To the Graduate Council:

I am submitting herewith a dissertation written by Angela L. McKinney entitled "Studies of the *Saccharomyces cerevisiae* α-factor mating pheromone and its receptor, the *STE2* gene product." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Science.

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Studies of the *Saccharomyces cerevisiae* α-factor mating pheromone and its receptor (the *STE2* gene product)

A Dissertation presented for the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Angela L. McKinney
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DEDICATION

This dissertation is dedicated to my parents, Lowell and Mary McKinney, and to the memory of my grandparents, Virginia and Tony Jones. I could never have achieved my goal without their constant love, support, prayers, and encouragement. Thanks Mom and Dad, I will always be grateful to both of you for the many sacrifices you made in order for me to receive the best education possible.

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ABSTRACT

The binding of α-factor to the α-factor receptor is the first step in a cascade of cellular events that initiates the mating response. In order to understand the molecular interactions involved in hormone-receptor binding, it is necessary to know the conformation of the messenger peptide in the active site of its receptor and the identity of the binding domain within its receptor.

Structure-function studies have been performed on α-factor. Previous studies indicated that a Type II β-turn spanning residues 7 through 10 of the pheromone is important for the biological activity. Based on those results, a series of synthetic analogs were prepared in the laboratory of Dr. Fred Naider (College of Staten Island, Staten Island, New York). The first set of analogs synthesized to study the β-bend region were the cyclic lactam analogs in which the ring size (residues 7-10) was systematically varied. Subsequently, cyclic analogs were synthesized in which residues 7 and 10 were replaced with cysteines to form a disulfide bond which restricted the conformational mobility of the peptide. These analogs were assayed for biological activity and for the ability to bind to the receptor. Although none of the analogs were as active as native α-factor, results suggest a Type II β-turn conformation spanning residues 7-10 with proper orientation of the amine and carboxyl termini around the turn region is required for optimal interaction of α-factor with its receptor and is necessary for biological activity (See Part II and III of dissertation).

Determination of the α-factor binding domain within its receptor (Ste2p) was attempted using photoaffinity labeling. α-factor analogs containing *p*-benzoyl-phenylalanine(BPA) were synthesized, tested for their biological activity and for the ability to bind to Ste2p (Part IV of dissertation). Photoaffinity labeling experiments were

conducted using both membrane proteins and partially purified Ste2p. The results of the photoaffinity labeling experiments indicated a 52 kDa protein which was verified to be Ste2p using antibodies (Part V of dissertation). These observations suggest that a photoactivatable α-factor analog is able to covalently label the α-factor receptor and may be suitable for isolating and characterizing the receptor binding site(s).

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PART I

GENERAL INTRODUCTION

CHAPTER I

LITERATURE REVIEW

Saccharomyces cerevisiae, commonly referred to as baker's yeast, exists in both a stable haploid anda diploid state (Sprague *et al* ., 1983; Thorner 1981). The haploid state consists of an **a**-cell and an α-cell. When a haploid **a**-cell mates with a haploid α-cell, a diploid **a**/α-cell is formed. Mating between the haploid **a** and α-cell is initiated by the reciprocal secretion of mating pheromones termed **a**-factor and α-factor. **a**-cells secrete **a**-factor, a dodecapeptide, which is farnesylated and carboxymethylated at the C-terminal end of the peptide [YIIKGVFWDPAC(Farnesyl)-OCH_3] (Anderegg *et al* ., 1988). This pheromone binds to its receptor, the *STE3* gene product, located on the α-cell. α-factor, a tridecapeptide (WHWLQLKPGQPMY), is secreted by α-cells and binds to its receptor, the *STE2* gene product, located on the **a**-cell (Figure 1).

When **a** and α-cells are exposed to the opposite mating pheromone a number of physiological changes occur which prepare the cells for mating. These changes include growth arrest in the G1 phase of the cell cycle, production of cell surface agglutinins, transcription of mating specific genes, and a morphological change termed shmooing (Hartwell 1973). Due to its stable haploid and diploid life cycle, the ease of laboratory manipulations, the abundant background of fundamental knowledge, and the extensive genetic and biochemical methodologies which have been developed for analysis of this yeast, *Saccharomyces cerevisiae* is an excellent model organism to study (Herskowitz 1989; Sprague *et al* ., 1983).

Two genes, *MFα1* and *MFα2*, encode the α-factor precursor protein (prepro-α-factor) which is proteolytically cleaved to generate mature α-factor (Kurjan &

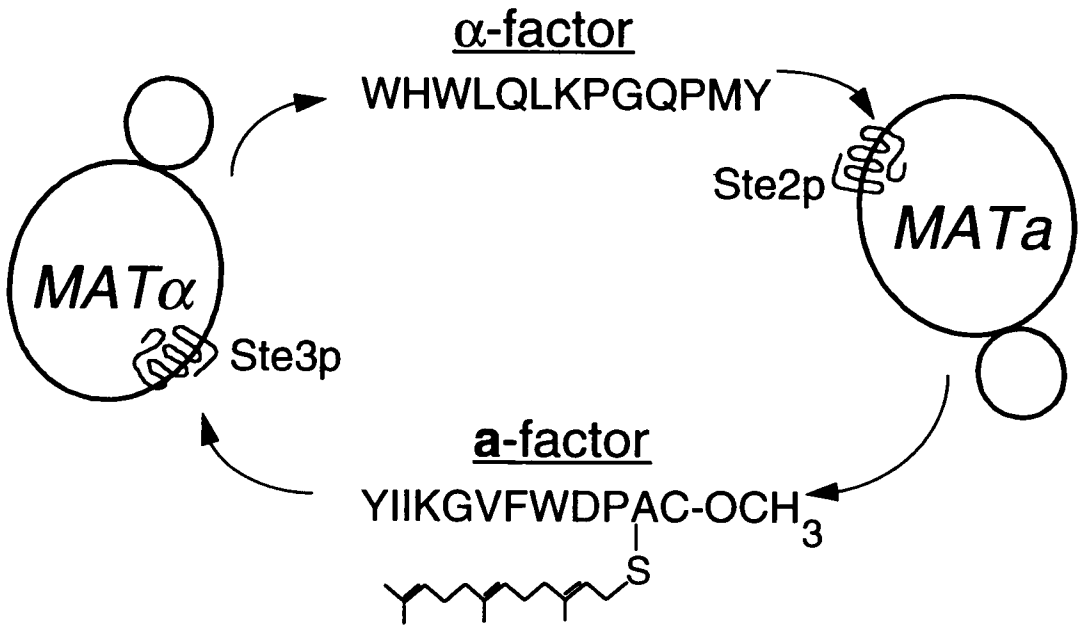


Figure 1. An overview of yeast mating. Two opposite *Saccharomyces cerevisiae* haploid cells (**a** and α) secrete mating pheromones (**a**-factor and α-factor) which interact with their respective receptor (Ste2p = α-factor receptor; Ste3p = **a**-factor receptor) in order to mate.

Herskowitz, 1982; Singh *et al*. 1983; Julius *et al*., 1983). Prepro-α-factor consists of an N-terminal hydrophobic signal, a hydrophilic leader region, and a C-terminal domain which contains repeats of the α-factor sequence separated from one another by a spacer region. This spacer region is comprised of either six or eight residues with the sequence - KRGAGA- or -KRGAG(or D)AGA. The sequence of the precursors encoded by *MFα1* and *MFα2* are closely related and differences in the two can be found in the C-terminal domain. The precursor encoded by *MFα1* contains four identical copies of α-factor (WHWLQLKPGQPMY) in the C-terminal domain whereas the precursor encoded by *MFα2* contain only two copies of α-factor. One copy is identical to that found in the *MFα1* precursor and the sequence of the second copy differs in residue five and seven (WHWL**NLR**PGQPMY). Biologically both forms of mature α-factor are identical and the variant form produced by *MFα2* does not appear to have a special function (Raths *et al*. 1986). *MFα1* is expressed at a higher level than *MFα2* and therefore the majority of extracellular α-factor is derived from *MFα1* (Brake *et al*., 1983; Kurjan 1985).

α-factor is processed by the classical secretory pathway comprised of the endoplasmic reticulum, the Golgi apparatus, and secretory vesicles. Processing of prepro-α-factor to mature α-factor consists of four proteolytic events. The first proteolytic event is removal of the signal sequence by signal peptidases after prepro-α-factor enters the endoplasmic reticulum (Waters *et al*., 1988). Each of the α-factor repeats are then liberated by cleavage of prepro-α-factor at the carboxyl side of the KR residues in the spacer region by the *KEX2* protease (Julius *et al*., 1984; Achstetter & Wolf, 1985; Mizuno *et al*., 1987, Brenner & Fuller 1992). The exposed C-terminal KR residues are then removed from the pro-α-factor repeats by a specific carboxypeptidase encoded by the *KEX1* gene (Dmochowska *et al*., 1987; Wagner & Wolf 1987) and the

final step in maturation of α-factor is removal of the alternating -GA/DA- residues at the N-terminus of the spacer region by dipeptyl aminopeptidase encoded by the *STE13* gene (Julius *et al..*, 1983; Figure 2).

The binding of α-factor to the α-factor receptor is the first step in a cascade of cellular events that initiates the mating response. In order to understand chemical signalling by peptides between cells, tissues, and organisms, it is necessary to know the conformation of the messenger peptide in the active site of its receptor. Previous studies of α-factor have suggested that in its active conformation the peptide forms a Type II β-bend spanning residues 7 through 10 (KPGQ) of the peptide (Masui *et al* ., 1979; Naider *et al* ., 1986; Jelick *et al* ., 1988 & 1989). These studies include the synthesis and bioactivity of α-factor analogs in which Gly^9 was replaced with an *L*-Ala, *L*-Leu, *D*-Ala, or *D*-Leu. It was found that the Gly to *L*-residue changes resulted in an inactive peptide whereas the Gly to *D*-residue changes remained biologically active. From this observation it was concluded that the Gly^9 to *D*-residue changes would retain the Type II β-turn but the *L*-residue changes would disrupt this turn (Shenbagamurthi *et al* ., 1985; Gounarides *et al* ., 1993) Proton NMR analysis also suggested that in its active conformation α-factor assumes a β-bend (Jelick *et al* ., 1989). From the observations that biologically active α-factor assumes a bent conformation spanning residues 7-10, a cyclic α-factor analog was synthesized in which the side chains of Lys^7 and Gln^{10} were covalently linked through an amide bond causing a bend to be maintained in this region of the peptide. This analog was found to be biologically active within the same concentration range found for the wild type α-factor (Xue *et al* ., 1989). Based on those results, further refinement of structure-function was attempted by the synthesis of cyclic lactam analogs in which the ring size (residues 7-10) was systematically varied. Additional cyclic analogs were synthesized in which residues 7 and 10 were replaced with

