-Leu-Glu-Leu-Arg-Gln-Cys-Leu-Pro-Cys-Gly-Pro-Gly
Guinea pig (VP)    ----Leu---------Thr-------------------------------------------
Bovine (OT)        ----Val-Leu---------Asp-Val-----Thr-----------------------------
Human (OT)         ----Ala-Pro---------Asp-Val-----Lys-----------------------------
Bufo toad (VT)     Ser-Tyr-Pro-----Thr-Ala-Val-------------Ile--------------------
Sucker fish (VT)Ser-Gln-Pro-----Thr-[ ]-Ser-----Glu-----Val-Ser----------------


17  18  19  20  21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36
Gly-Lys-Gly-Arg-Cys-Phe-Gly-Pro-Ser-Ile-Cys-Cys-Gly-Asp-Glu-Leu-Gly-Cys-Phe-Val
----Gln-----------------------------------------Ala-----Ala--------------------
-------------------------------------------------------------------------------
--------------------------------Asn-------------Ala-Glu-------------------------
Asn-Arg-----Asn-----------------Asn-----------------Glu-Asp-------------Tyr----
Asn-Ala-------------Tyr----------------------------Ala-Ala-------------Leu----


37  38  39  40  41  42  43  44  45  46  47  48  49  50  51  52  53  54  55  56
Gly-Thr-Ala-Glu-Ala-Leu-Arg-Cys-Gln-Glu-Glu-Asn-Tyr-Leu-Pro-Ser-Pro-Cys-Gln-Ser
-------------------------------------------------------------------------------
-------------------------------------------------------------------------------
-------------------------------------------------------------------------------
--------Pro-----Thr-------------Val---------Thr-------------------------Glu-Ala
----Ser-Pro-----Thr-Met-Ser-----Met-------------His---------------------Glu-Thr


57  58  59  60  61  62  63  64  65  66  67  68  69  70  71  72  73  74  75  76
Gly-Gln-Lys-Pro-Cys-Gly-Ser-Gly-Gly-Arg-Cys-Ala-Ala-Ala-Gly-Ile-Cys-Cys-Asn-Asp
-----------------------------------------------------Asn-----Val----------------
-----------------------------------------------------------------------Ser-Pro
------------Ala-----------------------------------Val-Leu-----Leu---------Ser-Pro
----Gly-------------Ser-----------------------------Pro-----Val---------Ser----
----Gly-Arg-------------Asp-Glu---------------------Pro-----Val---------Asp-Ser


77  78  79  80  81  82  83  84  85  86  87  88  89  90  91  92  93  94  95
Glu-Ser-Cys-Val-Thr-Glu-Pro-Glu-Cys-Arg-Glu-Gly-Val-Gly-Phe-Pro-Arg-Arg-Val
----------------Ile-------------------------Glu-Phe-His-[     ]-----Pro----
Asp-Gly-----His-Glu-Asp-----Ala-----Asp-Pro-Glu-Ala-Ala-----Ser-Gln
Asp-Gly-----His-Ala-Asp-----Ala-----Asp-Ala-Glu-Ala-Thr-----Ser-Gln
Asp-Thr---------Val-Asp-Ser-Ser-----Leu-Asp-Glu-Asp-Ser-Glu-Arg----
Val-------------Met-Asp-Ser---------Leu-----Asp-----Arg-Ser-Asp-Gln-Ser-Glu-
```

Figure 1.4. Amino acid sequences of neurophysins from various species. Standard three letter symbols are used for amino acid residues. Dashes show a residue identical to the corresponding one in the top (bovine) sequence; brackets indicate a gap introduced to optimise alignment. Sequences from precursors of vasopressin (VP), oxytocin (OT) and vasotocin (VT) are shown. References: bovine, guinea pig and human, Breslow & Burman (1990); toad, Nojiri *et al* (1987); sucker fish, Heierhorst *et al* (1989).

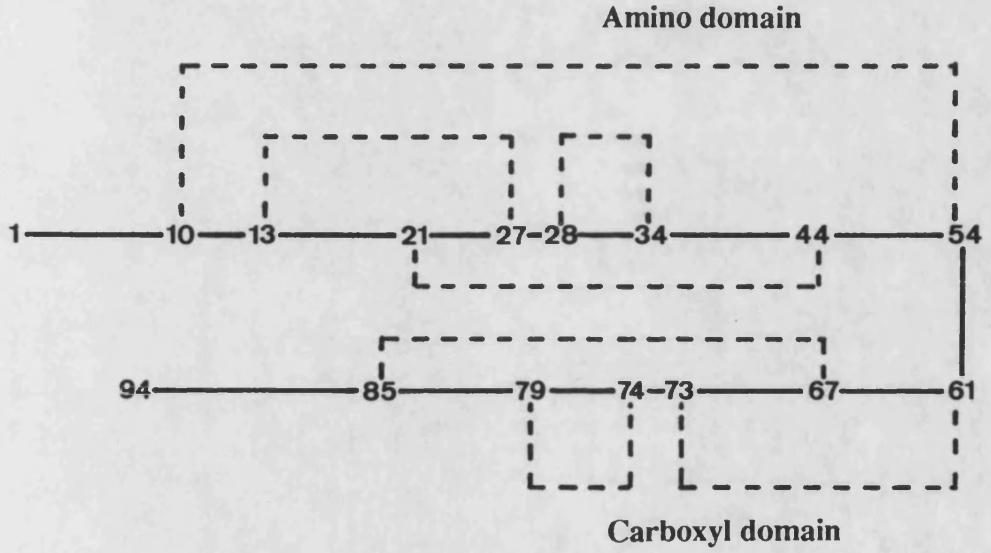


Figure 1.5. Disulphide pairing in bovine neurophysins forming two separate domains. From Burman *et al* (1989).

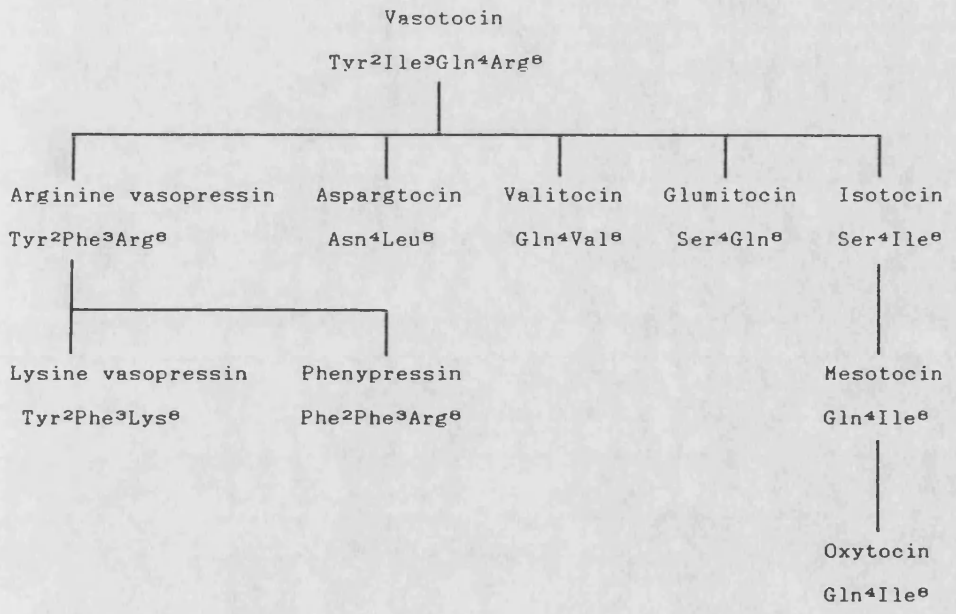


Figure 1.6. A putative evolutionary pathway of neurohypophyseal hormones.

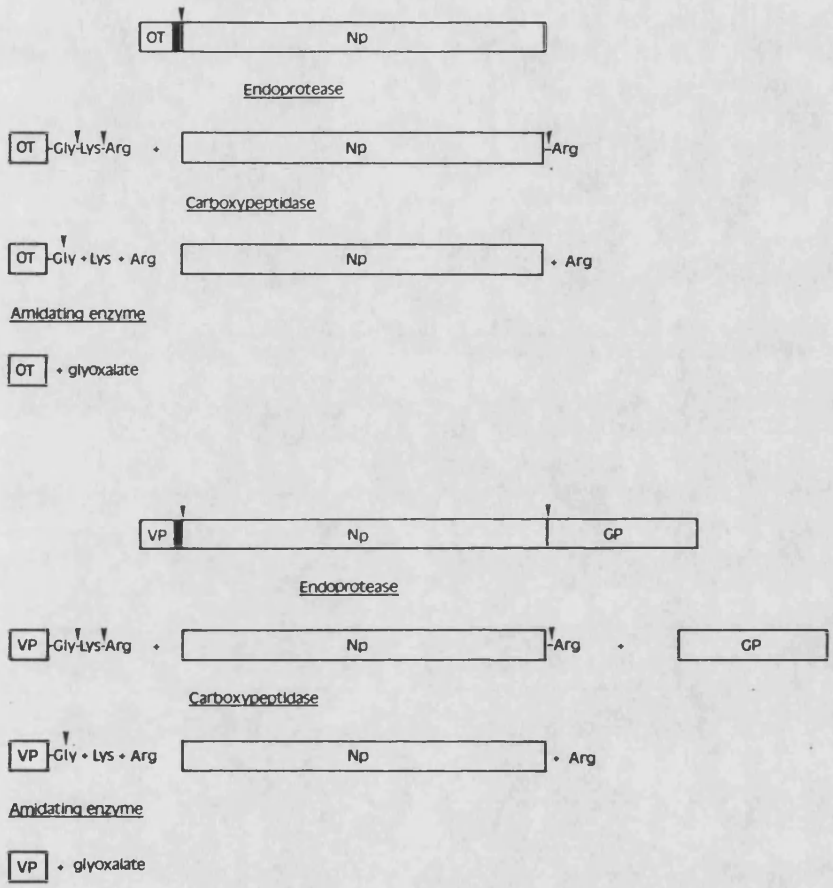


Figure 1.7. Processing of prooxytocin and provasopressin to generate active hormones, neurophysins and glycopeptide. The major functional domains are separated by endoproteolysis, then the basic residues which signal that cleavage are removed by carboxypeptidase activity. Finally the hormones are activated by C-terminal amidation.

Oxytocin-related	
Oxytocin	Cys-Tyr-Ile-**Gln**-Asn-Cys-Pro-**Leu**-Gly-NH_2
Mesotocin	Cys-Tyr-Ile-**Gln**-Asn-Cys-Pro-**Ile**-Gly-NH_2
Isotocin	Cys-Tyr-Ile-**Ser**-Asn-Cys-Pro-**Ile**-Gly-NH_2
Glumitocin	Cys-Tyr-Ile-**Ser**-Asn-Cys-Pro-**Gln**-Gly-NH_2
Valitocin	Cys-Tyr-Ile-**Gln**-Asn-Cys-Pro-**Val**-Gly-NH_2
Aspargtocin	Cys-Tyr-Ile-**Asn**-Asn-Cys-Pro-**Leu**-Gly-NH_2
Vasopressin-related	
Arginine vasopressin	Cys-**Tyr**-**Phe**-Gln-Asn-Cys-Pro-**Arg**-Gly-NH_2
Lysine vasopressin	Cys-**Tyr**-**Phe**-Gln-Asn-Cys-Pro-**Lys**-Gly-NH_2
Phenypressin	Cys-**Phe**-**Phe**-Gln-Asn-Cys-Pro-**Arg**-Gly-NH_2
Vasotocin	Cys-**Tyr**-**Ile**-Gln-Asn-Cys-Pro-**Arg**-Gly-NH_2

Table 1.1. Sequences of the neurohypophyseal hormones.

Vertebrate type	Oxytocin-like	Vasopressin-like
Cyclostomes		Vasotocin
Rays	Glumitocin	Vasotocin
Sharks	Valitocin	Vasotocin
	Aspargtocin	
Teleosts	Isotocin	Vasotocin
Lungfish	Mesotocin	Vasotocin
Amphibians	Mesotocin	Vasotocin
Reptiles	Mesotocin	Vasotocin
Birds	Mesotocin	Vasotocin
Prototherian mammals	Oxytocin	Arginine vasopressin
Eutherian mammals	Oxytocin	Arginine vasopressin
Metatherian mammals	Oxytocin	Arginine vasopressin
		Lysine vasopressin
		Phenypressin
Pig	Oxytocin	Lysine vasopressin

Table 1.2. The presence of neurohypophyseal hormones in vertebrates.

CHAPTER 2:

PREPARATION AND METHOD DEVELOPMENT

2.1. Introduction

In order to study the effects of substrate form and environmental conditions on CPE activity, it was necessary to produce a preparation of the enzyme, appropriate peptide substrates, a means of separating and quantifying substrates and products, and to devise a suitable method to assay the enzyme activity.

The major requirement for the enzyme preparation was that it should be rich in CPE activity with minimal contamination from other carboxypeptidases. The starting material of choice was therefore the soluble fraction from a preparation of bovine neurosecretory granules, obtained by ultracentrifugation. Similar preparations have been used previously to study CPE activity (Hook & Loh, 1984, Kanmera & Chaiken, 1985a, Ando *et al*, 1988 and Plevrakis *et al*, 1989). If the effects of adding neurophysin to the reaction are to be assessed, it is essential to remove this protein from the initial enzyme preparation, and gel filtration provided a simple means of achieving this aim. Finally, the preparation needed to be characterized in terms of type of activity, pH spectrum and effectors to confirm that it was in fact CPE activity that was being observed.

Following the overwhelming majority of evidence that the initial fragment for CPE activity in this system is the extended hormone sequence oxytocin-Gly-Lys-Arg, this peptide was chosen for these investigations. Again, this peptide has been used in previous studies as a substrate for prohormone processing enzymes (Kanmera & Chaiken, 1985a, Clamagirand *et al*, 1987b and Ando *et al*, 1988). The most convenient method of preparation of such a molecule in appropriate quantities is solid-phase synthesis. To assess any effect of ionic binding between this substrate and neurophysin on CPE activity, a control substrate is required which should be as similar as possible to the original substrate, but unable to interact with neurophysin. Treatment of oxytocin-Gly-Lys-Arg with performic acid was chosen, to yield a peptide with minimal chemical differences from the original and which would hopefully remain a viable substrate for CPE, but not bind to neurophysin. A neurophysin affinity column was used as a simple and rapid means of observing interaction between neurophysin and the two forms of substrate.

In order to develop a method to separate the substrates and products involved in the study, a series of standards was required. These were prepared from the cyclic and oxidised forms of substrate by digestion with commercial enzymes. Separation by reverse-phase high performance liquid chromatography (RP-hplc) was selected for its abilities to resolve several peptides in a single sample, rapidly, consistently, in both analytical and preparative quantities with simple and sensitive detection of peptide. In establishing the assay method, the important criteria were considered to be consistency, speed, economical use of reagents and throughput of large numbers of samples (up to 120). As the substrates were expensive to synthesize but a relatively high concentration of substrate was preferred, the minimum quantity of peptide that

could be reliably monitored and quantified in the separation system was used in the minimum practical volume, and quantities of all other reagents were adjusted as appropriate.

2.2. Materials and Methods

Unless stated otherwise, all reagents were analytical grade, purchased from BDH Chemicals Ltd., Poole, UK.

2.2.1. Enzyme preparation.

Soluble and membrane fractions of bovine neurosecretory granule preparations were kind gifts of Sonia Birkett and Professor Brian Pickering at the University of Bristol. The soluble fraction (8mg) was dissolved in 0.1M ammonium acetate pH 5.8, centrifuged in an MSE Micro Centaur (Fisons Scientific Equipment, Crawley, UK.) at 11,600 x g for 5min, and 500µl of the supernatant underlayed onto Sephadex-G50(80) (Pharmacia Fine Chemicals, Uppsala, Sweden; 1 x 30cm) equilibrated with the same buffer. Fractions of 0.5ml were collected at a flow rate of 12ml/h, then diluted to 1ml with buffer to allow the absorbance at 280nm to be measured. An aliquot (5µl) of each fraction was assayed for carboxypeptidase activity. The fractions high in activity were pooled and used as the "soluble enzyme preparation"; this was stored at -40°C.

2.2.2. Synthesis of oxytocin-Gly-Lys-Arg.

Oxytocin-Gly-Lys-Arg (OT-GKR) was prepared by solid-phase synthesis using the Fmoc-Polyamide method in a CRB Pepsynthesiser II semi-automatic peptide synthesizer (Cambridge Research Biochemicals Ltd., Cambridge, UK). The peptide was assembled from the carboxy terminus using 9-fluorenylmethoxycarbonyl (f-moc) to protect the α-amino groups and other suitable protecting agents on the side groups of the amino acids: methoxytrimethylbenzenesulphonyl for arginine, butyloxycarbonyl for lysine, triphenylmethyl for cysteine and butyl for tyrosine. All amino acid reagents were purchased from CRB and used in four-fold excess. The carboxy terminal arginine was mixed with dicyclohexylcarbodiimide (Aldrich Chemicals, Poole, UK) in dichloromethane to produce the reactive symmetrical anhydride, and linked to Pepsyn KA polydimethylacrylamide resin (CRB) with the aid of 4-dimethylaminopyridine as a catalyst at a loading of 0.095meq/g. Subsequent couplings utilized o-pentafluorophenyl esters of protected amino acids and the catalyst 1-hydroxybenzotriazole (Aldrich); the f-moc linkage is base-labile and was cleaved by treatment with 20% piperidine in dimethylformamide. The extent of each coupling was tested on a small sample of washed resin by the Kaiser test (Kaiser *et al*, 1970), using ninhydrin which forms a blue/purple colour with free amino groups.

When the sequence was complete, overnight treatment with trifluoroacetic acid (TFA, Aldrich) was used to cleave the peptide from the resin and remove the protecting groups; in the presence of phenol to assist deprotection of arginine, and 1,2-ethanedithiol (Sigma Chemical Co., Poole, UK) to prevent formation of intermolecular disulphide bonds. The resin was removed by filtration, and most of the TFA removed by rotary evaporation at room temperature. Water was then added

to give a final peptide concentration of 1mg/ml, and the phenol and ethanedithiol removed by extraction into diethyl ether. The peptide solution was de-gassed with nitrogen and left open at room temperature to cyclise overnight. The peptide was lyophilized for storage at 4°C before purification by RP-hplc, using the peptide separation system detailed below. The peptide fraction from HPLC was desalted on a Sep-Pak C18 cartridge (Waters Chromatography Div., Massachusetts, USA), eluted with 80% acetonitrile (ACN) in 0.1% TFA, lyophilized and stored at 4°C.

