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CHEMICAL SYNTHESIS, BACTERIAL EXPRESSION, AND CHARACTERIZATION OF PRO-GNRH/GAP, A PRECURSOR PROTEIN OF TWO BIOLOGICALLY ACTIVE PEPTIDE HORMONES

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CHEMICAL SYNTHESIS, BACTERIAL EXPRESSION, AND CHARACTERIZATION OF PRO-GNRH/GAP, A PRECURSOR PROTEIN OF TWO BIOLOGICALLY ACTIVE PEPTIDE HORMONES

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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LIST OF ABBREVIATIONS

2-Br-Z	2-bromo-benzyloxycarbonyl
2-Cl-Z	2-chloro-benzyloxycarbonyl
Boc	tert-butyloxycarbonyl
BSA	bovine serum albumin
Bzl	benzyl
CD	circular dichroism
DCM	dichloromethane
DIC	diisopropylcarbodiimide
DMF	dimethylformamide
DMS	dimethylsulfide
DMSO	differential scanning calorimetry
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
Fmoc	9-fluorenylmethyloxycarbonyl
FSH	follicle stimulating hormone
GAP	GnRH associated peptide
GnRH	gonadotropin-releasing hormone
HBc	hepatitis B virus core protein
HBTU	2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HF	hydrogen fluoride
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
LH	lutenizing hormone
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser enhancement spectroscopy
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	prohormone convertase
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
TFE	trifluoroethanol
TGN	trans golgi network
TOCSY	total correlation spectroscopy
Tos	toluenesulfonyl

ABSTRACT

CHEMICAL SYNTHESIS, BACTERIAL EXPRESSION, AND CHARACTERIZATION OF PRO-GNRH/GAP, A PRECURSOR PROTEIN OF TWO BIOLOGICALLY ACTIVE PEPTIDE HORMONES

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Two biologically active peptides, gonadotropin releasing hormone (GnRH) and GnRH associated peptide (GAP) are both derived from a common prohormone precursor protein, pro-GnRH/GAP. Both peptides are cosecreted from hypothalamic neurosecretory granules and are involved in the regulation of mammalian reproduction. A calcium dependent, neutral pH serine protease discovered in this laboratory, GAP-releasing enzyme, is the most likely processing enzyme of pro-GnRH/GAP. GAP-releasing enzyme is immunologically related to PC1/3, a member of the

prohormone convertase (PC) class of processing endoproteinases.

GAP-releasing enzyme recognizes the eight residue processing site within pro-GnRH/GAP, $G^{6}LRPGGKR^{13}$, and correctly cleaves the R^{13}-D^{14} bond to yield bioactive GAP and a three residue extension of GnRH. We and others have postulated that the recognition site for GAP-releasing enzyme forms a defined structural element at the surface of the substrate protein and that this structural element helps mediate limited endoproteolysis.

In the work reported here, hundred mg quantities of pro-GnRH/GAP were prepared by novel methods of both chemical synthesis and bacterial expression. Large amounts of pure protein are required for enzymatic and biophysical studies of pro-GnRH/GAP, which are intended to establish whether or not the processing site within the prohormone exists as a defined structural element that plays a central role in endoproteolytic processing. Synthetic pro-GnRH/GAP was prepared in high yield but proved difficult to purify to homogeneity. Recombinant pro-GnRH/GAP was prepared in

sufficient yield and purity to perform all subsequent experiments.

An immunoassay was developed against a processing site epitope within pro-GnRH/GAP. Both synthetic and recombinant pro-GnRH/GAP proteins are immunoreactive, consistent with the idea that the epitope, and, thus, the processing site, is located on the surface of the molecule. Proteolysis of synthetic or recombinant pro-GnRH/GAP by trypsin or kallikrein caused immediate loss of immunoactivity, showing that the processing site is susceptible to proteolysis and that the integrity of the processing site is required for immunoactivity. One of the kallikrein hydrolytic products was identified as GAP. Therefore, kallikrein cleaves at the R^{13}-D^{14} bond.

The intrinsic fluorescence yield of the Trp residue near the processing site region of pro-GnRH/GAP is sensitive to changes in pH, but not to changes in ionic strength or calcium concentration; its fluorescence yield is maximal at neutral pH. This suggests that the processing site displays maximum structure at neutral pH. This finding is coincident with the fact that GAP-releasing

enzyme is optimally active at neutral pH. However, the relative contribution of secondary structural elements, as discerned by circular dichroism, remains constant over the range of pH 5.2-10.7. Only at pH <5.0 are significant changes in secondary structure apparent. This indicates that the pH effects on the structure of pro-GnRH/GAP are rather subtle.

Thermal denaturation of pro-GnRH/GAP follows a simple two-state transition at neutral pH, as assessed by differential scanning calorimetry. This shows that pro-GnRH/GAP assumes a protein-like tertiary structure at neutral pH. 1D NMR data obtained at variable pH showed changes in resonance position and spectral resolution which are consistent with pH mediated conformational change and with the assumption of organized structure at neutral pH. However, the lack of through space correlations in the 2D NOESY experiment indicates that determination of the three-dimensional structure of pro-GnRH/GAP at neutral pH may be problematic.

INTRODUCTION

Peptide hormones and neuropeptides mediate the communication between cells in neuroendocrine systems. These peptides are secreted into the extracellular environment, through which they reach their target tissues and exert their biological functions. Most peptide hormones are synthesized as larger precursor proteins which must undergo post-translational processing to yield the biologically active hormone (for reviews, see Docherty and Steiner, 1982; Gainer et al, 1985; Harris, 1989; Rouillé et al, 1995). Yet, relatively little is known about the molecular mechanisms of hormone precursor processing and its regulation. Recent evidence suggests that the structure of the precursor protein itself plays a role in the regulation of the processing pathway.

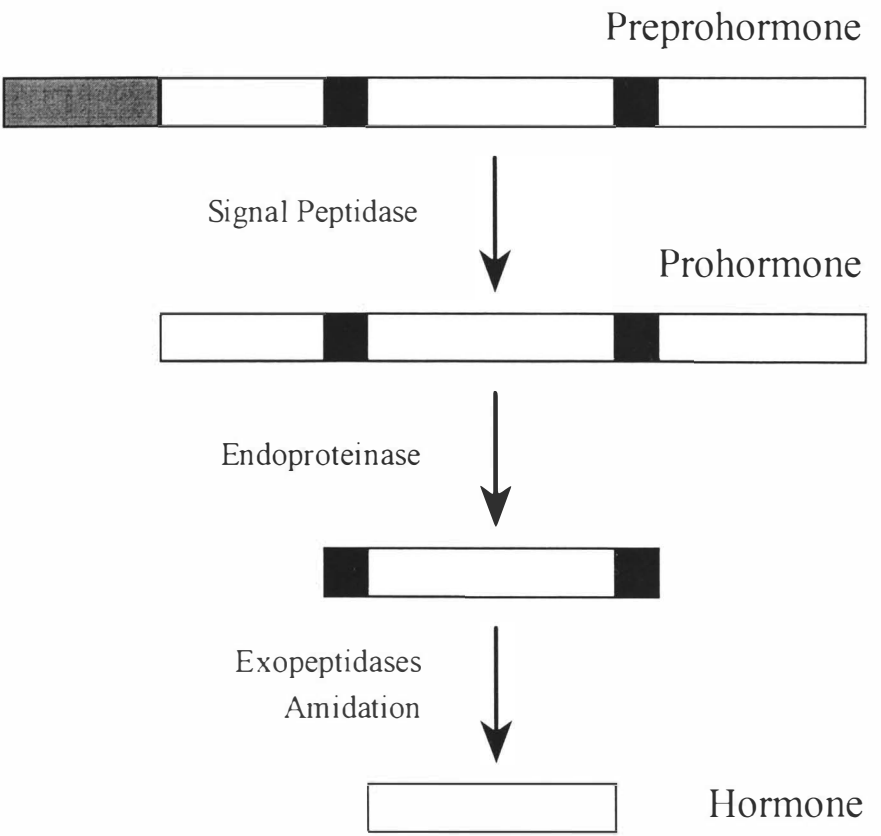
Biosynthesis and secretion of peptide hormones. Peptide hormones are initially translated as large preprohormones which contain an N-terminal signal sequence of 15-30 amino acid residues. These precursors are

processed in a highly ordered and stepwise fashion (Figure 1). Processing begins with the removal of the signal peptide to yield the prohormone. The prohormone may then be modified by disulfide bond formation, phosphorylation, sulfation, and/or glycosylation. All prohormones undergo limited endoproteolysis at specific loci. The resulting peptides may require the action of exopeptidases, amidating, and/or acetylating enzymes to produce the biologically active hormone.

Proteins that are destined for secretion follow either the constitutive or the regulated pathway (for reviews, see Kelly, 1985; Burgess and Kelly, 1987). In both pathways, proteins are targeted for the endoplasmic reticulum (ER) and subsequently translocated to the golgi apparatus. The two pathways diverge in the trans golgi network (TGN).

Constitutively secreted proteins are packaged into transport vesicles at concentrations which are no more than 2-fold higher than their respective concentrations in the ER (Hearn et al, 1985). These vesicles move continuously to the plasma membrane and release their contents within minutes of leaving the golgi.

Figure 1: Pathway of peptide hormone biosynthesis. Peptide hormones are translated as preprohormones. The signal peptide is removed in the ER. The resulting prohormone may undergo futher modification such as disulfide formation, glycosylation, phosphorylation, and/or sulfation in the ER and golgi. Endoproteolysis occurs in either the TGN or in the secretory granule. Final maturation occurs in the secretory ganule, where the hormone is stored until it is released in response to the appropriate stimulus. Adapted from Hook et al (1994).


Preprohormone
Signal Peptidase
Prohormone
Endoproteinase
Exopeptidases
Amidation
Hormone

In the regulated pathway, proteins become highly concentrated in the TGN and are packaged into dense core vesicles called secretory granules. In endocrine cells, the protein concentration inside these granules may be as much as 200-fold higher than in the ER (Salpeter and Farquhar, 1981). These secretory granules are stored in the cytoplasm for hours or days. In response to an extracellular stimulus, the granules fuse with the plasma membrane and release their contents.

Biosynthesis and processing of preprohormones and the subsequent release of their biologically active products occur via the regulated secretory pathway (c.f., Gumbiner and Kelly, 1982; Moore and Kelly, 1985). Translation of the preprohormone begins on cytoplasmic ribosomes. As the signal peptide emerges, it binds to a signal recognition particle which targets the ribonucleoprotein complex to the ER (for reviews, see Walter and Lingappa, 1986; Skach and Lingappa, 1993). The ribosome binds to the ER membrane and the nascent polypeptide is translocated across the membrane into the cisternal space. The N-terminal signal peptide is then removed by a membrane-bound signal peptidase, giving

rise to the prohormone (Jackson and Blobel, 1977; Evans et al, 1986).

The ER cisterna is the location for disulfide bond formation and N-glycosylation as appropriate for a given prohormone (for review, see Bennett et al, 1993). Proper folding of secretory proteins also occurs in the ER. It has not been conclusively demonstrated that prohormones assume defined structures or that such structures are physiologically important. However, it is logical to assume that any existing prohormone structural features are formed in the ER. The prohormone then moves via transport vesicles from the ER to the cis face of the golgi apparatus (for review, see Rothman and Orci, 1992).

Phosphorylation, sulfation, and oligosaccharide maturation occur in the golgi stack (for review, see Bennett et al, 1993). Sorting of the prohormone into the regulated secretory pathway occurs in the TGN by selection mechanisms which are unclear. It has been suggested that sorting into the regulated pathway occurs via selective aggregation (c.f., Tooze et al, 1993). According to this idea, the chemical environment of he TGN allows segregation of proteins on the basis of their physicochemical

properties. As secretory proteins move into the TGN, they encounter an increase in protein and calcium ion concentrations and a decrease in pH. Proteins that aggregate under these conditions are selected for the regulated pathway; and proteins that remain freely soluble continue on the constitutive pathway. Whatever the mechanism, prohormones or their proteolytic products condense into electron-dense spherical cores that bud from the TGN to form secretory granules.

The enzymes responsible for endoproteolytic processing of the prohormone and final maturation of the biologically active hormone are colocalized with the prohormone in the TGN and are copackaged into the secretory granules. Endoproteolysis may begin in the TGN (Schnabel et al, 1989) or after packaging into secretory granules (Orci et al, 1987). In either case, final maturation of the biologically active peptide most likely occurs in secretory granules. Here, specific carboxypeptidases, aminopeptidases, and/or N-acetylating and α-amidating enzymes act to generate the mature hormone which is stored in the secretory granules at high concentration.

In response to an extracellular stimulus, the secretory granules move along cytoskeletal elements toward the plasma membrane. The granules fuse with the plasma membrane, thus releasing their contents into the extracellular environment.

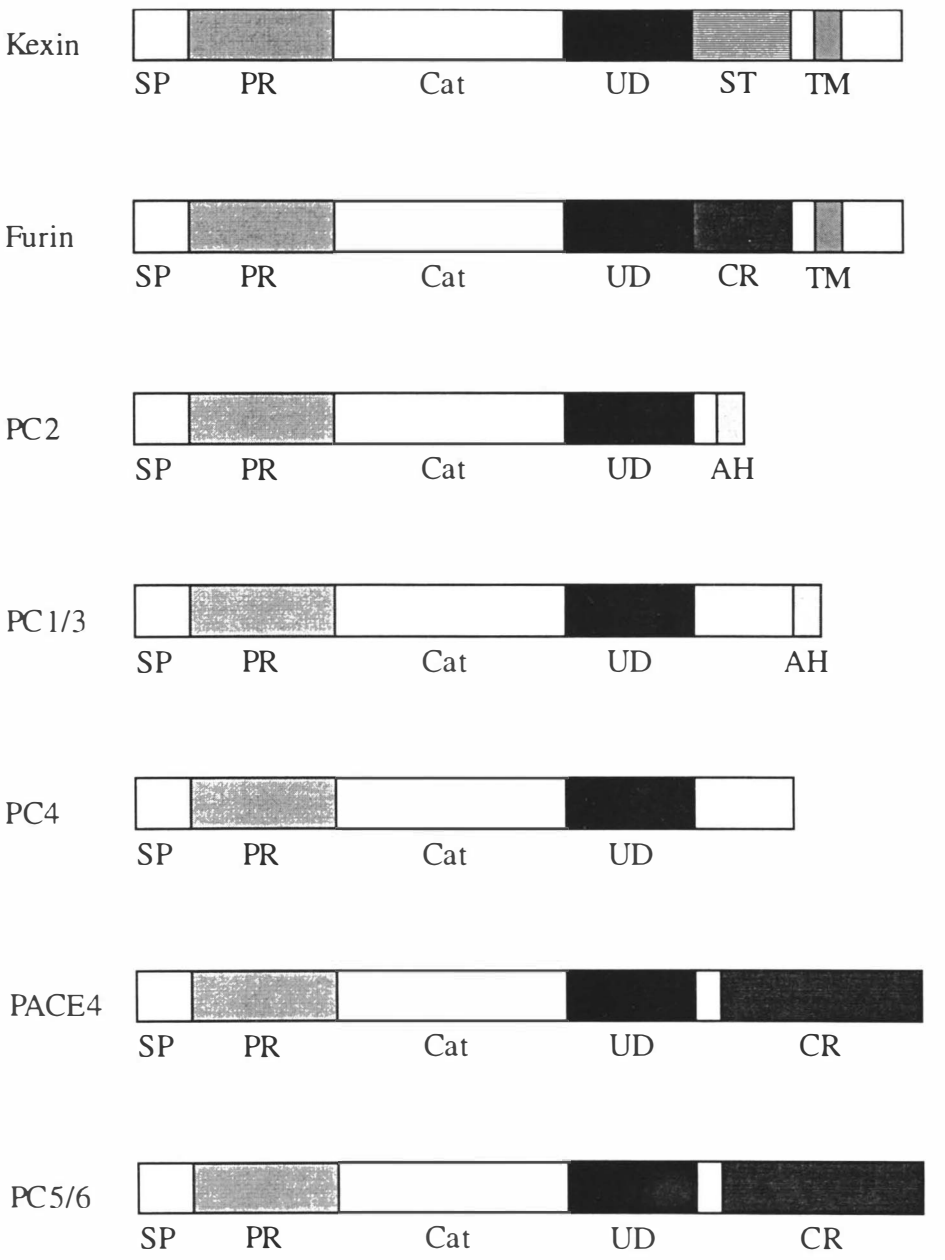
Endoproteolysis of prohormone precursor proteins. All prohormones must undergo endoproteolysis at one or more loci to liberate their biologically active peptides. The processing enzymes responsible for these endoproteolytic events are called prohormone convertases (PC's). Although it has been difficult to unequivocally identify the physiologically relevant processing enzyme for a particular endoproteolytic event, a number of enzymes have been implicated in the processing of a wide variety of precursor proteins (for reviews, see Seidah et al, 1991a; Steiner et al, 1992; Seidah and Chrétien, 1994; Rouillé et al 1995). It has been suggested that a physiologically relevant prohormone convertase should 1) cleave the prohormone *in vitro* at the physiologically relevant site(s), 2) be located in the subcellular compartment in which processing takes place, and 3) function in the physiological

conditions of that subcellular compartment (Docherty and Steiner, 1982; Harris, 1989).

The first unambiguously identified processing enzyme was kexin, whose physiological substrate is pro-α-mating factor in the yeast *Saccharomyces cerevisiae* (Julius et al, 1984; Fuller et al, 1988). Kexin is a calcium dependent serine protease with a catalytic domain related to the bacterial subtilisins (Mizuno et al, 1988; Fuller et al, 1989; Mizuno et al, 1989). Knowledge of the nucleotide and predicted amino acid sequence of kexin made it possible to search for mammalian homologues on the basis of the presumed sequence conservation in the catalytic region of the enzyme. This led to the discovery of a new family of mammalian endoproteases called the subtilisin-like proprotein convertases (Figure 2) (cf Rioullé et al, 1995).

These enzymes share several common structural features including an N-terminal signal peptide, an 80-90 residue propeptide sequence, a subtilisin-like catalytic domain of approximately 240 residues, and a well conserved domain that is required for activity, but whose function is unknown.

Figure 2: Schematic diagram of the subtilisin-like proprotein convertases. All these enzymes share an N-terminal signal sequence (SP), a pro-region (PR), a subtilisin-like catalytic domain (Cat), and a well conserved domain of unknown function (UD). Some of these enzymes have a Ser/Thr rich region (ST), a Cys rich region CR, a transmembrane domain (TM), and/or an amphipathic helix (AH). Adapted from Rouillé et al (1995).


Kexin
SP
PR
Cat
UD
ST
TM
Furin
SP
PR
Cat
UD
CR
TM
PC2
SP
PR
Cat
UD
AH
PC1/3
SP
PR
Cat
UD
AH
PC4
SP
PR
Cat
UD
PACE4
SP
PR
Cat
UD
CR
PC5/6
SP
PR
Cat
UD
CR

Furin, PACE4, and PC5/6 all share an additional cysteine rich region. Furin and an isoform of PC6 with a much longer cysteine rich domain (Nakagawa et al, 1993a) both have a transmembrane domain near their respective C-termini.

Processing by furin is generally directed by the canonical sequence R-X-[K/R]-R↓ (where ↓ indicates the site of proteolysis); however, it has been shown that furin is capable of processing the minimal recognition motif R-X-X-R↓ (Molloy et al, 1994). PACE4 (Creemers et al, 1993; Rehemtulla et al, 1993) and PC5/6 (Nakagawa et al, 1993b) exhibit similar substrate specificities to that of furin, but with a greater dependence on a basic residue in the P2 position relative to the scissile bond.

Furin is localized primarily in the TGN; however, it does move to the plasma membrane via constitutive vesicles and back to the golgi via endosomes (Vidricaire et al, 1993; Molloy et al, 1994; Bosshart et al, 1994). Furin possesses a neutral pH optimum which is consistent with its potential role as a processing enzyme, and it is expressed in many different tissues. Furin is thought to process a

variety of precursor proteins in the constitutive pathway (cf Rioullé et al, 1995).

PACE4 is also expressed nearly ubiquitously, but at particularly high levels in the anterior lobe of the pituitary gland (Johnson et al, 1994). The PACE4 levels in the pituitary are regulated by thyroid status, suggesting an involvement in processing of some neuroendocrine peptides. However, any definitive role of PACE4 in prohormone processing has yet to be demonstrated.

Both isoforms of PC5/6 are expressed at very high levels in intestinal mucosa and the adrenal glands and to a lesser extent in many other tissues (cf Rouillé et al, 1995). It has been suggested that PC5/6 may process cellular growth factor precursors or intestinal regulatory peptide precursors.

PC1/3, PC2, and PC4 all lack a cysteine rich domain and a transmembrane domain. However, PC1/3 and PC2 both have a putative amphipathic helix at their respective C-termini, which may be involved in membrane association and/or sorting into secretory granules (Smeekens et al, 1991; Seidah et al, 1991b).

All three enzymes cleave at pairs of basic amino acids which contain an Arg residue in the P1 position (K-R↓ or R-R↓) (c.f., Seidah and Chrétien, 1994), and PC2 is capable of processing dibasic residue sites which contain a P1 Lys residue (K-K↓ or R-K↓) (Zhou et al, 1993). These dibasic sites are the predominant cleavage motifs found in prohormones and proneuropeptides.

PC4 is expressed exclusively in the testis (Nakayama et al, 1992), and it is likely involved in processing the pituitary adenylate cyclase-activating polypeptide precursor (Li et al, 1998). PC1/3 and PC2 are expressed exclusively in the brain and the extended neuroendocrine system (Smeekens et al, 1991; Seidah et al, 1991b). They have both been localized to the TGN and secretory granules where prohormone processing takes place (Lindberg et al, 1994). PC1/3 and PC2 exhibit mildly acidic pH optima (5.0-7.0) (Zhou and Lindberg, 1993; Lindberg et al, 1995; Azaryan et al, 1995), which is consistent with proteolytic activity in the latter stages of the TGN and in secretory granules. Both enzymes process prohormones to physiologically relevant products (c.f., Benjannet et al,

1991). Thus, PC1/3 and PC2 are thought to be the enzymes responsible for the processing of most prohormones and proneuropeptides.

Role of prohormone structure in endoproteolytic specificity and regulation. The primary sequence of most peptide hormones is flanked by pairs of basic amino acid residues in the intact prohormone. It is generally accepted that endoproteolysis occurs at these sites to release the peptides from the framework of the prohormone. However, there are many examples of dibasic sites in prohormones that are not processing sites; and such dibasic sites may exist in the biologically active peptides. Therefore, the presence of a pair of basic amino acid residues is not necessarily sufficient to direct prohormone processing.

It is likely that the proteolytic recognition site in a given prohormone is a sequence of several residues on one or both sides of the dibasic cleavage site. Studies of the primary sequences of a number of prohormones revealed no concensus sequence in the neighboring residues of the cleavage sites (Rholam et al, 1986). However, most recognition sequences include a strongly polar residue

between the P4 and P8 positions relative to the cleavage site (Harris et al, 1989). This polar residue and the pair of basic residues enhance the hydropathy of the recognition sequence and probably serve to bring the processing site to the external surface of the prohormone. Thus, it is likely that the structure of the processing site, rather than the specific sequence of amino acids, imparts processing enzyme specificity (Harris et al, 1989).