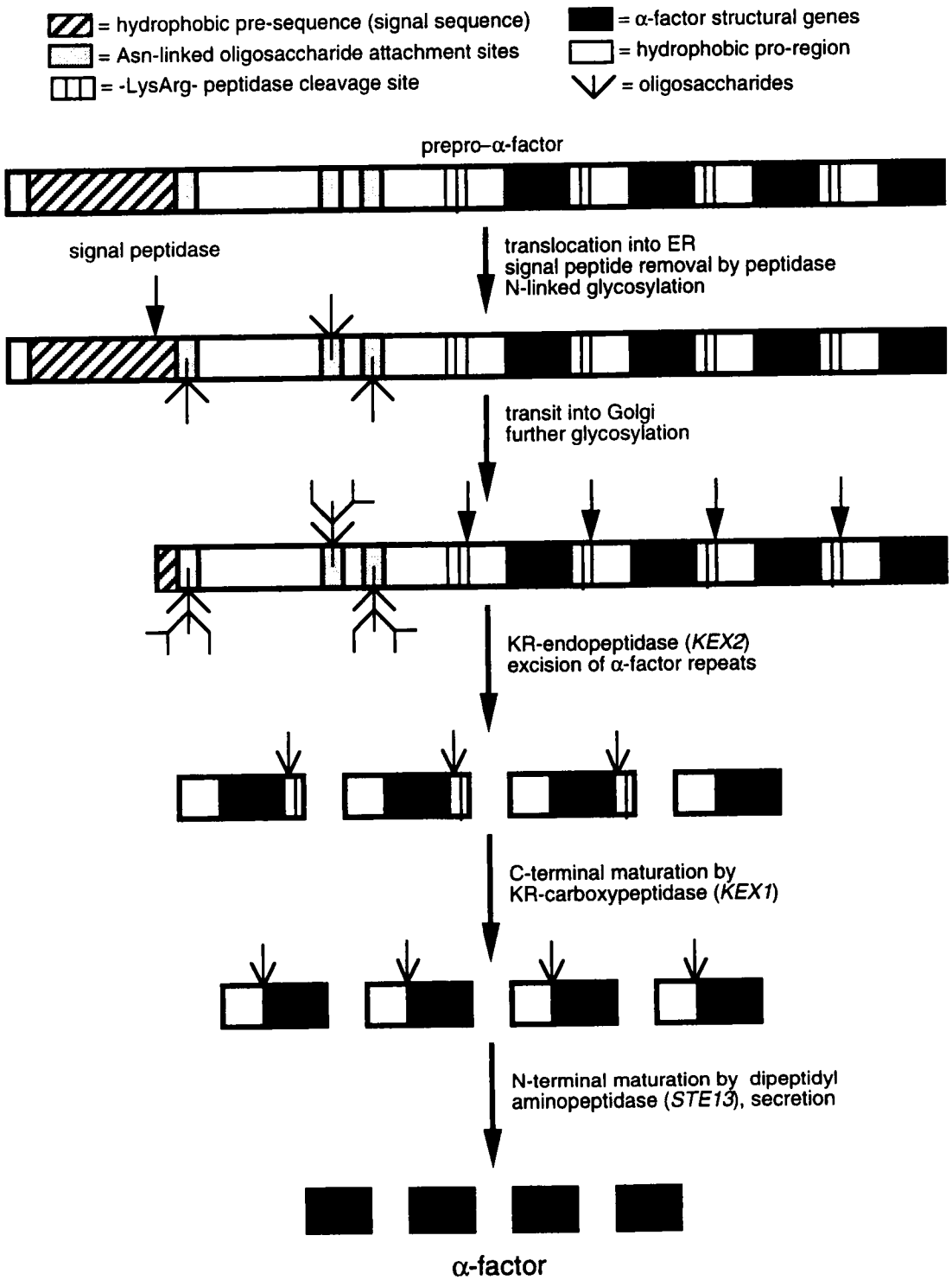


Figure 2. Processing of prepro-α-factor to give mature α-factor.

cysteines to form a disulfide bond in order to restrict the conformational mobility of the peptide. Part II and III of this dissertation discuss the synthesis and bioactivity of these cyclic analogs.

The α-factor receptor is encoded by the *STE2* gene. This gene has been cloned and from the gene sequence it has been determined that the gene product is a 431 residue protein (Ste2p) (Burkholder & Hartwell, 1985; Nakayama *et al.*, 1985). Hydropathy analysis suggests that the protein contains seven transmembrane domains and is part of a large family of seven transmembrane domain G-protein coupled receptors which include the mammalian β-adrenergic, the muscarinic acetylcholine and the rhodopsin receptors (Julius *et al* ., 1988; Dixon *et al* ., 1986; Kobilka *et al* ., 1987; Nathan & Hogness, 1984).

Genetic evidence suggesting that the *STE2* gene product is the α-factor receptor came from studies of *ste2* mutants which were unable to mate and did not respond to α-factor. It was also observed that expression of *STE2* in cell types which normally do not express this gene allowed those cells to become responsive to α-factor (Bender & Sprague, 1986). Biochemical evidence to support the *STE2* gene product being the α-factor receptor came from ligand binding studies which showed α-factor bound specifically to **a**-cells with a Kd $\approx 10^{-9}M^{-1}$ and that **a**-cells with a temperature sensitive *ste2* mutation exhibited temperature-sensitive pheromone binding (Moore 1983; Jenness *et al* ., 1983; Jenness *et al* ., 1986). Crosslinking experiments in which α–factor was chemically crosslinked into membrane fractions prepared from **a**-cells indicated that Ste2p resides in the plasma membrane and is consistent with the view that Ste2p is a membrane bound protein (Blumer *et al* ., 1988).

The pheromone response pathway is controlled by several genes. Genetic analysis has identified those genes whose products play a role in transmitting the signal once α-factor has bound to Ste2p located on the cell surface. Those genes involved include *STE2, STE4, STE5, STE7, STE11, STE12, STE18, STE20, FUS3/KSS1,* and *GPA1* (Sprague & Thorner, 1992; Table 1). As a result of the cloning and sequencing of these genes, information has been obtained as to the biochemical function of each gene product which helps in understanding the molecular events that occur as the signal is transmitted from the cell surface to the nucleus.

Peptide hormones are involved in many biological processes which include growth (growth hormone), reproduction (oxytocin and gonadotropin releasing hormone), basal metabolism (insulin and glucagon), and memory and pain perception (endorphins and enkephalins). The biological effect of these peptide hormones is generally produced when the peptide hormone interacts with its membrane bound receptor. Valuable information can be gained by studying the initial interaction of hormones with their receptors and how this interaction triggers signal transduction. Two attributes essential to understanding the interaction of peptide hormones with their receptors are the active conformation of the peptide hormone when bound to its receptor and determination of the binding domain within its receptor. In order to study the active conformation of peptide hormones it is necessary to design, synthesize and test peptide hormone analogs for their biological activity and ability to bind to the appropriate receptor. Determination of the binding domain within the receptor can be achieved either biochemically or genetically. Biochemical methods include chemical crosslinking and photoaffinity labeling using photoactivatable hormone analogs. Genetic approaches include using chimeric receptors, mutant receptors, and alanine-scanning of specific regions of the receptor. The information obtained from the structure-function studies of analogs coupled with the

Table 1. Genes controlling the pheromone response pathway.

Gene	Function
STE2	α-factor receptor
STE4	β-subunit of the G-Protein
STE5	Scaffolding Protein
STE7	Protein Kinase
STE11	Protein Kinase
STE12	Pheromone responsive transcriptional activator
STE18	γ-subunit of the G-Protein
STE20	Protein Kinase
FUS3/KSS1	Protein Kinase
GPA1	α-subunit of the G-Protein

identification of specific residues within the receptor involved in ligand binding will help to elucidate the molecular interactions involved in hormone-receptor binding. This information will also be used to design hormone agonist and antagonist which will be beneficial in treating human diseases caused by malfunctioning peptide hormone/receptor systems. With regard to the work discussed in this dissertation, the *Saccharomyces cerevisiae* α-factor mating pheromone is being used as a prototypical peptide hormone to investigate the interaction of peptides with cells and it is believed that the information obtained from studying this pheromone can be applied to other peptide hormone systems.

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PART II

SYSTEMATIC ANALYSIS OF THE *SACCHAROMYCES CEREVISIAE* α-FACTOR CONTAINING LACTAM CONSTRAINTS OF DIFFERENT RING SIZE*

*Part II was published in its entirety as **Wei, Y., A. McKinney, J. Becker, and F. Naider.** 1995. Systematic analysis of the *Saccharomyces cerevisiae* α-factor containing lactam contraints of different ring size. *Biochemistry.* **34**, 1308-1315. The research for Part II was done in collaboration with the laboratory of Dr. Fred Naider (College of Staten Island, Staten Island, NY). Dr. Naider's laboratory was responsible for the synthesis, purification and characterization of the cyclic α-factor analogs, which include HF cleavage of the peptides and the circular dichroism studies. Angela McKinney was responsible for the biological studies which include the growth arrest assay and binding competition assay.

CHAPTER I

INTRODUCTION

The search for the biologically active conformation of a peptide entails the application of a combination of physical, biochemical and synthetic methodologies. Extensive studies using spectroscopic techniques such as circular dichroism and nuclear magnetic resonance spectroscopy have investigated conformational aspects of linear peptides in solution and in the presence of lipid. Although these procedures often result in conclusions regarding peptide conformation, the inherent flexibility of many linear peptides makes suspect the relationship of the solution structure to the biologically relevant conformation. Attempts to surmount these uncertainties have used a variety of solvent conditions and studies in the presence of lipids, cryoscopic solvents at low temperature, or solvents of high viscosity to mimic the binding environment (Motta *et al.*, 1988).

An alternative to the above procedures is to constrain the flexibility of the peptide by covalent modification (Veber *et al.*, 1979; Rizo and Gierasch, 1992). In particular, backbone and side chain cyclization can result in rigidified peptides that may closely mimic the bound state (Veber *et al.*, 1981; Hruby *et al.*, 1982). A number of such studies have resulted in superactive agonists and antagonists which have excellent potential in both drug design and in the quest for understanding the bound conformation of biologically active peptides. Cyclic peptides have the obvious advantage that they are more amenable to conformational analysis because the ϕ,ψ distributions are significantly narrowed.

The α-factor mating pheromone of *Saccharomyces cerevisiae* is a linear tridecapeptide (WHWLQLKPGQPMY) involved in the sexual conjugation between opposite haploid cells of this yeast. The peptide has been extensively studied as a model for understanding the biochemistry of mammalian peptide hormones (Sprague and Thorner, 1992). Spectroscopic studies on this peptide indicate that the Lys^{7}—Q^{10} fragment assumes a Type II ß-turn and that this conformational feature is an important factor in the bioactive state of the molecule (Jelicks *et al.*, 1988; Gounarides *et al.*, 1993). Recently, we showed that cyclo^{7,10}[Nle^{12}]α-factor, which contains a side chain lactam, retains significant, but not full, biological activity (Xue *et al.*, 1989). The rigidification of the pheromone by lactamization between the side chains of the seventh and tenth residues with retention of biological activity led to the conclusion that the pheromone must be bent when it binds to its receptor.