The expected amino acid content of the synthetic peptide was confirmed by amino acid analysis.

2.2.3. Preparation of standards oxytocin-Gly-Lys and oxytocin-Gly.

These methods were adapted from Kanmera & Chaiken (1985a). Oxytocin-Gly-Lys (OT-GK) was prepared from 1mg OT-GKR by digestion with 2 units of TPCK treated trypsin immobilized onto agarose (Sigma T4019) in 200µl 0.1M ammonium bicarbonate, pH 8.6, for 2h at 37°C. The enzyme was removed by centrifugation in an MSE Micro Centaur at 11,600 x g for 5min. 1mg OT-GKR was also digested with 14 units of carboxypeptidase B type I-DFP (Sigma C7261) in 200µl 0.1M ammonium bicarbonate pH 8.3 for 30min at 37°C, to produce oxytocin-Gly (OT-G). Both peptides were purified by RP-hplc, desalted on a Sep-Pak C18 cartridge and lyophylized.

2.2.4. Performic acid oxidation of peptides.

Performic acid was prepared by mixing 0.1ml 30%v/v hydrogen peroxide with 0.9ml 90%v/v formic acid and standing at room temperature for 1h. The acid was

then cooled on ice and added to the appropriate dry peptide (0.1ml/mg) and the mixture left on ice for 1h. The peptide was separated from the reaction mixture using a Sep-Pak C18 cartridge, dried, then purified by RP-hplc, using the same conditions as for purification of the synthetic OT-GKR. Performic acid oxidises the cysteine residues to cysteic acid, irreversibly opening the ring formed by the disulphide bridge.

To assess binding to neurophysin of the cyclic and oxidised forms of OT-GKR, 20μg of each peptide, in 200μl 0.1M ammonium acetate, pH 5.8, were applied to an affinity column consisting of bovine neurophysin covalently linked to agarose (0.75 x 5cm, prepared by Dr RW Swann using the method of Robinson & Walker, 1979). The column was washed with 10ml acetate buffer, then eluted with 10ml 0.1M formic acid. Fractions of 1ml were collected, and elution of the peptides from the column was monitored by RP-hplc analysis of 500μl samples of each fraction.

The ability of oxidised OT-GKR to act as a substrate for the enzyme preparation was tested by substitution of this peptide for untreated OT-GKR in the standard assay.

2.2.5. Separation of peptide standards by RP-hplc.

A single RP-hplc method was developed to both purify and separate all of the synthetic peptides used in this study. The system comprised two Gilson model 302 pumps, model 802 manometric module, model 231 sample injector and model 621 data module (all from Gilson Medical Electronics, Villiers Le Bel, France), an ABI 1000S Diode Array Detector (Applied Biosystems Inc., Cheshire, UK) controlled by

Gilson 714 software installed on an Opus V personal computer which was also used to store and manipulate the data.

Separation was achieved on Spherisorb C18 (0.46 x 20cm, HPLC Technology Ltd., Macclesfield, UK). Buffer A was 67mM o-phosphoric acid (Aristar) adjusted to pH 3.0 with triethanolamine; buffer B was 40% buffer A, 60% ACN (HPLC grade S, Rathburn Chemicals Ltd., Walkerburn, UK). The gradient used for separation was 25% to 40% buffer B in 15min, followed by a 5min wash of 100% buffer B; the flow rate was 1ml/min.

2.2.6. Assay of carboxypeptidase activity.

Unless stated otherwise, assays were conducted in 0.1M ammonium acetate, pH 5.8, with 1-2µl of enzyme preparation and 50µM peptide substrate in a total volume of 30µl, and the reaction proceeded for 1h at 37°C. All reagents were first dissolved in assay buffer; solutions were equilibrated at room temperature before being dispensed into 1.5ml polypropylene microcentrifuge tubes (Elkay Products Inc., Shrewsbury, MA., USA.), with a Microman 1-25µl positive displacement pipette (Gilson). Reactions were started by the addition of enzyme preparation to the tubes at 15s intervals, vortex mixing for 5s and placing in a 37°C waterbath. The reaction was terminated by the addition of 200µl 0.1M HCl at the same time interval, again mixing for 5s, and 200µl of each sample were analysed by RP-hplc as described above. Details of the automatic hplc sample delivery are given in the Appendix.

Automatic peak detection and integration enabled quantification of remaining substrate and newly-formed product in each sample, and thus calculation of the percentage of substrate converted to product in the reaction time, which was used as a

measure of enzyme activity. Three or four replicates were prepared for each treatment. To allow statistical analysis to be performed, all individual percentage results were subjected to arcsin transformation, then results displayed as mean activity ± standard error of the mean (standard deviation/$\sqrt{n}$).

2.3. Results

2.3.1. Separation of peptide standards by RP-hplc.

OT-GKR eluted at approximately 11.5min under the conditions used, while the linear, oxidised form eluted earlier at about 10.6min. The digestion products of OT-GKR, (OT-GK and OT-G) had progressively longer retention times, while the oxidised forms eluted earlier than the substrate peptide and in the reverse order (figure 2.1). Synthetic oxytocin (UCB Bioproducts SA, Braine L'Alleud, Belgium), which was used as a standard for quantitative estimation of peptides, co-eluted with OT-G at approximately 15.2min in this system.

2.3.2. Activity of the soluble enzyme preparation.

Gel filtration of the soluble fraction of bovine neurosecretory granules using Sephadex-G50 resulted in elution of a single peak of carboxypeptidase activity at the void volume of the column (figure 2.2). The six fractions with highest activity (numbers 19-24 inclusive) were pooled and the activity of this preparation assessed. The enzyme preparation was shown to cleave the carboxy terminal arginine from synthetic OT-GKR with an activity of approximately 560pmol/h/µl at pH 5.8, 37°C, in the presence of 1mM cobalt chloride. This activity was optimal around pH 5.6

(figure 2.3), was stimulated by cobalt and inhibited by copper, cadmium, p-chloromercuriphenyl-sulphonic acid (PCMPS) and ethylenediaminetetra-acetic acid (EDTA) (table 2.1). Suppression of CPE activity by guanidinoethylmercaptosuccinic acid (GEMSA, 1μM), resulted in a reduction of arginine cleavage activity to 16% of the control value.

2.3.3. Binding of cyclic and oxidised OT-GKR to agarose-linked bovine neurophysin.

At pH 5.8, all the untreated OT-GKR in the sample bound to the affinity column and was finally eluted with 0.1M formic acid; the oxidised peptide, however, was washed straight through the column without retardation (figure 2.4).

When both OT-GKR and oxidised OT-GKR were incubated with the enzyme preparation, the oxidised peptide was indeed found to act as a substrate, at least as effectively as did the cyclic form (figure 2.5); the relative cleavage efficiencies of these two substrates are considered in Chapter 4.

2.4. Discussion

The hplc separation system developed for this study allowed a single column and gradient combination to be used for all preparative and analytical procedures for the peptides involved. Use of the automatic sample injector and communication between the PC and the hplc modules allowed unattended analysis of up to eighty samples in each run, with a cycle time of 30min/sample.

Complete resolution of all the substrates and products of the carboxypeptidase reaction made study of all relevant digestions possible. Co-elution of OT-G and oxytocin, however, remained an unresolved problem and prevented extension of the experimental work to the amidation reaction. This would have been an interesting addition to the study, but not possible without spending considerable time to establish another separation procedure.

Exclusion of the enzyme activity from Sephadex-G50 revealed the molecule(s) involved to have a molecular weight in excess of 30kD. The soluble enzyme preparation was shown to have significant activity in removal of the carboxy terminal arginine from OT-GKR; subsequent removal of the carboxy terminal lysine, however, was not observed. Although CPE is reported to exhibit lower affinity for carboxy terminal lysine residues than for arginine in the case of enkephalin precursors, this is only a twofold (Supattapone *et al*, 1984) or threefold (Fricker & Snyder, 1983 and Fricker & Herbert, 1988) difference. Removal of lysine from both vasopressin and oxytocin precursors by neurosecretory granule preparations has been reported (Hook & Loh, 1984, Kanmera & Chaiken, 1985a and Ando *et al*, 1988), albeit 7 times more slowly than removal of arginine in the case of extended oxytocin (Kanmera & Chaiken, 1985). However, despite using prepared OT-GK as the initial substrate, with increased quantities of enzyme preparation and extended incubation times, removal of lysine could not be achieved with this enzyme preparation.

Despite the problem of inactivity against lysine, the identity of the major carboxypeptidase activity in the preparation was confirmed as CPE by its pH profile as well as the effects of cobalt ions and various inhibitors. Comparison with previous studies of bovine pituitary preparations shows these results to be consistent with

published findings. The pH optimum of 5.6 found here corresponds with reported values of 5.5-6.0 (Fricker & Snyder, 1982), 5.4-5.8 (Supattapone *et al*, 1984), 5-6 (Kanmera & Chaiken, 1985a) and 5.6 (Fricker & Herbert, 1988).

CPE was originally identified as cobalt-stimulated activity at pH 5.6 (Fricker & Snyder, 1982 and Fricker *et al*, 1982), and the extent of stimulation of the bovine pituitary enzyme by cobalt ions has been reported to be between 2 and 12 times the activity in the absence of cobalt (Fricker *et al*, 1982, Supattapone *et al*, 1984 and Fricker & Herbert, 1988); in this study the addition of 1mM cobalt chloride resulted in approximately 3.5 times the control activity.

Reduction of enzyme activity by EDTA, cadmium and copper ions to 18.5%, 31.5% and <5% of control activity, respectively, is again consistent with published observations. Studies by Fricker *et al* (1982) and Supattapone *et al* (1984) on bovine pituitary preparations report inhibition by EDTA to 13% and 15%, by cadmium ions to 33% and 12% and by copper ions to 4% and 9% of control activities respectively. Carboxypeptidase activity was shown to be reduced to less than 5% by addition of 1mM PCMPS both in this study and by Supattapone *et al*, (1984).

The definitive measure of CPE activity is now considered to be cobalt-stimulated carboxypeptidase activity at pH 5.6 which is abolished by low concentrations of GEMSA (Fricker & Herbert, 1988, Thiele & Fricker, 1988 and Vilijn *et al*, 1989). The activity of the preparation used for these experiments was reduced to 16% in the presence of 1μM GEMSA, so it was concluded from this and the above results that the major carboxypeptidase activity of this preparation was due to CPE.

Neurophysin has been reported to possess similar affinities for oxytocin and the extended forms OT-GKR, OT-GK and OT-G (Kanmera & Chaiken, 1985a and Ando *et al*, 1988), so binding of OT-GKR to the neurophysin affinity column as described above was expected; as with neurophysin-oxytocin binding, the interaction between neurophysin and OT-GKR breaks down at low pH and so the peptide was eluted in formic acid. Oxidation of OT-GKR apparently abolished the interaction with neurophysin, as was hoped, and the peptide passed through the column without retardation. Performic acid treatment did not prevent the peptide from being cleaved by the enzyme preparation, however, so this substrate appeared to be a suitable control for investigations of the effect of substrate-neurophysin binding on CPE activity.

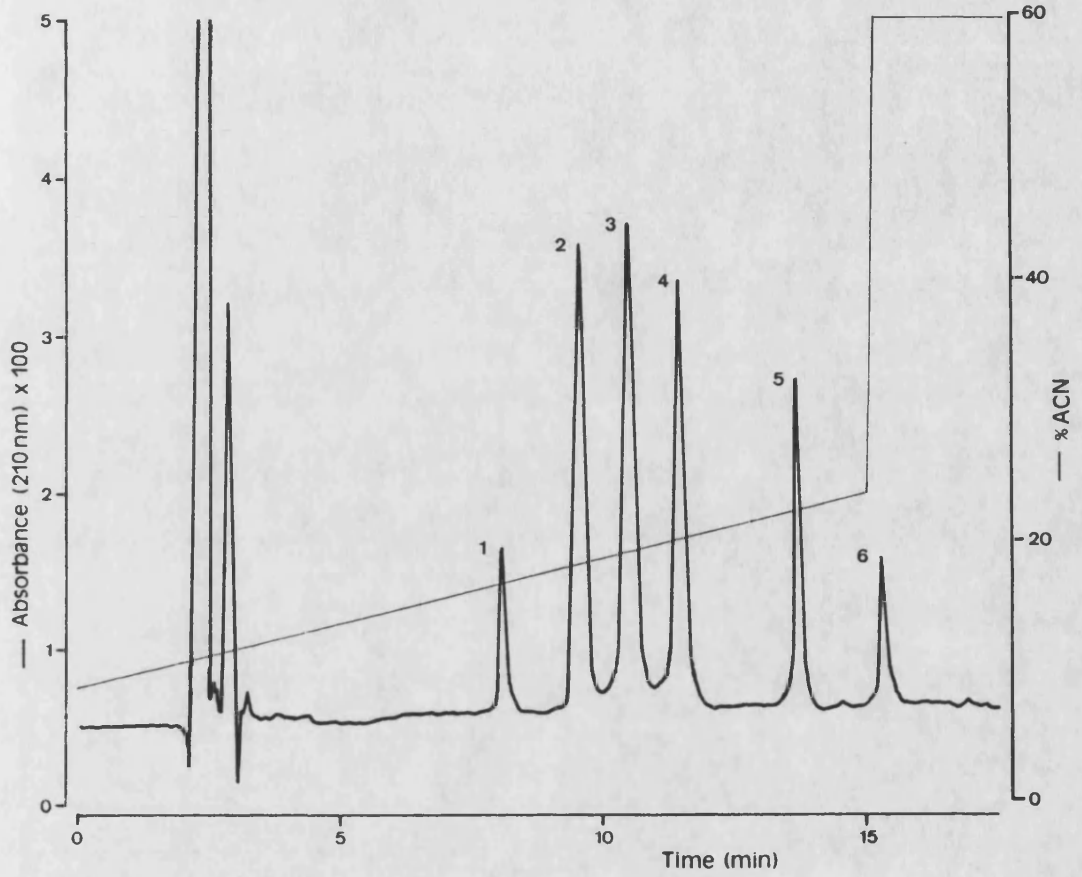


Figure 2.1. Separation of peptides by RP-hplc. Separation was achieved on Spherisorb C18 (0.46 x 20cm, HPLC Technology) with a gradient of 15% acetonitrile, 85% TEAP (67mM triethanolamine phosphate, pH3.0) to 24% acetonitrile, 76% TEAP in 15min, at a flow rate of 1ml/min. Numbered peaks correspond to oxidised OT-G (1), oxidised OT-GK (2), oxidised OT-GKR (3), OT-GKR (4), OT-GK (5), oxytocin and OT-G (6) which co-eluted in this system.

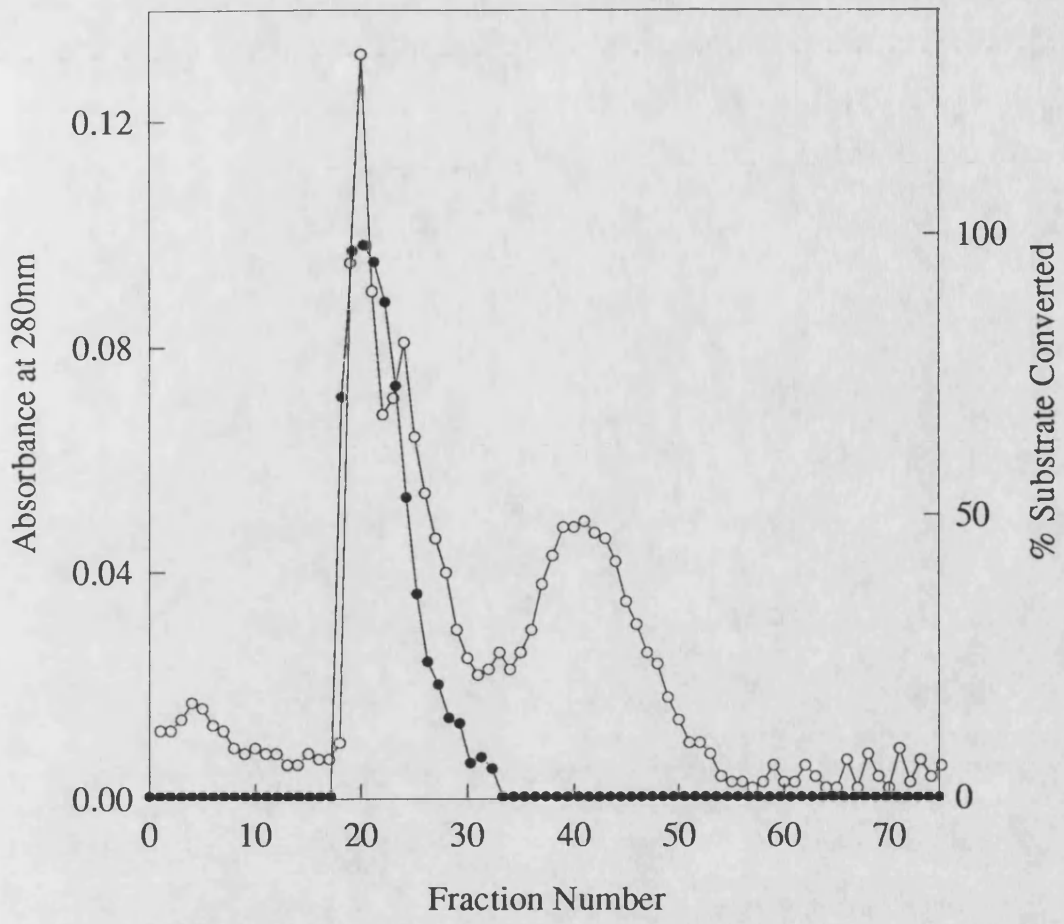


Figure 2.2. Gel filtration of bovine neurosecretory granule soluble fraction. 8mg of the soluble fraction from bovine neurosecretory granules were dissolved in 600µl 0.1M ammonium acetate, pH 5.8, and centrifuged in an MSE Micro Centaur (Fisons) at 11,600 x g for 5min. 500µl of supernatant were underlayed onto Sephadex-G50(80) (Pharmacia, 1 x 30cm) equilibrated with acetate buffer. Fractions of 0.5ml were collected at a flow rate of 12ml/h and diluted to 1ml with buffer; protein content was monitored by measuring the absorbance at 280nm (open circles). A 5µl aliquot of each fraction was assayed for carboxypeptidase activity (solid circles) by incubation with 50µM OT-GKR and 1mM cobalt chloride in 0.1M ammonium acetate, pH 5.8, at 37°C for 1h. The total incubation volume was 30µl. Reactions were terminated by the addition of 200µl 0.1M HCl, and samples analysed by RP-hplc as described in the text.