Secondary structure predictions of prohormone sequences indicate that dibasic cleavage sites exist either in or adjacent to β-turns (Rholam et al, 1986) or Ω-loops (Bek and Berry, 1990). In contrast, dibasic sites which are not cleaved in vivo are predicted to be associated with ordered structures such as α-helices, β-sheets, or higher order organization (Rholam et al, 1986).

The prediction of secondary structure at the processing site is supported by structural evidence. The processing site at the CA junction in proinsulin has been shown to form a defined structural feature which has been designated the "CA knuckle" (Weiss et al, 1990). Studies of prooxytocin/neurophysin have shown that the tetrapeptide sequence immediately preceding the K11-R12 processing site

(P7-G10) organizes as a β-turn and that the neighboring residues on the C-terminal side of this site tend to form an α-helical structure (Paolillo et al, 1992). This β-turn structural motif has been shown to be important in substrate recognition by the putative processing enzyme of prooxytocin/neurophysin (Brakch et al, 1993).

These results support the hypothesis that the structure of the processing site in prohormones plays a role in processing enzyme specificity. However, the physiological relevance of these processing site structures remains to be demonstrated. The proinsulin "CA knuckle" was observed in a solution containing 20% acetic acid at pH 3.0 (Weiss et al, 1990). The β-turn motif in prooxytocin/neurophysin was observed in peptide fragments in the presence of TFE, a structure promoting solvent (Paolillo et al, 1992; Brakch et al, 1993).

It is well known that chemical parameters such as pH and ionic strength can influence protein structure; and it is well documented that proteins experience environmental changes with respect to these parameters as they move through the various compartments of the regulated secretory

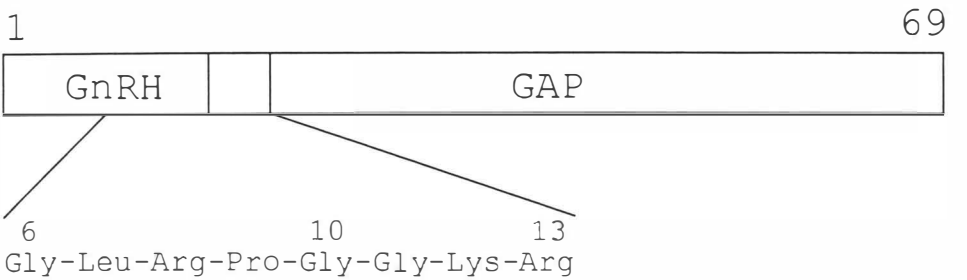
pathway. Changes in pH and/or $CaCl_2$ concentration control the compartment specific processing of proinsulin (Davidson et al, 1988) and proopiomelanocortin (Schmidt and Moore, 1995). It is logical to assume that such changes in the chemical environment could affect prohormone structure, and that any resulting conformational changes may be relevant to processing.

Biophysical analysis of an intact prohormone is necessary to assess the structural organization of its processing site(s). These studies must be performed under physiological conditions in order to establish the biological relevance of prohormone structure. Structural information must be obtained under various chemical conditions to determine the response of prohormone conformation to the distinct chemical environments encountered in the subcellular compartments of the regulated secretory pathway.

Pro-GnRH/GAP as a model for prohormone processing.

Pro-GnRH/GAP provides a useful model system for the study of prohormone structure and processing (see Figure 3). Endoproteolysis of a single processing site is sufficient for the release of two biologically relevant

Figure 3: Primary amino acid sequence of pro-GnRH/GAP. Pro-GnRH/GAP is a 69 residue prohormone containing the sequences of GnRH and GAP. GAP-releasing enzyme recognizes the sequence Gly6-Arg13 and cleaves the Arg13-Asp14 bond. This endoproteolytic event yields functional GAP and a three residue C-terminal exstension of GnRH, which must undergo further processing to become biologically active.



GAP-releasing enzyme

QHWSYGLRPGGKR DAENLIDSFQEIVKEVGQLAET

QRFECTTHQPRSPLRDLKGALESLIEEETGQKKI

peptide sequences. The recognition sequence for this processing site is well characterized (Palen et al, 1987; Rangaraju et al, 1991; Rangaraju and Harris, 1991). Proximal to the processing site recognition sequence, pro-GnRH/GAP contains a single Trp residue which facilitates fluorescence analysis of the processing site region.

Hypothalamic gonadotropin-releasing hormone (GnRH) is an amidated decapeptide which is released from neurosecretory granules. GnRH stimulates the secretion of leutenizing hormone (LH) and follicle stimulating hormone (FSH) from anterior pituitary gonadotroph cells via receptor mediated activation of phosphoinositide hydrolysis (Morgan et al, 1987).

The primary sequence of pre-pro-GnRH/GAP has been deduced from its cDNA (Seeburg and Adelman, 1984; Adelman et al, 1985). The prohormone (pro-GnRH/GAP) contains the sequence of GnRH and a 56 residue peptide, termed GnRH associated peptide (GAP) (Figure 3). GAP inhibits the release of prolactin and stimulates the release of gonadotropic peptides from cultured rat pituitary cells (Nikolics et al, 1985). GnRH and GAP are co-secreted into the hypothalamo-hypophyseal portal blood of ovariectomized

ewes (Clark et al, 1987), indicating that GAP is also a secretory product of neurosecretory granules. This concept is supported by immunocytochemical evidence which shows that GnRH and GAP are co-localized in hypothalamic neurosecretory granules of the rat (King and Anthony, 1983; Phillips et al, 1985) and primate (Song et al, 1987).

GAP-releasing enzyme was discovered in this lab and subsequently identified as a candidate processing enzyme of pro-GnRH/GAP by its ability to specifically cleave peptide substrates (Palen et al, 1986). GAP-relasing enzyme is a membrane bound protease found in hypothalamic neurosecretory granules. Its recognition sequence has been mapped to eight residues ($G^{6}LRPGGKR^{13}$; Figure 3), and its character is consistent with that of other subtilisin-like proprotein convertases (Palen et al, 1987; Rangaraju and Harris, 1991). GAP-releasing enzyme has not yet been sequenced, but it is immunologically related to PC1/3 (Rangaraju and Harris, 1992). Thus, GAP-releasing enzyme is likely a member of the subtilisin-like proprotein convertase family and is considered the physiologically relevant processing enzyme of pro-GnRH/GAP.

Three distinct enzyme activities are necessary to process pro-GnRH/GAP to functional GnRH and GAP. All three activities have been localized in, and purified from, hypothalamic neurosecretory granules (Rangaraju et al, 1991). The three enzymes are GAP-releasing enzyme, Carboxypeptidase E, and α-Amidating enzyme. Endoproteolysis by GAP-releasing enzyme occurs initially and yields equimolar amounts of GAP and a three residue C-terminal extension of GnRH. Carboxypeptidase E and α-Amidating enzyme serve to further process this C-terminal extension of GnRH.

GAP-releasing enzyme is active only in the neutral pH range and has optimal activity at pH 7.2. Carboxypeptidase E has maximal activity at pH 5.5 and is inactive at pH > 7.5. α-Amidating enzyme is optimally active at neutral pH but has substantial activity at low pH (4.5-6.5) and high pH (9-10.5). These data are consistent with the hypothesis that endoproteolysis takes place in the TGN, where the pH is near neutrality, and that further processing occurs in secretory granules, where the pH is approximately 5.5.

GAP-releasing enzyme specifically cleaves peptide substrates encompassing its recognition sequence on the C-terminal side of the Lys-Arg doublet. This occurs whether the recognition sequence is situated within the framework of pro-GnRH/GAP or pro-ANF, the precursor to atrial natriuretic factor (ANF) (Rangaraju and Harris, 1991). However, GAP-releasing enzyme does not act at the recognition sequence for atrial granule serine proteinase (AGSP), the putative endoproteolytic processing enzyme of pro-ANF, even when this sequence is situated within the framework of pro-GnRH/GAP. Also, AGSP does not act at the recognition sequence of GAP-releasing enzyme in either context (pro-GnRH/GAP or pro-ANF). The recognition sequences for GAP-releasing enzyme (GLRPGGKR) and AGSP (APRSLRR) are similar in that both contain a dibasic site as well as an upstream basic amino acid residue. These results are consistent with the idea that the processing site recognition sequence for GAP-releasing enzyme exists as a defined structural feature in pro-GnRH/GAP and that this feature contributes to processing enzyme specificity.

Pre-pro-GnRH/GAP and GAP have been successfully assembled in a stepwise solid-phase cosynthesis using Boc

chemistry and an optimized acylation schedule (Milton et al, 1992). Recombinant GAP has been produced in bacteria and has been shown to stimulate the release of LH and FSH and to inhibit the release of prolactin from cultured rat pituitary cells (Nikolics et al, 1985; Seeburg et al, 1987). Chemically synthesized GAP has been shown to exert the same biological activity as the bacterially derived peptide (Milton et al, 1992;). These results indicate that synthesis and/or expression of pro-GnRH/GAP is/are feasible and that peptides derived from such preparations are likely to possess the appropriate biological activities.

We have assessed the conformational properties of synthetic pre-pro-GnRH/GAP (You et al, 1993a). The fluorescence emission maximum due to the two Trp residues is blue shifted relative to N(Ac)-Trp-amide indicating that these residues are buried within the protein. Fluorescence polarization measurements indicate that the microenvironment(s) of the two Trp residues is(are) resistent to thermal induced conformational change. CD spectroscopy confirmed these results; the protein retains nearly 50% of its initial helical character at 90°C. However, both fluorescence and CD spectroscopies revealed

that the protein structure is sensitive to changes in pH. Intrinsic fluorescence due to Trp is severely quenched on either side of the neutral pH range. Likewise, the relative mean residue ellipticity at 222 nm, an index of helical content, decreases markedly outide the neutral pH range. Thus, pre-pro-GnRH/GAP displays maximum structure only over the pH 7.1-7.9 range.

Within the pH 7.8-8.2 range, GAP-releasing enzyme hydrolyzes pre-pro-GnRH/GAP to yield GAP. Trypsin, kallikrein, and plasmin all yield multiple products under similar conditions. Thus, it appears that only GAP-releasing enzyme displays limited specificity.

There is a strong correlation between the structural integrity of pre-pro-GnRH/GAP in the neutral pH range and the optimal endoproteolytic activity of GAP-releasing enzyme at neutral pH. This supports the hypothesis that the processing site recognition sequence must exist as part of a stable structural feature which exposes the processing site to GAP-releasing enzyme in order for specific cleavage to occur. It is possible that changes in cellular conditions such as pH serve to order and regulate the processing pathway by affecting protein structure.

Goals of the current research. Comprehensive biophysical and enzymatic analysis of intact pro-GnRH/GAP is necessary to discern the structural properties of the processing site and to establish their potential biological significance. Hundred mg quantities of prohormone are needed for structural studies and for the eventual determination of its solution conformation by multidimensional NMR protocols.

Thus, the immediate goals of this project were: 1) to produce intact pro-GnRH/GAP in sufficient yield and purity to permit biophysical analysis, and 2) to assess the structural organization of pro-GnRH/GAP and its response to changes in chemical conditions such as ionic strength, calcium concentration, and pH.

We describe the large scale preparation of full length pro-GnRH/GAP and subsequent biophysical studies. We show that pro-GnRH/GAP is amenable to total chemical synthesis, although its purification has proven problematic. We developed a novel strategy for bacterial expression of pro-GnRH/GAP, which yielded large amounts of pure prohormone. We show that the structure of pro-GnRH/GAP is sensitive to changes in pH, but not to changes in calcium concentration

or ionic strength. We further demonstrate that intact pro-GnRH/GAP contains protein-like tertiary structure at neutral pH. Preliminary NMR data indicate that determination of the three-dimensional structure of pro-GnRH/GAP may be problematic at neutral pH.

CHEMICAL SYNTHESIS OF PRO-GNRH/GAP

Experimental Strategy

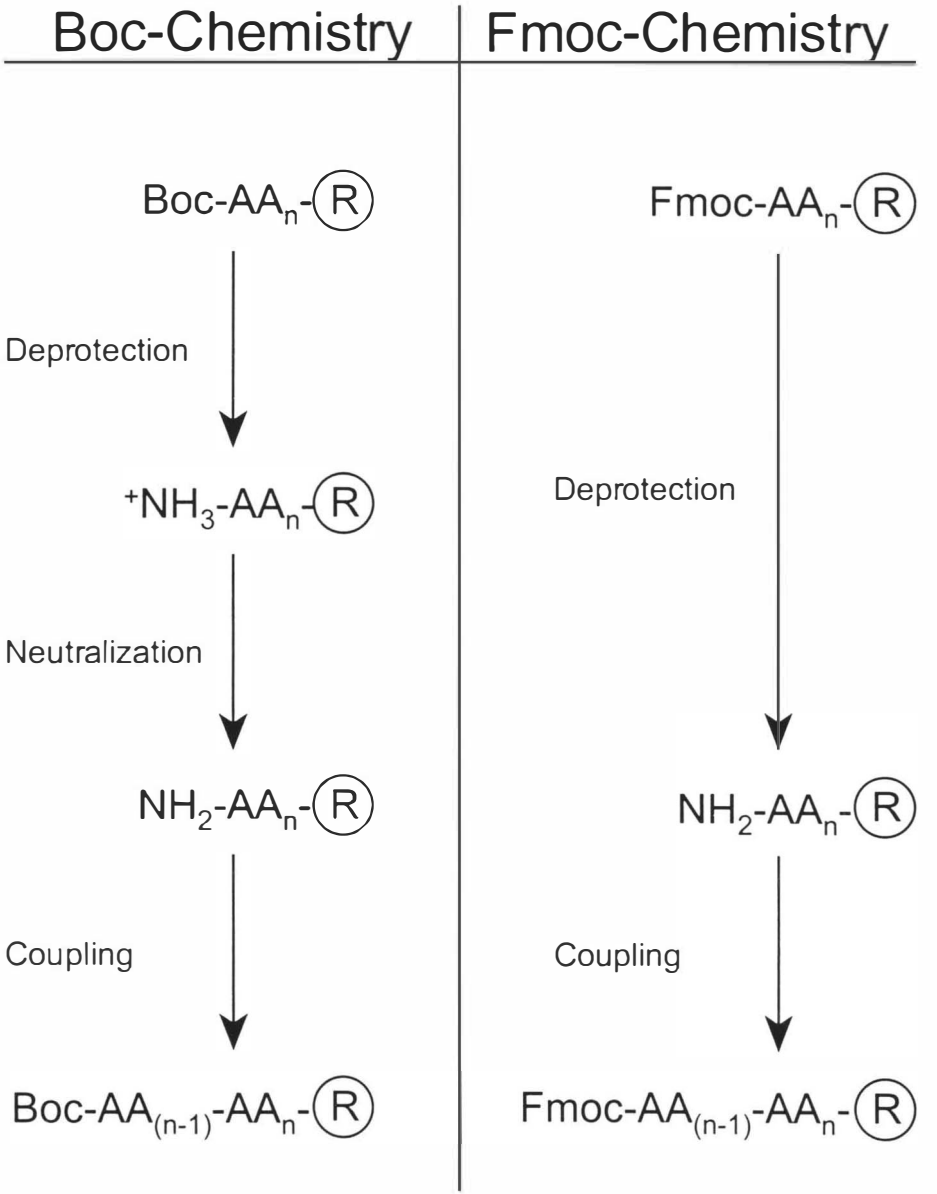
Since its inception, solid-phase peptide synthesis has been a viable method for the preparation of highly purified peptides of known structure (Merrifield, 1963). The development of standard methods (c.f., Stewart and Young, 1984) has made possible the routine preparation of peptides up to 35 amino acid residues in chain-length. However, conventional methods of stepwise solid-phase synthesis are usually unsuccessful when applied to many longer-chain peptide sequences. The primary sequence of pro-GnRH/GAP contains 69 amino acid residues; therefore, we attempted its synthesis by non-conventional protocols.

Stepwise solid-phase peptide synthesis. Stepwise solid-phase peptide synthesis involves the assembly of a peptide chain while it is covalently attached to a solid resin support (for review, see Kent, 1988). Synthesis begins with the attachment of the C-terminal residue to the

resin and ends with the incorporation of the N-terminal residue. Chemically reactive functional groups of the amino acid side chains are protected during synthesis with "blocking groups" which can be removed under defined chemical conditions. Amide bond formation is facilitated by the use of reagents such as carbodiimides or various uronium salts. When the synthesis is complete, the linkage between the C-terminal residue and the resin is cleaved and the blocking groups are simultaneously removed to yield the desired peptide.

There are two basic chemistries commonly used in solid-phase peptide synthesis (Figure 4), which differ primarily with respect to the blocking groups used to protect the α-amino group of the individual amino acids. In Boc chemistry protocols, the N-α Boc group is removed with TFA, giving rise to a trifluoroacetate salt. The protonated amine is subsequently neutralized with a hindered organic base. In Fmoc chemistry protocols, the N-α-Fmoc group is removed in a β-elimination reaction initiated by piperidine, giving rise to a neutral amine.

Figure 4: Standard strategies for solid-phase peptide synthesis. In Boc chemistry, the α-amino group is deprotected with TFA. The resulting protonated amine is neutralized with a hindered organic base prior to coupling. In Fmoc chemistry, the α-amino group is deprotected with piperidine, which yields a neutral amine directly. In both protocols, peptide bond formation is accomplished via coupling of an activated amino acid derivative. Adapted from Kent (1988).


Boc-Chemistry
Fmoc-Chemistry
Boc-AA$_n$-(R)
Fmoc-AA$_n$-(R)
Deprotection
$^+$NH$_3$-AA$_n$-(R)
Deprotection
Neutralization
NH$_2$-AA$_n$-(R)
NH$_2$-AA$_n$-(R)
Coupling
Coupling
Boc-AA$_{(n-1)}$-AA$_n$-(R)
Fmoc-AA$_{(n-1)}$-AA$_n$-(R)

In either procedure, there are limitations to the peptide chain-length that can be synthesized, presumably due to the cumulative effect of incomplete peptide bond formation. Nonquantitative coupling leads to the accumulation "deletion peptides" missing one or more amino acid residues but retaining properties very similar to those of the desired product. As these deletion peptides accumulate, the effective yield of the target peptide decreases, and purification becomes increasingly difficult or, in extreme cases, impossible.

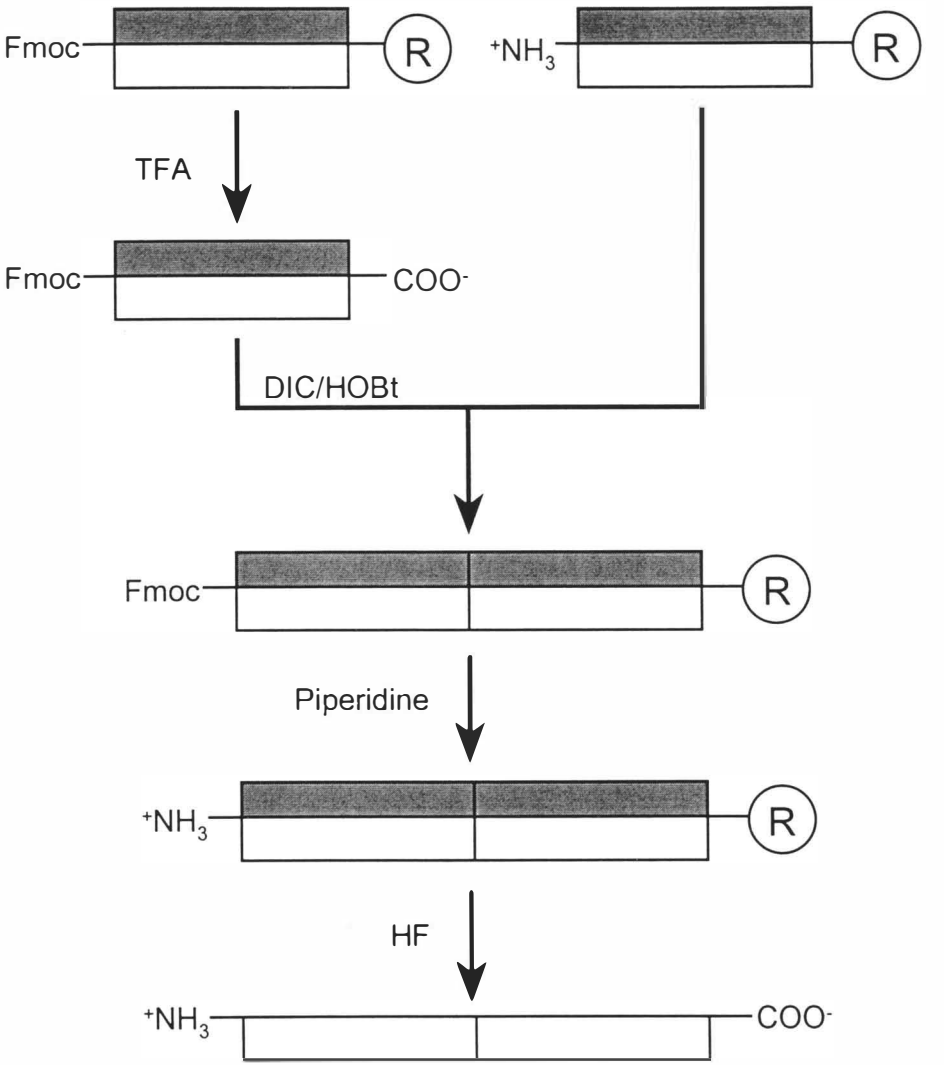
Some of these purification problems may be minimized by "capping" unreacted resin-bound peptide chains with agents such as acetylimidazole, thus terminating the further extension of these sequences (Barany and Merrifield, 1980). Acetylation of these "termination sequences" often facilitates their separation from the target peptide on the basis of differences in charge and/or solubility.

The problems associated with incomplete coupling are exacerbated by the occurrence of so-called "difficult sequences" (Merrifield, 1967; Kent, 1985; Shoemaker, 1987), which exhibit decreased coupling yields leading to a

greater accumulation of shorter-chain peptide impurities. Difficult sequences arise primarily when the resin-bound peptide forms secondary structure and/or intermolecular aggregates (Meister and Kent, 1984; Deber et al, 1989), both of which may hinder the solvent accessibility of the N-terminal amino acid. Coupling efficiencies in some difficult sequences can be improved by simply treating the synthesis resin with a chaotropic agent such as KSCN prior to the coupling step (Stewart and Klis, 1990). This is expected to disrupt any organized structure assumed by the resin-bound peptide.