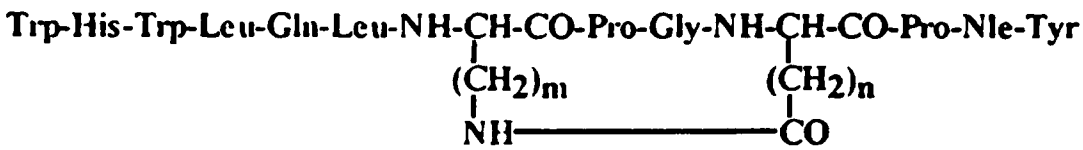
In recent years, a number of laboratories have used side chain lactamization to constrain regions of linear peptide hormones (Al-Obeidi *et al.*, 1989; Struthers *et al.*, 1990; Roques, 1992). Important insights into the biologically active conformation of growth hormone-releasing hormone have been achieved using i - (i + 4) lactams to stabilize an α-helix (Fry *et al.*, 1992). Since a ß-turn involves four residues, i - (i + 3) lactamization might be expected to stabilize this conformation. Side chain cyclization between the ε-amine of Lys^{7} and the γ-carboxyl of Glu^{10} in cyclo^{7,10}[Nle^{12}]α-factor results in an 18 membered ring that spans the middle of the pheromone. It is possible that this ring causes significant distortions in the backbone ϕ,ψ angles, resulting in an analog with good but not equal potency to the native pheromone. In order to study the influence of side chain lactam formation on both the bioactivity of the α-factor and the conformation of the peptide in the cyclic ring, we have initiated a systematic investigation of the effect of ring size on the bioactivity of the pheromone. In this dissertation, the synthesis,

purification and biological characterization of eight cyclic α-factor analogs in which the ring size is varied from 18 to 14 atoms is discussed.

CHAPTER II

MATERIALS AND METHODS

Except for N^{α}-Boc-Orn(N^{δ}Fmoc), N^{α}-Boc-Dab(N^{γ}-Fmoc), and Nα -Boc-Dpr(N^{β}-Fmoc), all protected amino acids were purchased from BaChem Inc. (Torrance, CA). N^{α}-Boc-Orn-(δ-Fmoc) and N^{α}-Boc-Dab(N^{γ}-Fmoc) were prepared in 90% and 63% overall yields, respectively, from Boc-Gln and Boc-Asn (Waki *et al.*, 1981; Stanfield *et al.*, 1990). N^{α}-Boc-Dpr(N^{β}-Fmoc) was prepared by the procedure of Stanfield *et al.*, (1990). N^{α}-Boc-Glu(γ-OFm) and N^{α}-Boc-Asp(β-OFm) were prepared from NαBoc-Glu(γ-OBzl) and N^{α}-Boc-Asp(β-OBzl) as previously described (Bolin *et al.*, 1989). All synthetic derivatives were purified to over 99% homogeneity as judged by reversed-phase HPLC, and characterized by 200 MHz ^{1}H NMR spectroscopy before being used in solid-phase peptide synthesis. All peptides were synthesized with Nle in place of Met^{12} of the native α-factor. Previous studies have shown Nle to be an isosteric substitution with full retention of biological activity of [Nle^{12}]α-factor in comparison to α-factor (Raths *et al.*, 1988). In this dissertation the designation α-factor and [Nle^{12}]α-factor is used interchangeably. All cyclic analogs synthesized are given a **C** prefix, with the corresponding linear homologs given a **L** prefix. The numbers following the prefix indicate the number of CH_2 groups on the side chains of residues 7 and 10, respectively. For example, the cyclo^{7,10}[Orn^{7},Glu^{10},Nle^{12}]-α-factor is designed **C32** (Figure 1), whereas the corresponding linear peptide [Orn^{7},Glu^{10},Nle^{12}]-α-factor is designed **L32**.



C(4)2: m = 4; n = 2	**C41**: m = 4; n = 1
C32: m = 3; n = 2	**C31**: m = 3; n = 1
C22: m = 2; n = 2	**C21**: m = 2; n = 1
C12: m = 1; n = 2	**C11**: m = 1; n = 1

FIGURE 1: Chemical structures of cyclic analogs of α-factor. **C(4)2** represents a D-Lys[7] residue. In other analogs, all residues are L.

Peptide Synthesis

N^{α}-Boc-Tyr(2-BrZ) was reacted with 4-(bromomethyl)-phenylacetic acid phenacyl ester to produce Nα-Boc-Tyr(2-BrZ)-$OCH_2PhCH_2COOCH_2COPh$. This product was reduced using Zn/HOAc to N^{α}-Boc-Tyr(2-BrZ)-OCH_2PhCH_2COOH (yield 77%) (Tam *et al*, 1979). The free acid was coupled with aminomethyl polystyrene resin (0.759 mmol/gram of resin) using BOP in DMF to give N^{α}-Boc-Tyr(2-BrZ)-OCH_2-PAM-resin.

Solid Phase Peptide Synthesis

The solid-phase syntheses of the α-factor analogs were carried out manually starting with N^{α}-Boc-Tyr(2-BrZ)-OCH_2-PAM-resin (0.54 mmol/g of resin), following the procedures described by Xue *et al.*. (1989). The Boc group was used for all N^{α} protections. The side chain protecting groups were Trp(For), His(Tos), D-Lys(Fmoc), Glu(OFm), Orn(Fmoc), Dab(Fmoc), Dpr(Fmoc), and Lys(Fmoc), and Asp(OFm). A typical cycle consisted of the following procedures (15 mL of solvent/g of resin): (1) CH_2Cl_2, 3 x 1 min; (2) 50% TFA in CH_2Cl_2 or 48% TFA in CH_2Cl_2 (2% DMS) (from Trp^3 on), 1 x 1 min; (3) 50% TFA in CH_2Cl_2 or 48% TFA in CH_2Cl_2 (2% DMS) (from Trp^3 on), 1 x 30 min; (4) CH_2Cl_2, 3 x 1 min; (5) Kaiser test; (6) 10% DIEA in CH_2Cl_2, 1 x 2 min; (7) 10% DIEA in CH_2Cl_2, 1 x 5 min; (8) CH_2Cl_2, 3 x 1 min; (9) N^{α}-Boc-amino acid (3 equiv) in CH_2Cl_2 added to the vessel followed by DIPC (3 equiv), 3 h; (10) CH_2Cl_2, 6 x 1 min; (11) kaiser test; (12) 5% DIEA in CH_2Cl_2, 1 x 2 min; (13) CH_2Cl_2, 3 x 1 min; (14) N^{α}-Boc-amino acid (1 equiv) in CH_2Cl_2 added to the vessel followed by DIPC (1 equiv), 1 h; (15) CH_2Cl_2, 6 x 1 min; (16) Kaiser test.

In cases where the N^{α}-Boc-amino acids did not dissolve well in CH_2Cl_2, such as for Boc-Glu(OFm), Boc-Leu, Boc-His(Tos), and Boc-Trp(For), the couplings were carried out in CH_2Cl_2/DMF (1;1, v/v) and the resin was washed with DMF prior to and after the coupling step, 2 x 1 min. The procedures of the cycle immediately following the

incorporation of Boc-Gln were different from the preceding protocol: (1) dioxane, 3 x 1 min; (2) 4 N HCl in dioxane, 1 x 1 min; (3) 4 N HCl in dioxane, 1 x 30 min; (4) dioxane, 3 x 1 min; (5) $CHCl_3$, 3 x 1 min; (6) Kaiser test; (7) 10% DIEA in $CHCl_3$, 1 x 2 min; (8) 10% DIEA in $CHCl_3$, 1 x 5 min; (9) $CHCl_3$, 3 x 1 min; (10) CH_2Cl_2, 3 x 1 min; (11) DIPC (3 equiv) in CH_2Cl_2 added to the vessel followed by Boc-amino acid (3 equiv) in CH_2Cl_2, 3 h; (12) CH_2Cl_2, 3 x 1 min; (13) Kaiser test; (14) $CHCl_3$, 3 x 1 min; (15) 5% DIEA in $CHCl_3$, 1 x 2 min; (16) $CHCl_3$, 3 x 1 min; (17) CH_2Cl_2, 3 x 1 min; (18) DIPC (1 equiv) in CH_2Cl_2, 1 h; (19) CH_2Cl_2, 6 x 1 min; (20) Kaiser test. In the cases in which a third coupling was required, as revealed by the Kaiser test, 1 equiv each of Boc-amino acid, DIPC, and HOBt in DMF were added, and the reaction was allowed to proceed for 1-3 h. This approach always resulted in a negative Kaiser test.

Cyclization

Cyclization was carried out on the resin-bound peptide prior to HF cleavage to reduce any intermolecular cross-links using BOP to effect amide bond formation. For example, the Boc-Trp(For)-His(Tos)-Trp(For)-Leu-Gln-Leu-D-Lys(Fmoc)-Pro-Gly-Glu(OFm)-Pro-Nle-Tyr(2-BrZ)-PAM-resin (1.53 g) was washed with DMF (3 x 1 min), reacted with 20% piperdine in DMF (1 x 2 min and 1 x 30 min) to remove the Fmoc and OFm groups, and washed with DMF (2 x 1 min), MeOH (2 x 1 min), and CH_2Cl_2 (2 x 1 min). The free amino groups were indicated by a positive Kaiser test. The resin was washed with DMF (2 x 1 min) and shaken with BOP (5 equiv) in DMF (containing 1.5% DIEA) for 3 h to effect the amide bond formation. The resin was subjected to the Kaiser test after being washed with DMF (2 x 1 min), MeOH (2 x 1 min), and CH_2Cl_2 (2 x 1 min). A positive Kaiser test indicated incomplete cyclization, and additional BOP (3 equiv) in DMF (containing 1.5% DIEA) was added and the mixture was shaken overnight (total coupling time, 12 h). A negative Kaiser test was obtained, which indicated the

absence of free amino groups. The on-resin cyclization of other cyclic peptides followed the preceding protocol. Increased difficulty with amide bond formation was encountered as the size of the lactam ring decreased. For example, cyclization to form **C11** required a total of 28 h of coupling time.