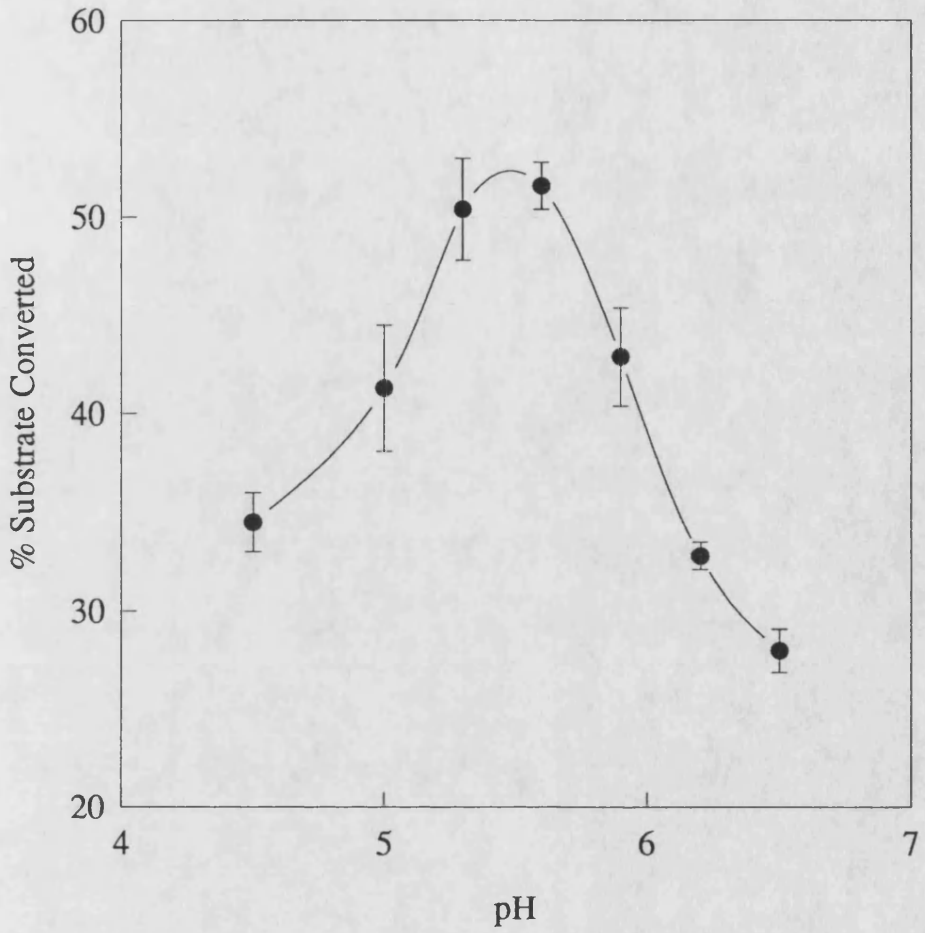


Figure 2.3. pH activity profile of the soluble enzyme preparation. 50μM OT-GKR was incubated with 3μl of enzyme preparation and 1mM cobalt chloride, in 0.1M ammonium acetate adjusted to the appropriate pH with acetic acid. The total incubation volume was 30μl and reactions proceeded for 1h at 37°C, then were terminated by the addition of 200μl 0.1M HCl and analysed by RP-hplc as described in the text. Individual values were subjected to arcsin transformation before calculation of means and standard errors; n=3.

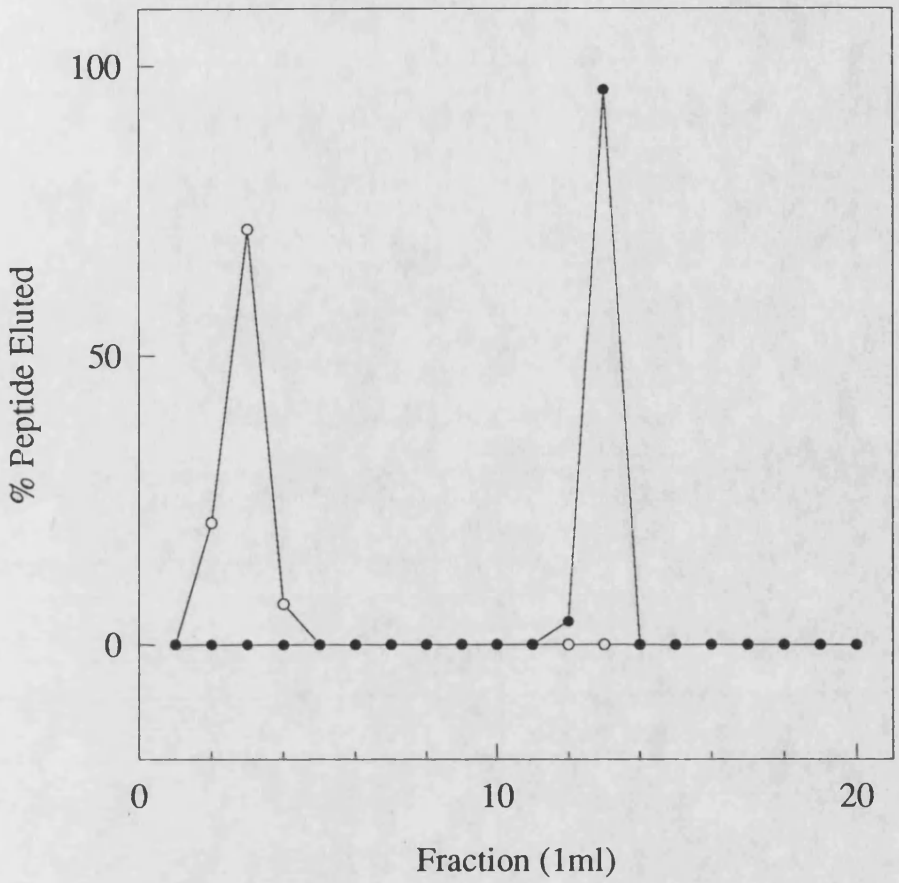


Figure 2.4. Interaction of cyclic and oxidised forms of OT-GKR with agarose-neurophysin. 20μg each of cyclic and oxidised OT-GKR in 200μl 0.1M ammonium acetate, pH 5.8, were applied to a column (0.75 x 5cm) containing agarose-linked bovine neurophysin in the same buffer. The column was washed with 10ml buffer then 10ml 0.1M formic acid to elute any ionically bound material. Fractions of 1ml were collected and 500μl of each fraction examined for peptide content by RP-hplc, as detailed in the text.

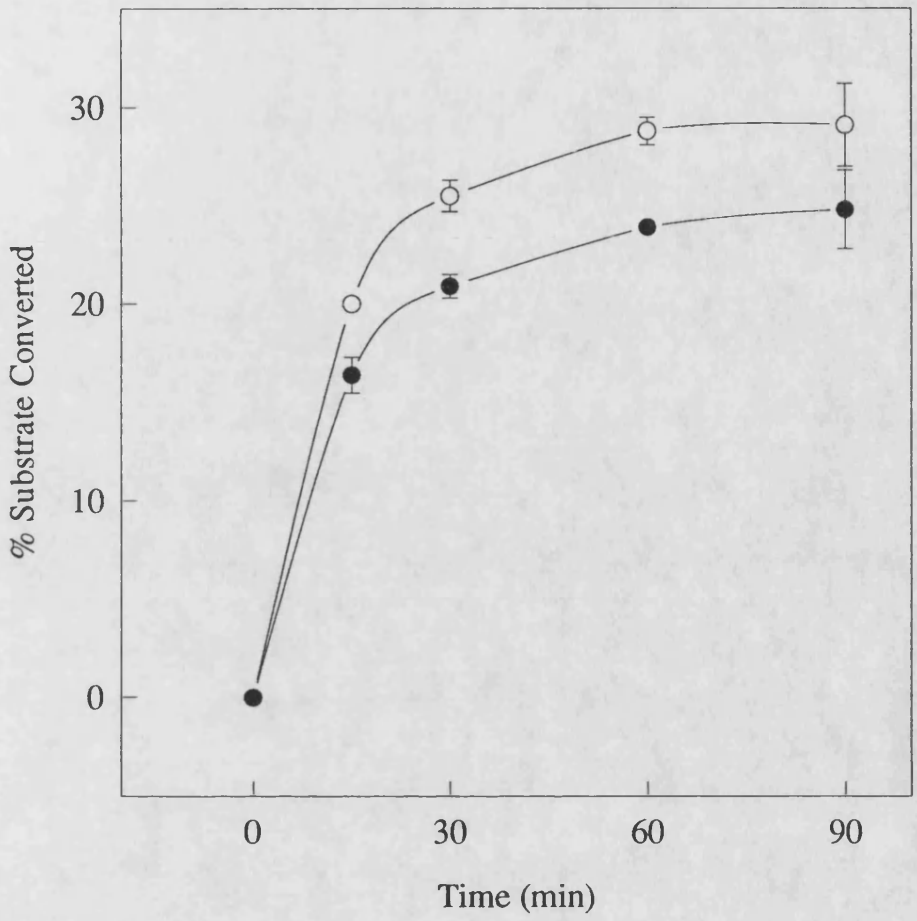


Figure 2.5. Cleavage of oxidised OT-GKR by the soluble enzyme preparation. 50μM OT-GKR (solid circles) and 50μM oxidised OT-GKR (open circles) were incubated with 2.5μl enzyme preparation and 1mM cobalt chloride in 0.1M ammonium acetate, pH 5.8, in a total volume of 30μl. Reactions were terminated at the stated times by addition of 200μl 0.1M HCl and samples analysed by RP-hplc as detailed in the text. Individual values were subjected to arcsin transformation before calculation of means and standard errors; n=4.

Treatment		% Control Activity
Cobalt chloride	1mM	348
EDTA	1mM	18.5
Cadmium chloride	0.1mM	31.5
Copper chloride	0.1mM	<5.0
PCMPS	1mM	<5.0

Table 2.1. Characterization of carboxypeptidase E activity of the soluble enzyme preparation. 50μM OT-GKR was incubated with 3μl enzyme preparation in 0.1M ammonium acetate, pH 5.8, in a total volume of 30μl at 37°C for 1h. Reactions were terminated by the addition of 200μl 0.1M HCl, and samples analysed by RP-hplc as described in the text; n=4.

CHAPTER 3:
THE EFFECTS OF NEUROPHYSIN AND PROTEIN CONCENTRATION ON CARBOXYPEPTIDASE E ACTIVITY

3.1. Introduction

As explained in Chapter 1, the initial *in vivo* substrate for CPE activity in oxytocin biosynthesis is probably an ionic complex of OT-GKR and neurophysin. Studies have shown that neurophysin can influence the *in vitro* processing rates of extended oxytocin intermediates by carboxypeptidase and amidating enzymes (Kanmera & Chaiken, 1985a, Ando *et al*, 1988). At a substrate concentration of 710μM, concentrations of 1420, 710 and 350μM of neurophysin caused specific inhibition of carboxypeptidase activity, although processing still occurred at 50-70% of the control rate (Ando *et al*, 1988). Addition of a lower concentration of neurophysin (180μM), however, resulted in a slight increase in carboxypeptidase activity, which was also observed when neurophysin was replaced by RNase S protein, which is similar in size to neurophysin, so the stimulatory effect of neurophysin on the enzyme was deemed to be non-specific. Similar stimulation of both carboxypeptidase and amidating activities was found using tenfold lower substrate and neurophysin concentrations of 71μM; again these increases in processing rates could also be achieved with RNase S protein in the place of

neurophysin. It was proposed that dimerisation of neurophysin-substrate complexes at the higher concentrations could impose stearic constraints on enzymic processing and thus act as a control mechanism to limit the production of active hormone; however the stimulation of the enzyme activities by neurophysin at lower substrate and protein concentrations was not persued.

Stimulation of catalytic activity by the addition of protein - the protein effect - has been demonstrated for the enzymes proline hydroxylase and tryptophan hydroxylase (Rhoads *et al*, 1967, Popenoe *et al*, 1969 and Rhoads & Udenfriend, 1970), as well as phenylalanine hydroxylase (Guroff *et al*, 1970). In the case of proline hydroxylase, the increase in enzyme activity was shown to be related both to the sulphydryl content of the added protein, and to a factor that was acting in addition to the stimulation by thiol groups (Rhoads *et al*, 1967). In the study into the effects of neurophysin on processing enzymes, stimulation of activity was caused at low substrate concentrations both by neurophysin, which is rich in cysteine residues, and by RNase S protein, which has a significant sulphydryl content, when these proteins were added at the same concentration as the substrate (Ando *et al*, 1988). It seems possible, therefore, that neurophysin and RNase S protein were exerting their effects on the enzymes, at least in part, by a mechanism similar to that seen occurring with proline hydroxylase, that is, by a contribution from their sulphydryl groups. If the enzymes involved in biosynthesis of oxytocin are stimulated by sulphydryl groups, this may go some way to explaining the extraordinarily high cysteine content of neurophysin, the protein which is present during processing.

In view of the high protein concentration found within the neurosecretory granules the experiments described here were designed to investigate the effects of

neurophysin on CPE activity, specifically to examine the importance of neurophysin-substrate binding, and any general contribution to stimulation of the enzyme by protein molecules.

3.2. Materials and Methods

Preparation of the enzyme, peptide substrates and assay procedure are detailed in Chapter 2.

3.2.1. Preparation of OT-GKR affinity column.

The method of Robinson *et al* (1975) was used to covalently attach synthetic OT-GKR to CNBr-Sepharose 4B (Sigma). 45mg OT-GKR were reacted with 20ml 67%v/v acetone at room temperature for 18h to protect the α-amino group of the peptide. The solution was rotary evaporated to dryness and the peptide redissolved in 20ml reaction buffer (0.1M sodium carbonate in 0.5M sodium chloride, pH 8.5). 15g CNBr-Sepharose 4B was swollen in 100ml 1mM HCl for 15min, then washed on a glass sinter with 1000ml 1mM HCl. Immediately before use, the gel was washed with 150ml reaction buffer, resuspended in 80ml buffer and added to the peptide solution. The suspension was mixed end-over-end for 3h at room temperature to allow the coupling reaction to take place. The gel was drained on a glass sinter and washed with 0.2M glycine in coupling buffer, pH 8.5, then mixed in 100ml of this solution for 2h to block any unreacted groups on the gel. After draining, the gel was washed alternately with 100ml each of coupling buffer and 0.1M sodium acetate in 0.5M sodium chloride, pH 3.9, three times, to remove any adsorbed protein. The gel-

peptide complex was then transferred to a glass column and washed with 0.5M acetic acid for 72h (12ml/h) to remove the acetone from the peptide. The column was stored at 4°C in 0.1M ammonium acetate, pH 5.8, containing 0.001% sodium azide as preservative.

3.2.2. Purification of bovine neurophysin.

Fresh bovine pituitaries were collected from Bath Wholesale Meats Ltd. (Bath, UK.) within 2h of slaughter and transported on ice to the laboratory, where the posterior lobes were dissected and extracted. Eight neural lobes were homogenized in 10 volumes (30ml) of 15%v/v TFA, 5%v/v formic acid and 1%w/v NaCl in 1M HCl (Bennett *et al*, 1978), using a Citenco homogenizer with a teflon pestle in a glass tube at speed setting 3.5, and protein was extracted into the acid preparation by mixing for 1h at room temperature. The suspension was cleared by centrifugation at 8,000 x g for 10min at 4°C in an MSE High Speed 18 centrifuge (Fisons). The supernatant was desalted using a Sep-Pak C18 cartridge, the bound material eluted with 80% ACN/0.1% TFA and lyophilized.

This protein mixture was redissolved in 0.1M ammonium acetate, pH 5.8 (0.5ml/neural lobe), centrifuged as above and the supernatant applied to the OT-GKR affinity column. After washing with 10 column volumes of acetate buffer, the bound material was eluted with the same volume of 0.1M formic acid; 3ml fractions were collected at 12ml/h. The unbound and bound material in the fractions was detected by absorbance at 280nm and the appropriate fractions pooled into two peaks; these were desalted using a Sep-Pak C18 cartridge, lyophilized, and stored at -40°C. Samples of extract before and after affinity purification were examined on

polyacrylamide gels. Purity of the neurophysin preparation (bound peak) was confirmed by eluting as a single peak on hplc (using the standard RP-hplc method as described in Chapter 2).

3.2.3. Analysis of the affinity purification of neurophysin by polyacrylamide gel electrophoresis.

Pituitary extracts were analysed on a 7.5% mini-gel, with a 4% stacking gel and discontinuous buffer system (Davis, 1964). A 30% stock solution of monomer was prepared by dissolving 29.2g acrylamide (Bio-Rad Laboratories Inc., California, USA) and 0.8g N,N-methylene bisacrylamide (Bio-Rad) in 100ml water; this was filtered and stored at 4°C in the dark. For the separating gel, 9.9ml water, 5ml monomer stock solution and 5ml 1.5M Tris-HCl, pH 8.8, were mixed and degassed for 15min. 100µl 10% ammonium persulphate and 10µl N,N,N',N'-tetramethyl-ethylenediamine (TEMED) were added and the solution mixed by gentle swirling. The gel was poured into the gel plate assembly (Protean II Electrophoresis Cell, Bio-Rad) and overlayed with water. The stacking gel was prepared by mixing 6.2ml water, 1.3ml monomer stock solution and 2.5ml 0.5M Tris-HCl, pH 6.8, and again degassing for 15min. 50µl 10% ammonium persulphate and 10µl TEMED were added, the solution carefully mixed and poured onto the surface of the separating gel with the comb (which formed the sample wells) in place. The separating gel was 9cm wide, 7cm high and 0.1cm thick; the stacking gel measured 0.5cm from the base of the sample wells to the separating gel.

Two gels were inserted into the gel tank which was then charged with running buffer, containing 3g Tris, 14.4g glycine and 1mg bromophenol blue per litre. The

samples were dissolved in 50mM Tris-HCl pH 7.4 and applied to the gels. Electrophoresis was started at 100v (Bio-Rad Model 2197 Electrofocusing Constant Power Supply) for 15min, to allow the samples to enter the stacking gel, then at 150v for 60min until the ion front (indicated by a line of bromophenol blue) approached the bottom of the gels. After disassembly of the apparatus, the gels were carefully removed from the plates and stained for 30min in 0.2% Coomassie brilliant blue, 10% glacial acetic acid and 30% propan-2-ol. Destaining was in 10% glacial acetic acid and 10% methanol overnight. The destained gels were photographed (plate 1) then dried with a vacuum gel drier (Bio-Rad).