The method of fragment condensation. The total synthesis of long-chain peptides may be accomplished by the condensation of two or more shorter-chain peptide fragments (Kubiak et al, 1987). In a strategy developed in this lab (You et al, 1993b), the "C-most" terminal fragment is synthesized with Boc chemistry, using strong acid labile blocking groups (Figure 5). This fragment remains attached to its synthesis resin during condensation with other peptide fragments. All other fragments are synthesized with Fmoc chemistry, also using strong acid labile blocking groups. These fragments are cleaved from their synthesis

Figure 5: Strategy for the use of fragment condensation in solid-phase peptide synthesis. The C-terminal fragment is synthesized by Boc chemistry protocols. Other fragments are synthesized by Fmoc chemistry protocols using strong acid labile blocking groups (shaded areas) and are cleaved from the resin in dilute TFA. Fully protected Fmoc-peptides are coupled as their -OBt esters, and the N-terminal Fmoc group is removed with piperidine. The full length peptide is cleaved from the resin and simultaneously deprotected with HF. Adapted from You et al (1993b).

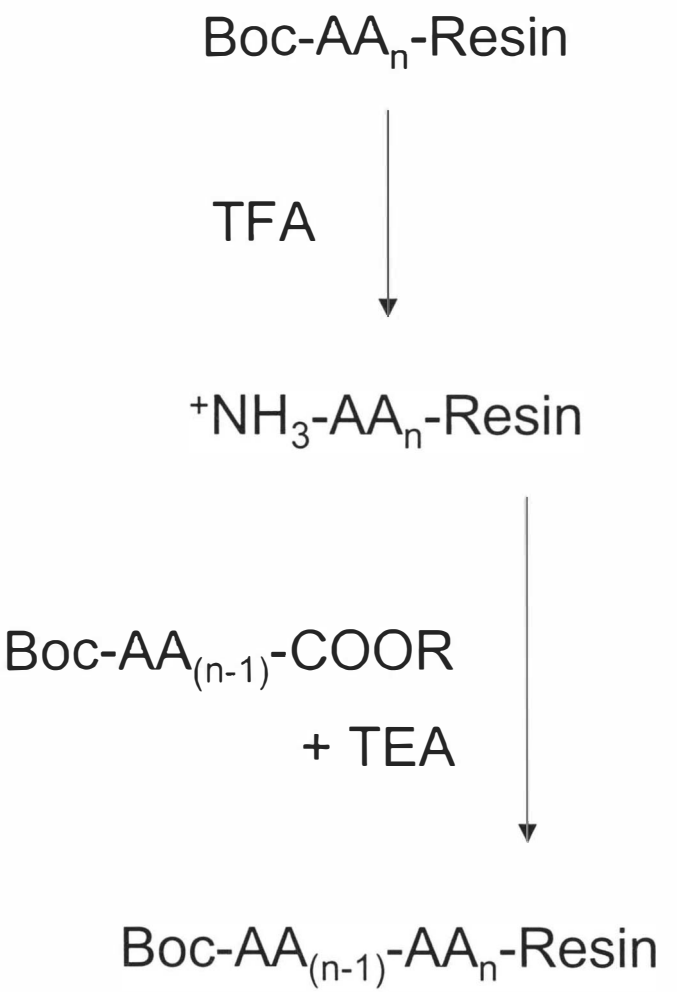

Fmoc
R
+NH3
R
TFA
Fmoc
COO-
DIC/HOBt
Fmoc
R
Piperidine
+NH3
R
HF
+NH3
COO-

resins in dilute TFA, leaving the N-terminal Fmoc group and the side chain protecting groups in place. Fully protected peptide fragments are activated with a coupling reagent such as DIC/HOBT and sequentially condensed with the resin-bound peptide. The full-length peptide is then deprotected and purified according to traditional protocols.

Coupling via *in situ* neutralization in stepwise solid-phase peptide synthesis. Another approach to the synthesis of long chain peptides is to improve the efficiency of stepwise procedures by minimizing the aggregation that might occur, creating difficult sequences. Aggregation generally occurs when the terminal amino group of the resin-bound peptide is in its neutral charge state (Schnölzer et al, 1992; Milton et al, 1990; Nakaie, 1990). Therefore, the problems caused by aggregation may be minimized by limiting the time that the free amino group exists as a neutral species.

The use of *in situ* neutralization has been shown to improve the efficiency of the coupling step in Boc chemistry peptide synthesis (Schnölzer et al, 1992). In this procedure (Figure 6), the terminal amino group is deprotected in concentrated TFA which disrupts any

Figure 6: Strategy for the use of *in situ* neutralization in solid-phase peptide synthesis. The N-α-Boc group is removed with neat TFA. The active amino acid is added to the protonated resin along with sufficient base to neutralize the resin bound amine. Adapted from Schnölzer et al (1992).


Boc-AA$_n$-Resin
TFA
$^+$NH$_3$-AA$_n$-Resin
Boc-AA$_{(n-1)}$-COOR
+ TEA
Boc-AA$_{(n-1)}$-AA$_n$-Resin

secondary structure or intermolecular aggregates, and neutralization does not occur prior to coupling. Rather, the neutralizing base for the resin-bound peptide is added together with the activated amino acid to the coupling reaction. Thus, coupling proceeds quickly, and the exposure time of the free amine is minimized. This method has been applied successfully to the stepwise synthesis of the HIV-1 protease, a 99 amino acid protein (Schnölzer et al, 1992).

Chemical synthesis of pro-GnRH/GAP. The methods of fragment condensation and stepwise *in situ* neutralization coupling were applied separately in an attempt to chemically synthesize sufficient amounts of pro-GnRH/GAP protein for structural studies.

It has been shown that the disulfide bridged dimer of GAP is biologically inactive (Abrahamson et al, 1987). Thus, for chemical synthesis, Cys 40 was replaced by Ser to preclude the formation of interchain disulfides during synthesis and purification. Ser shares many of the same physicochemical properties as Cys, and this substitution was not expected to affect the structure of the final purified protein.

A Met residue was added to the N-terminus, immediately before Gln, to prevent rearrangement to pyroglutamic acid (pGlu), and to allow N-terminal sequence analysis of the final product. This Met residue can also function as an internal standard for amino acid compositional analysis. Our intention was to remove Met with BrCN after the synthetic protein was fully characterized, thus forming the native protein structure.

Development of pro-GnRH/GAP immunoassay. We needed an assay that could detect pro-GnRH/GAP in impure mixtures and that could distinguish full length prohormone from cleavage products generated by processing enzymes. Amino acid analysis and N-terminal sequence analysis are useful methods for characterization of pure proteins. However, such experiments usually yield ambiguous results when they are applied to complex peptide mixtures. SDS-PAGE and HPLC are often useful in detecting proteins in complex mixtures. However, SDS-PAGE may not offer sufficient sensitivity to distinguish full length pro-GnRH/GAP from the expected cleavage products (GnRH and GAP). Pro-GnRH/GAP and GAP have similar chain lengths (69 and 56 residues, respectively), and GnRH may not stain sufficiently to be

visible in SDS gels. Unequivocal identification of full length pro-GnRH/GAP by HPLC analysis would require previous knowledge of its retention time obtained through the use of a standard.

We developed an ELISA to identify full length pro-GnRH/GAP and to monitor its proteolysis. A peptide immunogen was synthesized whose sequence spans the processing site and encompasses the entire putative recognition sequence (Figure 7). We postulate that the processing site of pro-GnRH/GAP exists as part of a defined structural feature at the surface of the proprotein. Therefore, antisera raised against the immunogen were expected to recognize intact pro-GnRH/GAP. Further, the immunoactivity of pro-GnRH/GAP was expected to be abolished by proteolysis of the processing site.

Figure 7: Primary amino acid sequence of pro-GnRH/GAP processing site immunogen. The recognition sequence for GAP-releasing enzyme is indicated by underlined bold-face type. The N- and C-termini were acetylated and amidated, respectively, to better represent the structure of this sequence in the intact prohormone. A Cys residue was added to the C-terminus to facilitate haptenization.

Ac-S-Y-**G-L-R-P-G-G-K-R**-D-A-C-NH_2

Materials and Methods

Peptide synthesis. All peptides were synthesized by solid-phase methods (c.f., Stewart and Young, 1984) on a Model 9600 peptide synthesizer (Milligen/Biosearch), a Coupler 1000 semi-automated synthesizer (Vega/Dupont), or a manual bench-top apparatus, using 0.2-1.0 g of the appropriate synthesis resin. Boc synthesis was done on phenylacetamidomethyl (PAM) resin (Advanced Chem Tech; 0.35-0.82 mmol/g resin substitution level). Fmoc synthesis was done on dilute acid susceptible (DASRIN) resins (The Peptide Laboratory; 0.1-0.96 mmol/g substitution level). Diisopropylcarbodiimide (DIC), hydroxybenzotriazole (HOBT), and 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were used as coupling reagents. Dichloromethane (DCM) and dimethylformamide (DMF) were the typical reaction solvents.

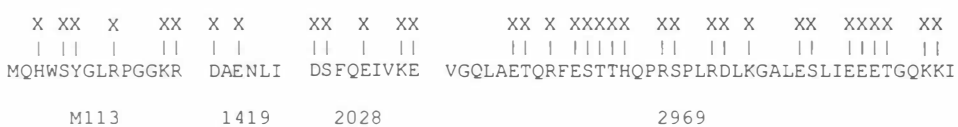
Synthesis of pro-GnRH/GAP by fragment condensation. Total synthesis of pro-GnRH/GAP was attempted using a previously described method for condensation of individual peptide fragments (You et al, 1993b). Several assembly schemes were devised (Figure 8).

Figure 8: Fragment condensation schemes for synthesis of pro-GnRH/GAP. Each fragment is named pGnRH followed by a four digit number which represents the numbers of the first and last residues relative to the primary sequence of full length pro-GnRH/GAP. The N-terminal fragment is named pGnRHM113 to identify the Met residue at the -1 position. Where appropriate, side chain blocking groups are indicated by "X". The blocking groups used were 2-Br-Z (Y), 2-Cl-Z (K), Bzl (D,E,S,T), and Tos (H,R).

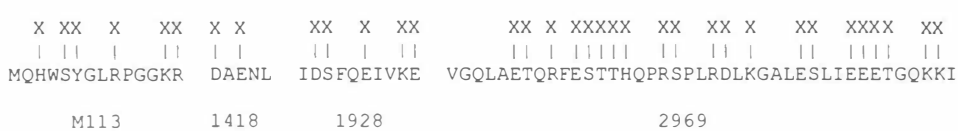
Scheme 1

Scheme 2

Scheme 3



Scheme 4



C-terminal fragments were synthesized by Boc chemistry protocols. The peptide-resin was washed with 0.8 M KSCN in DMF prior to each coupling step to disrupt any secondary structure or intermolecular aggregates. Acetylimidazole (0.3 M) was used to "cap" unextended sequences following each coupling step. Middle and N-terminal fragments were assembled using N(acyl)Fmoc derivatized amino acids whose side chains, where appropriate, were blocked with strong acid labile protecting groups (Advanced Chem Tech). The N-terminal Fmoc groups were intentionally left on these fragments by escaping the normal synthesis protocol prior to the final deprotection step.

Following synthesis, the fully protected Fmoc peptides were cleaved from their synthesis resins using 30% (v/v) TFA-2.5% anisole in DCM (room temperature, 2 hr). 1 mg/ml indole was added to the cleavage cocktail of the N-terminal fragment as a scavenger for Trp. The resin-peptide mixtures were filtered, and the solvents were removed by rotary evaporation under reduced pressure. The residues were washed several times with absolute methanol. Finally, the peptides were precipitated from methanol with diethylether. The peptides were recovered by filtration.

The Fmoc peptides were dissolved in DMF, DMF/DMSO (1:1), or DMSO and converted to their -OBt active esters for coupling to the resin-bound C-terminal peptide. Coupling was attempted using a 2.5-fold molar excess of Fmoc peptide over the resin-bound peptide (room temperature, 2 hr). The progress of the coupling reaction was monitored by ninhydrin assay (Sarin et al, 1981).

Stepwise synthesis of pro-GnRH/GAP with *in situ* neutralization. Synthesis of full length pro-GnRH/GAP was attempted in a stepwise fashion using an adaptation of a recently described Boc chemistry protocol (Schnölzer et al, 1992) in which coupling is accomplished via *in situ* neutralization. For automated synthesis, unique programs were written in the language of the Model 9600 to accomplish the desired protocols.

The N-terminal Boc group was removed with neat TFA or 95% TFA-5% anisole (2 X 1 min). HBTU was used as the primary coupling reagent. Prior to coupling, amino acids (0.4 M) were activated with HBTU (0.38 M) and triethylamine (TEA) (0.75 M) for 2 min. The amount of TEA used was sufficient for the neutralization of the Boc-amino acid plus 2 equivalents relative to the protonated resin. HOBT

was included in an equimolar amount for coupling of Asn and Gln. The synthesis resin was washed with 0.8 M KSCN in DMF between steps to minimize the potential formation of secondary structure or intermolecular aggregates. After conjugation of Gln, the resin was washed with DCM immediately before and after TFA-catalyzed removal of the Boc group to prevent conversion to pyrrolidone carboxylic acid (Schnölzer et al, 1992).

Cleavage, deprotection, and purification of synthetic pro-GnRH/GAP. The peptide was cleaved from the resin with simultaneous removal of all side chain protecting groups following the low-high HF procedure (Tam et al, 1983). First, the peptide-resin was treated with a mixture consisting of anhydrous HF (25%, condensed from the gas), dimethyl sulfide (DMS) (65%), p-cresol (7.5%), and p-thiocresol (2.5%) for 2 hr at 0°C. Then, HF and DMS were removed in vacuo, and the reaction vessel was recharged with anhydrous HF for an additional 45 min at 0°C. HF was removed in vacuo, and the resin/peptide mixture was collected by filtration.

The peptide was extracted from the spent resin with successive washes of methanol, 50% (v/v) methanol in water,

water, and 50% (v/v) acetic acid in water (20 ml per gram resin). The solvent was removed by rotary evaporation under reduced pressure. The peptide was solubilized in water and recovered by lyophilization. The lyophilysate was then redissolved in water and subjected to preparative reverse-phase HPLC.

High performance liquid chromatography (HPLC). Preparative and analytical reversed-phase HPLC was done on a Shimadzu LC-8A (preparative) or LC-6A (analytical) Binary Gradient System. Separations were achieved on C-18 columns which were typically developed in a linear gradient of 0.1% (v/v) TFA in water to 80% (v/v) acetonitrile in 0.1% TFA.

Amino acid compositional analysis. Samples were subjected to acid hydrolysis in 6 N HCl in sealed, evacuated glass tubes for 20 hr at 110°C. Alternatively, samples were subjected to vapor phase acid hydrolysis (Savant AP100 AminoPrep Autohydrolyzer). The hydrolysis mixture was dried (Speedvac) and redissolved in 0.1 N HCl. Samples were analyzed by reversed-phase HPLC (Hewlett Packard AminoQuant System). Amino acids were detected fluorimetrically after derivitization with o-phthaldehyde

(Benson and Hare, 1975). All analyses were performed at Commonwealth Biotechnologies, Inc., Richmond, VA.

N-terminal sequence analysis. Peptide sequences were determined by automated Edman degradation chemistry (Edman and Begg, 1967; Niall, 1973). Samples were loaded on a C-18 sequencing cartridge in 2% TFA in water. The sequence analysis was performed on a Hewlett Packard HP G1005A Protein Sequencer. All analyses were performed at Commonwealth Biotechnologies, Inc., Richmond, VA.

SDS-PAGE. Samples were subjected to SDS-PAGE analysis according to the method of Laemmli (1970). Samples were reconstituted in the appropriate loading buffer (with or without 2-mercaptoethanol), heated at 90°C for 2 min, and subjected to PAGE at 10-20 mA constant current. Proteins were visualized with either Coomassie Brilliant Blue or Silver Stain. The apparent molecular weight was determined by the relative mobility in comparison to molecular weight standards (Integrated Separation Systems).

Preparation of pro-GnRH/GAP antisera. The peptide immunogen was synthesized by solid-phase methods. It was then haptenized to keyhole limpet hemocyanin. Rabbit

antisera raised against this preparation were provided by Commonwealth Biotechnologies, Inc., Richmond, VA.

Pro-GnRH/GAP ELISA. The integrity of the processing site in pro-GnRH/GAP was assessed in proteolyzed and nonproteolyzed samples by ELISA. Individual wells of a microtiter plate were passively coated (2 hr, room temperature) with 50 µl of prohormone preparation diluted in phosphate buffered saline solution (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2). The prohormone samples were removed, and the wells were washed 2 times with PBS. The wells were then blocked (overnight, room temperature) with 300 µl BSA/PBS (3% w/v bovine serum albumin, 0.02% w/v sodium azide in PBS). The blocking solution was removed, and the wells were washed 4 times with PBS.

Rabbit antiserum was typically diluted 1:1,000 in BSA/PBS and 50 µl added to each well. The primary antiserum was removed, and the wells were washed 4 times with PBS. Alkaline phosphatase labelled goat anti-rabbit-IgG (Biosource International) was typically diluted 1:1,000 and 50 µl added to each well (2 hr, room temperature). The

secondary antibody was removed, and the wells were washed 4 times with PBS. The wells were then washed with substrate buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$) and developed with 1 mg/ml p-nitrophenyl-phosphate (Sigma) in substrate buffer (50 µl).

Color development was allowed to proceed for no longer than 1 hr. The optical density of each well was measured as the absorbance at 405 nm minus the absorbance at 650 nm by a microtiter plate reader. The absorbance at 650 nm was subtracted to correct for light scattering.

Results

Synthesis of pro-GnRH/GAP by fragment condensation.

We attempted the total chemical synthesis of pro-GnRH/GAP by condensation of three individual peptide fragments (Figure 8, Scheme 1). Each fragment was synthesized by solid-phase methods as described. The middle (pGnRH1440) and N-terminal (pGnRHM113) fragments were obtained in sufficient yield to allow coupling in excess relative to the resin bound C-terminal (pGnRH4169) peptide.

In an effort to choose a suitable solvent for the condensation reaction, the solubility of fully protected pGnRH1440 was assessed in a number of organic solvents and mixtures (Table 1). This fragment was insoluble in most solvents that are compatible with peptide synthesis. However, the peptide appeared to be freely soluble in DMSO and DMF/DMSO.

We attempted coupling of pGnRH1440 to resin bound pGnRH4169 in DMF/DMSO, and the progress of the reaction was monitored by ninhydrin assay (Sarin et al, 1981). After 2 × 2 hr of coupling, no significant reduction in ninhydrin reactivity was observed, indicating that the resin bound

Table 1

Solubility of pGnRH1440 in peptide synthesis solvents

Solvent	Observation
Dimethylformamide (DMF)	Forms gel
Dichloromethane (DCM)	Insoluble
Chloroform ($CHCl_3$)	Insoluble
Dimethylsulfoxide (DMSO)	Soluble
DMF/DCM (1:1)	Forms gel
DMF/$CHCl_3$ (1:1)	Insoluble
DMF/DMSO (1:1)	Soluble
DCM/$CHCl_3$ (1:1)	Insoluble
DCM/DMSO (1:1)	Slightly soluble
$CHCl_3$/DMSO (1:1)	Slightly soluble

amine had not been acylated. Also, there was no weight gain on the synthesis resin, indicating that coupling was unsuccessful. Amino acid analysis confirmed that only pGnRH4169 was bound to the resin.

When the coupling mixture was removed from the resin, it was visibly turbid; and it became semi-crystalline after two days at room temperature. This indicates that, although fully protected pGnRH1440 was initially soluble in DMF/DMSO, its -OBt active ester falls out of solution with time. Similar results were obtained with neat DMSO.

We attempted to circumvent the problems of solubility by devising new condensation schemes (Figure 8, Schemes 2-4). Elongation of the C-terminal fragment (pGnRH2969) allowed condensation with a shorter-chain middle fragment (pGnRH1428). However, fully protected pGnRH1428 was similarly insoluble in all solvents tested. Therefore, we tried using even smaller fragments (Figure 8, Schemes 3 and 4). Both fully protected fragments pGnRH1419 (Scheme 3) and pGnRH1928 (Scheme 4) were insoluble in all solvents tested. It appeared unlikely that we would solve these solubilty problems; thus, we abandoned this approach in favor of the *in situ* neutralization procedure.

Stepwise synthesis of pro-GnRH/GAP with *in situ* neutralization. Following synthesis, pro-GnRH/GAP was deprotected, cleaved, and extracted from the resin as described (Materials and Methods). The crude peptide product was analyzed by SDS-PAGE before and after purification by reversed-phase HPLC (Figure 9). As shown, a 6-8,000 molecular weight band was visualized in the crude and purified peptide preparations. This molecular weight is consistent with that predicted for the full length peptide (8,009).

The purified peptide preparation was characterized by N-terminal sequence analysis (Table 2). Through 15 cycles of sequence analysis, only the primary sequence of the full length peptide was observed. This result indicates that only the full length peptide possesses a free N-terminus. If any shorter-chain sequences are present, they are presumably acetylated from the capping reaction; thus, they do not sequence.

The purified pro-GnRH/GAP preparation was further characterized by amino acid compositional analysis (Table 3). Here, the single Met residue (at the -1 position of pro-GnRH/GAP) served as a normalization standard against

Figure 9: SDS-PAGE analysis of synthetic pro-GnRH/GAP. Potein samples were subjected to electrophoresis at 15 mA constant current in an 18% SDS-PAGE slab gel. Lane 1 contains protein standards (low range kit, Integrated Separation Systems). Lanes 2 and 3 contain crude and HPLC purified synthetic pro-GnRH/GAP, respectively. Proteins were visualized with siver stain.

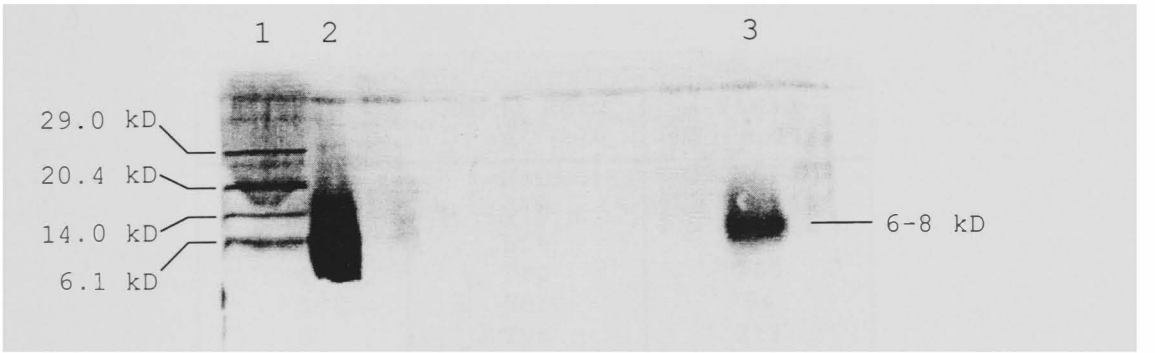

1
2
3
29.0 kD
20.4 kD
14.0 kD
6.1 kD
6-8 kD

Table 2

N-terminal sequence analysis of synthetic pro-GnRH/GAP

Cycle	Amino acid	Yield (pmol)
1	Met	367
2	Gln	317
3	His	307
4	Trp	160
5	Ser	54
6	Tyr	211
7	Gly	179
8	Leu	179
9	Arg	260
10	Pro	152
11	Gly	171
12	Gly	173
13	Lys	100
14	Arg	167
15	Asp	100

Table 3

Amino acid compositional analysis of synthetic pro-GnRH/GAP

Amino acid	Theoretical Res/mol	Actual Res/mol	Actual/ Theoretical
Asx	4	5.6	1.40
Glx	15	24.6	1.64
Ser	5	5.2	1.04
His	2	2.2	1.10
Gly	6	12.4	2.07
Thr	4	7.8	1.95
Ala	3	7.0	2.33
Arg	5	8.9	1.78
Tyr	1	1.3	1.30
Val	2	3.2	1.60
Met	1	1.0	1.00
Trp	1		
Phe	2	3.3	1.65
Ile	4	12.7	3.18
Leu	7	19.1	2.73
Lys	5	12.9	2.58
Pro	3	4.9	1.63

which the molar equivalents of all other residues were calculated. Similarly, the molar amount of this Met residue was assumed to represent the molar amount of full length pro-GnRH/GAP.