HF Cleavage

It was found to be beneficial to deprotect other side chain protecting groups before HF cleavage (Xue *et al.*, 1989). In particular, cleaner crude products were obtained when the tosyl group on His was removed using HOBt. The tosyl group on His^2 was deprotected with 5% HOBt in DMF (1 x 2 min and 1 x 30 min), and the amino-terminal Boc group was deprotected with 50% TFA in CH_2Cl_2 (1 x 2 min and 1 x 30 min) . The formyl groups on Trp^1 and Trp^3 were deprotected during the treatment with 20% piperidine in DMF used to remove the Fmoc and OFm groups prior to cyclization (see above). The resin was washed with CH_2Cl_2 (2 x 1 min), MeOH (2 x 1 min), CH_2Cl_2 (2 x 1 min) , and MeOH (2 x 1 min) and dried in vacuo overnight. One gram of the resin was mixed with 1 mL of anisole in a Kel-F HF reaction vessel, and HF was condensed at -78 °C under reduced pressure to a total volume of 15 mL (HF + anisole + resin). The HF cleavage was allowed to proceed at -5 to °0 C for 1 h. After evaporation of HF, the residue was washed with ethyl ether to remove scavenger, extracted with 20-50% acetic acid, and lyophilized. The yields of the crude peptides, based on the starting amine content of the aminomethyl resin, were in the ranges 32-58% for the cyclic analogs and 35-83% for the linear analogs.

Tritiated [Nle^{12}]α-factor was prepared as previously described (Raths *et al.*, 1988). Briefly, α-factor containing dehydroproline (Dhp) in place of proline in positions eight and eleven and norleucine at position twelve in place of methionine was synthesized

by standard methods of solid phase peptide synthesis. [Dhp^{8}, DHP^{11}, Nle^{12}]α-factor was tritiated by Amersham Corp. by the T-3 method, involving reduction by tritium gas. The crude tritiated peptide was purified to homogeneity by HPLC as previously described (Raths *et al.*, 1988). Purity and specific activity were monitored by TLC, HPLC, amino acid analysis, and biological activity. Control reductions with hydrogen gas indicated that [Nle^{12}]α-factor was fully regenerated (Raths *et al.*, 1988).

Purification and Characterization

The crude peptides were analyzed by reversed-phase analytical HPLC on a Waters μBondapak C_{18} Column (3.9 mm x 300 mm). Crude peptides usually were dissolved in 20-40% CH_3OH/H_2O/ 0.025%TFA and purified by reversed-phase, semi-preparative HPLC on a Waters μBondapak C_{18} Column (19 mm x 150 mm or 19 mm x 300 mm). The purification was carried out with a linear gradient of H_2O (0.025% TFA) and CH_3CN (0.025% TFA), from about 20% CH_3CN to about 60% CH_3CN over 60 min. at a flow rate of 6 ml/min. Up to 200 mg crude material could be purified in a single run. The linear analogs were usually over 99% pure after a single purification. Cyclic analogs needed additional chromatographic separations. All analogs exhibited one spot on silica thin layers in two solvent systems using UV and ninhydrin detection (Table 1 and 2). Amino acid analyses were carried out at the Wistar Institute, and FABMS was measured at the University of Tennessee Mass Spectrometry Center. ^{1}H NMR spectra were acquired on a 400 MHz JEOL GX-400 spectrometer or a Bruker 200 MHz spectrometer. Data processing was performed on a SUN Sparc IPC workstation utilizing the NMRI 1.4.1 software package.

Table 1: Chemical and Physical Properties of Cyclic Analogs

	amino acid analysis										R_f[b]				
peptide	Asx	Glx	Gly	His	Leu	Lys	Nle	Pro	Tyr	Trp[a]	solvent A	solvent B	K'[c]	FAB-MS[d]	calculated mass (amu)
C(4)2		1.86	0.94	0.88	2.21	1.02	0.96	2.05	1.07	ND[e]	0.46	0.68	6.92	1649.0	1648.8
C32		2.03	1.20	0.91	1.92		0.94	2.01	1.07	1.92	0.43	0.65	6.90	1635.0	1634.8
C22		1.75	0.97	1.10	2.18		0.93	1.93	1.02	2.13	0.44	0.64	6.85	1620.1	1620.8
C12		1.82	0.99	1.02	1.86		ND	2.11	1.21	ND	0.44	0.68	6.84	1607.4	1606.8
C41	0.92	1.04	1.12	0.89	2.03	0.91	0.98	2.04	1.07	ND	0.51	0.69	7.14	1635.0	1634.8
C31	0.93	0.91	1.01	1.04	1.87		ND	2.10	1.15	ND	0.54	0.67	6.99	1621.5	1620.8
C21	1.08	0.88	0.97	1.01	1.80		ND	2.09	1.16	ND	0.51	0.66	6.89	1607.3	1606.8
C11	1.16	0.92	0.96	1.01	1.81		ND	2.02	1.14	ND	0.51	0.66	6.87	1593.3	1592.8

[a] In two cases (**C32** and **C22**), special care was taken during hydrolysis to obtain the Trp content. In the other peptides, the presence of Trp residues is verified by the FAB-MS values. [b] Solvent system A, butanol/acetic acid/water (4:1:2, v/v/v); solvent system B, butanol/acetic acid/water/pyridine (4:1:2:1, v/v/v/v). R_f values were determined using TLC on silica. [c] K' values were determined for a μBondapak C_{18} column using a 20−60% acetonitrile gradient (0.025% TFA) over 20 min. [d] The values represent monoisotopic masses. [e] ND, not done.

Table 2: Chemical and Physical Properties of Linear Analogs

	amino acid analysis										R_f[b]				
peptide	Asx	Glx	Gly	His	Leu	Lys	Nle	Pro	Tyr	Trp[a]	solvent A	solvent B	K'[c]	FAB-MS[d]	calculated mass (amu)
L(4)2		1.93	1.01	0.91	2.22	1.12	0.86	1.97	0.99	ND[e]	0.43	0.59	5.99	1666.9	1666.8
L42		2.24	1.03	0.84	1.95	0.98	1.04	1.96	0.96	ND	0.34	0.53	5.33	1666.9	1666.8
L32		1.93	1.01	0.89	1.96		0.95	2.18	1.09	1.91	0.44	0.59	6.06	1653.0	1652.8
L22		1.97	1.04	0.98	2.15		0.86	2.07	1.10	1.88	0.45	0.61	6.14	1638.1	1638.8
L12		1.77	1.00	1.05	1.87		ND	2.13	1.19	ND	0.47	0.63	6.19	1625.4	1624.8
L41	1.07	1.02	1.13	0.88	1.83	1.01	0.84	2.20	1.03	ND	0.48	0.59	6.11	1653.0	1652.8
L31	1.09	0.87	0.97	1.00	1.77		ND	2.18	1.13	ND	0.49	0.58	6.11	1639.4	1638.8
L21	1.21	0.98	1.09	1.06	2.02		ND	2.35	1.26	ND	0.44	0.60	6.13	1625.3	1624.8
L11	1.08	0.87	0.97	1.01	1.85		ND	2.07	1.15	ND	0.47	0.61	6.20	1611.2	1610.8

[a] In two cases (**L32** and **L22**), special care was taken during hydrolysis to obtain the Trp content. In the other peptides, the presence of Trp residues is verified by the FAB-MS values. [b] Solvent system A, butanol/acetic acid/water (4:1:2, v/v/v); solvent system B, butanol/acetic acid/water/pyridine (4:1:2:1, v/v/v/v). R_f values were determined using TLC on silica. [c] K' values were determined for a μBondapak C_{18} column using a 20−60% acetonitrile gradient (0.025% TFA) over 20 min. [d] The values represent monoisotropic masses. [e] ND, not done.

CD Measurements

All CD measurements were performed on a Jasco J-500 spectropolarimeter using circular quartz cells, with a path length of 0.01 cm at ambient tmeperature (~25 °C). The instrument was calibrated with *d*-(+)-10-camphorsulfonic acid. Spectra were obtained in the range 190-260 nm, at a scan speed of 20nm/min and using a time constant of 16 s. Peptide solutions were prepared in HPLC grade water and methanol. All spectra were corrected by subtracting the solvent absorption recorded under identical conditions. The CD spectra are reported in terms of molar ellipticity, $[\theta]_M$ (deg cm^2 $dmol^{-1}$), wherein the actual molecular weight of the peptide and not the mean residue molecular weight was used to calculate the ellipticity. The CD additivity experiment was carried out following a method described by Fasman and co-workers (Perczel *et al.,* 1993). Specifically, Ac-cyclo[Orn-Pro-Gly-Glu]-NH_2 (0.44 mg, 1μmol) and **L32** (2.00 mg, 1 μmol) were dissolved in a mixture of 1 mL of water and 1 mL of MeOH. In the additivity experiment, for molar ellipticity calculations, the average concentration, 0.61 mg/ml, was used with a modified molecular weight of 1217 (average molecular weight of the two peptides).

Growth Arrest Assay

The biological activity of the α-factor analogs was assayed using a growth arrest assay (Halo) as described previously (Raths *et al.*, 1988). Briefly, peptides to be tested are added to sterile filter disks (diameter = 6 mm) placed on a lawn of S. cerevisiae RC629. The amount of peptide is systematically lowered until no growth arrest is observed indicated by a halo around the disk. Activity was measured as the diameter (mm) of growth arrest halo on a lawn of *S. cerevisiae* cells. At least three determinations were made and averaged. The values of each experiment were within 2 mm of each other. Results for a typical experiment are given in Figure 2, where the end points for

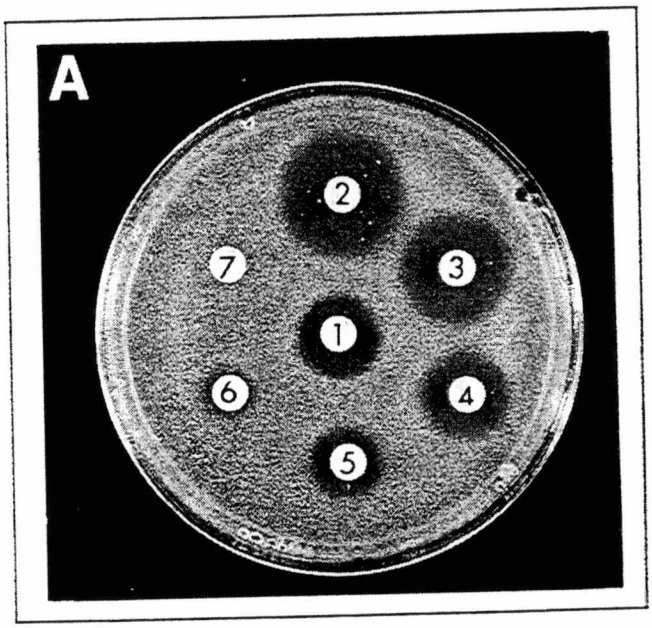
C12 and **L12** are 0.5μg/disk for this determination, although the zone diameters are clearly different. All activities were examined using a supersensitive mutant (RC629 [*sst1*]) of *S. cerevisiae*. The *sst1* mutation results in the loss of *BAR1* protease, a secreted enzyme, which cleaves the α-factor between residues 6 and 7 (Chan and Otte, 1982). Thus, the biological activities observed are not influenced by peptide degradation.