3.3. Results

3.3.1. Stimulation of CPE activity by neurophysin.

Addition of bovine neurophysin caused a concentration-dependent increase in cleavage of arginine from OT-GKR by the enzyme preparation; this apparent dose effect was confirmed when a similar pattern was obtained using a higher enzyme concentration (figure 3.1). When neurophysin was added to incubations containing oxidised OT-GKR, activity against this substrate was also increased (figure 3.2). Conducting the assay in phosphate rather than acetate buffer did not alter the general effect of neurophysin on enzyme activity (figure 3.3).

3.3.2. The effect of protein on CPE.

Addition of BSA from 0.01 to 100 μM resulted in a quantitatively similar increase in activity against both forms of substrate (figure 3.4) to that seen on

addition of 3 to 50μM neurophysin. A selection of proteins was tested for their effects on activity and all were found to significantly increase cleavage of the cyclic substrate at a concentration of 2mg/ml (figure 3.5). The degree of stimulation caused by each protein, however, was unrelated to its cysteine content. Enzyme activity was equally stimulated by wheat germ agglutinin, which is cysteine-rich, and collagen, which has no sulphydryl residues; similarly, neurophysin, with 15% cysteine, caused approximately the same increase in conversion as did catalase, where cysteine is again absent.

As stimulation of enzyme activity appeared to be independent of the presence of sulphydryl groups in the proteins, the influence of hydrophobicity on CPE was examined. t-Butanol was added to the incubations, and again stimulation was observed in a concentration-dependent manner (figure 3.6).

3.3.3. Inhibition of CPE activity by neurophysin.

To examine the reported effects of high concentrations of neurophysin causing inhibition of CPE activity, experiments similar to those described in the published studies (Kanmera & Chaiken, 1985 and Ando *et al*, 1988) were conducted. At a concentration of 50μM OT-GKR, 50μM neurophysin caused the expected increase in activity, as did RNase S protein (figure 3.7a). A higher concentration of either protein, however, led to a reduced level of stimulation (figure 3.7a). Activity against the oxidised substrate followed a similar pattern (figure 3.7b). When a higher concentration (500μM) of OT-GKR was used, both neurophysin and RNase S protein at 50μM caused an increase in activity; however when 500μM protein was present,

CPE activity was reduced to less than the control activity by both proteins (figure 3.7c).

3.4. Discussion

The previously reported ability of neurophysin to stimulate CPE activity at low concentrations (Kanmera & Chaiken, 1985a and Ando *et al*, 1988) was confirmed by these experiments, as was the non-specific nature of the effect; similar increases in activity were obtained with all the proteins tested. The stimulation was also shown conclusively to be independent of any ability of neurophysin to bind the substrate, as similar profiles were obtained for untreated OT-GKR, which would be expected to interact with neurophysin under the assay conditions, and oxidised OT-GKR, which would not. Phosphate concentration has been reported (Tellam & Winzor, 1980) to influence the extent of neurophysin-hormone binding, but replacing the acetate assay buffer with a phosphate buffer had no apparent effect on stimulation of CPE activity by neurophysin. If the stimulation was related to ionic binding between neurophysin and the peptide substrate, a factor that altered the strength of this binding, such as phosphate concentration, would also be expected to affect the influence of neurophysin on the carboxypeptidase reaction. As no change in the effect of neurophysin was observed when phosphate was used, this provides more evidence that stimulation of CPE activity is not related to neurophysin-substrate binding.

The mechanism by which these proteins act to enhance CPE activity is unlikely to be related to thiol groups, as proteins lacking in cysteine residues (myoglobin, collagen and catalase) were seen to stimulate the enzyme to a similar extent as did

those with substantial sulphydryl content (neurophysin and wheat germ agglutinin). Assays were also conducted in the presence of dithiothreitol (0.01 to 0.1mM) and L-cysteine (1mM); this had no apparent effect on enzyme activity, so it would seem unlikely that protein could be enhancing catalysis by providing additional sulphydryl groups when a small, sulphydryl-containing molecule such as dithiothreitol was ineffective.

The peptidase activity of carboxypeptidase B has been shown to be stimulated by low concentrations of 1-butanol (0.03-0.3M), and to a lesser extent by 1-propanol, ethanol and methanol (Folk *et al*, 1962); the effect of butanol was thought to be due to reversible modification of structure at the active site. In the present study, addition of t-butanol to the incubations caused an increase in CPE activity. As proteins have a hydrophobic component, they may be acting on the enzyme by increasing the hydrophobic nature of the environment and causing the enzyme to adopt a more active conformation; many factors, such as small changes in pH or ionic concentration, or the presence of other molecules, can have subtle effects on the tertiary structure of an enzyme, and small alterations of structure at the active site can have major effects on activity. Such a mechanism may be related to the high protein concentration found at the in vivo site of CPE activity, the neurosecretory granule, allowing maximum activity of processing enzymes.

Evidence to the contrary, however, comes from the observations of reduced CPE stimulation, and even inhibition, when high concentrations of protein were added. If the effects of protein seen in the last set of experiments were occurring in the granule, the extremely high levels of protein there would be expected to greatly reduce enzyme activity, so perhaps the effects of protein seen in vitro are not relevant

to the *in vivo* situation. Inhibition of activity by high levels of protein could prove problematical when attempting to re-create an intragranular environment for *in vitro* study of the processing enzymes.

Reduced stimulation by a high concentration (500μM) of neurophysin, and actual inhibition of activity when combined with a high substrate concentration, again confirmed earlier findings (Kanmera & Chaiken, 1985a and Ando *et al*, 1988). However, similar results were obtained both when neurophysin was replaced by RNase S protein, and when oxidised substrate was used. As there is no evidence to suggest interaction occurring between RNase S protein and either substrate, and the oxidised substrate has been shown not to bind to immobilized neurophysin (Chapter 2), it appears that reduced stimulation or inhibition by high concentrations of neurophysin are unrelated to substrate-neurophysin binding. This contradicts the findings of the studies referred to above, where high levels of RNase S protein did not have the same effect in reducing CPE activity as did neurophysin, and inhibition was thought to be due to dimerisation of neurophysin-substrate complexes imposing stearic constraints on processing reactions (Ando *et al*, 1988). The precise mechanism whereby high concentrations of neurophysin inhibit processing must await further investigation but since this clearly is not related to neurophysin-substrate binding it might involve a direct interaction between neurophysin and the enzyme.

The question of high protein concentration within the granule may not be a problem for enzyme activity if this protein is not all in solution. The osmotic potential across the granule membrane would be considerable if all of the precursor protein was free in solution. Some mechanism must therefore exist to prevent the

granule from swelling due to inflow of water. Microscopic studies have suggested that the intragranular contents (mainly precursor protein) are present in a semi-crystalline state (Nordmann & Morris, 1984); this would reduce the osmotic potential inside the granule (only molecules in solution can contribute to this effect), and would also solve the problem of inhibition of processing enzymes by very high protein concentrations, if the actual dissolved protein concentration is a small proportion of the total protein content of the granule.

Another aspect to be considered is the state of the enzyme as it is acting in the granule. The preparation used for the experiments described here was a dilute aqueous solution of enzyme, which may be relatively vulnerable to effects from surrounding molecules, including other proteins. If the enzyme were associated with the inner surface of the granule membrane, it may be protected from such conformational alterations and thus remain fully active in the presence of high protein concentrations. Such a phenomenon could occur through several mechanisms. For example, the nature of the interaction between the enzyme molecule and the membrane components may increase the structural stability of the enzyme by "holding" it in an active conformation. Alternatively, a microenvironment may exist close to the membrane surface, due to chemical differences between the lipid membrane and the aqueous granule contents, which results in conditions more amenable to enzyme activity. Either of these mechanisms may result in continued enzyme processing in the presence of high concentrations of hormone and precursor molecules. These considerations led to a series of experiments to compare the soluble and membrane bound forms of CPE.

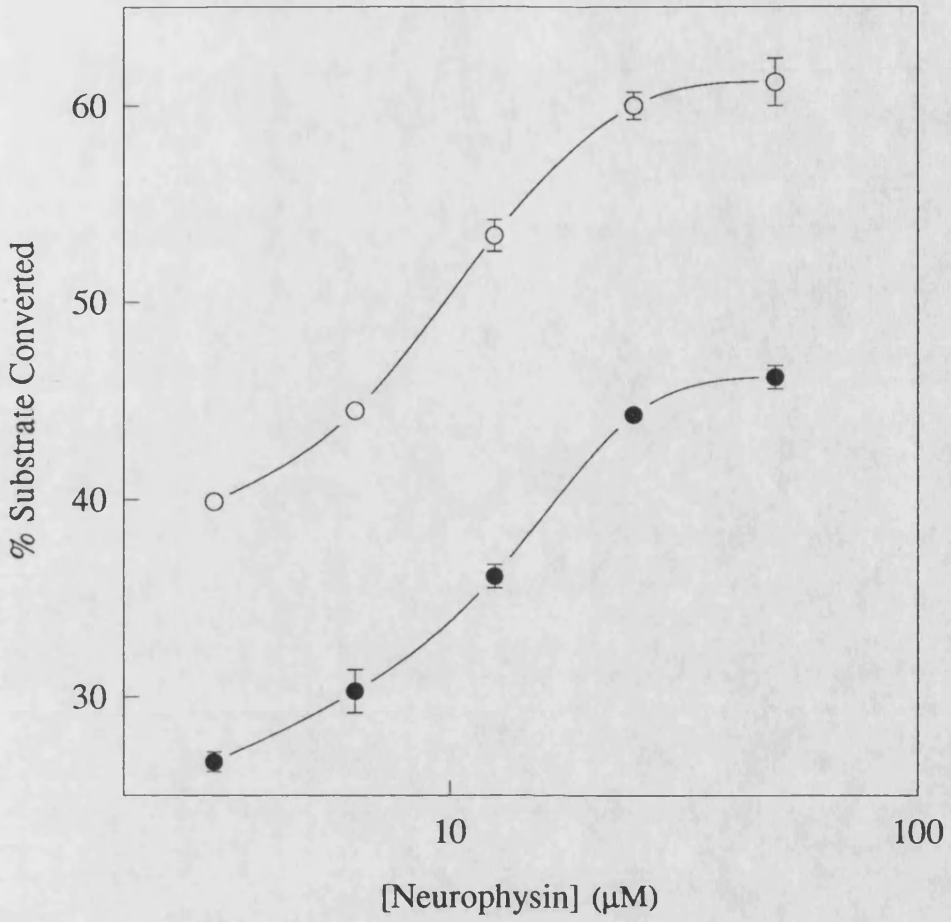


Figure 3.1. The effect of neurophysin concentration on cleavage of OT-GKR. 50μM OT-GKR was incubated with 1.5μl (solid circles) or 2.5 μl (open circles) of enzyme preparation and increasing concentrations of bovine neurophysin, in 1mM cobalt chloride, 0.1M ammonium acetate, pH 5.8, in a total volume of 30μl at 37°C. After 1h, reactions were terminated by addition of 200μl 0.1M HCl, and samples analysed by RP-hplc as detailed in Chapter 2. In the absence of neurophysin, 25% of the substrate was converted to product by 1.5μl enzyme preparation and 41% converted by 2.5μl in 1h. All values were subjected to arcsin transformation before calculation of means and standard errors; n=4.

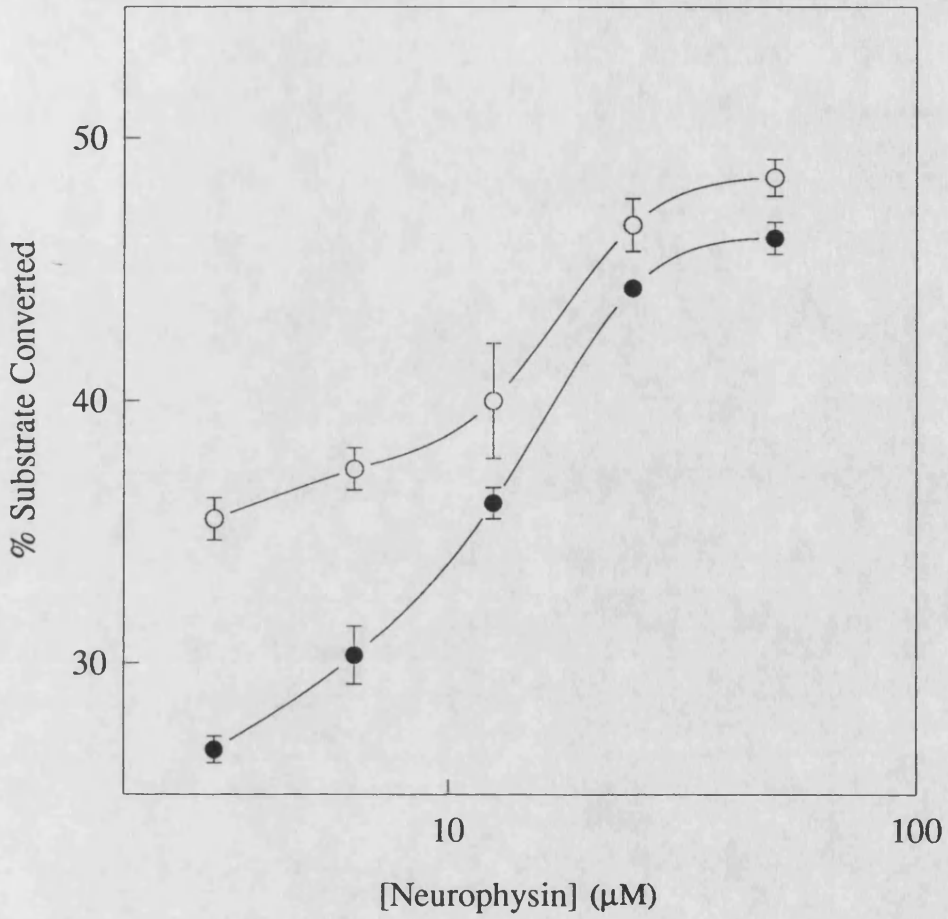


Figure 3.2. The effect of neurophysin concentration on cleavage of oxidised OT-GKR. 50μM OT-GKR (solid circles) or 50μM oxidised OT-GKR (open circles) were incubated with 1.5μl enzyme preparation under the same conditions as detailed in figure 3.1. Enzyme activity on the oxidised substrate with no added neurophysin was 33%; n=4.

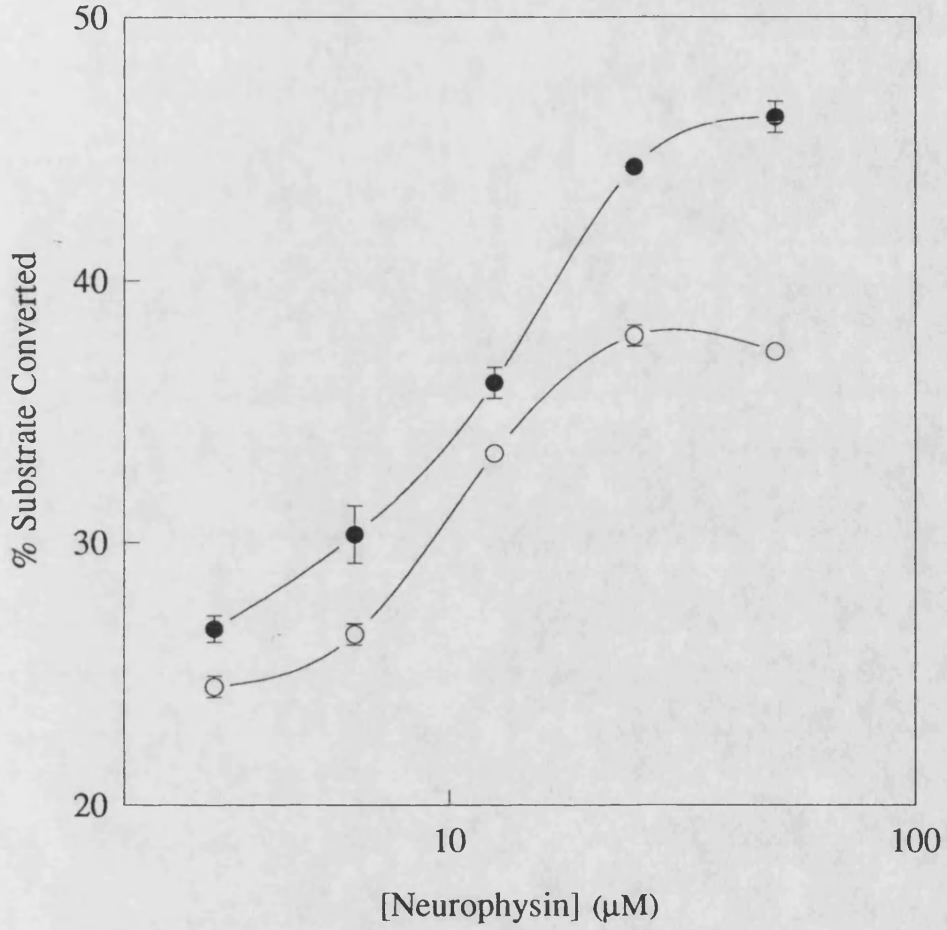


Figure 3.3. The effect of neurophysin in phosphate buffer. 50μM OT-GKR was incubated with 1.5μl enzyme preparation and 1mM cobalt chloride in 0.1M ammonium acetate (solid circles) or 0.15M sodium phosphate (open circles) at pH 5.8, 37°C, for 1h. Samples were treated as detailed in figure 3.1. In the absence of neurophysin, 23% of substrate was cleaved in samples containing phosphate buffer; n=4.