As shown, from this analysis, it is clear that termination peptides are present in the preparation. Based on the amount of Met present, it was apparent that our preparation of pro-GnRH/GAP contained 8.7 μmol of full length peptide. This corresponds to approximately 70 mg of intact prohormone, enough for some structural studies. However, the preparation was not sufficiently pure for structural work. Based on the relative abundance of the various amino acids, full length pro-GnRH/GAP was estimated to comprise 50% of the peptidacious material in the preparation. Much greater purity (preferably >95%) would be necessary for structural studies.

Further purification of pro-GnRH/GAP was attempted by isoelectric focusing and by gel flitration, hydrophobic interaction and ion exchange chromatographies. None of these methods yielded significant enrichment of full length pro-GnRH/GAP, as determined by amino acid analysis and analytical HPLC. Although our synthetic preparation of

pro-GnRH/GAP was suitable for immunological and enzymatic characterization, we turned to a recombinant approach in hopes of obtaining material of sufficient purity for structural work.

Immunological characterization of the processing site of synthetic pro-GnRH/GAP. Regardless of the presence of termination sequences, if full length pro-GnRH/GAP is present and its processing site is surface exposed, then the antiserum raised against the processing site immunogen would be expected to react with the peptide preparation. Further, proteolysis of the processing site would be expected to abolish any immunoactivity. Hence, the immunoactivity of the purified preparation was assessed by ELISA before and after endoproteolysis with trypsin.

The preparation was incubated with increasing amounts of trypsin for either 2 or 24 hours and then aliquots of the digest mixture were assayed for immunoactivity. The non-proteolyzed preparation is strongly immunoreactive (Table 4), indicating that the processing site epitope is intact and is surface exposed. After only 2 hours, treatment with as little as 2 µg trypsin (a weight ratio of 1:500 trypsin:peptide) essentially abolished the

Table 4

Immunological characterization of trypsin catalyzed proteolysis of synthetic pro-GnRH/GAP

Weight ratio Trypsin: Pro-GnRH/GAP	Relative immunoactivity(%)	
	2 hr	24 hr
0	100	100
1:500	4.2	3.4
1:200	2.8	2.4
1:100	1.0	0.4
1:50	0.7	0.9
1:20	0.7	0.1
1:10	0.7	0.2

immunoactivity. Thus, the eptopic sequence is subject to degradation by trypsin, consistent with the idea that the endoproteolytic processing site is readily hydrolyzed.

Discussion

Structural analysis of intact prohormones requires their preparation in high yield and purity. Until now, only one intact prohormone has been subjected to structural studies (Weiss et al, 1990). This is primarily due to the fact that intact prohormones have not been readily prepared in sufficient yield and purity for biophysical analysis. We have accomplished the total chemical synthesis of pro-GnRH/GAP, a 69 amino acid prohormone. We obtained synthetic pro-GnRH/GAP in high yield (70 mg) but only marginal purity (50%). Although our preparation is not sufficiently pure for structural studies, we were able to characterize this preparation immunologically and enzymatically. Alternatively, recombinant pro-GnRH/GAP has been prepared in sufficient yield and purity for structural studies, as described in the following section.

Fragment condensation. The solubility of protected peptide chains is often a problem in condensation reactions (Kent, 1988). We encountered such problems in our attempts to assemble pro-GnRH/GAP by the method of fragment condensation. Although we obtained the individual peptide

fragments in high yield, we were unable to effect condensation of these fragments.

After the attempted coupling of fully protected pGnRH1440 to resin bound pGnRH4169 (Figure 8, Scheme 1), no acylation of the resin bound amine was detected by ninhydrin, and no incorporation of pGnRH1440 was detected by amino acid analysis of the resin bound peptide.

The failure of this condensation reaction was likely due to the limited solubility of fully protected pGnRH1440. This fragment exhibited solubilty problems in many organic solvents which are compatible with peptide synthesis (Table 1). Although fully protected pGnRH1440 initially appeared to be freely soluble in DMSO and DMF/DMSO, its active ester falls out of solution with time. It is likely that this fragment aggregates at the high concentration required for peptide synthesis (0.4 M), and thus, becomes unavailable for condensation.

We attempted to solve the solubility problem by designing shorter peptide fragments and using alternative organic solvents. Regardless, we were unable to prepare any peptide fragments of pro-GnRH/GAP which, when fully

protected, are freely soluble in the organic solvents that are compatible with peptide synthesis.

The solubility of protected peptide chains is largely sequence dependent, and only limited methodology is available for the design of freely soluble peptide fragments (Sakakibara, 1995). Due to the empirical nature of these problems and the exhaustive efforts potentially required for their solution, we abandoned the fragment condensation strategy in favor of a novel approach to the stepwise synthesis of pro-GnRH/GAP.

In situ neutralization. We have successfully synthesized full length pro-GnRH/GAP protein using a stepwise solid-phase method which involves coupling of amino acid derivatives via *in situ* neutralization. This procedure has been shown previously to be effective in the preparation of long chain peptides and in reducing the problems associated with difficult sequences (Schnölzer et al, 1992). The stepwise nature of this procedure circumvents the solubility problems often associated with fragment condensation strategies.

After HPLC purification of synthetic pro-GnRH/GAP, SDS-PAGE revealed a single, diffuse major band,

corresponding to the predicted molecular weight of 8,009 Da. Only the target peptide was detected by N-terminal sequence analysis. However, amino acid compositional analysis revealed the presence of a significant number of termination peptides which proved impossible to remove. During the synthesis, unextended sequences were capped with acetylimidazole after coupling of each amino acid derivative. This explains the existence of termination peptides in the preparation.

Capping procedures are routinely used in solid phase peptide synthesis to reduce the occurrence of deletion peptides which cause severe purification problems (Barany and Merrifield, 1980). When capping procedures are used in the synthesis of short chain peptides, termination peptides usually comprise only a small fraction of the preparation. Also, the N-terminal acetyl group on these peptides introduces new physicochemical properties which facilitate their separation from the target peptide.

However, when such procedures are applied to the synthesis of long chain peptides, the resulting preparations may be more complex and more difficult to purify. As chain length increases, so does the number of

coupling steps required for complete chain assembly. Therefore, the potential number and relative abundance of termination sequences resulting from capping procedures increase with peptide chain length. Also, the physicochemical effects of the N-terminal acetyl group probably become less significant as peptide chain length increases. Thus, long termination peptides may be virtually inseparable from the full length target peptide.

Repeated attempts to further purify the synthetic pro-GnRH/GAP preparation by gel filtration, hydrophobic interaction, and ion exchange chromatographies or by isoelectric focusing failed. In all cases, broad peaks were observed on HPLC and the protein was not proven pure by amino acid analysis. Thus, we abandoned further attempts to purify synthetic pro-GnRH/GAP.

Pro-GnRH/GAP ELISA. An immunoassay was developed which is specific for the processing site region of pro-GnRH/GAP protein. A peptide immunogen was designed to span the processing site (R13-D14) and to wholly encompass the recognition sequence for GAP-releasing enzyme (G6-R13). Antisera raised against this immunogen were expected to recognize intact pro-GnRH/GAP by interaction with residues

at or near the processing site. Further, disruption of the processing site epitope by proteolysis was expected to abolish this immunoactivity.

We showed that our preparation of synthetic pro-GnRH/GAP is immunoreactive in the ELISA. This demonstrates that the processing site epitope is intact and that it is exposed on the surface of the protein. We also showed that trypsin digestion of the synthetic pro-GnRH/GAP preparation abolishes its immunoactivity. It is likely that trypsin hydrolyzes the processing site in pro-GnRH/GAP, thus, disrupting the epitope that is recognized in the ELISA.

BACTERIAL EXPRESSION OF RECOMBINANT PRO-GNRH/GAP

Experimental Strategy

As an alternative to chemical synthesis of pro-GnRH/GAP, we considered the feasibility of a recombinant approach to expression of the prohormone. Because prohormones are subject to processing, the anticipated pitfall of overexpression of prohormones is proteolysis by endogenous proteases of the host organism in which the expression vector has been placed. It is not surprising, then, that few reports of successful overexpression of intact prohormone proteins exist.

Thus, in designing an expression system for pro-GnRH/GAP, we needed to address the issue of proteolysis. It may be possible to express intact prohormone proteins in protease deficient bacterial strains or in cell-free translation systems. For example, α-, β-, and γ-preprotachykinins have been expressed in wheat germ and rabbit reticulocyte cell-free systems (MacDonald et al,

1988); but the yields were not sufficient for structural studies. Therefore, we adopted a novel approach to the expression of pro-GnRH/GAP in bacterial cultures. Our strategy involves the coexpression of hepatitis B virus core protein (HBc) and pro-GnRH/GAP as a single fusion protein, with subsequent limited chemical cleavage at a site between the fusion partners to release HBc and intact pro-GnRH/GAP.

Hepatitis B virus (HBV) is a member of a family of hepatotropic animal viruses known as the hepadnaviruses, all of which are characterized by circular DNA genomes that are replicated by reverse transcription (Summers, 1981; Marion and Robinson, 1983). The intact virion consists of a spherical outer envelope and a nucleocapsid, which contains the genomic DNA and reverse transcriptase (for reviews see Tiollais et al, 1985; Ganem and Varmus, 1987).

The major structural component of the HBV nucleocapsid is a 22 kDa core protein (HBc) (Feitelson et al, 1982). This core protein has a nucleic acid-binding domain that exists as an arginine-rich sequence near the carboxyl terminus (Pasek et al, 1979). HBc assembles into a 28 nm

core particle to encapsulate the nuclear components of the virus (Hirschman et al, 1974; Onodera et al, 1982).

Recombinant HBc has been successfully overexpressed in E. coli (Pasek et al, 1979). Recombinant HBc forms core particles even when significant modifications are made to the core protein monomer. A 16 kDa truncated core protein resulting from the deletion of 39 amino acid residues at the carboxyl terminus of HBc is still capable of forming core particles (Birnbaum and Nassal, 1990; Gallin et al, 1989). Also, HBc mutants in which any or all cysteine residues are replaced retain the ability to form core particles (Zheng et al, 1992).

We thought that HBc may still form core particles even if a low molecular weight fusion partner was added to its C-terminus. If this were true, then this fusion protein should be amenable to the relatively simple purification schemes used to purify HBc (Zheng et al, 1992), thus obviating the need for unique procedures. The 3-dimensional structure of the HBc monomer and its orientation within the core particle are not known. However, since the nucleic acid is encapsulated by the core particle in the intact virion, it is logical to assume that

the C-terminal nucleic acid binding domain is oriented toward the interior of the particle. A fusion partner at the C-terminus of HBc might, therefore, be buried within the core particle. If so, the fusion partner might be protected from proteolysis throughout purification of the fusion protein.

Design of HBc-pro-GnRH/GAP fusion protein. We intended to express pro-GnRH/GAP as a carboxyl terminal fusion partner of HBc. However, it was necessary to devise a strategy for separating intact pro-GnRH/GAP from HBc after expression and purification. To this end, we designed the fusion protein to contain a single BrCN sensitive site (Met) between HBc and pro-GnRH/GAP.

Native pro-GnRH/GAP contains no Met residues and, therefore, is not susceptible to BrCN degradation. A Met residue was placed immediately preceeding the amino terminal Gln residue of pro-GnRH/GAP. Thus, treament of the purified HBc-pro-GnRH/GAP fusion protein with BrCN would be expected to yield intact pro-GnRH/GAP and HBc.

On the other hand, HBc contains an initiator Met residue and two internal Met residues. Cleavage of HBc with BrCN would, thus, be expected to yield three low

molecular weight products, any one of which could possibly interfere with purification of pro-GnRH/GAP. It was, therefore, advantageous to use a Met(-) mutant HBc, in which these internal Met residues were replaced by Thr and Ile residues, respectively. A block diagram of the desired fusion protein is shown in Figure 10.

Design of Met(-) HBc-pro-GnRH/GAP expression vector. HBc and various mutants have been expressed successfully from a modified pUC18 vector (pUC18N) (Zheng et al, 1992). A pUC18N plasmid with a Met(-) HBc gene was generously provided by Professor Darrell Peterson (Dept. of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Richmond, VA). The Met(-) HBc gene was situated between NcoI and SacI restriction sites in pUC18N (Figure 11). Extraneous DNA was present between the SacI and HindIII sites. Therefore, the gene for the HBc-pro-GnRH/GAP fusion could be constructed by replacing the SacI-HindIII DNA sequence with a pro-GnRH/GAP pseudogene in the appropriate reading frame (Figure 11).

Design and synthesis of the pro-GnRH/GAP pseudogene. cDNA encoding full length pro-GnRH/GAP is not available, and so a synthetic gene was constructed according to

Figure 10: Schematic diagram of HBc-pro-GnRH/GAP fusion protein. The protein contains a Met(-) mutant HBc sequence in which the two internal Met residues have been replaced by Thr and Ile, respectively. The sequence of pro-GnRH/GAP is located at the C-terminus of the fusion protein. A single internal Met residue is included immediately preceding the pro-GnRH/GAP sequence to facilitate release of the prohormone by BrCN cleavage.

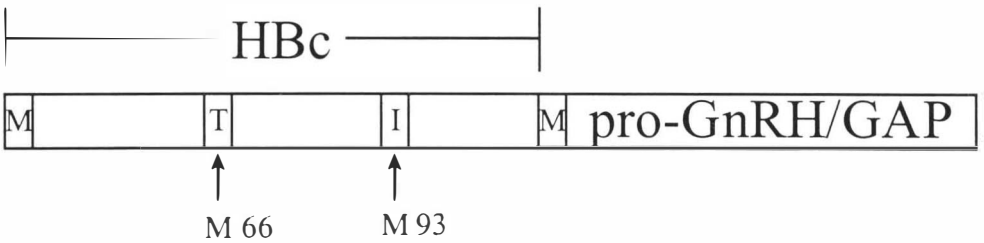
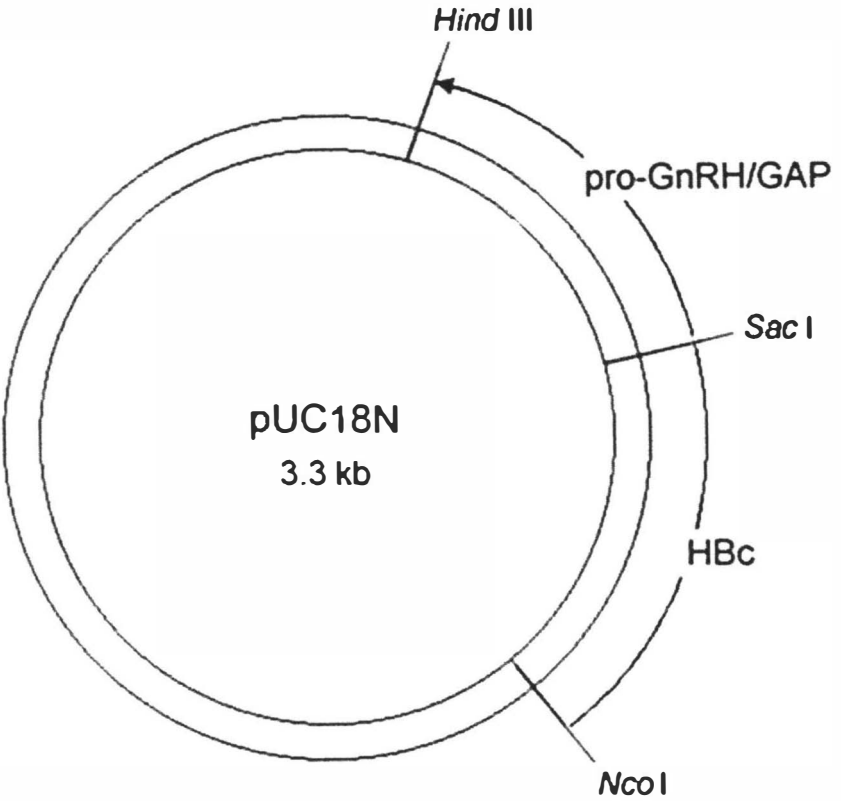

HBc
M
T
I
M
pro-GnRH/GAP
M 66
M 93

Figure 11: Schematic diagram of HBc-pro-GnRH/GAP expression plasmid. pUC18N vector was derived from pUC18 by introducing an NcoI restriction site between the promoter and the multiple cloning site. DNA encoding HBc was subcloned into the vector via the NcoI and SacI restriction sites. The pro-GnRH/GAP pseudogene was subcloned via the SacI and HindIII sites to complete the fusion protein gene construct.


Hind III
pro-GnRH/GAP
Sac I
pUC18N
3.3 kb
HBc
Nco I

recently described methods (Birkett, 1994). In this procedure, double stranded DNA fragments are ligated through appropriate restriction sites to assemble an intact pseudogene. Because expression of the fusion protein would ultimately take place in E. coli, the pro-GnRH/GAP pseudogene was designed such that each amino acid would be encoded by the codon most frequently used by E. coli (Wada et al, 1991). SacI and HindIII restriction sites were included at the appropriate ends of the pro-GnRH/GAP pseudogene to facilitate its ligation into the plasmid. The pseudogene encoding pro-GnRH/GAP is shown in Figure 12.

Double-stranded DNA can be made by PCR extension of overlapping oligonucleotide primers (Rossi et al, 1982), but effective synthesis of such primers is limited to about 100 bases in chain length. Thus, allowing for a 20 base pair annealing region, the upper limit for synthetic DNA fragments is about 180 base pairs. Because the target pro-GnRH/GAP gene has over 200 base pairs, it had to be constructed from two separate DNA fragments. There is a single TaqI restriction site near the midpoint of the target gene sequence (Figure 12); thus, two DNA fragments

Figure 12: DNA and predicted amino acid sequence of pro-GnRH/GAP pseudogene. The synthetic gene encodes full length pro-GnRH/GAP with a Cys40 to Ser substitution and a Met residue added to the N-terminus. A single TaqI restriction site facilitates construction of the pseudogene via ligation of two gene fragments. The coding sequence for pro-GnRH/GAP is bracketed by SacI and HindIII restriction sites to facilitate subcloning; additional nucleotides were included outside the restriction sites to allow digestion by the endonuleases, which are less active on sites at the ends of DNA fragments. The SacI site and pro-GnRH/GAP DNA are juxtaposed such that, after subcloning into the pUC18N HBc vector, the coding sequence of pro-GnRH/GAP will be in the same reading frame as that of HBc. Restriction sites are indicated by underlined bold-face type. The stop codon is indicated by Ø.


SacI
CCCCGAGCTCCATGCAGCATTGGAGCTATGGCCTGCGTCCGGGCGGCAAACGTGATGCGG
M Q H W S Y G L R P G G K R D A E
GGGGCTCGAGGTACGTCGTAACCTCGATACCGGACGCAGGCCCGCCGTTTGCACTACGCC
AAAACCTGATTGATAGCTTTCAGGAAATCGTGAAAGAAGTGGGCCAGCTGGCGGAAACCC
N L I D S F Q E I V K E V G Q L A E T Q
TTTTGGACTAACTATCGAAAGTCCTTTAGCACTTTCTTCACCCGGTCGACCGCCTTTGGG
TaqI
AGCGTTTCGAAAGCACCACCCATCAGCCGCGTAGCCCGCTGCGTGATCTGAAAGGCGCGC
R F E S T T H Q P R S P L R D L K G A L
TCGCAAAGCTTTCGTGGTGGGTAGTCGGCGCATCGGGCGACGCACTAGACTTTCCGCGCG
HindIII
TGGAAAGCCTGATTGAAGAAGAAACCGGCCAGAAAAAAATCTAAGCTTGGGG
E S L I E E E T G Q K K I Ø
ACCTTTCGGACTAACTTCTTCTTTGGCCGGTCTTTTTTTAGATTCGAACCCC

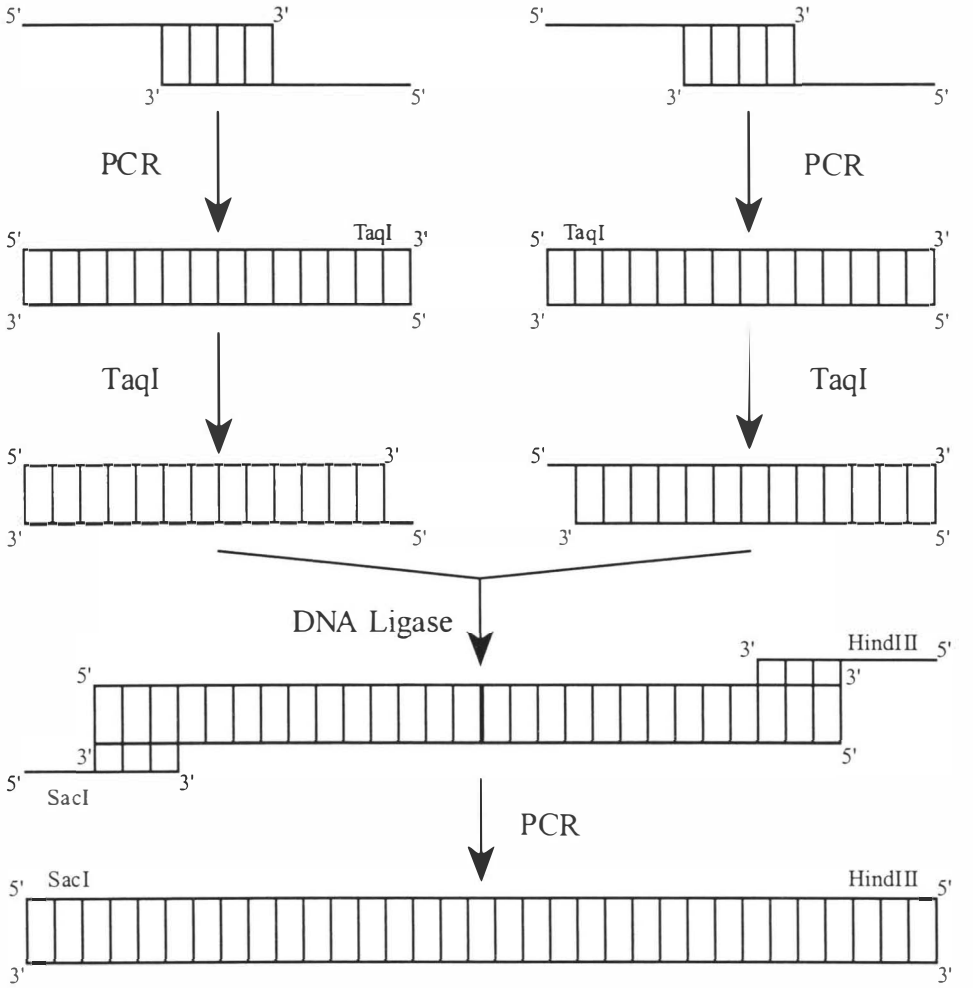
were selected such that ligation at the TaqI site would yield the full length gene.