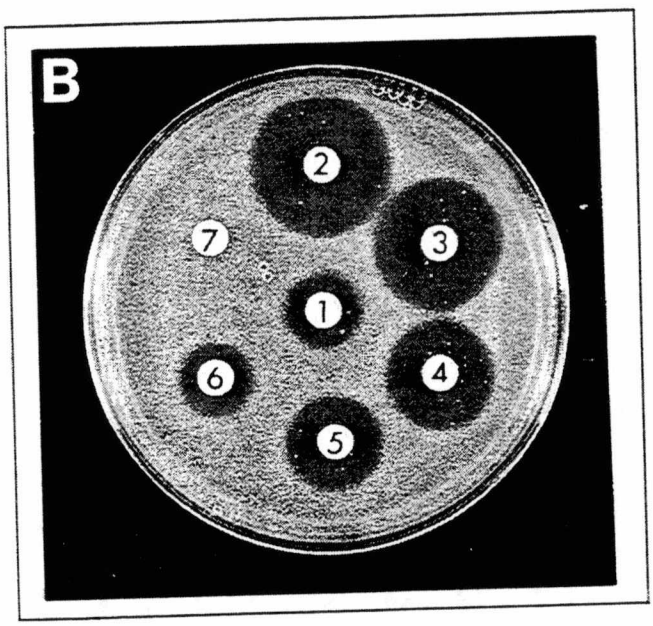
Binding Competition Assay

Competition of bound tritiated [Nle^{12}]α-factor by unlabeled α-factor analogs was measured by the following protocol. *S. cerevisiae* strain 4202-15-3 (*MATa cryl bar1-1 ade2-1 his 4-580 lys2 tyrl SUP4-3*) was grown in YM-1 medium [yeast extract (Difco), 5 g/1; peptone (Difco), 10 g/1; yeast nitrogen base without amino acids (Difco), 6.7 g/1; adenine, 0.01 g/1 uracil, 0.01 g/1, succinic acid, 10 g/ml; sodium hydroxide, 6 g/1; and glucose, 10 g/ml, at a final pH of 5.8] overnight at 30°C with shaking at 120 rpm to 1 x 10^7 cells/ml. Cells were harvested by centrifugation (6000 x g for 10 min. at 4°C), and the resultant pellet washed twice with ice-cold YM-1+i medium (YM-1 containing 10mM sodium azide, 10mM KF, and 10mM *p*-tosyl-L-arginine methyl ester) and resuspended to 1.25 x 10^9 cells/ml in YM-1+i. Final assay concentrations of the unlabeled competitors were between 3 x 10^{-5} to 1 x 10^{-9} M as determined from the A_{287} for each peptide and the extinction coefficients calibrated for [Nle^{12}]α-factor from quantitative elemental analysis. The reaction was started by the addition of 100 μl of analog/[^{3}H]α-factor mix (120 μl of appropriate unlabeled α-factor analog mixed with 120 μl of labeled α-factor 9.3 x 10^{-7} M, 9.24 Ci/mmole) to 400 μl of cell suspension. Identical competition curves were obtained using a ten-fold lower concentration of radioactive ligand.

After the reaction was started, two 20 μl samples were removed and counted to determine total amount of radioactivity present. Duplicate reactions were started 3 min.

Figure 2. Growth arrest of *S. cerevisiae* RC629 (**MATa** *sst1-2*) by α-factor analogs. Peptides were added to sterile disks placed on a lawn of RC629 cells and incubated until well-developed halos of growth inhibition could be observed. (A) [Nle^{12}]-α-factor at 0.2μg (disk 1) and **C12** at 10, 5, 2, 1, 0.5, and 0.2 μg (disks 2-7, respectively). (B) [Nle^{12}-α-factor at 0.2 μg (disk 1) and **L12** at 10 , 5, 2, 1, 0.5, and 0.2 μg (disks 2-7, respectively).


A
2
7
3
1
6
4
5


B
2
7
3
1
6
4
5

after initial reaction for each analog tested. At 30 min., two 200 µl portions of cell suspension were added separately to 2 ml of YM-1+i and filtered over GN-6 Metricel filters (Gelman Sciences, Inc., Ann Arbor, MI) presoaked in 1% bovine serum albumin. The reaction tubes were then rinsed twice with 2 ml ice-cold YM-1+i medium, with each rinse filtered over the same filter. Finally, each filter was rinsed twice with 2 ml YM-1+i medium. The non-specific binding of labeled α-factor in the absence of cells was less than 100 cpm. This represented less than 5% of the total counts. The filters were counted in 5 ml of Budget-solve counting cocktail (Research Products International Corp., Mt. Prospect, IL). All assays and manipulations were done in siliconized borosilicate tubes or vials. Equilibrium binding was reached after a 30 min incubation of cells and pheromone, with or without competitor, as indicated by identical binding and competition curves in control experiments using a 90 min incubation time (data not shown). The binding of the radio-labeled [Nle^{12}]-α-factor was highly specific as the radioactive counts were reduced to background levels by the addition of an excess of unlabeled pheromone. The binding curve for α-factor has been previously done by the infinite dilution experiment by us and others (Raths *et al.*, 1988; Blumer *et al.*, 1988; and Jenness *et al.*, 1986) to show that there is no evidence of cooperactivity in the binding between α-factor and its receptor.

CHAPTER III

RESULTS

Synthesis and Characterization of Cyclic α-Factor Analogs

The lactam containing α-factor analogs were all synthesized on PAM resins using the strategy previously described for the cyclo^{7,10}[Nle^{12}]α-factor (Xue *et al.*, 1989). Cyclization was carried out on the resin using BOP as a coupling reagent. All crude peptides (both cyclic and linear) contained one major product and similar side products. In a parallel investigation of the synthesis of corresponding model tetrapeptides (e.g., Ac-cyclo[Lys-Pro-Gly-Glu]-NH_2), we observed a major impurity in the crude product (Rao *et al.*, 1995). This impurity has been isolated and shown to be the cyclic dimer that results from interpeptide amide bond formation. Similar interchain reactions have been reported during the syntheses of cyclic analogs of enkephalin (Schiller *et al.*, 1985). In the present case, it appears that inclusion of an Xxx-Pro-Gly-Zzz sequence in the center of a large peptide (such as the [Nle^{12}]-α-factor) impedes interchain reactions. This may be a consequence of a general steric effect that prevents interchain contacts or of the fact that cyclization is favored when the Xxx-Pro-Gly-Zzz sequence is present in the center of the tridecapeptide. The cyclic lactam-containing tridecapeptides were purified to near homogeneity using preparative reversed-phase chromatography. Yields of the final products varied from 5% to 20 %. The yields of the analogous linear peptides were 8% to 35%.

Chemical and Spectroscopic Characterization of α-Factor Analogs

All peptides subjected to bioassay were >99% pure as judged by acetonitrile/water/trifluoroacetic acid gradients on reversed phase HPLC and greater than

97% pure using methanol/water/ trifluoroacetic acid gradients (Figure 3). The HPLC analysis permits us to conclude that the cyclic analogs contained less than 0.1% of the corresponding linear homolog. We also coinjected [Nle^{12}]-α-factor with the purified analogs and ascertained that the parent pheromone was not present in any of these peptides (data not shown). Therefore, it is highly unlikely that the activity of the cyclic lactams is due to contamination by either [Nle^{12}]-α-factor or the corresponding linear homolog. This conclusion is supported by the more than 100-fold variation in the activity of the cyclic analogs and by the fact that several cyclic analogs (e.g., **C22** and **C12**) have as much as 10% of the activity of [Nle^{12}]-α-factor (see Table 3). Neither of these observations can be explained by spurious impurities that might be picked up during HPLC purification.

The peptides had the expected amino acid ratios and monoisotopic masses within 0.7 amu of the calculated values (Tables 1 and 2). The linear and cyclic analogs differ by 18 amu, indicating formation of the amide bond between the side chains of residues 7 and 10. Several of the peptides were characterized by 1D and 2D NMR spectroscopy and gave the expected resonances. The $K^7{}_\alpha$-$P^8{}_\partial$ connectivity which appeared in the NOESY spectra of **C32** and **C42** indicates that the amide bond involving Pro^8 is in a *trans* configuration in this 17- and 18-membered lactam-containing tridecapeptide (data not shown). A detailed conformational analysis of all of these cyclic tridecapeptides, using ^{1}H NMR and quantitative NOE approaches, is currently in progress.

Circular Dichroism Studies on Cyclic and Linear Tridecapeptides

In order to gain insights into the conformational preferences of the lactam-containing α-factor analogs, we measured the circular dichroism spectra of **C32** and appropriate controls. Previous CD studies on α-factor concluded that this linear peptide is predominantly unstructured in aqueous buffer and becomes more ordered in organic

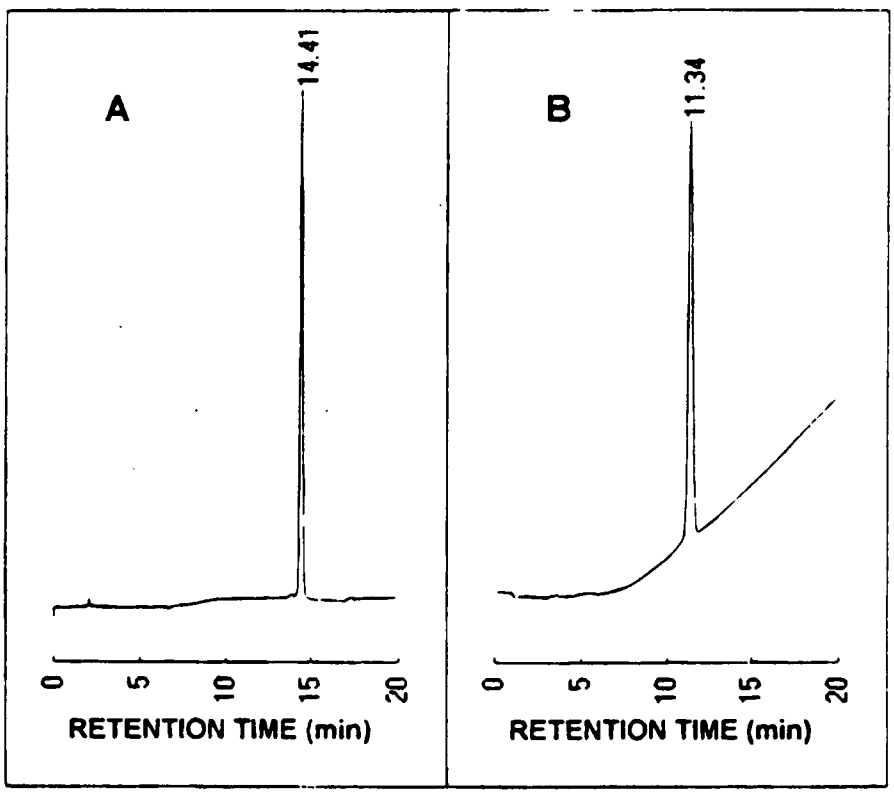


FIGURE 3: (A) HPLC of **C22** in a H_2O/CH_3CN system. Chromatography was performed on a C_{18} column using a 20−60% CH_3CN gradient (0.025% TFA) over 20 min. (B) HPLC of **C22** in a H_2O/MeOH system. Chromatography was performed on a C_{18} column using a 50−90% MeOH gradient (0.025% TFA) over 20 min.