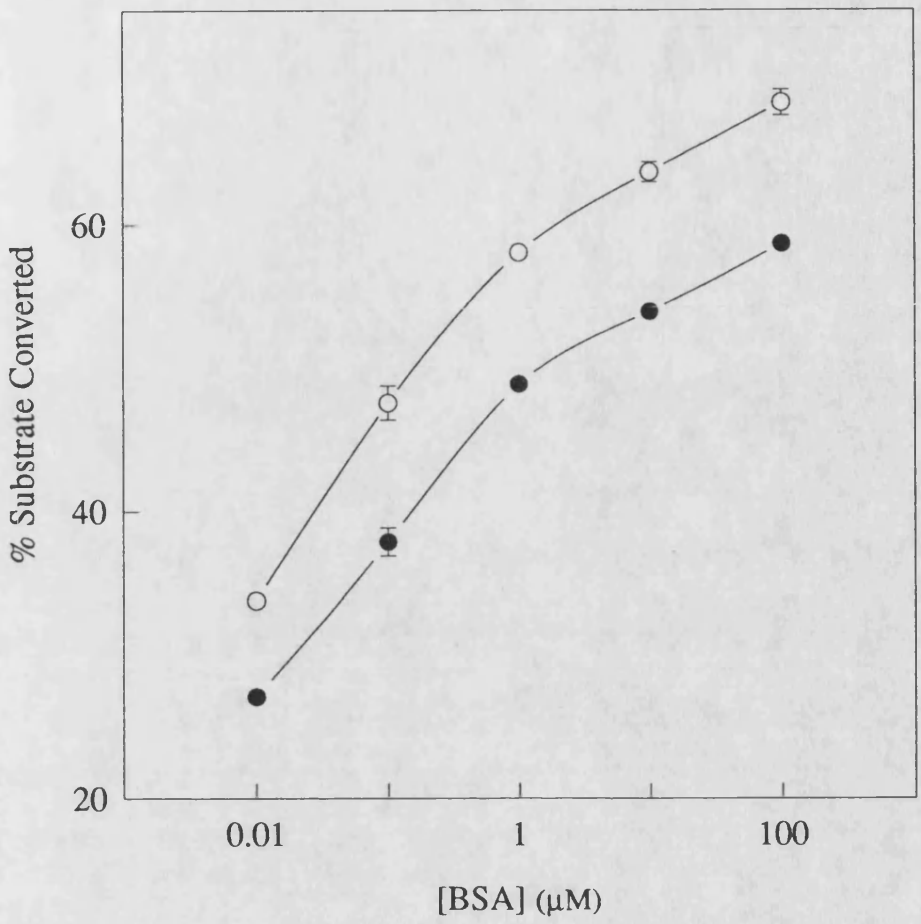


Figure 3.4. The effect of BSA concentration on CPE activity. 50μM OT-GKR (solid circles) and 50μM oxidised OT-GKR (open circles) were incubated with 2μl enzyme preparation and increasing concentrations of BSA, with 1mM cobalt chloride in 0.1M ammonium acetate, pH 5.8, at 37°C, for 1h. Reactions were terminated by addition of 200μl 0.1M HCl, and samples analysed by RP-hplc as described in Chapter 2. Substrate conversion in the absence of BSA was 25% of the cyclic substrate and 32% of oxidised OT-GKR. All values were subjected to arcsin transformation before calculation of means and standard errors; n=4.

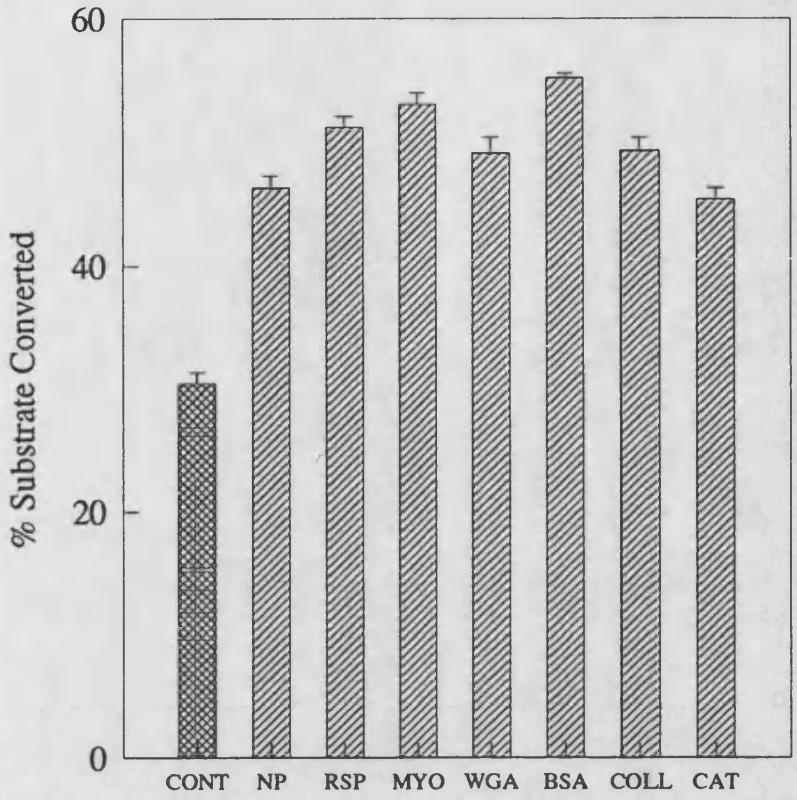


Figure 3.5. The effects of various proteins on CPE activity. 50μM OT-GKR was incubated with 2μl enzyme preparation and 1mM cobalt chloride, in the presence of 2mg/ml added protein. Assays were conducted in 0.1M ammonium acetate, pH 5.8, at 37°C for 1h, then terminated by addition of 200μl 0.1M HCl and samples analysed by RP-hplc as described in Chapter 2. Key to proteins: NP, neurophysin; RSP, ribonuclease S protein (Sigma, bovine pancreas); MYO, myoglobin (Sigma, horse skeletal muscle); WGA, wheat germ agglutinin (Sigma, Tritium vulgaris); BSA, bovine serum albumin (Sigma, 96-99%); COLL, collagen (Sigma, human placenta); CAT, catalase (Sigma, bovine liver). Values from individual samples were subjected to arcsin transformation before calculation of means and standard errors; n=4.

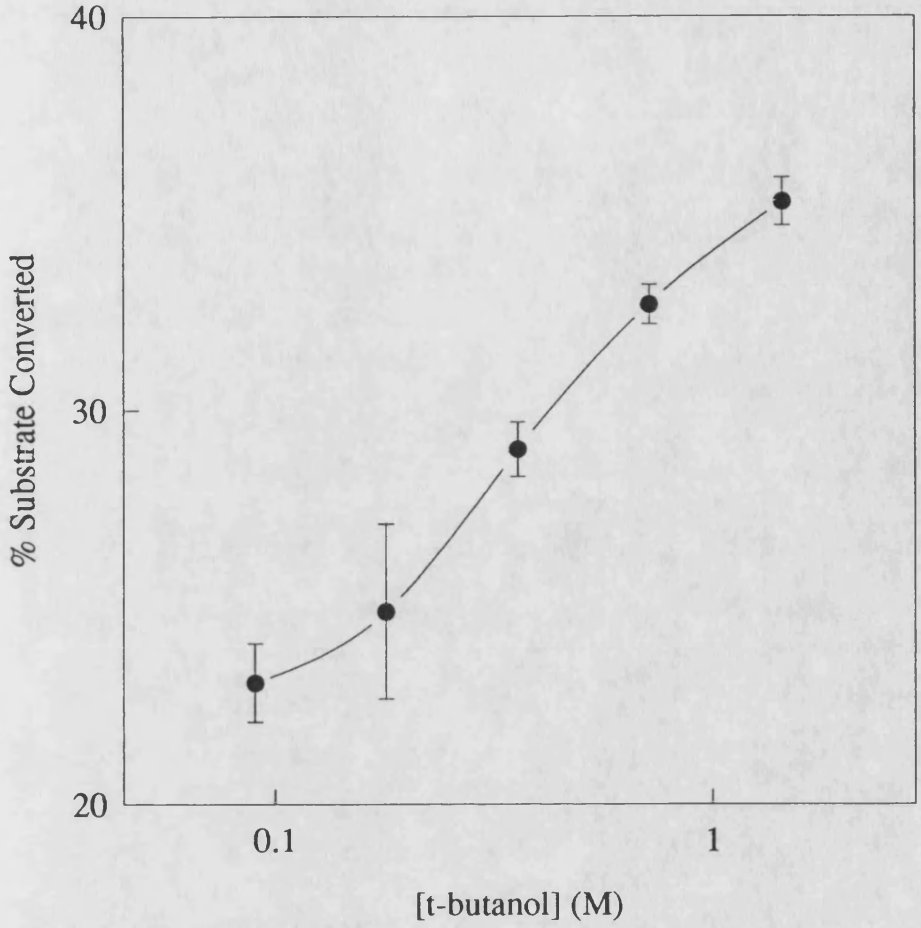


Figure 3.6. The effect of 3-butanol on CPE activity. 50μM OT-GKR was incubated with 2μl enzyme preparation and 1mM cobalt chloride, with increasing concentrations of 3-butanol. The total reaction volume was 30μl in 0.1M ammonium acetate, pH 5.8, and reactions proceeded for 1h at 37°C before termination by addition of 200μl 0.1M HCl and analysis by RP-hplc as detailed in Chapter 2. Individual values were subjected to arcsin transformation before calculation of means and standard errors; n=4.

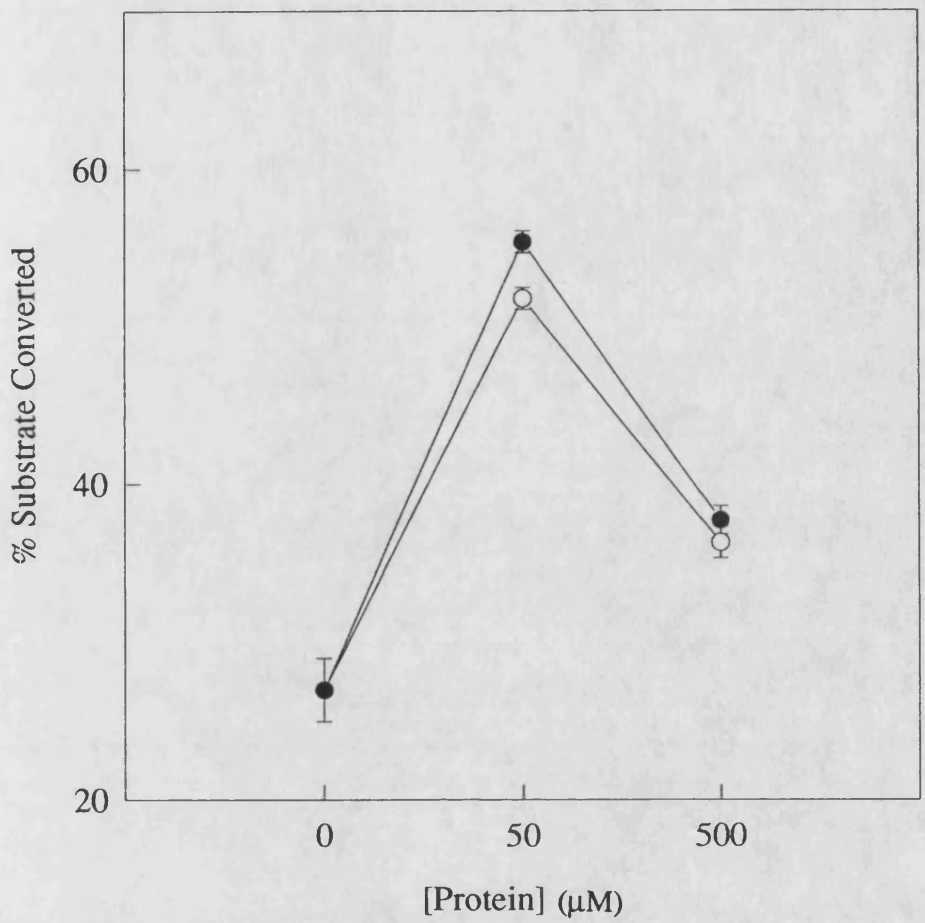


Figure 3.7a. The effect of high neurophysin concentration on CPE activity. 50μM OT-GKR was incubated with 1.5μl enzyme preparation, 1mM cobalt chloride and 0, 50 or 500μM neurophysin (solid circles) or RNase S protein (open circles). The total incubation volume was 30μl in 0.1M ammonium acetate, pH 5.8, and reactions were for 1h at 37°C. Digestions were halted by the addition of 200μl 0.1M HCl, and samples analysed by RP-hplc as described in Chapter 2. Individual results were subjected to arcsin transformation before calculation of means and standard errors; n=3.

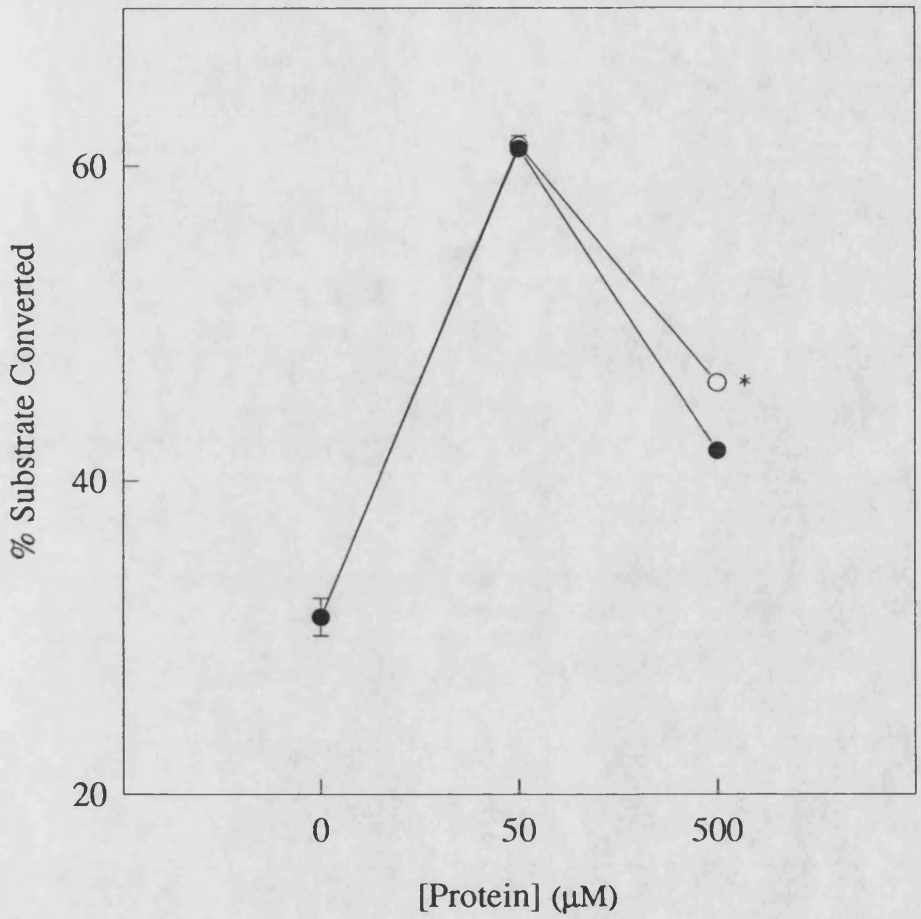


Figure 3.7b. The effect of high neurophysin concentration on CPE activity against oxidised substrate. 50μM oxidised OT-GKR was incubated as figure 3.7a; n=3 (*n=1; two samples were lost due to a malfunction of the autosample injector).

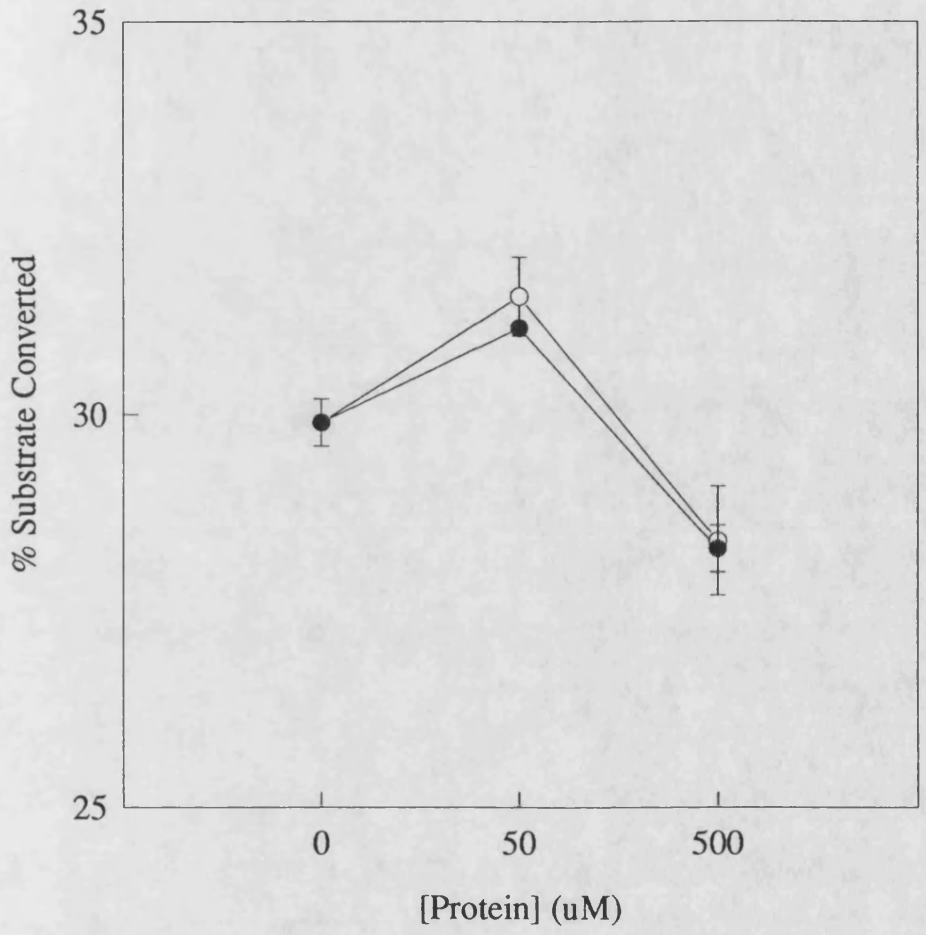


Figure 3.7c. The effect of high neurophysin concentration on CPE activity with high substrate concentration. 500μM OT-GKR was incubated as figure 3.7a. Reactions were terminated by addition of 1ml 0.1M HCl, and 200μl each sample were analysed; n=3.