Materials and Methods

Synthesis of the pro-GnRH/GAP pseudogene. The overall strategy for the synthesis of the pseudogene encoding pro-GnRH/GAP is shown in Figure 13. Two pairs of synthetic oligonucleotide primers were used to construct the two fragments of the pro-GnRH/GAP gene. These primers did not include the SacI and HindIII restriction sites, but these sites were added during PCR amplification of the intact pseudogene. Each pair of primers shared 21 base pairs of overlapping complementary sequence at their respective 3' ends to allow the primers to anneal. Each pair of primers was subjected to five cycles of PCR, during which the DNA became fully double-stranded (Figure 13). No amplification is achieved by this procedure.

The two gene fragments were digested with TaqI (New England Biolabs) restriction endonuclease to create "sticky" ends for ligation (Figure 13). Both fragments were digested in a single reaction held at 65°C for 2 hours. The two fragments were then ligated together using T4 DNA ligase (New England Biolabs) at 15°C for 16 hours.

Figure 13: Strategy for synthesis of pro-GnRH/GAP pseudogene. Double stranded gene fragments were made by PCR extension (5 cycles) of overlapping synthetic oligonucleotide primers. The fragments were digested with TaqI and ligated. The intact pseudogene was amplified and the SacI and HindIII restriction sites simultaneously added by 35 cycles of PCR.


5'
3'
PCR
TaqI
TaqI
DNA Ligase
HindIII
SacI
PCR
SacI
HindIII

Two short oligonucleotide primers were prepared which would anneal to the 3' ends of the two strands of the pro-GnRH/GAP gene, respectively. These primers contained extra unpaired nucleotides, encompassing the respective SacI and HindIII restriction sites (Figure 13). The pro-GnRH/GAP gene was then amplified, and the restriction sites were simultaneously added by 35 cycles of PCR.

Preparation of Met(-) HBc-pto-GnRH/GAP expression vector. pUC18N expression plasmid containing the Met(-) HBc gene and the pro-GnRH/GAP pseudogene were digested separately with SacI and HindIII restriction endonucleases at 37°C for 2 hours. The pro-GnRH/GAP gene construct was then ligated into the plasmid with T4 DNA ligase (15°C, 16 hr) at a 10:1 ratio of insert to plasmid.

The ligation mixture was used directly to transform TB1 E. coli competent cells. Transformants were grown on agar supplemented with 50 μg/ml ampicillin, and individual colonies were screened for the insert. Plasmid mini-preps (see DNA Purification) from each colony were subjected to digestion by NcoI and HindIII restriction endonucleases, which was predicted to yield two DNA fragments of 2.6 kbp and 720 bp, respectively. Plasmids yielding digestion

products with the expected chain lengths were subjected to DNA sequence analysis to ensure proper selection of clones. A large-scale plasmid preparation was generated from a single clone containing the correct Met(-)HBc-pro-GnRH/GAP gene sequence.

The DNA sequence and corresponding protein translation of the Met(-)HBc-PG gene is shown in Figure 14. In the protein, Met66 of HBc is changed to Thr, Met93 to Ile, and Cys107 to Ala, and Leu108 to Val. Further, HBc is encoded with a 19 residue C-terminal deletion and a bridge of 5 extraneous amino acids (INSSS) at its C-terminus. A Met residue is located between the fusion partners. In the sequence of pro-GnRH/GAP, Cys40 is changed to Ser to preclude the possibility of unwanted disulfide bond formation.

Expression and purification of Met(-)HBc-pro-GnRH/GAP fusion protein. Protocols for expression and purification were the same as those used previously for HBc (Zheng et al, 1992). The pUC18N Met(-)HBc-pro-GnRH/GAP plasmid preparation was used in a fresh transformation of TB1 E. coli and grown in medium containing 10 g casein enzymatic hydrolysate, 10 g yeast extract, 5 g NaCl, 1 g glucose, and

Figure 14: DNA and predicted amino acid sequence of HBc-pro-GnRH/GAP construct. The fusion construct encodes HBc with four amino acid substitutions (Met66 to Thr, Met93 to Ile, Cys107 to Ala, and Leu108 to Val) and a 19 residue C-terminal deletion, a bridge of five extraneous amino acids (INSSS), a single internal Met residue, and pro-GnRH/GAP with a single amino acid substitution (Cys40 to Ser). Restriction sites are indicated by underlined bold-face type. The stop codon is indicated by Ø.

NcoI

CCATGGACATCGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTCGTTTTTGC
M D I D P Y K E F G A T V E L L S F L P
GGTACCTGTAGCTGGGAATATTTCTTAAACCTCGATGACACCTCAATGAGAGCAAAAACG

CTTCTGACTTCTTTCCTTCAGTACGAGATCTTCTAGATACCGCCTCAGCTCTGTATCGAG
S D F F P S V R D L L D T A S A L Y R E
GAAGACTGAAGAAAGGAAGTCATGCTCTAGAAGATCTATGGCGGAGTCGAGACATAGCTC

AAGCCTTAGAGTCTCCTGAGCATTGTTCACCTCACCATACTGCACTCAGGCAAGCAATTC
A L E S P E H C S P H H T A L R Q A I L
TTCGGAATCTCAGAGGACTCGTAACAAGTGGAGTGGTATGACGTGAGTCCGTTCGTTAAG

TTTGCTGGGGGGAGTTAACCACTCTAGCTACCTGGGTGGGTGTTAATTTGGAAGATCCAG
C W G E L T T L A T W V G V N L E D P A
AAACGACCCCCCTCAATTGGTGAGATCGATGGACCCACCCACAATTAAACCTTCTAGGTC

CGTCTAGAGACCTAGTAGTCAGTTATGTCAACACTAATATAGGCCTAAAGTTCAGGCAAC
S R D L V V S Y V N T N I G L K F R Q L
GCAGATCTCTGGATCATCAGTCAATACAGTTGTGATTATATCCGGATTTCAAGTCCGTTG

TCTTGTGGTTTCACATTTCTGCAGTCACTTTTGGAAGAGAAACAGTTATAGAGTATTTGG
L W F H I S A V T F G R E T V I E Y L V
AGAACACCAAAGTGTAAAGACGTCAGTGAAAACCTTCTCTTTGTCAATATCTCATAAACC

TGTCTTTCGGAGTGTGGATTCGCACTCCTCCAGCTTATAGACCACCAAATGCCCCTATCC
S F G V W I R T P P A Y R P P N A P I L
ACAGAAAGCCTCACACCTAAGCGTGAGGAGGTCGAATATCTGGTGGTTTACGGGGATAGG

TATCAACACTTCCGGAGACTACTGTTGTTAGACGACGAGGCAGGTCCCCTAGAATTAATT
S T L P E T T V V R R R G R S P R I N S
ATAGTTGTGAAGGCCTCTGATGACAACAATCTGCTGCTCCGTCCAGGGGATCTTAATTAA

SacI

C**GAGCTC**CATGCAGCATTGGAGCTATGGCCTGCGTCCGGGCGGCAAACGTGATGCGGAAA
S S M Q H W S Y G L R P G G K R D A E N
G**CTCGAG**GTACGTCGTAACCTCGATACCGGACGCAGGCCCGCCGTTTGCACTACGCCTTT

ACCTGATTGATAGCTTTCAGGAAATCGTGAAAGAAGTGGGCCAGCTGGCGGAAACCCAGC
L I D S F Q E I V K E V G Q L A E T Q R
TGGACTAACTATCGAAAGTCCTTTAGCACTTTCTTCACCCGGTCGACCGCCTTTGGGTCG

GTTTCGAAAGCACCACCCATCAGCCGCGTAGCCCGCTGCGTGATCTGAAAGGCGCGCTGG
F E S T T H Q P R S P L R D L K G A L E
CAAAGCTTTCGTGGTGGGTAGTCGGCGCATCGGGCGACGCACTAGACTTTCCGCGCGACC

HindIII

AAAGCCTGATTGAAGAAGAAACCGGCCAGAAAAAAATCT**AAGCTT**
S L I E E E T G Q K K I ∅
TTTCGGACTAACTTCTTCTTTGGCCGGTCTTTTTTTAGA**TTCGAA**

50 mg ampicillin per liter. An 80 L culture was grown in a manually constructed fermentation system. Cells were harvested at 42 hours after innoculation, and 340 g of wet cell mass was recovered.

The cells were sheared under high pressure in 2 L of distilled water, using a continuous flow microfluidizer (Avestin). Insoluble debris was removed by centrifugation (26,000 × g, 1 hr). Solid ammonium sulfate was added to 45% saturation (277 g/L), and the protein precipitate was recovered by centrifugation (10,000 × g for 30 min). The precipitate was redissolved in 800 ml and dialyzed against two changes of distilled water (10 L) at 4°C. The dialysate was clarified by centrifugation (10,000 × g for 1 hour) and applied to a 1.2 L column of hydroxyapatite, prepared according to standard methods (Jenkins, 1962), and pre-equilibrated in 50 mM phosphate buffer pH 6.8. The fusion protein was eluted in 7 L of equilibration buffer and was precipitated with ammonium sulfate as before.

The protein was then dissolved in approximately 250 ml distilled water and applied to a 6 L Sepharose CL-4B column which was pre-equilibrated in 50 mM phosphate buffer pH

6.8. Elution of the protein was monitored by measuring the absorbance at 280 nm of individual fractions (25 ml). Peak fractions were assayed for the presence of HBc by ELISA using rabbit antiserum (Zheng et al, 1992). The peak fractions were pooled and lyophilized. The lyophilysate was redissolved and dialyzed against distilled water. Finally, the fusion protein was recovered by lyophilization.

Immunological determination of HBc-pro-GnRH/GAP and pro-GnRH/GAP concentration. The HBc-pro-GnRH/GAP fusion protein concentration was estimated using the anti-HBc ELISA (Zheng et al, 1992). We assumed that anti-HBc antibodies bind HBc and HBc-pro-GnRH/GAP with equal affinity and that all HBc immunoactivity in the HBc-pro-GnRH/GAP samples corresponds to intact fusion protein.

Serial dilutions of HBc-pro-GnRH/GAP solutions and of a 1.4 mg/ml standard HBc solution were analyzed simultaneously. The optical density of the standard HBc samples was used to construct a standard curve from which the HBc concentration (mg/ml) in HBc-pro-GnRH/GAP samples was estimated. The approximate molecular weights of HBc-pro-GnRH/GAP, HBc, and pro-GnRH/GAP are 26, 18, and 8 kDa,

respectively. Thus, the molecular weight ratios of HBc:HBc-pro-GnRH/GAP (18:26) and HBc:pro-GnRH/GAP (18:8) were used to estimate the concentration (mg/ml) of HBc-pro-GnRH/GAP and pro-GnRH/GAP, respectively.

Release and purification of intact pro-GnRH/GAP. The HBc-pro-GnRH/GAP fusion protein was treated with BrCN to separate the fusion partners. The fusion protein was dissolved in 70% (v/v) formic acid at a concentration of 30 mg/ml. BrCN was added to a 1000 fold molar excess over Met residues, assuming two Met residues per molecule and 100% purity of the fusion protein. The reaction was allowed to proceed in the dark for 24 hours at room temperature, after which the solution was diluted 10 fold with distilled water, and lyophilized. The extent of reaction was monitored by SDS-PAGE.

Following digestion with BrCN, the solubility of the components of the reaction mixture was investigated using successive extractions with water, 50 mM phosphate buffer, pH 6.8, and SDS-PAGE sample buffer (2%[w/v] SDS, 10%[v/v] glycerol, 5%[v/v] 2-mercaptoethanol in 62.5 mM Tris buffer, pH 6.8). The protein components in each extract were determined by SDS-PAGE.

Intact pro-GnRH/GAP was separated from HBc and other reaction byproducts by extraction with phosphate buffer. The lyophilysate containing the cleavage products was suspended in 50 mM phosphate buffer, pH 6.8 and stirred overnight at room temperature. Insoluble products were removed by centrifugation, and the successful extraction of pro-GnRH/GAP was confirmed by SDS-PAGE.

The phosphate extract containing pro-GnRH/GAP was then lyophilyzed, dissolved in distilled water, and subjected to preparative reversed-phase HPLC. The purity of the HPLC fractions was assessed by SDS-PAGE, analytical reversed-phase HPLC, and electrospray mass spectrometry (ES/MS). Fractions containing pure pro-GnRH/GAP were pooled and lyophilized.

Western blot analysis. The HBc-pro-GnRH/GAP fusion protein and the products obtained after BrCN cleavage were visualized by Western blot analysis. Samples were subjected to SDS-PAGE (18%) and electroblotted onto PVDF membranes. Protein transfer was done at room temperature for 1 hr at a constant current of 2.5 mA per cm^2 membrane. Proteins were fixed to the membrane with 10% (v/v) formaldehyde in water, washed with PBS, and blocked with

5% (w/v) dry milk in PBS. The membrane was then incubated overnight, at room temperature, with the appropriate antibody (anti-pro-GnRH/GAP or horseradish peroxidase conjugated anti-HBc). Each primary antibody was diluted 1:2000 in PBS.

Anti-HBc blots were developed with 0.03%(v/v) hydrogen peroxide, 0.6 mg/ml chloro-naphthol (Sigma), 20%(v/v) methanol in PBS. Anti-pro-GnRH/GAP blots were blocked as before, washed with PBS, and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (Biosource International) diluted 1:2000. The blot was washed with PBS followed by 5 mM $MgCl_2$, 100mM Tris, pH 9.5, prior to development with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma).

Preparation of synthetic oligonucleotides.

Oligonucleotide primers were synthesized by the VCU Nucleic Acid Core Facility. Long primers used to generate individual fragments of the pro-GnRH/GAP pseudogene were purified by SDS-PAGE. The DNA was eluted from the gel with 0.5 M sodium acetate and desalted on a commercially available Sephadex G-25 column (NAP-10, Pharmacia). All primers were dried and redissolved in distilled water. The

extinction coefficient for each primer was calculated based on those of the individual bases (A:16,000; G:12,000; C:7,000; T:9,600 $M^{-1}cm^{-1}$). Thus, the concentration of each primer solution was determined from its absorbance at 260 nm.

Polymerase chain reaction (PCR). Each cycle of PCR consists of melting (94°C, 1 min), annealing (55°C, 2 min), and extension (72°C, 30 sec). PCR reactions were done in a volume of 100 µl containing 200 µM dNTP's, Vent buffer, and 1 unit of Vent DNA polymerase (all from New England Biolabs). PCR extension of long primers representing the two fragments of the pro-GnRH/GAP gene was done at an oligonucleotide concentration of 250 nM. Amplification of the intact pro-GnRH/GAP gene was done at an oligonucleotide concentration of 1.5 µM.

DNA purification. DNA products of PCR reactions and restriction digestions were routinely purified by agarose (0.8-2.0%) gel electrophoresis. DNA was recovered by excision of the appropriate bands and subsequent extraction using a commercially available kit (Qiagen). Miniature (mini-prep) and large scale plasmid preparations were made

using a commercially available plasmid isolation kit (Qiagen).

Results

Expression and purification of HBc-pro-GnRH/GAP fusion protein. The HBc-pro-GnRH/GAP fusion was expressed in transformed E. coli and purified as described. Approximately 340 g wet cell mass was harvested from 80 L bacterial culture, and the crude cell lysate was strongly immunoreactive to anti-HBc ELISA.

After ammonium sulfate precipitation and dialysis, the fusion protein yield was determined to be approximately 2.4 g by ELISA. This corresponds to an expression level of 30 mg/L culture and is consistent with normal HBc expression levels in this system (Zheng et al, 1992). Assuming that all fusion protein molecules were intact, the pro-GnRH/GAP content within the fusion protein was estimated to be 740 mg.

The fusion protein solution was subjected to hydroxyapatite chromatography, concentrated, and subsequently subjected to Sepharose CL-4B chromatography, as described. The absorbance at 280 nm of each fraction was measured, and peak fractions were subjected to anti-HBc ELISA. The bulk of the UV absorbance and HBc

immunoactivity resides in the large middle peak (Figure 15). This elution profile is very similar to that of HBc (Zheng et al, 1992) and is, thus, consistent with the formation of core particles.

Under reducing conditions, HBc migrates as monomers on SDS-PAGE (Zheng et al, 1992). SDS-PAGE of the middle peak fractions (Figure 15) revealed a single major band corresponding to a molecular weight of approximately 26 kDa, which is the predicted molecular weight of HBc-pro-GnRH/GAP (Figure 16).

These fractions were combined and the pool was subjected to anti-HBc ELISA. This revealed an HBc-pro-GnRH/GAP content of about 1460 mg, which corresponds to about 450 mg of pro-GnRH/GAP. Interestingly, HBc-pro-GnRH/GAP was found to be immunoreactive to anti-pro-GnRH/GAP ELISA, showing that the pro-GnRH/GAP processing site epitope is exposed on the surface of the core particle.

Release and purification of intact pro-GnRH/GAP. The HBc-pro-GnRH/GAP fusion was treated with BrCN as described, and the progress of the reaction was monitored by SDS-PAGE (Figure 17). After 24 hours, the 26 kDa band was nearly

Figure 15: Sepharose CL-4B chromatography of HBc-pro-GnRH/GAP fusion protein. The fusion protein preparation obtained after hydroxyapatite chromatography was applied to a Sepharose CL-4B column developed in 50 mM phosphate buffer, pH 6.8. The absorbance of each fraction (25 ml) was measured at 280 nm (line graph). Selected fractions (indicated by arrows) were subjected to anti-HBc ELISA (bar graph). Fractions containing the peaks of absorbance and immunoactivity (58-110, indicated by the bracketed line) were pooled.

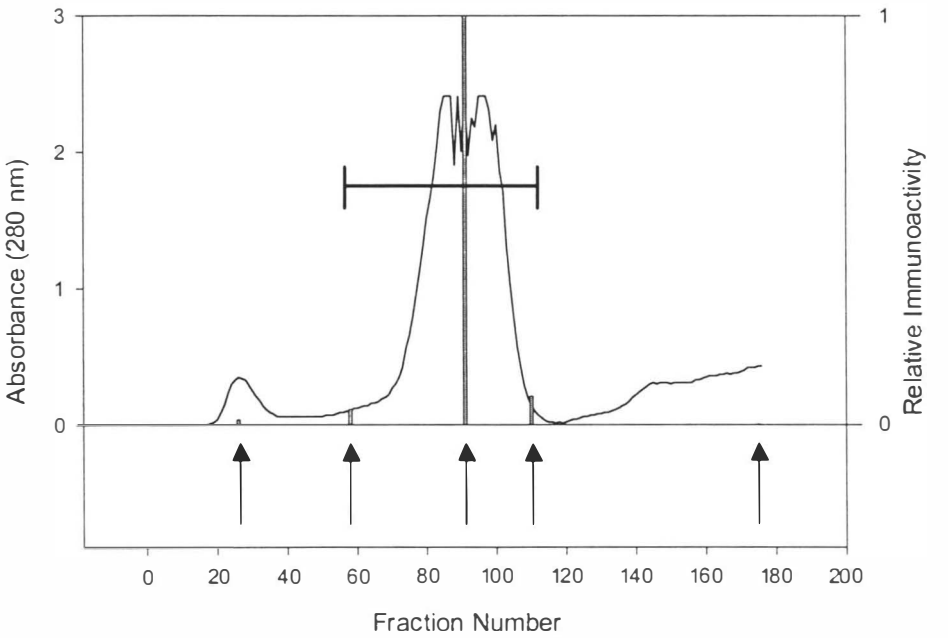

Absorbance (280 nm)
Relative Immunoactivity
Fraction Number
0
1
2
3
0
20
40
60
80
100
120
140
160
180
200

Figure 16: SDS-PAGE analysis of Sepharose CL-4B chromatography fractions. Individual fractions under the peak of absorbance and immunoactivity in the Sepharose CL-4B elution profile (Figure 15) were analyzed by SDS-PAGE. Samples were subjected to electrophoresis at 20 mA constant current in an 18% slab gel. Lane 1 contains protein standards (low range kit, Integrated Separation Systems). Lanes 2-8 contain Sepharose CL-4B fractions 58, 67, 76, 84, 92, 101, and 110, respectively. Proteins were visualized with Coommassie stain.

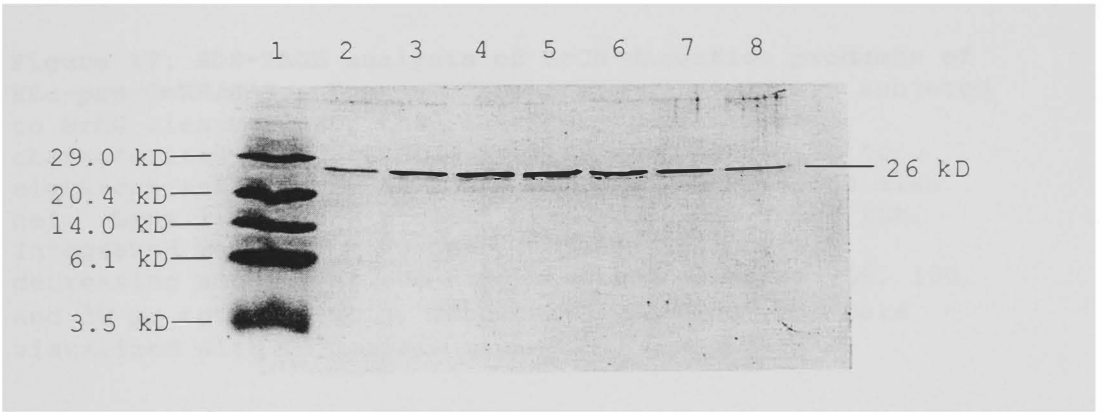
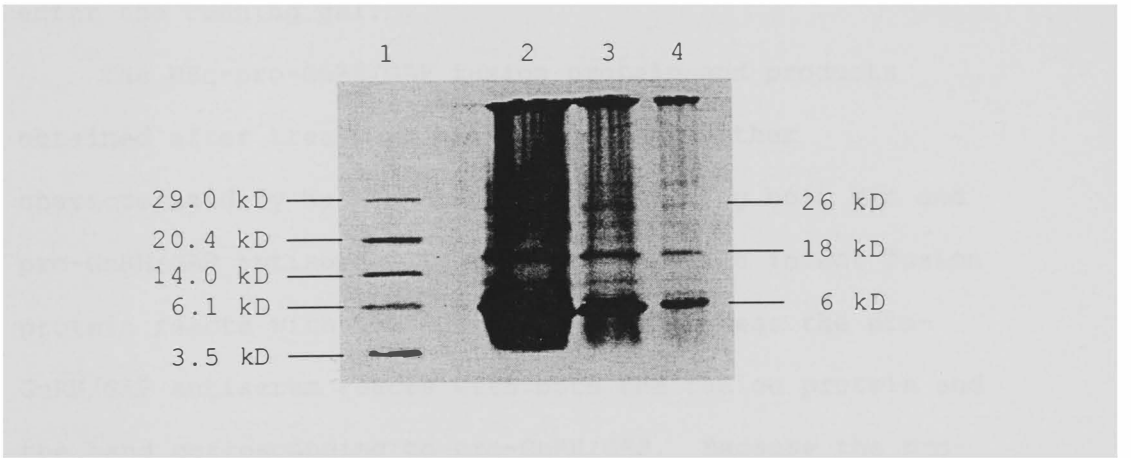

1
2
3
4
5
6
7
8
29.0 kD
20.4 kD
14.0 kD
6.1 kD
3.5 kD
26 kD

Figure 17: SDS-PAGE analysis of BrCN digestion products of HBc-pro-GnRH/GAP. The purified fusion protein was subjeted to BrCN cleavage, and the reaction products were characterized by SDS-PAGE. Samples were subjected to electrophoresis at 10 mA constant current in an 18% slab gel. Lane 1 contains protein standards (low range kit, Integrated Separation Systems). Lanes 2-4 contain decreasing amounts of the BrCN reaction mixture (350, 100, and 30 µg total protein, respectively). Proteins were visualized with Coommassie stain.