Table 3: Bioactivity and Receptor Affinity of Cyclic and Linear α-Factor Analogs

peptide	ring size	bioactivity[a] (μg/disk)	IC_{50}[c] (M × 10^7)	IC_{50} analog/IC_{50} ([Nle^{12}]-α-factor
C(4)2	18	>10 (>200)[b]	100	50
C42	18	0.5 (10)	150	75
C32	17	10 (200)	600	300
C22	16	0.5 (10)	2200	1100
C12	15	0.5 (10)	2000	1000
C41	17	>10 (>200)	>10000	>5000
C31	16	10 (200)	45000	22500
C21	15	5 (100)	10000	5000
C11	14	>50 (>1000)	>10000	>5000
L(4)2		5 (100)	700	350
L42		0.1 (2)	10	5
L32		0.1 (2)	30	15
L22		0.05 (1)	600	300
L12		0.1 (2)	260	130
L41		10 (200)	1400	700
L31		5 (100)	10000	5000
L21		5 (100)	2700	1350
L11		5 (100)	10000	5000

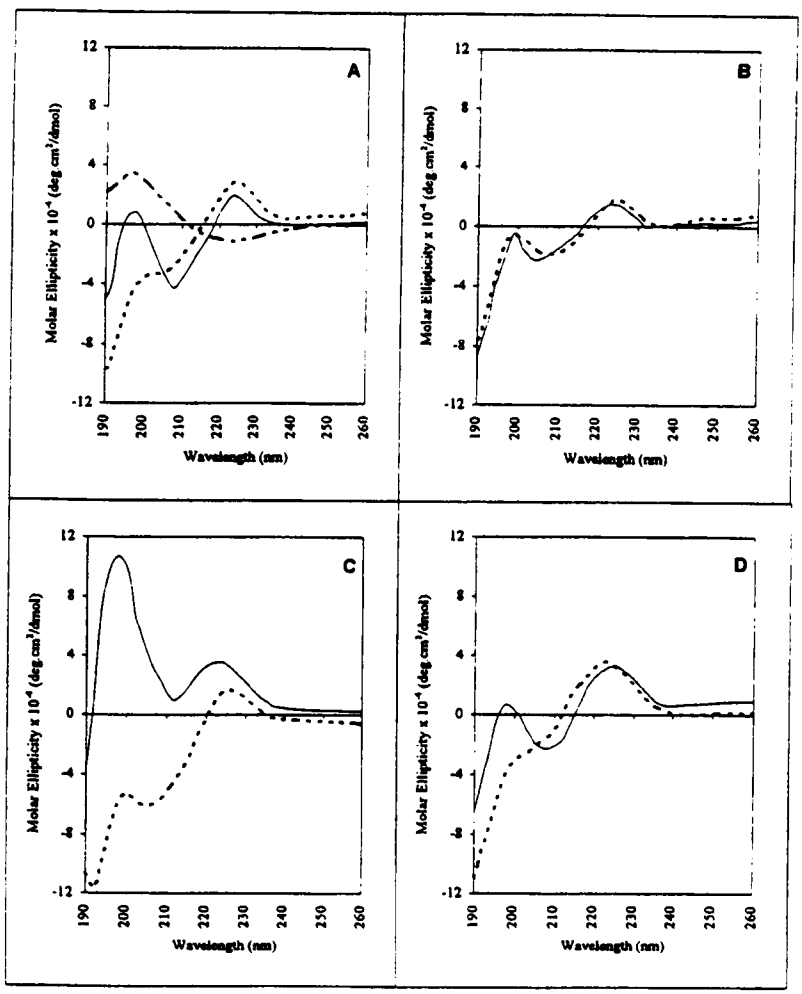
[a] The minimum amount of pheromone causing growth arrest of *S. cerevisiae* RC629. The quantities (μg/disk) represent the average of at least three determinations. [b] The numbers in parentheses are the ratio of the end point amounts for the analog to that of [Nle^{12}]-α-factor that caused growth arrest at a minimum of 0.05 μg/disk. [c] Peptide concentration needed to reduce binding by 50%. IC_{50} of [Nle^{12}]-α-factor = 2.0×10^{-7} M.

solvents or the presence of lipid (Higashijima *et al.,* 1983; Shenbagamurthi *et al.,* 1985). The CD pattern of **L32** in water/methanol (1:1, v/v) shows a broad minimum below 195 nm, a shoulder at 201 nm, and positive ellipticity between 217 and 235 nm (Figure 4A). Such a curve would be associated with a predominantly disordered peptide. In contrast, the CD pattern for a model tetrapeptide (Ac-cyclo-[Orn-Pro-Gly-Glu]-NH_2), which represents the cyclized central region of **C32**, exhibits a negative minimum at 223 nm and a maximum at 198 nm in methanol/water (Figure 4A). The general shape of this curve is typical of those found for peptides in β-turn structures (Woody, 1974). The CD spectrum for **C32** (Figure 4A) differs appreciably from those for **L32** and the cyclic tetrapeptide. However, CD spectra obtained on an equimolar mixture of **L32** and Ac-cyclo-[OrnProGlyGlu]-NH_2 or by computer summation of the patterns for **L32** and Ac-cyclo[OrnProGlyGlu]-NH_2 in Figure 4A are qualitatively similar to that found for **C32** (compare Figure 4A,B). On the basis of these results, it is reasonable to conclude that the central region of **C32** retains the turn structure found in the cyclic tetrapeptide. We also measured the CD spectra of the other cyclic analogs and their linear homologs in methanol/water (1:1, v/v). All of the linear analogs exhibit CD patterns that are similar to that of **L32** (for example, compare Figure 4A,C,D). In contrast, as was found for **C32**, the CD patterns for the cyclic lactam-containing tridecapeptides all differt markedly from that of the linear homolog (Figure 4C,D). A more detailed understanding of the conformational preferences of the center of the cyclic pheromones will be forthcoming upon completion of our NMR anlaysis.

Biological Activity of Lactam-Containing α-Factors Analogs

The biological activity of the lactam analogs was determined using *S. cerevisiae RC629*. The most potent cyclic analogs had end points in the growth arrest assay of 0.5 μg/disk (**C42**, **C22**, **C12**; Figure 2 and Table 3). The activities of the cyclic analogs

Figure 4. Circular dichroism of [Nle^{12}]-α-factor analogs in methanol/water: (A) 1.0 mg/ml **C32**(—), 1.0 mg/ml **L32**, (---), 1.0 mg/ml Ac-cyclo[Orn-Pro-Gly-Glu]-NH_2 (—- --); (B) experimental (—) and computed (---) CD spectra of equimolar mixtures of **L32** and Ac-cyclo[Orn-Pro-Gly-Glu]-NH_2; (C) 1.0 mg/ml **C12** (—), 1.0 mg/ml **L12** (---); (D) 1.0 mg/ml **C21** (—), 1.0 mg/ml **L21** (---).


A
B
C
D
Molar Ellipticity x 10⁻⁴ (deg cm²/dmol)
Wavelength (nm)
12
8
4
0
-4
-8
-12
190
200
210
220
230
240
250
260

varied from 10% (**C12**) to less than 0.1% (**C11**) of the activity of the parent [Nle^{12}]-α-factor (Table 3). In all cases, the series containing Glu in position 10 (compounds where n=2; see Figure 1) had higher potency than the corresponding analog with Asp. For example, the **C42** tridecapeptide was at least 20-fold more potent than the homologous **C41** pheromone. Except for the ornithine-containing peptides (**C32** and **C31**), the difference was usually at least one order of magnitude. The lactam containing D-Lys in position 10 (**C(4)2**) was inactive at all concentrations tested.

Biological Activity of Linear α-Factors

Linear model peptides identical to the lactam analogs except for a hydrolyzed lactam bond were evaluated using the same bioassays (Figure 2, Table 3). These peptides differ from the constrained peptides in that the amine and carboxyl side chains of residues 7 and 10 are ionizable. It is unclear which linear peptide most closely models the lactam, since the nitrogen in the lactam ring is acylated whereas the carbonyl is N-alkylated. The activities of [Nle^{12}]α-factor and [Glu^{10},Nle^{12}]α-factor (**L42**) are very similar. We decided, therefore, to use linear analogs with a free amine at the position 7 side chain and a free COOH at the position 10 side chain for comparative purposes in this investigation. We again found that, in all cases, the series with Glu at residue 10 was significantly more potent than corresponding peptides with Asp at this position. For example, **L32** was 50 times more potent than **L31** against *RC629*. All linear peptides within each series (Glu^{10} or Asp^{10}) had similar activities.

Competition for Binding to the *STE2* Receptor

The affinity for the α-factor receptor was determined by measuring the competition between the analogs and tritiated [Nle^{12}]α-factor. In all cases, the concentration of

analog required to displace 50% of the tritiated pheromone from the receptor was at least 15-fold higher than that of [Nle^{12}]α-factor and a minimum of 3-fold higher than the **L42** analog (Table 3). In general the lactams with Glu at position 10 exhibited higher receptor affinity than those with Asp at this position. This was also true for the corresponding linear homologs. The relative receptor affinities varied from nearly 300-fold (compare **L31** with **L32**) to as low as 5 (compare **C22** with **C21**). As the size of the lactam ring decreases from 18 members to 15 members in the Glu series, the affinity for the receptor decreased by approximately 15-fold. Interestingly, the cyclic compound containing *D*-Lys had a higher affinity for the receptor than the corresponding linear homolog. This is the only case where a cyclic lactam analog was a stronger binder than the linear homolog.

CHAPTER IV

DISCUSSION

Previous studies on a constrained congener of α-factor concluded that the pheromone was likely in a bent structure when bound to its receptor (Xue *et al.*, 1989). Complementary biophysical analysis on linear and disulfide constrained analogs suggested that the middle of the α-factor assumes a ß-turn (Jelicks *et al.*, 1988; Naider *et al.*, 1992; Gounarides *et al.*, 1994). In this dissertation, we report a biochemical analysis of 8 synthetic cyclic analogs (and their linear counterparts) of the mating pheromone containing a systematic variation in the size of the constrained region. The growth arrest and binding assays used in this investigation were carried out on strains lacking the BAR protease, the enzyme which cleaves α-factor. Thus, pheromone degradation does not influence our findings, and the results reflect the true potency of the analogs and their relative receptor affinities.