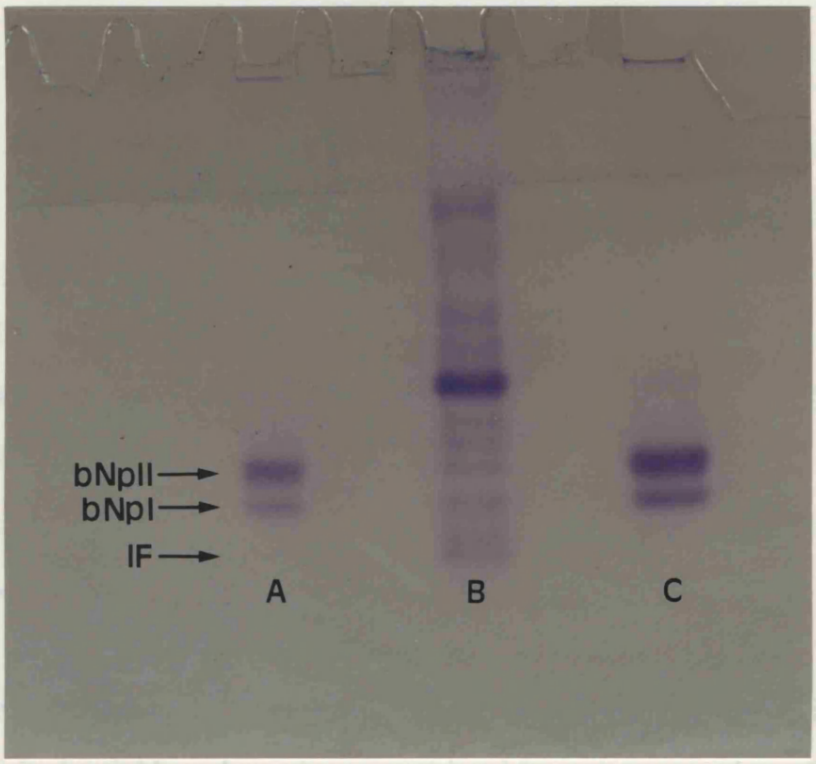


Plate 1. Analysis of the affinity purification of neurophysin by polyacrylamide gel electrophoresis. The gel was prepared and electrophoresis carried out as described in the text. The samples were lane A: 10ng bound fraction from affinity column (neurophysin fraction), lane B: 20ng pituitary extract prior to affinity purification, lane C: 20ng bound fraction. A larger quantity of the neurophysin fraction was applied to lane C so the preparation could be examined for contaminants. Labels: bNpI, bovine neurophysin I; bNpII, bovine neurophysin II; IF, ion front.

CHAPTER 4:
COMPARISON OF SOLUBLE AND MEMBRANE-ASSOCIATED FORMS OF CARBOXYPEPTIDASE E

4.1. Introduction

Carboxypeptidase activity on prohormones was shown in early studies to be present in both soluble and membrane-associated forms in preparations of adrenal medulla (Fricker & Snyder, 1982), insulinoma (Docherty & Hutton, 1983) and pituitary cells (Fricker & Snyder, 1982). Subsequent investigations of membrane-bound CPE have included pancreatic islet cells (Mackin & Noe, 1987), human phaeochromocytoma, mouse, shark and *Xenopus* brain, and tissues from *Aplysia* (Fricker & Herbert, 1988).

The relative amounts of CPE activity in soluble and membrane fractions varies widely both between species (Fricker & Herbert, 1988) and tissues (Strittmatter *et al*, 1984 and Supattapone *et al*, 1984). Lower vertebrates appear to possess a higher proportion of membrane-bound enzyme than do mammals (Fricker & Herbert, 1988); different regions of rat brain display between 30 and 60% of total CPE activity in the membrane form, while the pituitary has less, 26% of activity in the posterior lobe and only 10% in the anterior pituitary being found in the insoluble fraction (Supattapone *et al*, 1984).

Characterizations of membrane-associated CPE have shown this form of the enzyme to be similar to the soluble form in pH optimum, substrate specificity, activation and inhibition (Fricker & Snyder, 1983, Hook, 1984, Supattapone *et al*, 1984 and Fricker, 1988c). However, differences in kinetic parameters and an increase in total activity on solubilization suggest that although the two forms are believed to originate from the same precursor, there may be a difference in specific activity caused by interaction with the granule membrane (Fricker, 1988c and Hook, 1985), or by the presence of the additional sequence which interacts with the membrane and accounts for the larger size of the membrane-bound form (Fricker *et al*, 1990).

Association with a membrane can induce profound alteration of many characteristics of an enzyme, including kinetics, substrate specificity, cofactor requirements and pH optimum, and changes in the properties of the membrane can play an important role in regulating enzyme activity (Coleman, 1973). Although the membrane-bound form of CPE from bovine pituitary has been characterized (see above), this has only been carried out with small, synthetic substrates. In this study it was considered important to examine the relative activities of the two forms of enzyme against the natural substrate molecule.

4.2. Materials and Methods

4.2.1. Membrane enzyme preparation.

The membrane fraction of the neurosecretory granule preparation described in Chapter 2 was used as a source of membrane-associated CPE activity. Fresh

suspensions of 1mg/ml in 0.1M ammonium acetate, pH 5.8, were prepared for each investigation, and the mixture vortexed until homogenous.

4.2.2. Adaptations to assay procedure.

The assay procedure described in Chapter 2 for the soluble enzyme preparation was adapted for studies of the membrane enzyme preparation. The preparation was vortex mixed for 5sec after enzyme had been dispensed to each replicate set (3 or 4) of samples, to prevent sedimentation of the membranes. For incubations longer than 1h, the samples were vortex mixed for 5sec every hour. Finally, reactions were terminated by the addition of 210ml 0.1M HCl, vortex mixed, centrifuged in an MSE Micro Centaur (11,600 x g for 5min), and 230ml of supernatant removed into a clean tube for analysis. The RP-hplc procedure described in Chapter 2 was then followed.

4.3. Results

4.3.1. Characterization of activity in the membrane preparation.

The membrane enzyme preparation (1mg/ml) was shown to cleave the carboxy terminal arginine from OT-GKR at a rate of approximately 300pmol/h/mg at pH 5.8, 37°C, in the presence of 1mM cobalt chloride. As for the soluble preparation, the membrane enzyme was stimulated by cobalt and inhibited by copper, cadmium, EDTA and PCMPS (table 4.1). In the presence of 1mM GEMSA, only 5% of the carboxypeptidase activity remained.

Detailed pH profiles were obtained for soluble and membrane preparations using both acetate and phosphate buffers; both preparations show maximal activity at

about pH 5.6-5.8 irrespective of the buffer used, although the activity is generally slightly higher in acetate than in phosphate except at very low pH. The major difference between the two profiles is the maintenance of a high level of activity of the membrane preparation at pH values approaching neutrality in acetate buffer (figure 4.1a). This was not seen with the soluble preparation in either acetate or phosphate (figure 4.1b), nor with the same membrane source in phosphate buffer.

4.3.2. The effect of protein on membrane-associated activity.

On addition of 10mM BSA, the carboxypeptidase activity of the membrane preparation was increased to 176% of the control activity; this compares with stimulation to 213% of control achieved with the soluble preparation (Chapter 3). In the presence of 1.44M t-butanol, however, the membrane preparation was not significantly affected, whereas cleavage by the soluble preparation was increased to 187% of the control value by this treatment.

The presence of RNase S protein in incubations had similar effects on both enzyme preparations. While stimulation was seen at 50mM protein, this effect was decreased when the concentration of RNase S protein was raised to 500mM (figure 4.2); this was consistent with the results for the soluble preparation alone in Chapter 3.

4.3.3. Activity of the membrane preparation against oxidised substrate.

The activities of both enzyme preparations against OT-GKR and oxidised OT-GKR were measured over 90min. Both preparations were able to cleave the two forms of substrate to similar extents; however the soluble enzyme was more active

against the linear, oxidised form (figure 4.3a), while the membrane preparation processed the cyclic peptide with greater activity (figure 4.3b).

This apparent difference in substrate specificity between the soluble and membrane-bound forms of CPE was further examined with respect to pH. The soluble enzyme was more active against the oxidised peptide at all three pH values tested (4.8, 5.6 and 6.4), although the difference was slightly less at the higher pH (figure 4.4a). The membrane preparation, however, displayed a change in specificity with pH. At pH 4.8 there was a clear preference for the oxidised substrate; there was no significant difference at pH 5.6, and by pH 6.4 the enzyme was more active against the native, cyclic substrate (figure 4.4b).

4.4. Discussion

The effects of various activators and inhibitors on the membrane-associated form of CPE have been reported to be similar to those on the soluble enzyme (Fricker & Snyder, 1982), but figures have only been published for the enzyme purified from solubilized membrane (Supattapone *et al*, 1984). However, despite the difference in the preparations used, the results are similar; both studies found similar stimulation of both soluble and membrane enzymes by cobalt. Inhibition of the intact membrane preparation (this study) and the purified membrane enzyme (Supattapone *et al*, 1984) resulted in the following percentages of control activity respectively: EDTA 23% and 6%; copper 8% and 10%; cadmium 23% and 19%; PCMPS <5% and 11%. These results, together with the pH profile of the membrane-bound enzyme, and the reduction of carboxypeptidase activity to 5% in the presence of 1μM GEMSA,

confirm the identity of the activity as CPE, and are consistent with published findings (Fricker & Snyder, 1982 and Supattapone *et al*, 1984).

The difference in activities of the soluble and membrane preparations at pH values above pH 5.8 (in acetate buffer) may be a result of interaction between enzyme and membrane inducing some change in the enzyme itself, or by altering the microenvironment around the enzyme. Alternatively, the increase in activity may be due to loss of membrane-binding at increasing pH (Fricker *et al*, 1990), and higher specific activity of the solubilized form of CPE increasing the total activity of the preparation despite the higher pH. The unusually high activities of both enzyme preparations at very low pH in phosphate buffer may be caused by a loss of buffering capacity by phosphate at this level.

Stimulation of CPE activity in the membrane preparation by BSA, but not by t-butanol, is a very interesting result. The protein effect apparently still operates on the membrane-bound enzyme, although the increase in activity is somewhat less than with the soluble enzyme, but in this case cannot be attributed to an increase in the hydrophobicity of the environment as the membrane-bound enzyme is already in association with lipid, and butanol has no effect. If the protein is acting on the two enzyme forms in the same manner, this suggests that although there may be some contribution by a hydrophobic effect to the activity of the soluble enzyme, this does not occur with the membrane enzyme, perhaps because it is already in association with hydrophobic membrane components which are fully stimulating the enzyme by this mechanism. So the full effect on the membrane enzyme and perhaps a significant part of the stimulation of the soluble enzyme is occurring via another mechanism, independent of both hydrophobic and sulphydryl components. The

reduction in stimulation of the membrane enzyme at a higher concentration of protein is similar to the effect seen on the soluble enzyme. This rules out a possible advantage of membrane binding being to protect the enzyme from the high levels of protein *in granulo*, as suggested in Chapter 3.

A difference demonstrated between the soluble and membrane-bound activities was shown by the change in substrate specificity with pH. The efficiency of cleavage of the oxidised, linear peptide was greater than that of the cyclic peptide for both enzyme preparations up to about pH 5.6. Above this pH, while the specificity of the soluble enzyme did not change, the membrane-bound enzyme was more active against the native, cyclic peptide. There has been no other difference in substrate specificity reported for the two forms of CPE. Oxidation of OT-GKR, which converts both cysteine residues to cysteic acid, will clearly affect the pI of the peptide molecule, causing it to become more acidic. This may account for a difference in processing rates of the two forms of substrate by an enzyme, as both binding and catalysis can be influenced by small changes in charge on the substrate molecule. However, the change in specificity of the membrane-bound enzyme for these substrates at pH 6.4 is not seen with the soluble enzyme and must be due to a difference between the two enzyme forms. The possible relevance of this finding to processing *in vivo* is unclear and would require further investigation.

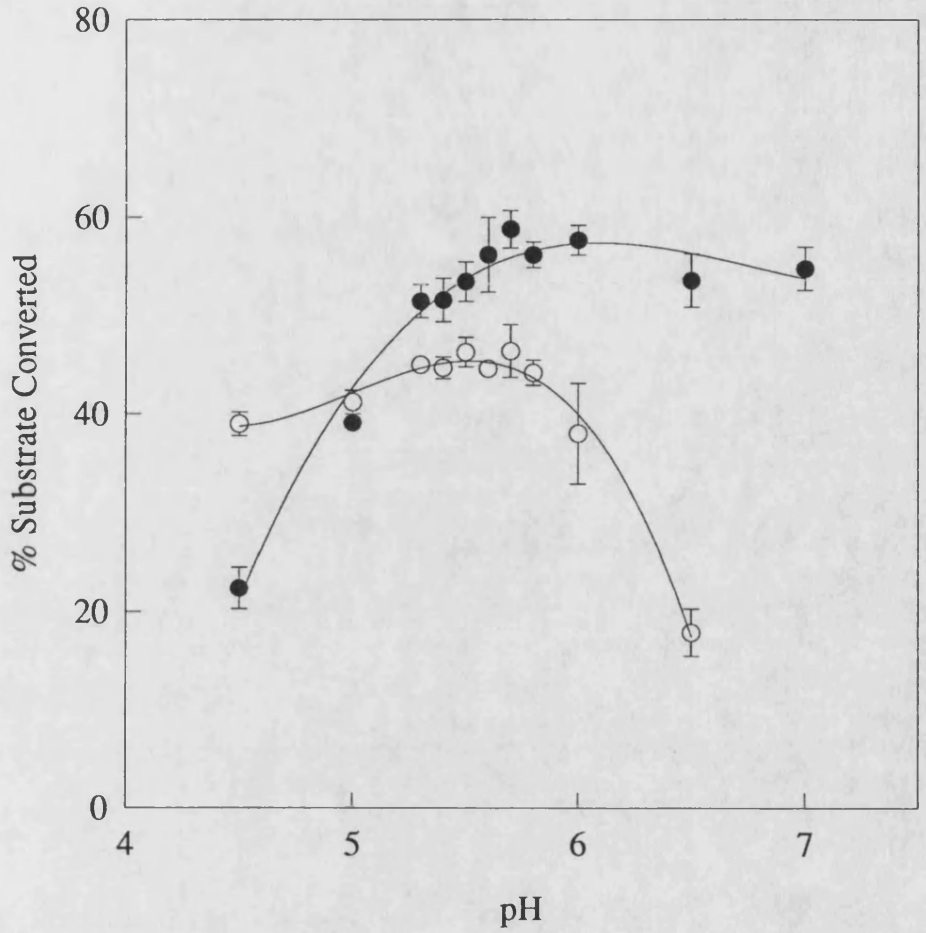


Figure 4.1a. pH activity profile of the membrane enzyme preparation. 50μM OT-GKR was incubated with 2μl membrane enzyme preparation (1mg/ml in assay buffer) and 1mM cobalt chloride, in 0.1M ammonium acetate (solid circles) or 0.15M sodium phosphate (open circles) for 1h at 37°C. The total incubation volume was 30μl. Reactions were terminated by addition of 110μl 0.1M HCl, and samples analysed as described in the text. Results obtained from individual samples were subjected to arcsin transformation before calculation of means and standard errors; n=4.

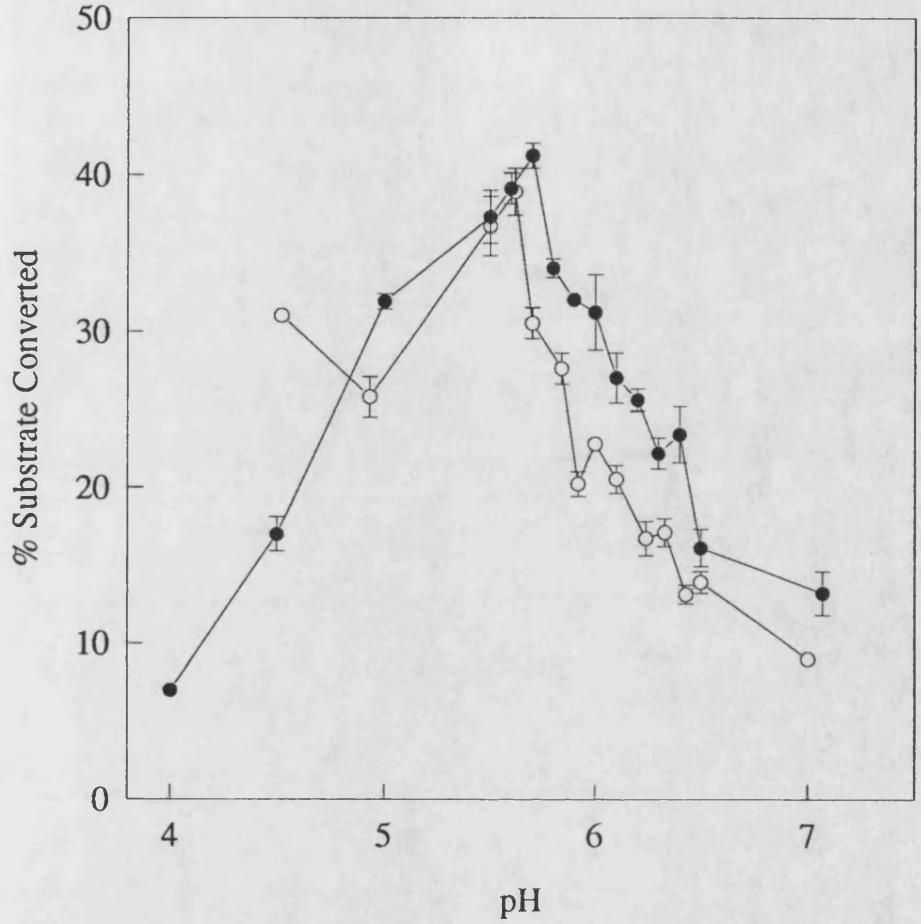


Figure 4.1b. pH activity profile of the soluble enzyme preparation. 2.5µl soluble enzyme preparation were incubated with 50µM OT-GKR as figure 4.1a. Reactions were terminated by addition of 100µl 0.1M HCl, and samples analysed as detailed in Chapter 2; n=4.