1
2
3
4
29.0 kD
20.4 kD
14.0 kD
6.1 kD
3.5 kD
26 kD
18 kD
6 kD

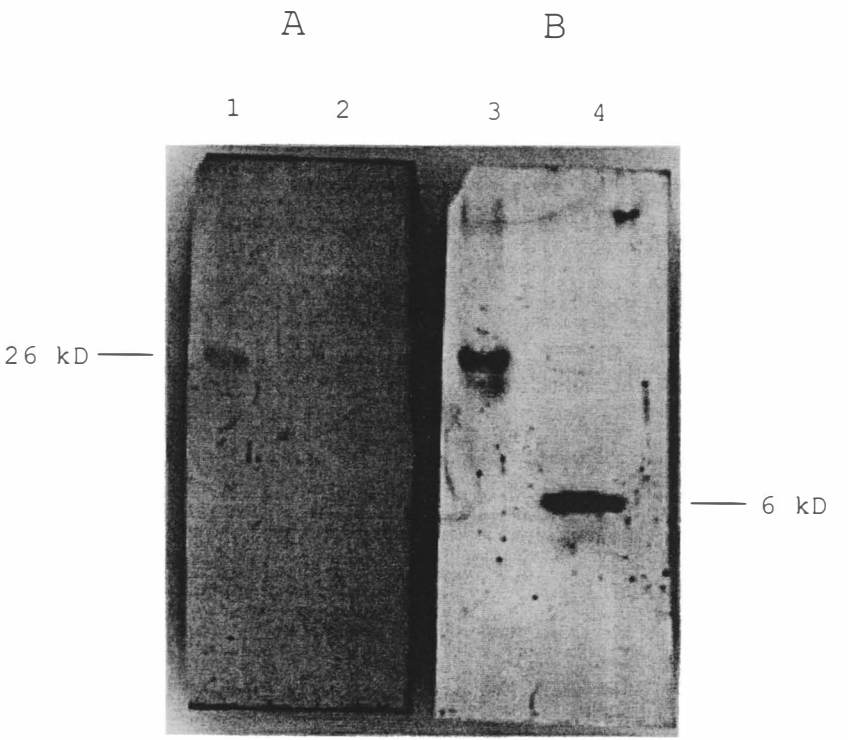
depleted and a new 6 kDa band, representing pro-GnRH/GAP, appeared, suggesting that the reaction is nearly complete.

An 18 kDa band, representing HBc, was also observed, but at much lower intensity than the pro-GnRH/GAP band. However, a substantial amount of Coomassie stained material remained at the top of the gel. It is possible that a large portion of cleaved HBc forms aggregates which do not enter the running gel.

The HBc-pro-GnRH/GAP fusion protein and products obtained after treatment with BrCN were further characterized by Western blot analyses using both HBc and pro-GnRH/GAP antisera (Figure 18). Only the intact fusion protein reacts with the HBc antiserum, whereas the pro-GnRH/GAP antiserum reacts with both the fusion protein and the band corresponding to pro-GnRH/GAP. Because the pro-GnRH/GAP antiserum was raised against a peptide immunogen that encompasses the pro-GnRH/GAP processing site, this finding shows that the recombinant prohormone obtained after BrCN treatment retains an intact processing site.

We investigated the separation of pro-GnRH/GAP and HBc from the BrCN reaction mixture using solvent extraction. A small portion of the BrCN digest mixture was extracted

Figure 18: Western blot analysis of BrCN digestion products of HBc-pro-GnRH/GAP. The intact fusion protein (lanes 1 and 3) and the products obtained after BrCN cleavage (lanes 2 and 4) were visualized by Western blot analysis. Proteins were subjected to SDS-PAGE (18% acrylamide) and electroblotted onto PVDF membranes. (A) Anti-HBc immunoblot. The blot was incubated with horseradish peroxidase conjugated anti-HBc rabbit serum and developed with chloro-naphthol (Sigma). B) Anti-pro-GnRH/GAP immunoblot. The blot was incubated sequentially with anti-pro-GnRH/GAP rabbit serum and alkaline phosphatase conjugated goat anti-rabbit IgG (Biosource International) and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma).


A
B
1
2
3
4
26 kD
6 kD

first with distilled water and then with phosphate buffer. The residual material was found to be soluble in SDS-PAGE sample buffer. All samples were analyzed by SDS-PAGE (Figure 19). The water extract contained no protein, but the phosphate extract contained a single band at 6-8 kDa. The remaining material contained two major bands at 26 and 18 kDa, corresponding to intact HBc-pro-GnRH/GAP fusion protein and to HBc, respectively. Thus, phosphate buffer was used to extract pro-GnRH/GAP from the reaction mixture.

The phosphate extract of pro-GnRH/GAP was desalted by preparative reverse-phase HPLC as shown (Figure 20). Individual HPLC fractions were analyzed by ES/MS (Figure 21). A single major peak at 7860 atomic mass units was observed in all HPLC fractions 52 through 55. Assuming cyclization of the N-terminal Gln residue to pGlu, the observed mass is in exact agreement with the calculated molecular weight of pro-GnRH/GAP. By virtue of the single peak, we can conclude that pro-GnRH/GAP was purified to homogeneity and was obtained as the intact proprotein.

HPLC fractions 52-55 were pooled and lyophilized, and 187 mg of pure prohormone was recovered. We attempted to confirm our results by N-terminal sequence analysis, but

Figure 19: SDS-PAGE analysis of extracts from the BrCN reaction. Following BrCN cleavage of the HBc-pro-GnRH/GAP fusion protein, the reaction products were successively extracted with water (lane 2), phosphate buffer (lane 3), and the residual material was solubilized in SDS-PAGE sample buffer (lane 4). Lane 1 contains protein standards (low range kit, Integrated Separation Systems). Samples were subjected to electrophoresis at 10 mA constant current in an 18% slab gel. Proteins were visualized with Coomassie stain.

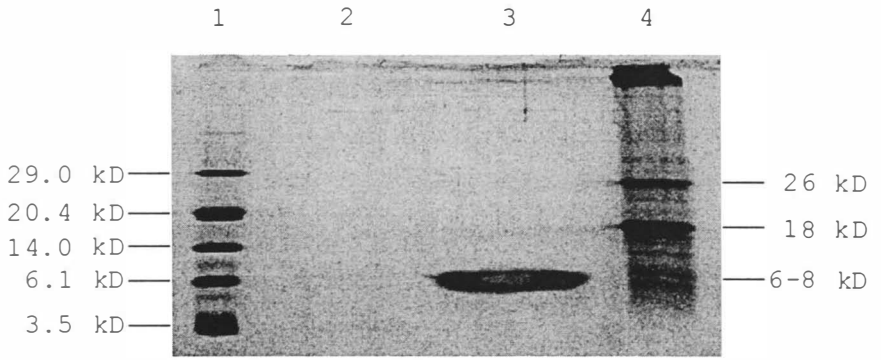
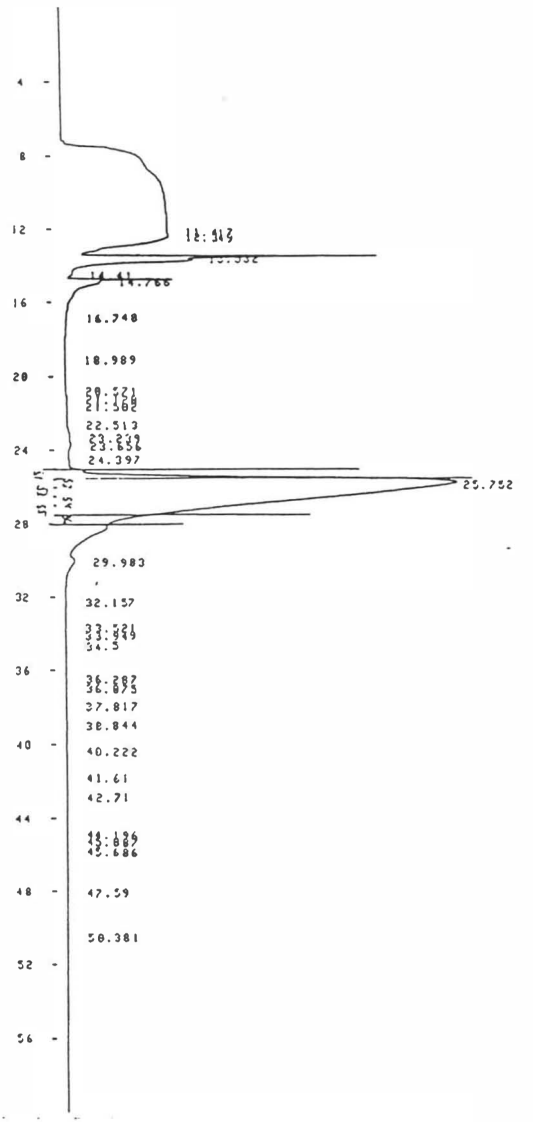

1
2
3
4
29.0 kD
20.4 kD
14.0 kD
6.1 kD
3.5 kD
26 kD
18 kD
6-8 kD

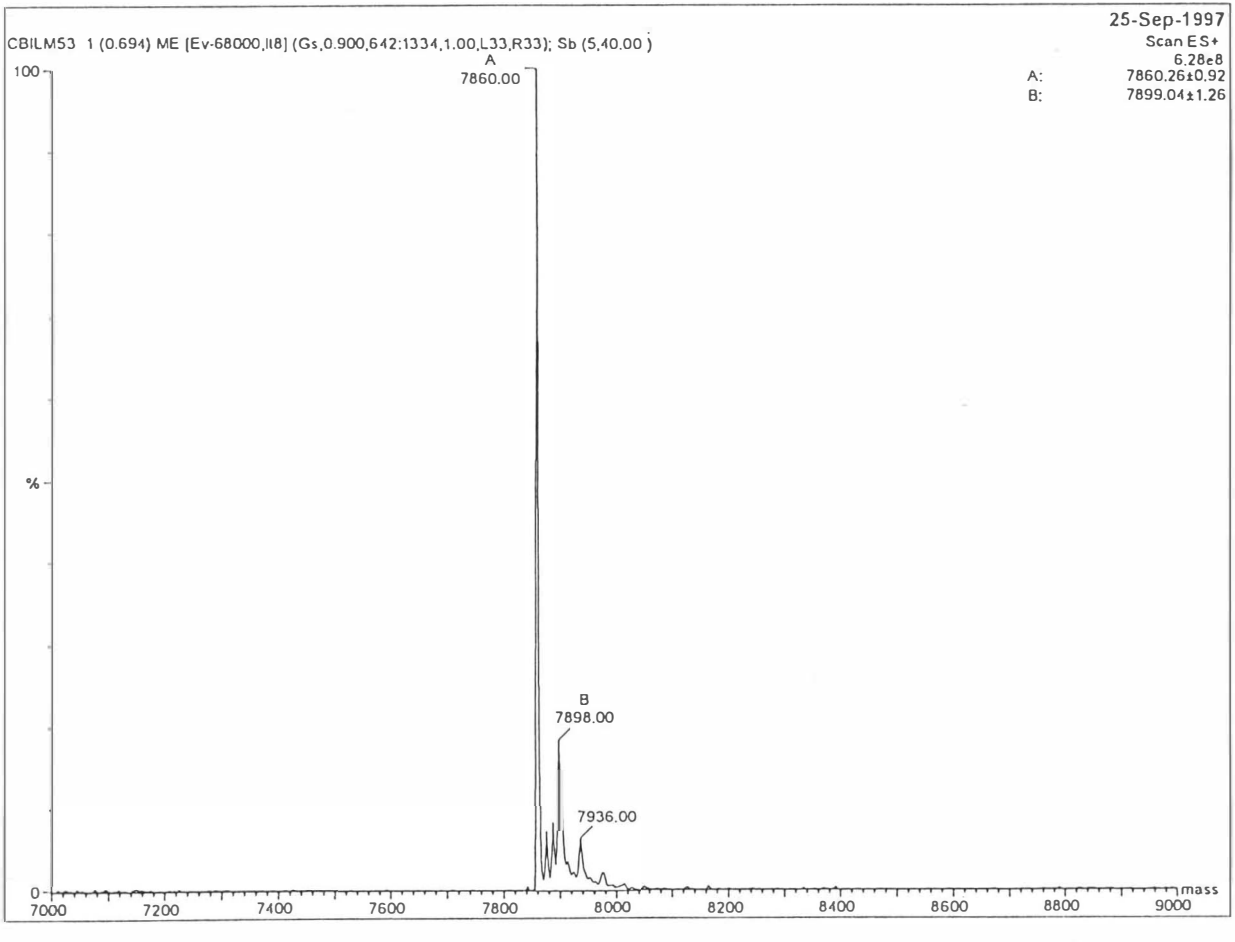
Figure 20: Preparative reverse-phase HPLC of recombinant pro-GnRH/GAP. Following extraction from the BrCN reaction mixture into phosphate buffer, pro-GnRH/GAP was purified by preparative reverse-phase HPLC. The protein was loaded on a C-18 column and eluted in a binary gradient from 20% to 80% solvent B (0.1% TFA, 80% acetonitrile in water) in solvent A (0.1% TFA in water). The absorbance at 220 nm was recorded and 0.5 min fractions were collected. Fractions 51-56 were analyzed by mass spectrometry (see Figure 21), and fractions 52-55 were subsequently pooled, as indicated by the bracketed line.

Absorbance (280 nm)



Retention Time (min)

Figure 21: Mass spectrum of HPLC purified recombinant pro-GnRH/GAP. HPLC fraction numbers 51-56 (Figure 20) were analyzed by electrospray mass spectrometry (ES/MS). The mass spectrum of fraction number 53 is shown.


CBILM53 1 (0.694) ME [Ev-68000,li8] (Gs,0.900,642:1334,1.00,L33,R33); Sb (5,40.00)
25-Sep-1997
Scan ES+
6.28e8
A: 7860.26±0.92
B: 7899.04±1.26
A
7860.00
B
7898.00
7936.00
100
%
0
7000
7200
7400
7600
7800
8000
8200
8400
8600
8800
9000
mass

the protein was not susceptible to Edman degradation. This is consistent with cyclization of the N-terminal Gln residue to pGlu.

The pure pro-GnRH/GAP preparation was subjected to amino acid analysis and was found to contain the predicted amino acid composition (Table 5). This confirms that the final preparation was homogeneous for the full length prohormone.

Table 5

Amino acid compositional analysis of recombinant pro-GnRH/GAP

Amino acid	Theoretical Res/mol	Actual Res/mol	Actual/ Theoretical
Asx	4	4.28	1.07
Glx	15	14.66	0.98
Ser	5	4.38	0.88
His	2	1.64	0.82
Gly	6	5.55	0.92
Thr	4	3.86	0.96
Ala	3	3.08	1.03
Arg	5	4.86	0.97
Tyr	1	1.10	1.10
Val	2	1.42	0.71
Trp	1	0.78	0.78
Phe	2	1.97	0.98
Ile	4	3.23	0.81
Leu	7	7.33	1.05
Lys	5	4.59	0.92
Pro	3	2.52	0.84

Discussion

Structural analysis of intact prohormone precursor proteins has seldom been reported, primarily due to the lack of methods for the routine preparation of prohormones in high yield and purity. We were able to chemically synthesize intact pro-GnRH/GAP in high yield, but the purity of our synthetic preparation was not sufficient for structural work.

Thus, we developed a unique strategy for the bacterial expression of recombinant pro-GnRH/GAP. This strategy involved the expression of pro-GnRH/GAP as a C-terminal fusion partner of HBc. This permitted purification of the fusion protein by the same techniques used for wild type HBc. After purification, the fusion protein was chemically cleaved with BrCN to yield intact pro-GnRH/GAP and HBc. Pro-GnRH/GAP was separated from HBc in a single purification step by solvent extraction.

The HBc-pro-GnRH/GAP fusion protein was expressed at a level of approximately 30 mg/L bacterial culture. This corresponds to an expression level of about 21 mg/L for HBc alone, which is commensurate with normal HBc expression

levels in this system (Zheng et al, 1992). Thus, the incorporation of pro-GnRH/GAP as a C-terminal fusion partner did not adversely affect the expression of the carrier protein.

We thought that the addition of a low molecular weight fusion partner at the C-terminus of HBc would not preclude the formation of core particles. Therefore we attempted to purify HBc-pro-GnRH/GAP according to the procedure normally applied to HBc purification (Zheng et al, 1992).

Like HBc, HBc-pro-GnRH/GAP was effectively precipitated by ammonium sulfate at 45% saturation. The fusion protein eluted in the breakthrough fraction of hydroxyapatite chromatography, and eluted at approximately the same position as HBc on Sepharose CL-4B chromatography. Thus, it appears that the HBc-pro-GnRH/GAP fusion retains the ability to form core particles and possesses physicochemical properties similar to those of HBc.

The HBc-pro-GnRH/GAP fusion protein is immunoreactive to both HBc and pro-GnRH/GAP antisera. It is expected that the fusion protein would retain anti-HBc immunoactivity. We expected HBc epitopes to be present on the external surface of the core particle, as they are in wild type HBc.

However, it is surprising that HBc-pro-GnRH/GAP possesses anti-pro-GnRH/GAP immunoactivity.

We thought that the pro-GnRH/GAP portion of the fusion would be sequestered within the interior of the HBc-pro-GnRH/GAP core particle and be inaccessible to solvent. However, the fact that we can detect the pro-GnRH/GAP processing site epitope in the fusion protein indicates that this is not the case. Rather, at least the processing site region of pro-GnRH/GAP is surface exposed even within the HBc-pro-GnRH/GAP core particle. This result also demonstrates that, despite its exposure on the core particle surface, the processing site is intact.

BrCN cleavage of HBc-pro-GnRH/GAP yielded intact pro-GnRH/GAP and HBc, as evidenced by the dissappearance of the 26 kDa band and the appearance of a 6 kDa band (pro-GnRH/GAP) and an 18 kDa band (HBc) on SDS-PAGE.

The identities of the 26 kDa and 6 kDa bands were confirmed by Western blot analysis with the appropriate antibodies. Anti-HBc reacted with the 26 kDa band, whereas anti-pro-GnRH/GAP reacted with both the 26 kDa and 6 kDa bands. The fact that the 6 kDa band was immunoreactive

indicates that BrCN cleavage did not affect the integrity of the pro-GnRH/GAP processing site.

The purity of pro-GnRH/GAP was assessed by N-terminal sequence analysis, amino acid analysis, and mass spectrometry. Amino acid analysis of the protein revealed the predicted amino acid composition and indicates that the preparation is essentially homogeneous.

The prohormone did not sequence, indicating that the N-terminus is likely to be blocked. Release of the prohormone was accomplished via BrCN cleavage of a Met-Gln bond, which generally causes cyclization of the Gln side chain to form pyroglutamic acid (pGlu). However, pGlu is not susceptible to sequence analysis by Edman degradation.

The purity of the preparation and the conversion of the N-terminal Gln residue to pGlu were both confirmed by mass spectrometry. The mass spectrum showed a single mass species of 7860 AMU, which agrees exactly with the calculated molecular weight of pro-GnRH/GAP after pGlu formation.

The formation of an N-terminal pGlu residue is of little consequence, and may in fact be appropriate. N-terminal Gln residues often rearrange spontaneously to form

pGlu. Also, conversion of the N-terminal Gln in pro-GnRH/GAP to pGlu is necessary for GnRH biological activity.

Our strategy was successful in producing pro-GnRH/GAP in sufficient yield and purity for biophysical and structural analyses. This strategy would likely be an effective method for the production of other prohormones and other intermediate chain length polypeptides in high yield and purity.

ENZYMATIC AND BIOPHYSICAL CHARACTERIZATION OF PRO-GNRH/GAP

Experimental Strategy

We hypothesize that the structure of pro-GnRH/GAP plays a pivotal role in the regulation of its own endoproteolysis. Cleavage of pro-GnRH/GAP by GAP-releasing is predicted to be specific for the R13-D14 peptide bond within pro-GnRH/GAP. The sequence, $G^{6}LRPGGKR^{13}$, is predicted to form an Ω-loop at the surface of the protein, which acts as a recognition element for GAP-releasing enzyme. Thus, we prepared hundred mg amounts of pro-GnRH/GAP in order to study its biophysical properties and structure.

Enzymatic characterization of pro-GnRH/GAP. Plasma kallikrein is a processing enzyme involved in the initiation of the intrinsic coagulation-kinin pathway (for review, see Kaplan and Silverberg, 1987). Kallikrein is a serine protease which cleaves after some, but not all, Arg residues in its protein substrates. For this reason, we

thought that kallikrein may exhibit limited endoproteolysis of pro-GnRH/GAP, including cleavage at the biologically relevant processing site. Thus, we studied the processing of pro-GnRH/GAP by plasma kallikrein.

Biophysical characterization of pro-GnRH/GAP. Most short-chain peptides (<30 residues) which lack intrachain constraints, such as disulfide bonds, have indefinite structures in solution. However, low molecular weight proteins (100-200 residues, 10-20,000 molecular weight) generally have well defined tertiary structure. Pro-GnRH/GAP contains 69 amino acid residues and has a molecular weight of 7,878 Da. We predict, however, that the structures of prohormone proteins contribute to the specificity of processing. Thus, we thought that pro-GnRH/GAP may contain elements of structure in solution. Furthermore, if the structures of prohormones are dynamic and regulate their own processing by undergoing conformational changes during different stages of the secretory pathway, it is likely that changes in the chemical environment contribute, in part, to these structural rearrangments. Thus, we thought that the

structure of pro-GnRH/GAP may be sensitive to changes in ionic strength, calcium concentration, or pH.