Earlier investigations on the α-factor have provided important insights into residues that influence the activity of the pheromone (Masui et al., 1979; Naider & Becker, 1986). Our goal in this investigation was to relate the activity of the pheromone to spatial constraints conferred by side chain cyclization in the center of the peptide. Before addressing this issue, we note that the results of the growth arrest analysis provide new knowledge concerning molecular groupings that influence the activity of the pheromone. Specifically, the γ-carbonyl of residue 10 was found to be a critical determinant of the potency of the pheromone. Thus, all compounds with Glu at this position are more active than those with Asp. This is true regardless of whether the pheromones are cyclic or linear. The growth arrest assay also gives evidence that the length of the side chain at position 7 is not decisive for optimal activity; in the linear series containing either Asp^{10}

or Glu^{10}, the activity of the pheromones varies very little as the side chain in position 7 is changed from $(CH_2)_4NH_3^+$ to $(CH_2)NH_2^+$ (Table 3). The fact that the ε-amine in positon 7 of the native α-factor is not essential for activity has been noted previously (Samokhin *et al.*, 1979; Shenbagamurthi *et al.*, 1983). Comparison of corresponding linear and cyclic analogs shows that the linear peptides were always the most potent. However, the ratio of activities varied from a high of 100 for **L32** versus **C32** to lows of 2 and 5 for the **L31/C31** and **L12/C12** homologs, respectively. The *D*-Lys-containing linear peptide had $^1/_{100}$ the activity of the parent compound.

The bioactivity of the cyclic analogs does not vary in a simple manner with the size of the lactam ring. Among the most potent α-factor analogs are those containing 18-, 16- and 15-membered rings (**C42**, **C22**, and **C12**). These peptides, which all contain Glu^{10}, show virtually no change in activity as the ring size decreases. In contrast, the 17-membered ring analog in this series (**C32**) has relatively low activity. The 17-membered ring analog in the Asp series (**C41**) also has very low activity. However, in the Asp series, the potency of all analogs is very low, with the 14-membered lactam analog (C11) having the lowest activity. In comparing the two series it is clear that the specific composition and conformation of the ring is more important than ring size in affecting biological activity.

The most striking outcome of the binding competition studies is that any change in the side chains of residue in position 7 or 10 leads to a decrease in affinity for the receptor. A decrease in the length of the position 7 side chain from four methylenes to three methylenes results in 3-fold and 7-fold drops in affinity in the linear Glu^{10} and the linear Asp^{10} series, respectively. A change in the side chain from Glu to Asp in the Lys- and Orn- containing linear pheromones results in 140- to 330-fold decreases in affinity, respectively. As was true for biological potency, the γ-carbonyl appears to have a greater

influence on receptor affinity than the ε-NH moiety. In Glu containing analogs, covalent linkage of the side chains of residues 7 and 10 further reduces the affinity for the receptor by 3- to 20-fold. Such modification in the Asp series, where receptor affinities are extremely low in both the linear and cyclic peptides, has at most a 4-fold effect.

The concept of ligand binding to a receptor and not eliciting a response has long been noted in the literature. The ultimate example is a pure competitive antagonist, which binds strongly to the agonist binding site but does not transduce signal. Less well understood would be a ligand that binds weakly, yet results in strong signal transduction. In the present study, we observed a clear dissociation between binding and biological potency, as defined by the absolute amount of a pheromone that results in a biological response. Specifically, **L42** and **L22** have nearly identical bioactivities against strain RC629, but the latter compound binds 60 times less effectively. Similarly, **C12** and **C22** have the same potency as **C42** in the halo assay, yet the latter compound binds more than an order of magnitued more avidly. For structurally similar compounds such as **L42** and **L22** to exhibit equal potency and a nearly 2 orders of magnitude variation in affinity would require the poorly bound pheromone (**L22**) to be an extremely efficent transducer of signal. This latter comparison attempts to normalize the potency of the pheromones at identical receptor occupancies. Although this approach is appealing, there are several specific problems associated with its application to our analysis: it requires the accurate determination of K_i values and a direct correlation of binding to bioactivity. The poor binding of several of our analogs (Table 3), even at millimolar concentrations, precludes the determination of their binding constants. Given the current lack of understanding of the atomic interactions between bound pheromone and receptor, we cannot offer an unequivocal explanation for the apparent highly efficient signalling by certain poorly bound analogs.

CD analysis of **C32** leads to the conclusion that the cyclized region of this tridecapeptide retains certain conformational features found in the corresponding model cyclic tetrapeptide (Figure 4A,B). Similar studies on the remaining analogs of [Nle^{12}]-α-factor showed that all of the cyclic peptides have significantly different CD patterns than their linear homologs (Figure 4C,D). We believe that these results suggest that the cyclization perturbs the conformation of the center of the pheromone and that the conformation of the cyclic lactam in the tridecapeptide is probably quite similar to that assumed by the corresponding model tetrapeptide. Detailed CD studies on the complete series of model tetrapeptides containing the constrained regions of the cyclic pheromones reported in this paper indicate that models of **C42** and **C22** assume structures that are nearly perfect type II β-turns (Rao *et al.*, 1995). The fact that the α-factor analogs corresponding to the tetrapeptide models that form the best type II β-turns are the most potent cyclic analogs reinforces our previous suggestion that a type II β-turn in the middle of the peptide may be an important determinant of the biologically active state (Shenbagamurthi *et al.*, 1983, 1985). Nevertheless, NMR studies in different solvents show that various amounts of conformational heterogeneity exist in these model tetrapeptides. Therefore, conclusions on the correlation between bioactivity and structure and on the exact conformations assumed by the central regions of the different cyclic tridecapeptides must await the outcome of detailed NMR, CD, and energy minimization studies that are currently in progress.

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PART III

PROBING THE FUNCTIONAL CONFORMATION OF THE TRIDECAPEPTIDE MATING PHEROMONE OF *SACCHAROMYCES CEREVISIAE* THROUGH STUDY OF DISULFIDE-CONSTRAINED ANALOGS*

*Part III was published in its entirety as **Xue, C.-B., A. McKinney, H.-F. Lu, Y. Jiang, J. Becker, and F. Naider.** 1996. Probing the functional conformation of the tridecapeptide mating pheromone of *Saccharomyces cerevisiae* through study of disulfide-constrained analogs. *Int. J. of Peptide & Protein Res.* **47**, 131-141. The research for Part III was done in collaboration with the laboratory of Dr. Fred Naider (College of Staten Island, Staten Island, NY). Dr. Naider's laboratory was responsible for the synthesis and analysis of the disulfide-constrained α-factor analogs. Angela McKinney was responsible for the biological studies which include the growth arrest assay and the competition binding assay.

CHAPTER I

INTRODUCTION

Introduction of covalent constraints into peptides has been used to reduce the conformational freedom of peptides (Rizo & Gierasch, 1992). These studies have led to the development of agonists and antagonists for small peptides active as hormones or neurotransmitters and have given insight into the active conformation of the peptide ligand. We have used the *Saccharomyces cerevisiae* mating pheromones and their receptors as a model system to study peptide-receptor interaction (Sprague & Thorner, 1992). This paradigm was chosen for many reasons including its ease of manipulation, an extensive knowledge of the peptide ligands and their receptors, and the apparent homology of the yeast system to those of other mammalian GTP-protein-coupled, seven-transmembrane-domain receptor systems.

Mating between haploid cells of *Saccharomyces cerevisiae* is controlled by diffusible peptide pheromones called **a**-factor and α-factor, which are secreted by MAT**a** and MATα cells, respectively. The **a**-factor is a lipopeptide with the sequence YIIKGVFWDPAC(farnesyl)OMe (Anderegg *et al.,* 1988; Xue *et al.,* 1989a). The α-factor is a simple tridecapeptide WHWLQLKPGQPMY; numerous studies on structure-activity relationships (Masui *et al.,* 1979; Naider & Becker, 1986) and conformational analyses (Wakamatsu *et al.,* 1986; Wakamatsu *et al.,* 1987; Jelicks *et al.,* 1988; Jelicks *et al.,* 1989; Gounarides *et al.,* 1993) of this peptide have been conducted. Investigations on analogs in which Gly^9 was replaced by *D* and *L* residues led to the suggestion that the α-factor exhibits a folded conformation in the -Lys^7-Pro^8-Gly^9-Gln^{10}- region when it binds to the α-factor receptor (Shenbagamurthi *et al.,* 1985). This

suggestion was supported by 2D NMR studies of α-factor in solution (Jelicks *et al.*, 1988) and in the presence of lipid (Jelicks *et al.*, 1989; Gounarides *et al.*, 1993), and from biochemical investigations on the constrained analog cyclo^{7,10}[Nle^{12}]α-factor (Xue *et al.*, 1989b; Yang *et al.*, 1995).

The disulfide loop is an important structural element which restricts the conformational mobility of peptides and proteins. Studies on two cyclic tetrapeptides Boc-Cys-Pro-X-Cys-NHMe (X = Tyr or Phe) established that the disulfide bridge enhances the tendency of Pro-X segments to form β-turn conformations that are stabilized by a 4-->1 hydrogen bond between the Cys(1) CO and Cys(4) NH groups (Kishore *et al.*, 1988). Conformational transitions from a type I β-turn in chloroform to a mixture of type I (major) and type II (minor) β-turns in DMSO were observed with these peptides. Recent studies on Ac-Cys-Pro-*D*-Val-Cys-NH_2 showed that this cyclic tetrapeptide adopted a favored type II β-turn conformation in DMSO (Garcia-Echeverria *et al.*, 1989; Garcia-Echeverria *et al.*, 1991). Given our previous findings that cyclization of the Lys^7-Gln^{10} region of α-factor via a side chain lactam resulted in retention of biological activity (Xue *et al.*, 1989b), and the hypothesis that the backbone assumes a type II β-turn, we were prompted to synthesize several cyclic [Nle^{12}]α-factor analogs containing disulfide rings (**Ia-Id**) (Figure 1) to further investigate the significance of a hairpin structure to the biological activity of the pheromone.