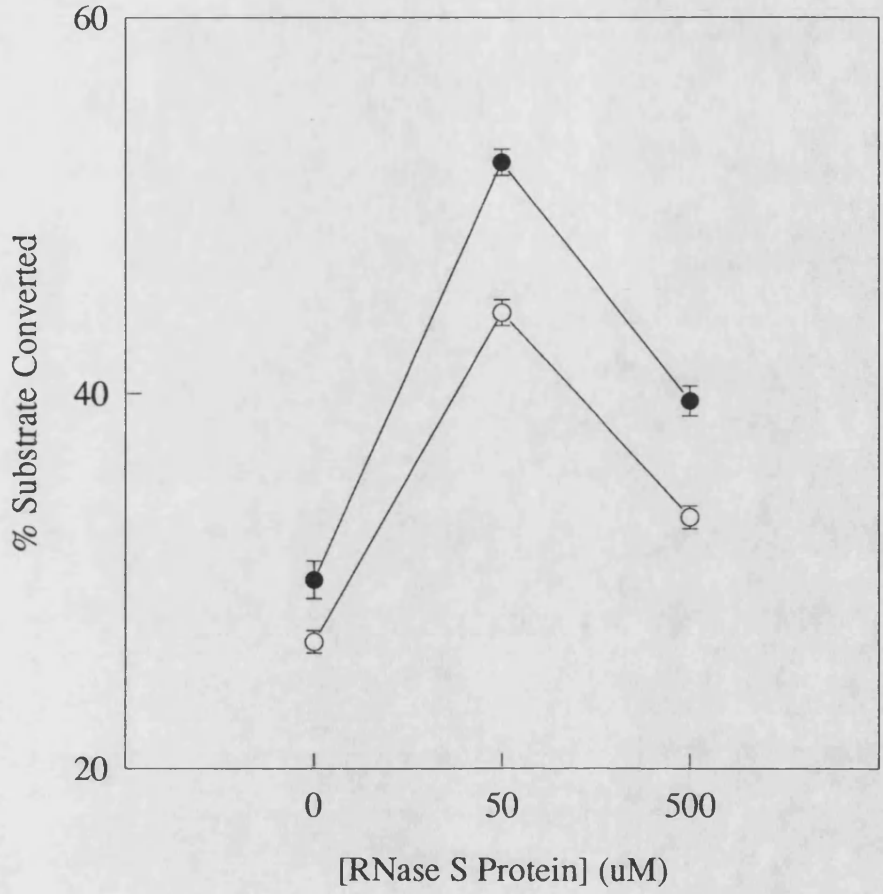


Figure 4.2. Effect of high protein concentration on the membrane enzyme preparation. 50μM OT-GKR was incubated with 3μl soluble (solid circles) or membrane (open circles) enzyme preparation and 1mM cobalt chloride in 0.1M ammonium acetate, pH 5.8, at 37°C for 1h. RNase S protein (Sigma) was dissolved in assay buffer and added to the stated concentrations, total incubation volume was 30μl and reactions were terminated by the addition of 200μl 0.1M HCl. Cleavage was assessed by RP-hplc analysis of samples. Results of individual samples were subjected to arcsin transformation before calculation of means and standard errors; n=3.

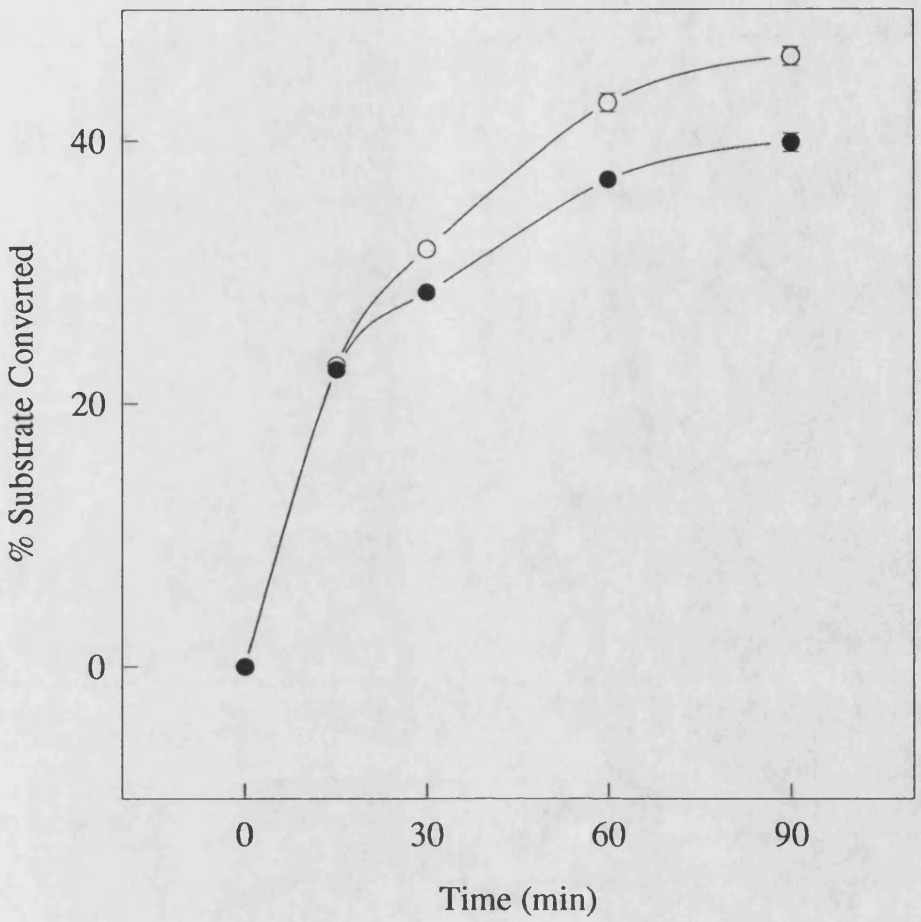


Figure 4.3a. Time course of cleavage of cyclic and oxidised OT-GKR by the soluble enzyme preparation. 50μM OT-GKR (solid circles) and 50μM oxidised OT-GKR (open circles) were incubated with 2.5μl soluble enzyme preparation and 1mM cobalt chloride, in 0.1M ammonium acetate, pH 5.8. The total volume was 30μl, and reactions proceeded for 1h at 37°C, before termination by addition of 200μl 0.1M HCl. Samples were analysed by RP-hplc as detailed in Chapter 2. Individual values were subjected to arcsin transformation before calculation of means and standard errors; n=4.

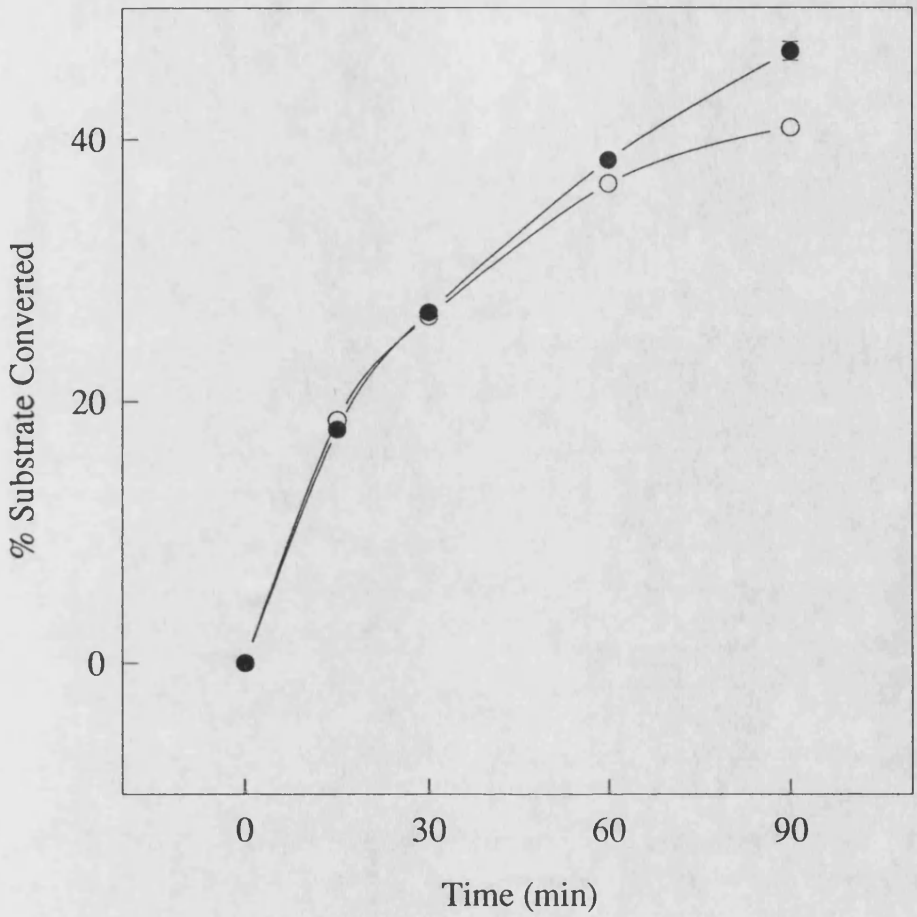


Figure 4.3b. Time course of cleavage of cyclic and oxidised OT-GKR by the membrane enzyme preparation. Conditions were as figure 4.3a, with 3µl membrane enzyme preparation. Reactions were terminated by addition of 210µl 0.1M HCl, and samples treated as detailed in the text; n=4.

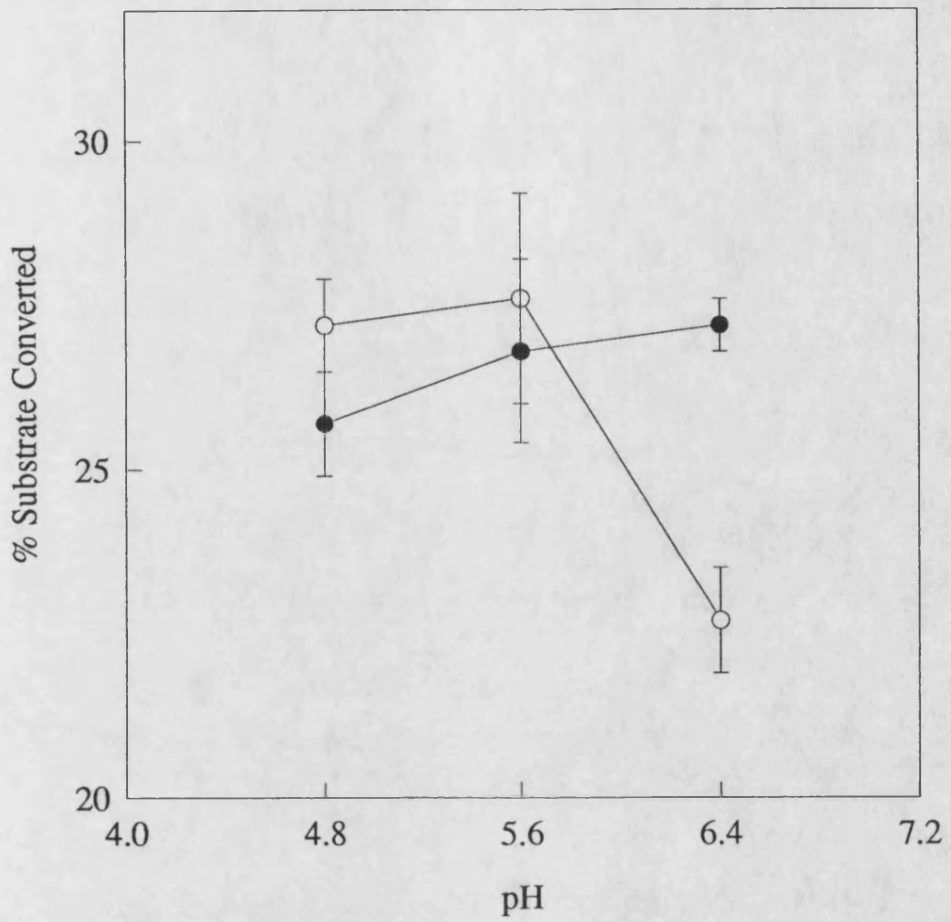


Figure 4.4a. Substrate specificity of the membrane enzyme preparation with pH. 50μM OT-GKR (solid circles) and 50μM oxidised OT-GKR (open circles) were incubated with 3μl membrane enzyme preparation and 1mM cobalt chloride in 0.1M ammonium acetate at the stated pH values. Total incubation volume was 30μl and reactions proceeded for 1h at 37°C before termination by addition of 210 μl 0.1M HCl, and treatment as described in the text. Individual values were subjected to arcsin transformation before calculation of means and standard errors; n=3.

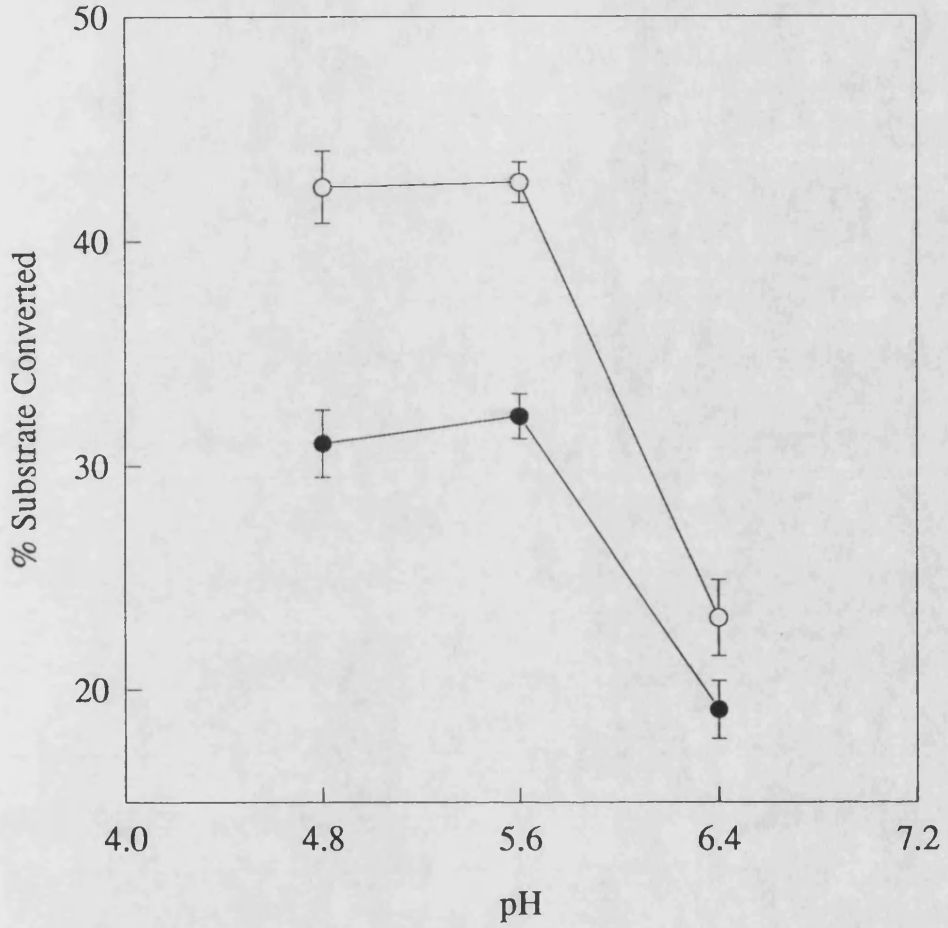


Figure 4.4b. Substrate specificity of the soluble enzyme preparation with pH. 3µl soluble enzyme preparation was used under the conditions given in figure 4.4a, and reactions terminated by addition of 200µl 0.1M HCl before analysis by RP-hplc as described in Chapter 2; n=4.

Treatment		% Control Activity
Cobalt chloride	1mM	300
EDTA	1mM	23
Cadmium chloride	0.1mM	23
Copper chloride	0.1mM	8
PCMPS	1mM	<5.0

Table 4.1. Characterization of carboxypeptidase E activity of the membrane enzyme preparation. 50μM OT-GKR was incubated with 3μl membrane enzyme preparation (1mg/ml in assay buffer) in 0.1M ammonium acetate, pH 5.8, in a total volume of 30μl at 37°C for 1h. Reactions were terminated by the addition of 210μl 0.1M HCl, and samples analysed by RP-hplc as described in the text; n=3.

CHAPTER 5:
THE CLEAVAGE OF CARBOXY TERMINAL LYSINE BY MEMBRANE-ASSOCIATED CARBOXYPEPTIDASE E

5.1. Introduction

Most investigations into CPE specificity have found this enzyme to display higher affinity for arginyl than for lysyl substrates. Early studies of enzyme from adrenal, brain and pituitary revealed that Leu- and Met-enkephalin-Arg were more potent inhibitors than Leu- or Met-enkephalin-Lys by a factor of three (Fricker & Snyder 1982 and Fricker & Snyder 1983). Both soluble and membrane-associated CPE from bovine pituitary were found to have twice the affinity for Leu-enkephalin-Arg than Leu-enkephalin-Lys (Supattapone *et al*, 1984). Cleavage rates of the oxytocinyl precursor by a neurosecretory granule lysate preparation have been reported (Kanmera & Chaiken, 1985a); although 95% of the initial substrate, OT-GKR, was converted to OT-GK in 8h under the conditions used, after 96h only 30% had been processed to OT-G.

Further evidence for the preference of arginyl substrates by CPE comes from inhibitor studies. GEMSA, which mimics the structure of arginine, is 45 times more potent an inhibitor of CPE than is APMSA, which is chemically similar to GEMSA, but is a structural analogue of lysine (Fricker *et al*, 1983).

One study of CPE purified from bovine pituitary and human hepatoma cells reported a 2:1 and 3:1 preference, respectively, for furylacrylic-Ala-Lys over furylacrylic-Ala-Arg by the two enzyme preparations (Grimwood *et al*, 1989); this is the only report of a preference for a lysyl substrate by CPE.

As removal of lysine from OT-GK is clearly necessary for maturation of the oxytocin precursor, this is an important reaction that is often overlooked in processing studies, and the lower rates of lysine cleavage that are usually seen have not been addressed. As stated in Chapter 2, cleavage of OT-GK was not achieved with the soluble enzyme preparation used in this study, although incubation times were not extended to 96h as in the report involving similar enzyme and substrate preparations where 30% conversion to OT-G was finally achieved (Kanmera & Chaiken, 1985). It was surprising, therefore, to discover the capability of the membrane enzyme preparation (described in Chapter 4) to cleave OT-GK to OT-G, although at a lower rate than the arginine cleavage occurred. This reaction was thus characterized to confirm that CPE was responsible for the catalysis, and comparisons were made with the conversion of OT-GKR to OT-GK by the same enzyme preparation.

5.2. Materials and Methods

The methods used in this chapter have been described in Chapters 2 and 4.

5.3. Results

5.3.1. Characterization of lysine cleavage by the membrane enzyme preparation.

Although cleavage of the carboxy terminal lysine could not be achieved under the conditions used with the soluble enzyme preparation (Chapter 2), it was found that the membrane preparation described in Chapter 4 was capable of catalysing this reaction, although at a much lower rate than the arginine cleavage. Incubations with OT-GK were conducted over 3h, and lysine was removed at approximately 30pmol/3h/μl membrane enzyme preparation, at 37°C in the presence of 1mM cobalt chloride.

Arginine and lysine cleavages by the membrane enzyme preparation displayed similar pH activity profiles (figure 5.1), with highest rates around pH 5.6, declining more rapidly at lower values than at those approaching neutrality. These results are similar to those for arginine cleavage alone (see Chapter 4).

In the absence of cobalt, no activity could be detected, so it was not possible to characterize the lysine cleavage in the same way as removal of arginine had been studied for the soluble and membrane preparations (Chapters 2 and 4 respectively). However, the activity seen in the presence of cobalt was completely abolished on addition of either PCMPS (1mM) or GEMSA (1μM). When the incubation was conducted in the presence of 10μM BSA, the activity was increased to 240% of the control; t-butanol (1.44M) did not affect activity.