In the present study, we employed biophysical methods to look for evidence of structure in pro-GnRH/GAP and to assess the response of this structure to changing chemical environments. We also performed preliminary experiments to assess the suitability of the prohormone for structure analysis by NMR protocols.

We first looked for evidence of structure in the region of the processing site. Pro-GnRH/GAP contains a single Trp residue which is in close proximity to the processing site. Thus, information regarding the Trp microenvironment is a good indicator of the structural organization of the processing site region of the prohormone.

We probed the Trp microenvironment by measuring the intrinsic fluorescence properties of pro-GnRH/GAP. The relative fluorescence intensity and emission spectral position of Trp residues are sensitive indicators of quenching caused by local groups and/or solvent. The maximum emission wavelength for N(Ac)-Trp-amide in solution is 355 nm (excitation wavelength 297 nm), where Trp is

fully solvated and free to rotate. If Trp is located in a relatively nonpolar environment, such as the interior of a protein, both the excitation and emission wavelengths are blue shifted relative to those of N(Ac)-Trp-amide. Also, as a Trp residue moves from a polar environment to a nonpolar one (for instance, as a consequence of changing conformation), its relative emission intensity increases. Structural perturbations in the region of the pro-GnRH/GAP processing site are expected to affect the Trp microenvironment and, thus, its intrinsic fluorescent properties.

Circular dichroism spectroscopy (CD) is a useful probe of protein secondary structure. Data from CD experiments can be used to calculate the relative contribution of the various secondary structural elements in a given protein or peptide. Also, changes in structure are reflected by changes in the CD spectrum. We used CD to look for evidence of and changes in secondary structure in pro-GnRH/GAP.

If pro-GnRH/GAP folds into a protein like structure, then it would be expected to undergo thermal induced transitions typical of low molecular weight proteins, but

absent in unordered peptides. Therefore, we used differential scanning calorimetry (DSC) to observe any thermal induced conformational changes in pro-GnRH/GAP.

Quantitative determination of solution-phase protein structure requires a comprehensive analysis of multidimensional NMR data leading to sequence specific resonance assignments and the delineation of distance constraints (c.f., Wütrich, 1986). Once sufficient information is obtained, computer modeling can be used to identify specific structural features or to solve the complete three-dimensional structure of the protein.

The usefulness of these methods is fundamentally dependent on the resolution of individual resonances in the NMR spectrum. The major limitation on the resolution of NMR spectra of macromolecules is spectral line broadening. This is due to the shorter relaxation times of macromolecular nuclei and is exacerbated by aggregation which sometimes occurs at the protein concentrations (≥1 mM) required for NMR experiments. Therefore, it is first necessary to define empirical chemical conditions to optimize the spectral resolution for a given protein.

We conducted preliminary 1D and 2D ^{1}H NMR experiments with pro-GnRH/GAP under different chemical conditions with the intent of defining conditions which would allow us to solve its solution structure by NMR protocols. These spectra were analyzed for spectral resolution of key regions, particularly the -NH (amide) proton region, which is a good index for structural stability. The spectra were also examined to assess the possibility of pH dependent conformational changes.

Materials and Methods

Proteolysis of pro-GnRH/GAP. Prohormone (32 μM) and plasma kallikrein (Enzyme Research Labs) were incubated (0-24 hr, 37°C) in a molar ratio of 10:1 in 50 mM Hepes buffer, pH 7.5. At the appropriate time, each reaction was stopped with 0.1 volume of 3N HCl. An aliquot of each timed hydrolysis sample was diluted 1:25 in PBS and subjected to ELISA with pro-GnRH/GAP antiserum. The extent of reaction was monitored by the disappearance of immunoactivity. Because the primary sequence of pro-GnRH/GAP is known, the sites of kallikrein catalyzed cleavage were determined by N-terminal sequence analysis of the reaction mixture, rather than of the separated peptide cleavage products.

Intrinsic fluorescence measurements. Measurements were made at room temperature on a Shimadzu RF-5000 spectrofluorometer according to the pH or $CaCl_2$ titration protocol. The protein solution (0.75-2.75 μM) was analyzed in a 3 mm path length optical cell. The band width for both excitation and emission was 5 nm. In all experiments, the excitation wavelength was 280 nm, and the emission wavelength was determined by spectral scanning.

Circular dichroism measurements. All measurements were made at room temperature on a Jasco J-500C spectropolarimeter according to the pH titration protocol. The protein solution (200 μM) was analyzed in a 1 mm path length optical cell. The spectra in the region 240-190 nm were analyzed to determine the fractional percent of secondary structural elements using the PROtein SECondary (PROSEC) structure program. This program calculates α-helix, β-strand, and β-turn elements, and all remaining structure is designated to be unordered, random coil.

$CaCl_2$ titration protocol. The protein was dissolved in either distilled water or 50 mM Hepes buffer, pH 7.5. 100 mM and 1 M $CaCl_2$ stock solutions were made in either water or 50 mM Hepes buffer, pH 7.5. An appropriate volume of the appropriate stock solution was added to the protein solution to reach the desired concentration.

pH titration protocol. The protein was dissolved in a buffer containing 40 mM Hepes, 25 mM sodium acetate, 25 mM glycine, and 1 mM EDTA at pH 7.0 (You et al, 1993a). Addition of small amounts of either dilute hydrochloric acid (1 mM) or dilute sodium hydroxide (1 mM) to this

solution results in a new pH, which is then determined by means of a pH probe (Chen et al, 1991).

Differential scanning calorimetry. Calorimetric experiments were performed on a Microcal Omega scanning calorimeter. Data were acquired at 100 μM protein in 50 mM Hepes, pH 7.5. Thermograms were generated by scanning upscale from 10-90°C at a rate of 1°C/min. All thermograms were baseline corrected and normalized for protein concentration. The DSC data analysis software is based on the statistical mechanical deconvolution of a thermally induced macromolecular melting profile as illustrated previously (Freire and Biltonen, 1978).

Nuclear magnetic resonance spectroscopy. 1D proton NMR experiments were performed on a Varian 500 spectrometer operating at a ^{1}H frequency of 500 MHz. All data were acquired at 25°C in 5 mm NMR tubes. The protein was dissolved in 1 ml H_2O/D_2O (90:10) to a concentration of 2 mM. The initial pH of this solution was 2.8. Subsequent pH adjustments were made by the addition of small amounts of sodium hydroxide.

2D TOCSY and NOESY experiments were performed on a Bruker spectrometer operating at a ^{1}H frequency of 500 MHz.

All data were acquired at 25°C in 5 mm NMR tubes. The protein was dissolved in 1 ml H_2O/D_2O (90:10) to a concentration of 1 mM, and the pH was adjusted to 6.85 using 50 mM phosphate buffer.

Results

Enzymatic processing of pro-GnRH/GAP. Processing of pro-GnRH/GAP by plasma kallikrein was monitored by immunoassay. Within 1 hour, all immunoactivity was abolished (Table 6), indicating that cleavage of the processing site epitope has occurred. The products of the 1 hr reaction were characterized by N-terminal sequence analysis. Because the primary sequence of pro-GnRH/GAP is known, we were able to determine the sequences resident in a mixture of peptides.

Five sequences were detected (Table 7) which are attributable to cleavage after Arg13, Arg37, Arg46, Arg50, and Lys53. Regardless, the presence of the amino acid sequence of GAP verifies that kallikrein cleaves pro-GnRH/GAP at its biologically relevant processing site. These results show that the processing site in recombinant pro-GnRH/GAP is exposed on the surface of the protein and that it is accessible for proteolysis.

Fluorescence spectroscopy. The structural organization in the processing site region of pro-GnRH/GAP and its response to changes in the chemical environment

Table 6

Immunological characterization of kallikrein catalyzed proteolysis of recombinant pro-GnRH/GAP

Time (hr)	OD_{405}
0	0.318
1	0
4	0
12	0
24	0

Table 7

N-terminal sequence analysis of products resulting from kallikrein catalyzed proteolysis of pro-GnRH/GAP

	Cycle				
Sequence *	1	2	3	4	5
1	Asp	Ala	Glu	Asn	Leu
2	Ser	Pro	Leu	Arg	N/D
3	Asp	Leu	Lys	Gly	Ala
4	Gly	Ala	Leu	Glu	Ser
5	Phe	Glu	Ser *	Thr	Thr

* Sequences were assigned based on the known primary sequence of pro-GnRH/GAP (Figure 3) with consideration of the Cys40 to Ser amino acid substitution (c.f., Figure 12).

were studied by intrinsic fluorescence emission spectroscopy. In separate experiments, the prohormone was titrated with respect to and pH and calcium chloride concentration (which represents both ionic strength and free calcium concentration), and the effects on fluorescence emission intensity were observed.

Titrations with calcium chloride were done in unbuffered solutions and in solutions buffered at pH 7.5 as described. In either case, titration up to a concentration of 500 mM yielded no significant effect on fluorescence intensity (Figure 22). This suggests that the structural organization in the region of the processing site is not sensitive to changes in ionic strength or free calcium concentration.

However, intrinsic fluorescence emission intensity is very sensitive to changes in pH. Emission intensity is maximal in the neutral pH range (7.0-8.0) and decreases dramatically outside this range (Figure 23). These data suggest that the Trp residue is least solvated (most buried) at neutral pH, which would be consistent with an increase in structural organization in this region of the prohormone.

Figure 22: Effect of $CaCl_2$ concentration on intrinsic fluorescence emission intensity of pro-GnRH/GAP. Recombinant pro-GnRH/GAP was titrated with calcium chloride over the concentration range of 0 to 500 mM (corresponding to an ionic strength range of 0 to 1.5 M). Samples were excited at 280 nm, and the emission intensity at 348 nm was measured. Emission intensity was normalized to protein concentration, which was diluted from 1.5 to 0.75 μM over the course of the titration.

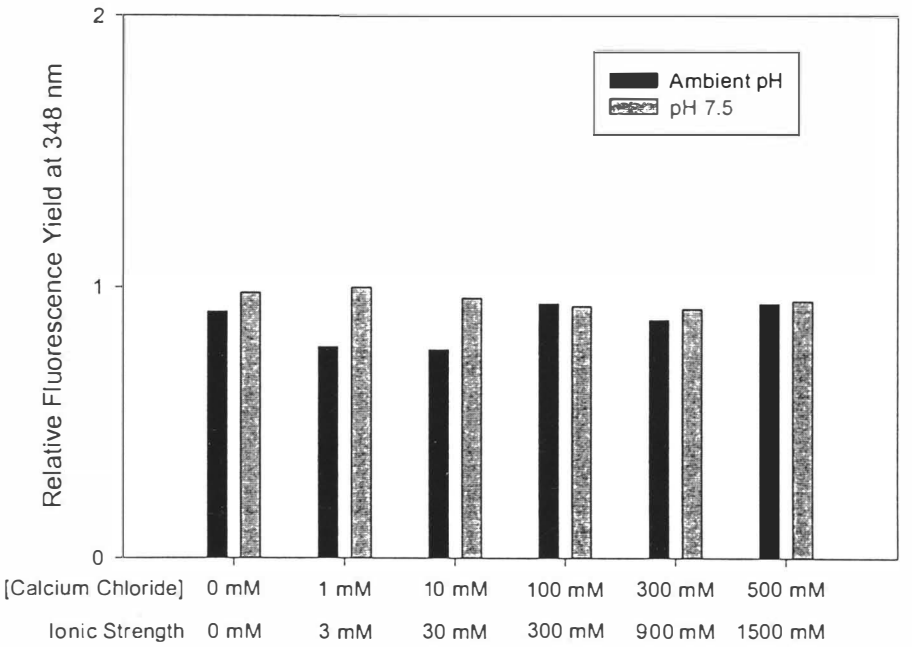
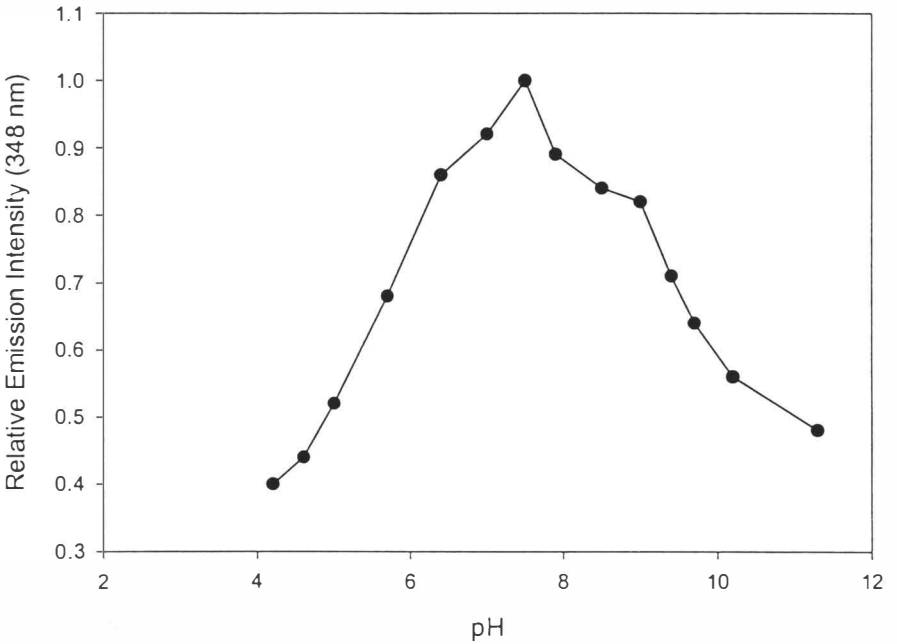

Relative Fluorescence Yield at 348 nm
2
1
0
Ambient pH
pH 7.5
[Calcium Chloride] 0 mM 1 mM 10 mM 100 mM 300 mM 500 mM
Ionic Strength 0 mM 3 mM 30 mM 300 mM 900 mM 1500 mM

Figure 23: Effect of pH on intrinsic fluorescence emission intensity of pro-GnRH/GAP. Recombinant pro-GnRH/GAP was titrated from pH 4.2 to 11.3. The protein (1.5 μM) was dissolved in buffer containing 40 mM Hepes, 25 mM sodium acetate, 25 mM glycine, and 1 mM EDTA at pH 7.0 (You et al,1993a). Addition of small amounts of hydrochloric acid or sodium hydroxide results in a new pH, which was measured with a pH probe (Chen et al, 1991). Samples were excited at 280 nm and the emission intensity at 348 nm was measured. Emission intensity was normalized to protein concentration, which was reduced by no more than 5% over the course of the titration.


Relative Emission Intensity (348 nm)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
2
4
6
8
10
12
pH

There appear to be at least two pKa's involved in the maintenance of the Trp microenvironment. Graphical interpretation of the data indicates that pKa's at 6.0-6.5 and 9.5-10.0 are likely important. These likely correspond to His and Lys side chains, respectively.

Circular dichroism spectroscopy. We used CD spectroscopy to look for secondary structure in the prohormone and to determine the dependence of secondary structural elements on changes in pH. As shown (Table 8), the prohormone displays predominantly β-strand character throughout the entire pH titration (pH 4.4-10.7). The fractional percent of each secondary structural element remains virtually constant over most of this pH range. However, at pH < 5.0, the relative distribution of the various elements changes; there is a slight but discernable decrease in β-strand content which coincides with an increase in α-helix, β-turn, and random coil elements.

Differential scanning calorimetry. The thermal stability of pro-GnRH/GAP was studied by differential scanning calorimetry. The fluorescence data showed maximum structure at neutral pH; therefore, the DSC experiments

Table 8

Effect of pH on secondary structure of pro-GnRH/GAP

pH	Secondary structure (%)			
	helix	strand	turn	coil
4.4	7.1	58.7	8.3	25.9
4.7	4.4	66.3	6.1	23.2
5.2	0	78.0	2.6	19.3
6.0	0	78.3	3.3	18.4
6.9	0	78.0	3.8	18.1
7.4	0	78.0	3.5	18.5
7.7	0	78.3	3.4	18.3
8.8	0	78.0	3.6	18.4
9.6	0	78.4	3.4	18.2
10.7	0	78.0	3.8	18.1

were performed at pH 7.5. The thermogram reveals a sharp transition with a midpoint temperature of 46°C and a transition enthalpy of 28.5 kcal/mol (Figure 24). The data are consistent with a theoretical fit of a two-state transition model of unfolding (native ↔ unfolded). This experiment shows that recombinant pro-GnRH/GAP has a definable structure in solution at neutral pH.

Nuclear magnetic resonance spectroscopy. The effect of pH on the structure of pro-GnRH/GAP was observed in a series of four 1D proton NMR experiments (pH 2.8-7.5). The spectra were analyzed for evidence of pH induced conformational changes and for resolution which may facilitate further structural analysis.

The 6.4-10.6 ppm region of each spectrum, which encompasses the aromatic, amide, and Trp indole proton resonances, is shown in Figure 25. The range of 6.4-8.8 ppm contains the aromatic (~6.4-7.5 ppm) and amide (~7.0-8.8 ppm) resonances. It is clear that the distribution of resonances changes in this region of the spectrum as a function of pH. This is unequivocal evidence for pH induced conformational change.

Figure 24: DSC thermogram of pro-GnRH/GAP at pH 7.5.
Recombinant pro-GnRH/GAP (100 μM) was dissolved in 50 mM Hepes pH 7.5 and scanned upscale from 10 to 90°C. The resulting curve (solid line) was compared with a theoretical two-stae transition model curve (dotted line), and thermodynamic parameters were determined from both curves (inset).

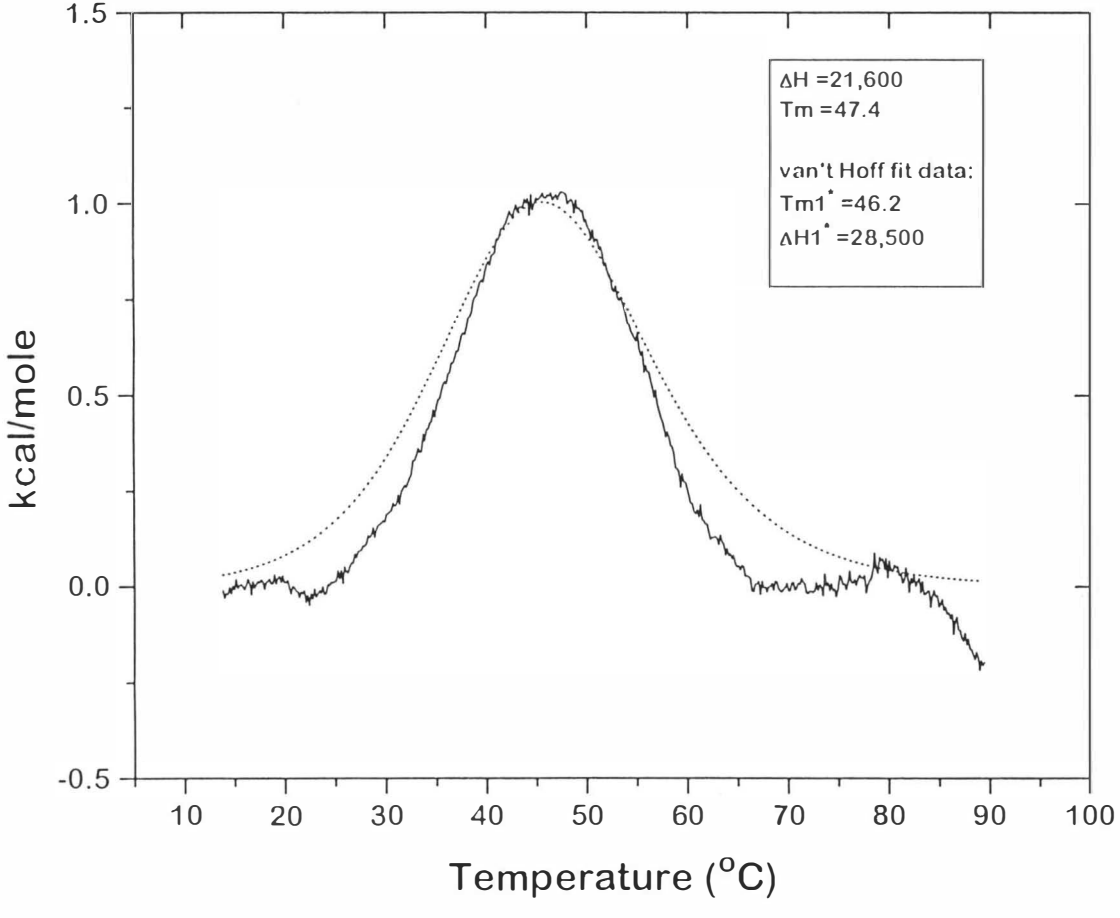
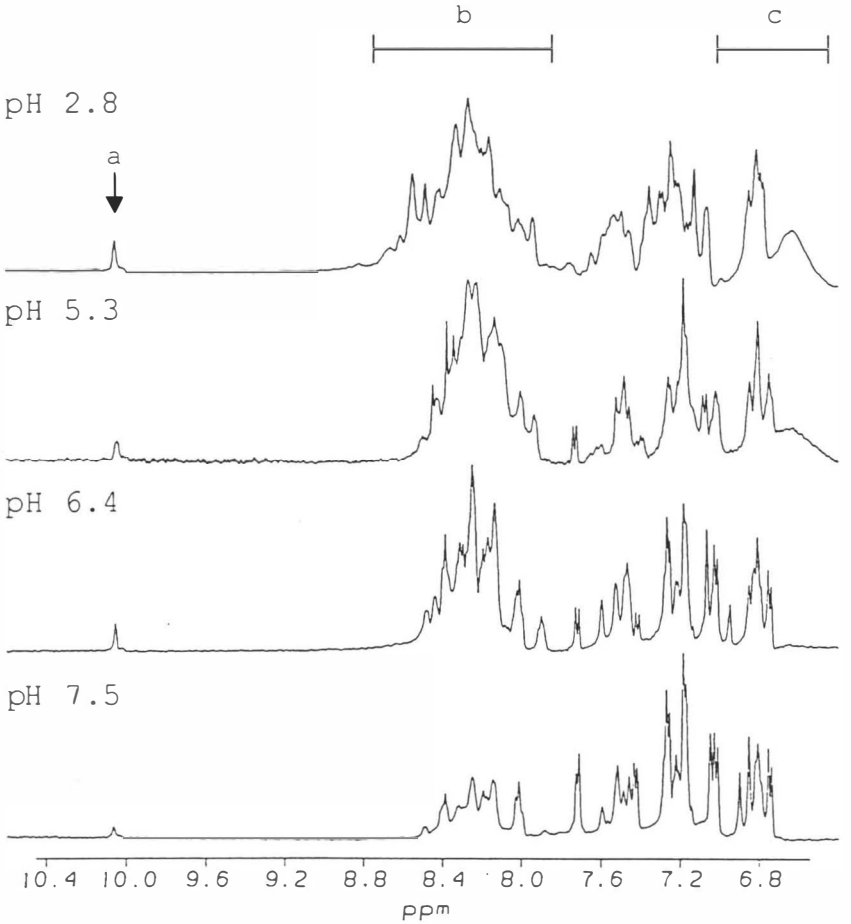

ΔH =21,600
Tm =47.4
van't Hoff fit data:
Tm1° =46.2
ΔH1° =28,500
kcal/mole
1.5
1.0
0.5
0.0
-0.5
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

Figure 25: 1D ^{1}H NMR spectra of pro-GnRH/GAP at variable pH. Recombinant pro-GnRH/GAP was dissolved in 1 ml H_2O/D_2O (90:10) to a concentration of 2 mM. The initial pH of this solution was 2.8; subsequent pH adjustments were made by adding small amounts of sodium hydroxide. Shown is the region of the 1D proton spectra encompassing the Trp indole -NH (a), amide (b) and aromatic (c) proton resonances.