5.3.2. Activity against oxidised substrate.

Under the conditions used for cleavage of the cyclic substrate, cleavage of oxidised OT-GK was not detected. However, the enzyme did appear to show some affinity for the oxidised form, as cleavage of cyclic peptide was slightly reduced in its presence (figure 5.2).

5.4. Discussion

As cleavage of lysine from OT-GK could not be detected in incubations carried out in the absence of cobalt, full characterization of this activity was not possible. However, the reaction did display many hallmarks of CPE activity, specifically: stimulation by cobalt ions (although this effect could not be quantified without a control value), optimal activity at pH 5.6, and abolition of cobalt-stimulated activity by 1mM PCMPS or 1μM GEMSA. This activity against the lysine residue was also affected by BSA but not by t-butanol, in a similar manner to the activity of the same preparation against carboxy terminal arginine, which was more completely characterized as bona fide CPE activity. These results, while not providing indisputable evidence of the identity of this enzyme activity, strongly suggest that the cleavage observed was due to the action of CPE.

Although the membrane-bound form of CPE may display lower specific activity against arginyl substrates than does the soluble form (Hook, 1985 and Fricker, 1988c), this may be compensated for by enhanced removal of the remaining carboxy terminal lysine residue. Alternatively, the enzyme may be present in both soluble and

bound states within the granule to allow cleavage of arginine and lysine by the two different forms, respectively.

If the prohormone is initially associated with the inner surface of the granule membrane, as discussed in Chapter 4, this could result in much higher activity of the membrane bound CPE due to the high local concentration of substrate in this region. The higher specific activity of the soluble form was observed in vitro using small, synthetic peptide substrates, which would not be expected to form any association with a membrane. However, if the native prohormone substrate were already attached to the membrane, as is likely following intragranular packaging, measurement of relative specific activities of the soluble and bound forms of enzyme using small, soluble substrates, would not necessarily be relevant to the in vivo situation. As detailed in the previous chapter, at the estimated intragranular pH of approximately 5.6, a high proportion of CPE would be able to associate with the inner membrane surface provided the amphipathic helix region of the enzyme (which is responsible for enzyme-membrane binding) had not been cleaved. It is not known how much of the enzyme is present in this state in the granules where processing is taking place, as some cleavage of this domain may occur during purification of the enzyme from granule preparations.

Unless the carboxypeptidase reactions function to limit the quantity of active hormone being produced, it would appear illogical to have the enzyme present in a form of relatively low activity. Therefore, assuming that a significant proportion of CPE remains bound to the granule membrane during processing, it seems possible that this binding has a particular function. This may be to allow the enzyme to act on locally high concentrations of membrane-associated precursor, or alternatively to

cause a subtle alteration in the substrate specificity of the enzyme, as seen in Chapter 4 with cyclic and oxidised forms of OT-GKR, and so increase the efficiency of removal of the lysine residue remaining after cleavage of the arginine by the soluble form of the enzyme.

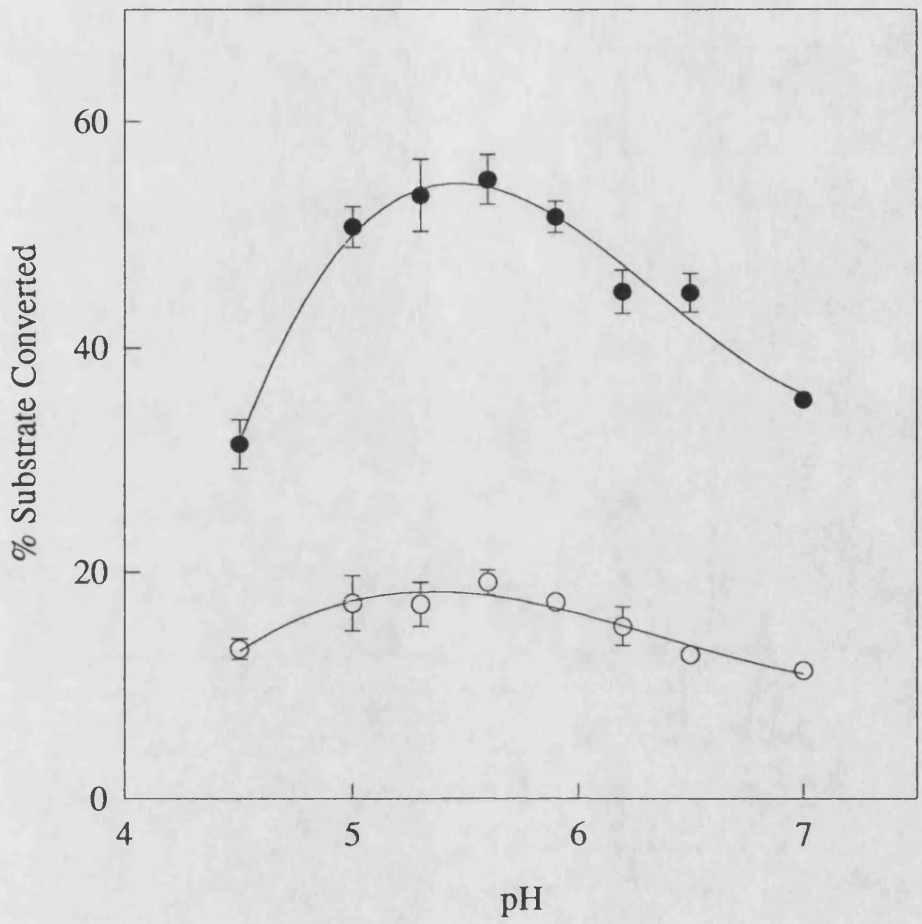


Figure 5.1. pH activity profiles of carboxy terminal arginine and lysine cleavages by the membrane enzyme preparation. 50μM OT-GKR (solid circles) or 50μM OT-GK (open circles) were incubated with 5μl membrane enzyme preparation and 1mM cobalt chloride in 0.1M ammonium acetate at the stated pH values. Total incubation volume was 30μl and reactions proceeded at 37°C for 1h for OT-GKR and 3h for OT-GK. Cleavage was halted by the addition of 210 μl 0.1M HCl and samples were analysed as described in the text. Individual results were subjected to arcsin transformation before calculation of means and standard errors; n=3.

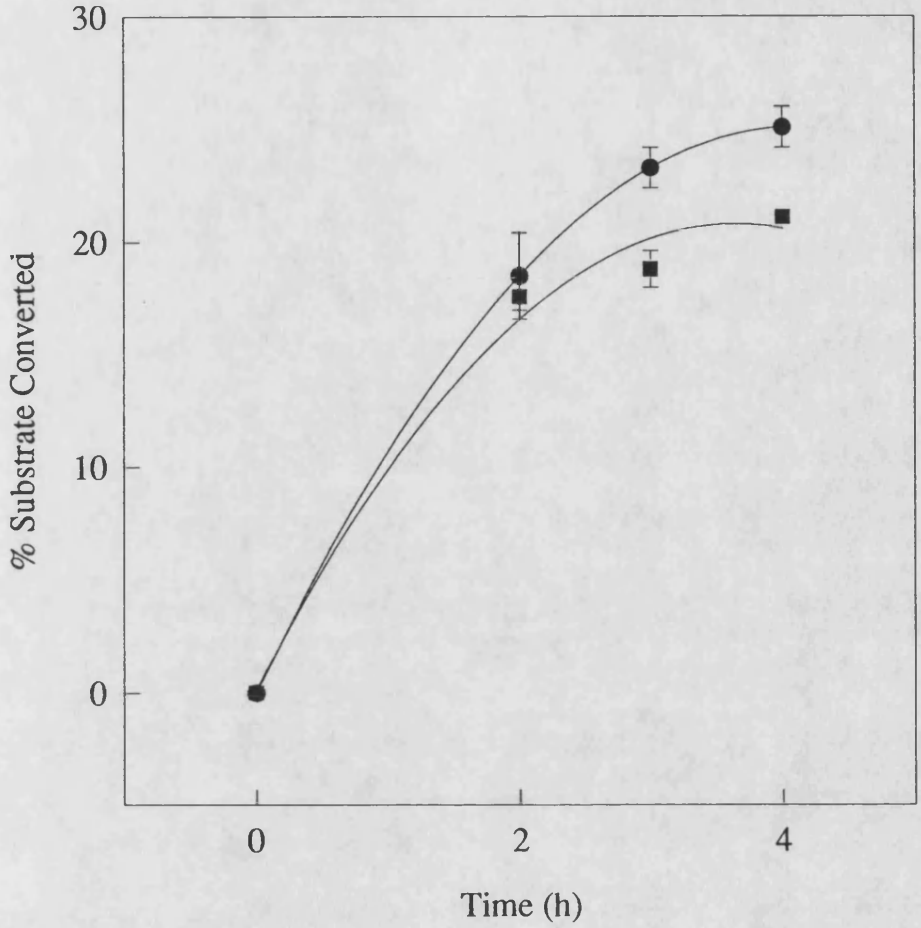


Figure 5.2. The effect of oxidised OT-GK on the cleavage of cyclic OT-GK by the membrane enzyme preparation. 50μM OT-GK were incubated with 5μl membrane enzyme preparation and 1mM cobalt chloride in the presence (squares) and absence (circles) of 50μM oxidised OT-GK. The total volume was 30μl with 0.1M ammonium acetate, pH 5.8; samples were incubated at 37°C and mixed every hour. At the stated times, reactions were stopped by addition of 210μl 0.1M HCl, and samples treated and analysed as detailed in the text. Individual values were subjected to arcsin transformation before means and standard errors were calculated; n=3.

CHAPTER 6:

DISCUSSION AND CONCLUSIONS

The work contained within this study has resulted in four significant observations:

1. CPE activity is influenced by the presence of protein;
2. Inhibition of CPE activity by high concentrations of neurophysin is not related to neurophysin-substrate binding;
3. The soluble and membrane-bound forms of CPE display different substrate specificities at certain pH values;
4. The membrane-bound preparation of CPE used in this study was more efficient than the soluble form in the cleavage of carboxy terminal lysine.

Studies of some hydroxylase enzymes have shown stimulation of activity by added protein (see section 3.1), but such effects on CPE by a wide range of proteins has not been previously reported. The increase in activity of proline hydroxylase seen on the addition of BSA and some other proteins was concluded in some reports to be mainly due to a sulphydryl effect, as proteins lacking in cysteine were not stimulatory, and the effect of BSA could be abolished by treating it to remove free sulphydryl groups, or largely substituted by sulphydryl reagents such as dithiothreitol (Rhoads *et al*, 1967 and Rhoads & Udenfriend, 1970). In the case of CPE, however,

all the proteins tested, whether or not they contained cysteine residues, had similar effects on enzyme activity (see Chapter 3). Unlike proline hydroxylase, the CPE preparation was not stimulated by the addition of thiol groups in the form of dithiothreitol or L-cysteine. An attempt was made to treat BSA to remove free sulphydryl groups so that this could be tested in the assay system; amino acid analysis, however, proved the reaction to have been unsuccessful.

Despite being unable to test the effects of BSA without thiol groups, the evidence gained from these experiments suggests that the protein effect on CPE activity is not due to stimulation by sulphydryl groups. Studies by Popenoe *et al* (1969) on proline hydroxylase suggested that only a small amount of stimulation of this enzyme by BSA was due to a thiol effect, but no alternative mechanism was proposed. Stimulation of phenylalanine hydroxylase by BSA was shown to be unrelated to sulphydryl availability, but probably resulted from binding of excess ferrous ions which could otherwise inhibit the enzyme (Guroff *et al*, 1970). Tryptophan hydroxylase is stimulated by BSA, and although the activity of this enzyme is also increased in the presence of dithiothreitol (Jequier *et al*, 1969), protein was not thought to affect the enzyme directly, by contributing sulphydryl groups (as highly purified enzyme is unaffected by BSA), but was believed to either stabilise the enzyme in crude preparations, or to remove an inhibitory component of such mixtures by binding to it (Hori & Ohotami, 1978). The CPE preparation which was used for the experiments detailed in this report was not highly purified, however any metals which could be inhibiting the enzyme and bound by BSA, for example copper, should have been removed by gel filtration. Despite this assumption, studies with BSA and metals such as copper may prove worthwhile. The possibility of a different inhibitory

compound remaining in the enzyme preparation after gel filtration, which can subsequently be removed by binding to BSA, could be investigated by testing for any effect of BSA on more highly purified preparations of CPE.

The activity of CPE in the presence of neurophysin has been previously examined as discussed in Chapter 3. Similar results are described here for low concentrations of neurophysin, which cause a non-specific enhancement of enzyme activity which is unrelated to neurophysin-substrate binding, and can be achieved with a wide range of proteins.

At higher concentrations of neurophysin, however, the observations detailed here led to different conclusions of how inhibition of activity occurs, when compared with published findings. In a previous report, while a high concentration of neurophysin was found to inhibit CPE activity, RNase S protein was not seen to reduce the reaction rate when added at any concentration (Ando *et al*, 1988). This led the authors to conclude that binding between neurophysin and the peptide substrate, combined with dimerisation of such complexes, resulted in stearic constraints on enzymatic processing of the peptide and thus led to a reduced reaction rate. In the present study, however, high concentrations of either protein resulted in similar degrees of reduced stimulation or inhibition of activity of both the soluble and membrane-bound preparations of CPE. The observation that activity against the oxidised peptide was equally affected shows conclusively that the mechanism of inhibition cannot be related to binding between neurophysin and substrate, as it was shown that oxidation prevented OT-GKR from binding to neurophysin. This conclusion is important if the strategy outlined in Chapter 1 is to be followed, that is, to attempt to study processing reactions under conditions approaching those found *in*

vivo. As the concentration of neurophysin within the neurosecretory granule is very high (0.06M, Nordmann & Morris, 1984), this would potentially be a major problem if neurophysin was indeed specifically inhibiting processing activity by binding the substrate. However, if the inhibition is non-specific, as suggested by this work, it may be that manipulation of other factors will overcome this problem and allow processing to be studied under the desired conditions.

The use of Southern blotting has established the presence of a single gene for CPE (Fricker *et al*, 1986), which is believed to code for a precursor molecule that is processed to the active, membrane-bound form (with the anchor region at the amino terminus) and then further cleaved to produce the soluble enzyme (Fricker, 1988b). Tissue extracts from various species reveal differing proportions of the two forms, and the function of membrane binding has not been established. Initial comparisons suggested that the soluble enzyme possessed higher specific activity, so membrane-association could be necessary for correct compartmentalization before cleavage to the soluble form for full activity occurred, alternatively it could be a mechanism to control CPE activity within the granule, by regulating the proportion of bound and free types (Fricker, 1988c). This study has shown differences in the characteristics of the two forms of CPE, both in response to changing environmental conditions (pH), and relative cleavage rates of arginyl and lysyl substrates; this introduces the possibility of different roles for the two forms of enzyme. Neurophysin has been isolated both in association with membrane lipids (Audhya & Walter, 1979) and membranes from neurosecretory granules (Swann & Pickering, 1976). It appears likely that the newly-formed granule contains both proenzyme and prohormone bound to the inner surface of the membrane; the proenzyme bound by the amino

terminal "amphiphilic helix", and the prohormone by associations between the neurophysin domain and membrane components. Cleavage of the carboxy terminal region of proCPE results in the active, membrane-bound form of the enzyme; endoprotease cleavage of prooxytocin may leave neurophysin in association with the membrane, also binding the extended hormone sequence, keeping it close to the processing enzyme on the inner surface of the granule. Much of the processing *in vivo* could thus be taking place at the membrane, but is not easily studied by disruption of the granules and separation of soluble and membrane components. This model would provide a function for neurophysin in retaining the extended forms of oxytocin and vasopressin close to the membrane surface after endoproteolytic cleavage has occurred, and may be an important area for further experimentation.

The findings of this study therefore have several implications for future investigations. The influence of protein concentration on the activity of CPE should be considered when *in vitro* studies of this and other processing enzymes are designed, with particular consideration given to the concentration found at the *in vivo* site of enzyme action. The presence of membrane components, and interactions between enzymes and granule membranes are also relevant in such studies, as this work has shown that such factors can alter enzyme characteristics, perhaps leading to different roles for soluble and membrane-associated forms of the same enzyme. Subtle changes in enzyme structure and microenvironment brought about by membrane binding may also help to overcome current problems of pH incompatability between purified processing enzymes, allowing complete processing of a prohormone by several different enzymes under a common set of conditions.

This study has shown that CPE activity is indeed influenced by environmental factors that are likely to be operating to some extent *in vivo*. These findings should be considered and persued in order to develop realistic investigations of how prohormone processing occurs within the cell.

APPENDIX

Automatic Sample Injector Programme

```
1  RACK CODE 0
2  INPUT B0/1
3  IF B0>80
4  GO TO 2
5  INPUT B2/30
6  IF B2>425
7  GO TO 5
8  B1=B2+15
9  INPUT B3/48
10 INPUT B4/47
11 INPUT B5/25
12 A1=0
13 FOR A=1/16
14 FOR B=1/5
15 A1=A1+1
16 IF A1>B0
17 HOME
18 PRINT A1/1
19 HEIGHT
20 ASPIR 0/3/0
21 TUBE A/B
22 HEIGHT -15
23 ASPIR 0/B1/0
24 TUBE 0/0
25 INJECT 1
26 DISP 0/15
27 INJECT 0
28 DISP 0/B2/0
29 WAIT /8
30 INJECT 1
31 WAIT
32 DISP 0/B3+3/5
33 WAIT B5
34 INJECT 0
35 RINSE
36 DISP 0/B4/9
37 NEXT B
38 NEXT A
39 HOME
```

Information entered for each run:

B0: number of samples (1-80)

B2: sample volume (typically 200µl)

B3: injection valve rinse volume (500µl)

B4: needle rinse volume

B5: cycle time for sample injector (27min)

This programme instructs the auto-sample injector to deliver samples for analysis in the following way. The samples (typically 230µl) are held in the 1.5ml polypropylene microcentrifuge tubes used for the assays, suspended in a rack of known dimensions. To prevent mixing of the sample with water in the sample tubing, 15µl of air are first aspirated into the tubing, followed by 215µl of sample. The needle moves to the injection port and 15µl of sample are dispensed with the injection valve in the "inject" position (by-passing the sample loop), to wash the system with the sample. The valve then moves to the "load" position, and the remaining 200µl of sample are dispensed into the sample loop. When a signal is received from the controller (the Gilson 714 programme on the PC) that the elution gradient has begun, the valve moves to the "inject" position, causing the mobile phase to flow through the sample loop and deliver the sample to the column. The needle and tubing are rinsed, and the system waits 27 minutes before repeating the cycle.

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