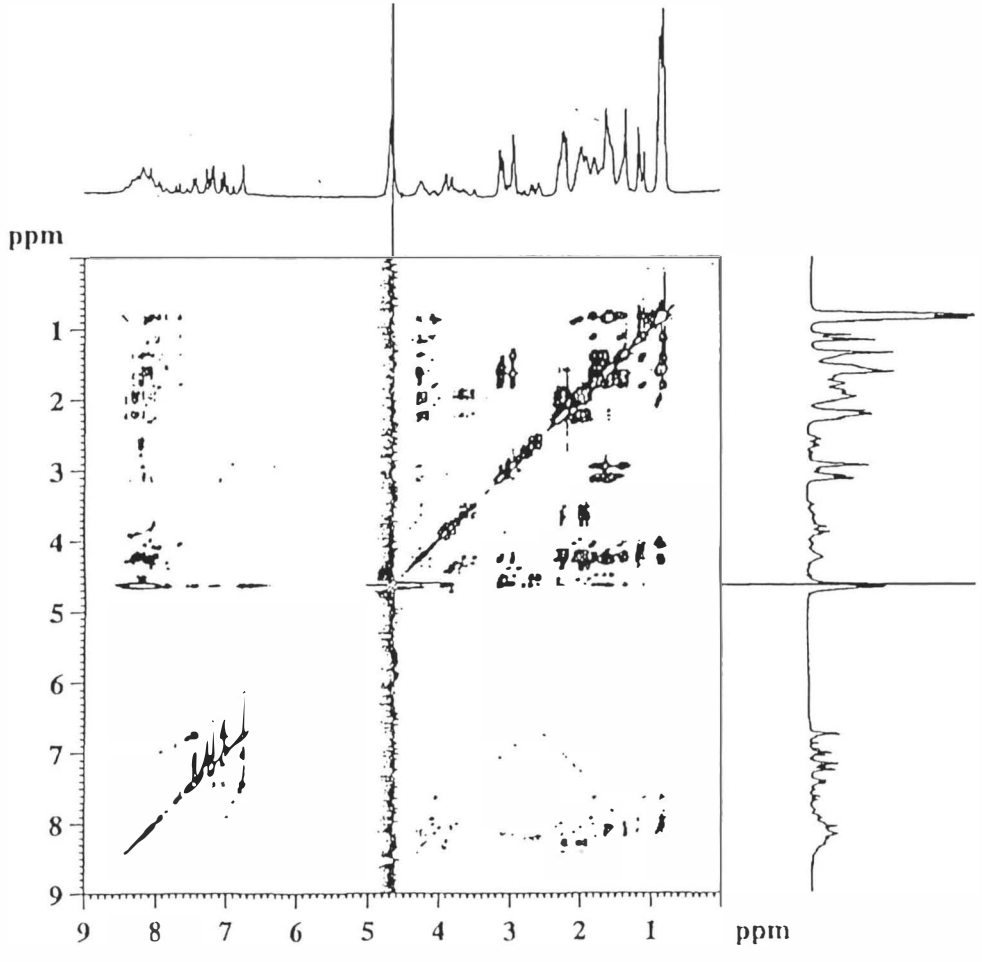

b
c
pH 2.8
a
pH 5.3
pH 6.4
pH 7.5
10.4 10.0 9.6 9.2 8.8 8.4 8.0 7.6 7.2 6.8
ppm

The resolution of the amide and aromatic resonances is dramatically improved as the pH is increased from 2.8 to 6.4, indicating an increase in structural organization of the protein. Similarly, the Trp resonance (~10.1 ppm) is more resolved at pH 6.4 than at pH 5.3, indicating less exposure to solvent. The resolution in the 6.4-7.6 ppm range, which contains aromatic and some amide resonances, continues to improve up to pH 7.5, indicating further structure enhancement. However, the Trp resonance and those in the 7.8-8.6 ppm range (primarily amides) are suppressed and exchange broadened at this pH.

These data are consistent with the idea that pro-GnRH/GAP assumes its most organized structure at neutral pH. However, subsequent NMR experiments were performed at pH < 7.0 in order to achieve sufficient resolution in the amide region for structural analysis.

2D TOCSY and 2D NOESY experiments were done at pH 6.85. The TOCSY data result from all correlations (through bond and through space). As shown (Figure 26), there are numerous corelations in the αβ-αβ and amide-amide regions. There are also significant correlations in the amide-αβ

Figure 26: 2D ^{1}H TOCSY NMR spectrum of pro-GnRH/GAP at pH 6.85. Recombinant pro-GnRH/GAP was dissolved in 1 ml H_2O/D_2O (90:10) to a concentration of 1 mM, and the pH was adjusted to 6.85 with phosphate buffer. The 2D contour plot shows all proton correlations (through bond and through space) with the exception of those involving the far downfield (10.1 ppm) Trp indole -NH proton resonance.


ppm
1
2
3
4
5
6
7
8
9
9
8
7
6
5
4
3
2
1
ppm

region (Figure 27). These data suggest that structural analysis may be feasible at pH 6.85.

However, the NOESY data are less favorable. The amide-αβ region of the 2D NOESY (pH 6.85, 100 msec mixing time) is shown in Figure 28. The lack of significant through space connectivities indicates that resonance assignments will be difficult at best. These preliminary NMR data demonstrate that structure analysis of pro-GnRH/GAP in the neutral pH range will be problematic.

Figure 27: Amide-alpha proton correlation region of the 2D ^{1}H TOCSY NMR spectrum of pro-GnRH/GAP at pH 6.85. The plot shows a more detailed view of the amide-alpha proton correlation region (upper left quadrant) of the plot in Figure 26.

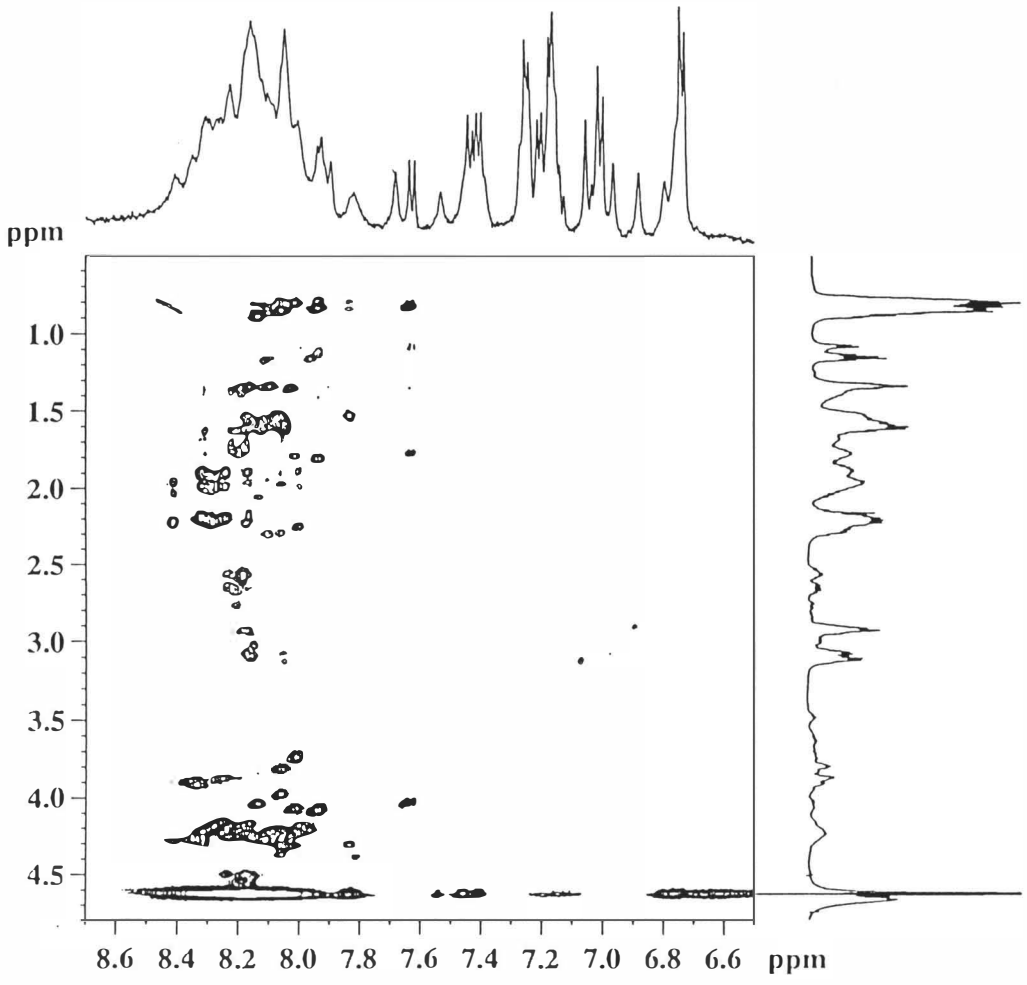
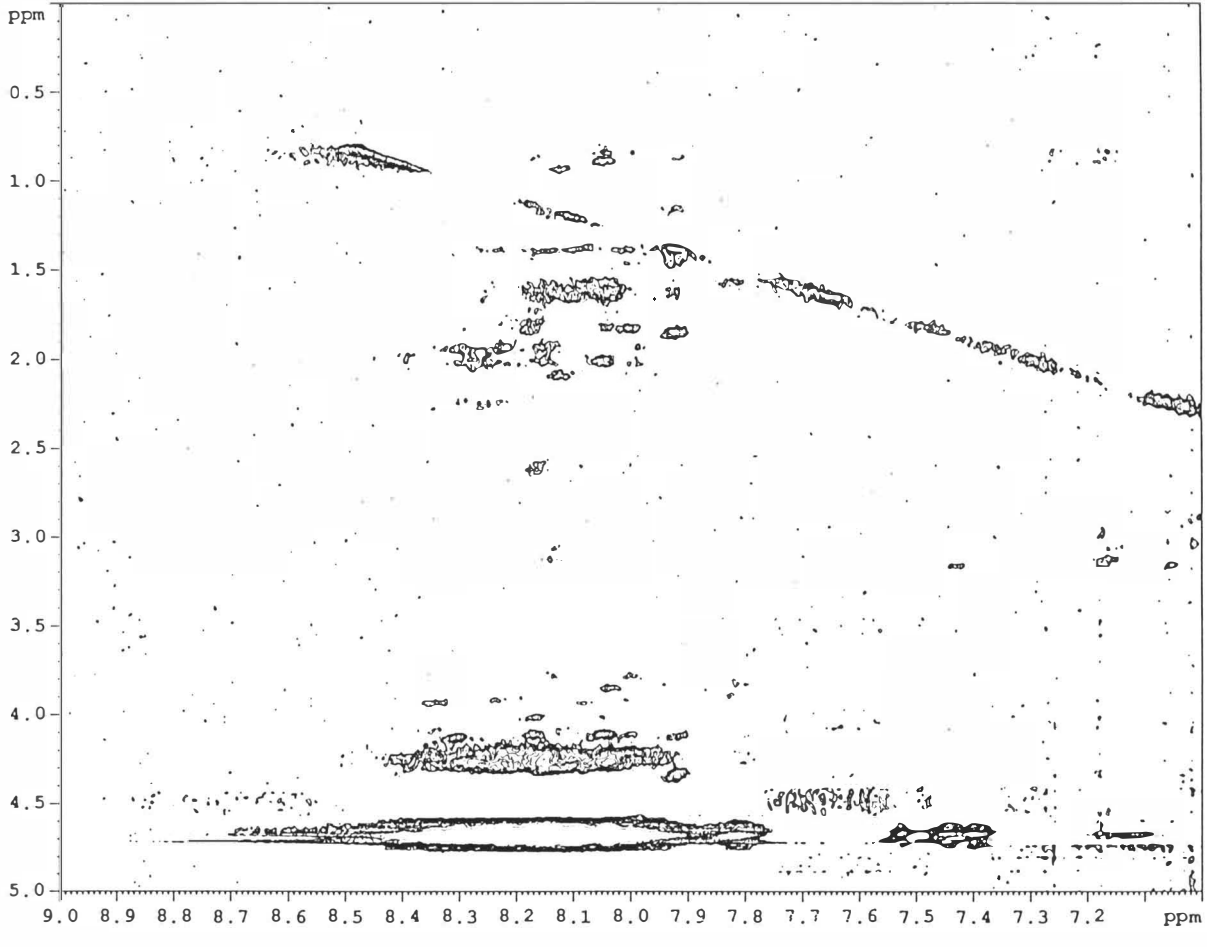

ppm
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
8.6 8.4 8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 ppm

Figure 28: Amide-alpha proton correlation region of the 2D ^{1}H NOESY NMR spectrum of pro-GnRH/GAP at pH 6.85. The same protein sample used in the TOCSY experiment (Figures 26 and 27) was used in the NOESY experiment (80 msec mixing time). The plot shows through space connectivities in the amide-alpha proton correlation region.


ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
9.0 8.9 8.8 8.7 8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2
ppm

Discussion

It has been suggested that cleavage sites within prohormones are associated with defined structural features which are exposed on the surface of the molecule (for reviews, see Harris, 1989; Harris et al, 1994). Further, it is likely that processing of prohormones is partly regulated by these structural features and their dynamics associated with changes in the chemical environment (pH, ionic strength, free calcium concentration) encountered in the various subcellular compartments of the regulated secretory pathway.

We showed that the processing site within recombinant pro-GnRH/GAP is surface exposed and that the appropriate cleavage site is susceptible to proteolysis. We demonstrated that the structure of the intact prohormone, and, in particular, that of the region encompassing the processing site, is sensitive to changes in pH, but not to changes in ionic strength or free calcium concentration. We further showed that the structural organization of the processing site region is maximal at neutral pH and that the intact prohormone assumes a defined tertiary structure

at neutral pH. However, preliminary NMR data suggest that structural analysis at neutral pH will be problematic.

Recombinant pro-GnRH/GAP is immunoreactive to anti-pro-GnRH/GAP antibody prior to cleavage by plasma kallikrein, which abolishes this immunoactivity. Therefore, the processing site epitope must be exposed on the surface of the prohormone, and it is disrupted by proteolysis.

Plasma kallikrein cleaves pro-GnRH/GAP after Arg13, Arg37, Arg46, Arg50, and Lys53,. Although mutiple cleavages occur, cleavage of the Arg13-Asp14 bond demonstrates the susceptibility of the biologically relevant cleavage site. Also, of the observed kallikrein sensitive sites, only Arg13 is in the vicinity of the processing site epitope. Thus, it is likely that cleavage at this site is sufficient to abolish anti-pro-GnRH/GAP immunoactivity.

Kallikrein cleavage of pro-GnRH/GAP was detected after every Arg residue in the prohormone except for Arg8. This is particularly significant because the Arg8-Pro9 bond remains intact in bioactive GnRH. Arg8 is also part of the putative recognition sequence for GAP-releasing enzyme, suggesting that it is surface exposed in the intact

prohormone. Therefore, it is likely that, at neutral pH, the processing site assumes a structure which simultaneously presents the Arg13-Asp14 bond for proteolytic cleavage and protects the Arg8-Pro9 bond from proteolysis, although X-Pro bonds are not generally susceptible to proteolysis.

Fluorescence data showed that the Trp microenvironment within pro-GnRH/GAP is sensitive to changes in pH, albeit unresponsive to changes in ionic strength or free calcium concentration. The intrinsic fluorescence emission intensity of pro-GnRH/GAP is maximal at neutral pH (7.0-7.9) and decreases dramatically outside this range. This indicates that the Trp residue, which is in close proximity to the processing site, is buried to a greater extent at neutral pH. Thus, it is likely that the structural organization of the processing site is maximal at neutral pH.

Analysis of the fluorescence pH titration curve indicates that His and Lys residues are likely involved in the maintenance of the processing site structure. The apparent pKa (6.0-6.5) in the acidic range is in aggreement with the expected value for the His side chain. The

observed pKa (9.5-10.0) in the basic range is close to the expected value for the Lys side chain (10.5). It is possible that the pKa of Lys is lowered by the presence of nearby charged residues. For example, Lys12 is immediately adjacent to an Arg residue. The charged Arg side chain could increase the local polarity relative to that of bulk water, causing a decrease in the apparent pKa of Lys.

The secondary structure of pro-GnRH/GAP is characterized predominantly by β-strand throughout the pH titration (pH 4.4-10.7). The relative distribution of secondary structural elements in pro-GnRH/GAP is constant over most of this range (pH 5.2-10.7). However, at pH <5.0, there is a significant decrease in β-strand character which corresponds to a significant increase in α-helix, β-turn, and random coil elements. The increase in helical content may be significant. Many predicted cleavage sites within prohormones, which are not cleaved *in vivo*, are located within sequences with a predicted propensity for helical structure (Rholam et al, 1986). At neutral pH, kallikrein cleaved GAP at multiple sites. However, the pH in secretory granules, in which GAP is stored *in vivo*, is

closer to pH 5.5, due to the action of a Na/K-ATPase in secretory granules. It is possible that the formation of α-helix at acidic pH protects key sites within GAP from further proteolysis in secretory granules.

Thermal denaturation of pro-GnRH/GAP at pH 7.5 occurs via a simple two-state transition, indicating that the prohormone assumes a defined tertiary structure at neutral pH. It is significant that a protein of this chain length (69 residues) is capable of forming a folded domain. In such a folded structure, it may be that only certain proteolytic sites are available on the suface of the prohormone, while others may be protected by virtue of the structural features in which they exist.

The NMR data clearly demonstrate that pro-GnRH/GAP undergoes pH induced conformational changes. This confirms the structural effects of pH we observed in the fluorescence and CD experiments. These results are consistent with the idea that prohormones may regulate their own processing by undergoing conformational changes during different stages of the secretory pathway.

Further analysis of the NMR data suggests that the structural organization of pro-GnRH/GAP increases as the pH

approaches neutrality. The aromatic, amide and Trp proton resonances become increasingly resolved as the pH increases from 2.8 to 6.4, indicating greater definition of structure. Although exchange broadening is apparent in the amide region at pH 7.5, the marked resolution in the aromatic region probably indicates futher structural organization. These results are consistent with the fluorescence data that indicated maximum structure of the processing site at neutral pH. There is a significant correlation between these results and the observation that GAP-releasing enzyme attains maximal activity at neutral pH.

Preliminary 2D NMR data revealed that pro-GnRH/GAP may not be amenable to structure determination under physiological conditions (i.e., isotonic, neutral pH conditions). Although there were substantial correlations observed in the TOCSY experiment, there were very few correlations apparent in the NOESY experiment. This demonstrates that the majority of correlations result from through-bond connectivities and that CαH-CβH connectivities predominate. Structure determination by NMR methods requires the resolution of through-space connectivities.

Therefore, structure determination of pro-GnRH/GAP at neutral pH appears to be problematic and will probably be best addressed using heteronuclear protocols to study ^{15}N and/or ^{13}C enriched pro-GnRH/GAP.

CONCLUSIONS AND SUMMARY

Pro-GnRH/GAP undergoes endoproteolysis at the Arg13-Asp14 bond as the initial event leading to the maturation of its biologically active products. It is likely that GAP-releasing enzyme is responsible for this processing event and that its proteolytic specificity is imparted by the structural organization of the prohormone processing site recognition sequence. To test this hypothesis, we conducted structural analysis of intact pro-GnRH/GAP. Hundred mg quantities of pure prohormone were needed for these studies. Thus, we devised protocols for the large scale preparation of pure pro-GnRH/GAP. We then studied the structural properties of the prohormone and assessed its response to changes in the chemical environment.

The chemical synthesis of pro-GnRH/GAP was accomplished, although we were unable to obtain the synthetic prohormone in sufficient purity for structural studies. An immunoassay was developed which is specific for the prohormone processing site. Using this assay, we

demonstrated that the processing site in synthetic pro-GnRH/GAP is exposed on the surface of the molecule and that the processing site epitope is disrupted by proteolysis.

Using a novel expression system, we obtained recombinant pro-GnRH/GAP in sufficient yield and purity to conduct our proposed studies. We showed that recombinant pro-GnRH/GAP contains a surface exposed processing site which is susceptible to proteolysis of the biologically relevant Arg13-Asp14 bond.

Fluorescence studies showed that the conformation of the processing site of pro-GnRH/GAP is not responsive to changes in ionic strength or calcium concentration. However, we showed that the processing site region of the prohormone attains maximal structural organization at neutral pH. This correlates strongly with the maximal activity of GAP-releasing enzyme at neutral pH. These data support the hypothesis that GAP-releasing enzyme is responsible for endoproteolysis of pro-GnRH/GAP and that the structure of the prohormone processing site helps impart proteolytic specificity.

It is apparent that the overall secondary structure of pro-GnRH/GAP is relatively resistent to changes in pH. It

is possible that the structure of the processing site is uniquely sensitive to pH over a narrow range relative to that which affects the global structure of the protein. It is also clear that the pH mediated effects on the conformation of the processing site are very subtle, but may be sufficient to alter the solvation state of the Trp residue.

We have shown that pro-GnRH/GAP possesses significant tertiary structure at neutral pH. Thermal denaturation of the prohormone occurs via a simple, irreversible, two-state transition. This is remarkable for a protein of this molecular weight (7,860 Da) and chain length (69 residues). This finding supports the idea that the global structure of pro-GnRH/GAP is relatively stable.

It is clear from our NMR results that the conformation of pro-GnRH/GAP is responsive to changes in pH. However, the precise nature of these changes in structure remains to be quantitated. Our ultimate goal is to solve the three-dimensional structure of the prohormone. Our preliminary experiments indicate that this may be problematic at neutral pH. We would like to determine the structure of pro-GnRH/GAP under physiologically relevant conditions.

However, these preliminary experiments indicate that it may be necessary to solve the structure of pro-GnRH/GAP under conditions that favor the resolution of NMR spectra (i.e. acidic pH), but which are clearly non-physiological. Alternatively, heteronuclear protocols using 15N and/or 13C labeled protein may give sufficient spectral resolution to allow structure determination at neutral pH.

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