The Lys^7 and Gln^{10} of [Nle^{12}]α-factor were replaced by cysteine residues and the position 9 residue was replaced with either *D*-Ala, Gly, *L*-Ala, or *D*-Val. Linear counterparts (**IIa-IId**) were synthesized for comparative purposes. We also incorporated the spacer 5-aminopentanoic acid [APA; $NH_2(CH_2)_4COOH$] in place of residues 8 and 9

[Nle^{12}]α-factor

Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Nle-Tyr

Ia Trp-His-Trp-Leu-Gln-Leu-Cys-Pro-*D*-Val-Cys-Pro-Nle-Tyr
S----------S

IIa Trp-His-Trp-Leu-Gln-Leu-Cys-Pro-*D*-Val-Cys-Pro-Nle-Tyr

Ib Trp-His-Trp-Leu-Gln-Leu-Cys-Pro-*D*-Ala-Cys-Pro-Nle-Tyr
S----------S

IIb Trp-His-Trp-Leu-Gln-Leu-Cys-Pro-*D*-Ala-Cys-Pro-Nle-Tyr

Ic Trp-His-Trp-Leu-Gln-Leu-Cys-Pro-Gly-Cys-Pro-Nle-Tyr
S----------S

IIc Trp-His-Trp-Leu-Gln-Leu-Cys-Pro-Gly-Cys-Pro-Nle-Tyr

Id Trp-His-Trp-Leu-Gln-Leu-Cys-Pro-*L*-Ala-Cys-Pro-Nle-Tyr
S----------S

IId Trp-His-Trp-Leu-Gln-Leu-Cys-Pro-*L*-Ala-Cys-Pro-Nle-Tyr

Ie Trp-His-Trp-Leu-Gln-Leu-Cys-NH(CH$_2$)$_4$CO-Cys-Pro-Nle-Tyr
S----------S

IIe Trp-His-Trp-Leu-Gln-Leu-Cys-NH(CH$_2$)$_4$CO-Cys-Pro-Nle-Tyr

IIf Trp-His-Trp-Leu-Gln-Leu-Cys(CH$_3$)-Pro-Ala-Cys(CH$_3$)-Pro-Nle-Tyr

IIg Trp-His-Trp-Leu-Gln-Leu-Cys(CH$_3$)-Pro-*D*-Ala-Cys(CH$_3$)-Pro-Nle-Tyr

IIh Trp-His-Trp-Leu-Gln-Leu-Ser-Pro-Ala-Ser-Pro-Nle-Tyr

IIi Trp-His-Trp-Leu-Gln-Leu-Ser-Pro-*D*-Ala-Ser-Pro-Nle-Tyr

FIGURE 1

Structure of α-factor analogs. (**Ia**) cyclo^{7,10}[Cys^7,D-Val^9,Cys^{10},Nle^{12}]α-factor; (**IIa**) [Cys^7,D-Val^9,Cys^{10},Nle^{12}]α-factor; (**Ib**) cyclo^{7,10}[Cys^7,D-Ala^9,Cys^{10},Nle^{12}]α-factor; (**IIb**) [Cys^7,D-Ala^9,Cys^{10},Nle^{12}]α-factor; (**Ic**) cyclo^{7,10},[Cys^7,Cys^{10},Nle^{12}]α-factor; (**IIc**) [Cys^7,Cys^{10},Nle^{12}]α-factor; (**Id**) cyclo^{7,10}[Cys^7,L-Ala^9,Cys^{10},Nle^{12}]α-factor; (**IId**) [Cys^7,L-Ala^9,Cys^{10},Nle^{12}]α-factor; (**Ie**) cyclo^{7,10}[Cys^7,APA^{8,9},Cys^{10},Nle^{12}]α-factor; (**IIe**) [Cys^7,APA^{8,9},Cys^{10},Nle^{12}]α-factor, where APA = 5-amino-pentanoic acid; (**IIf**) [Cys(CH$_3$)^7,L-Ala^9,Cys(CH$_3$)^{10},Nle^{12}]α-factor; (**IIg**) [Cys(CH$_3$)^7,D-Ala^9,Cys(CH$_3$)^{10},Nle^{12}]α-factor; (**IIh**) [Ser^7,L-Ala^9,Ser^{10},Nle^{12}]α-factor; (**IIi**) [Ser^7,D-Ala^9,Ser^{10},Nle^{12}]α-factor.

which constitute the corners of the turn, while maintaining the 14-membered disulfide ring spanning residues 7 and 10 (analogs **Ie** and **IIe**; Figure 1). In this dissertation, the synthesis, biological activity, and receptor binding of these peptides along with attempts to correlate bioactivity with structural characteristics are discussed. Our results provide further information on the biologically active conformation of α-factor.

CHAPTER II

MATERIALS AND METHODS

Analysis of Peptides

Reversed-phase HPLC was performed on a Waters HPLC system using Waters μ-Bondapak-C_{18} columns (3.9 mm x 300 mm for analytical analysis; 19 mm x 150 mm for preparative purifications). Analytical HPLC was run using a linear gradient of water (0.025% TFA) and acetonitrile (0.025% TFA) with acetonitrile from 20% to 60% over 20 min. or a linear gradient of water (0.025% TFA) and methanol (0.025% TFA) with methanol from 50% to 100% over 20 min. Preparative HPLC was run using a linear gradient of water (0.025% TFA) and acetonitrile with acetonitrile from 0% to 60% over 60 min. Detection was usually at 220 nm. Amino acid analysis was carried out at the Analytical Services Laboratory at the University of Tennessee and at Brigham and Women's Hospital, Boston, MA using a Waters Pico-Tag system. Fast atom bombardment mass spectrometry (FAB-MS) was performed at the Chemistry Department of the University of Tennessee. Spectra were acquired with a VG ZAB-EQ mass spectrometer equipped with a flow FAB ion source of an Ion Tech fast atom gun. Peptides were dissolved in water/acetonitrile/0.1% TFA and an aliquot of the solution was dispersed on a stainless steel target in a matrix of monothioglycerol. The accelerating voltage of the mass spectrometer was maintained at 8kV, whereas 8-keV xenon atoms at a discharge current of 1 mA were used to bombard the sample. Data were recorded in a 'multichannel acquisition' mode from *m/z* 800 to 1800 at 20s/decade by a VG 11-250J data system. Molecular ions [$M+H^+$] are reported as monoisotopic values rounded to a tenth of a mass unit.

Nuclear Magnetic Resonance Studies

All NMR spectroscopy was performed on the 400 MHz (^{1}H) JEOL GX-400 spectrometer of the CUNY NMR facility located at Hunter College according to methods published elsewhere (Gounarides *et al.*, 1994). Briefly, phase sensitive correlation spectroscopy together with nuclear Overhauser spectroscopy was used to make assignments and conformational deductions. Proton temperature coefficients were measured over the range of 5-35°C in DMSO-d_6/H_2O (80:20). Sample concentration was 6 mg/ml in DMSO-d_6/H_2O.

Synthesis of Peptides

All peptides were synthesized by the solid phase methodology using the procedures described previously (Xue *et al.*, 1989b). The peptides synthesized were analogs of the [Nle^{12}]α-factor in which norleucine is present as an isosteric replacement for methionine. Previous studies indicate that the [Nle^{12}]α–factor is equally active to the native pheromone and binds identically to the receptor (Raths *et al.*, 1988). Synthesis was started with a Boc-Tyr(2-BrZ)-OCH_2-PAM-resin (0.56 mmol/g of resin). The Boc group was used for protection of all N-α-groups, and formyl (For), tosyl (Tos), and acetamidomethyl (Acm) moieties were employed for protection of Trp, His, and Cys side chains, respectively. All Boc groups were deprotected using 45% TFA/2% dimethylsulfide/53% CH_2Cl_2 except for Gln where the Boc group was removed using 4N HCl in dioxane. Coupling was carried out twice for each residue using diisopropylcarbodiimide (DIPC) in CH_2Cl_2 for the first coupling and DIPC/HOBt in CH_2Cl_2/DMF for the second coupling. BocGln was coupled using DIPC/HOBt in CH_2Cl_2/DMF for the first and second couplings. Coupling was monitored using the Kaiser test (Kaiser *et al.*, 1970) and a third coupling was employed when necessary.

After completion of the assembly of the peptide chain, the N^{α}-Boc group was deprotected using 45% TFA/2% dimethyl sulfide/CH_2Cl_2, and the resin was treated with 3 equivalents of $Tl(TFA)_3$ in TFA-anisole (20:1, 30 ml/g of resin) at 0°C for 1 hour to form intramolecular disulfide bonds. The resin was washed three times each with TFA, CH_2Cl_2, DMF, and methanol, dried, and subjected to cleavage in HF/anisole (9:1) at 0°C for 1 h. After evaporation of the HF, the resin was washed with ether, and the peptide was extracted with three portions of DMF. The combined DMF filtrates were treated with piperidine (final concentration 20%) for 1 h at room temperature to remove the formyl groups. After the solution was concentrated at high vacuum, the residue was taken up in water/DMF, the solution was acidified with acetic acid to pH 3 and treated with 1 equivalent of HOBt to remove the incompletely deprotected tosyl group. The crude peptide was purified using reversed-phase HPLC to over 98% homogeneity as judged using acetonitrile/water/TFA and methanol/water/TFA gradients.

Linear peptides were generated from their corresponding cyclic counterparts by treatment with 10 equivalents of dithioerythritol in water/acetonitrile/1% piperidine for 30 min. HPLC analysis indicated the complete transformation of the disulfide in thiols. The solution was acidified with acetic acid to pH 3, and the crude product was purified on a preparative reversed-phase C_{18} column to give over 85% yield of linear peptide. Although purified thiol product was over 99% pure when checked by analytical HPLC immediately after collection, a small amount of disulfide product was observed after lyophilization and storage at low temperature for some time. For this reason, special procedures were taken for the bioassay of the linear peptides. Specifically, the -SH containing peptide was generated from its disulfide homolog immediately prior to either the bioassay or binding competition experiments. This insured minimal conversion back to the disulfide state.

Biological Assays of α-Factor Analogs

The biological activity of the α-factor analogs was assayed using a growth arrest assay (Halo) as described previously (Raths *et al.,* 1988). Activity was examined using a supersensitive mutant (RC629 [*sst1*]) of *S. cerevisiae*. The *sst1* mutation results in the loss of *BAR1* protease, a secreted enzyme, which cleaves the α-factor (Chan & Otte, 1982). In separate experiments, the ability of nonactive analogs to compete with the activity of the parent ([Nle^{12}]α-factor) α-factor was analyzed by placing the analog on a disk which was about 15 mm from a second disk containing the parent compound. Antagonism was evidenced by the appearance of bean-shaped zones of inhibition for the α-factor (Erioutou-Bargiota *et al.,* 1992).

Binding Competition Assay

Competition of bound tritiated [Nle^{12}]α-factor by unlabeled α-factor analogs was measured by the following protocol. *S. cerevisiae* strain 4202-15-3 (MATa *cry1 bar1-1 ade2-1 his 4-580 lys2 tyr1 SUP4-3*) was grown in YM-1 medium (yeast extract, 5 g/l; peptone, 10 g/l; yeast nitrogen base without amino acids, 6.7 g/l; adenine, 0.01 g/l; uracil, 0.01 g/l; succinic acid, 10 g/ml; sodium hydroxide, 6 g/l; and glucose, 10 g/ml, at a final pH of 5.8) overnight at 30°C with shaking at 120 rpm to 1 x 10^7 cells/ml. Cells were harvested by centrifugation (6000 x g for 10 min. at 4°C), and the resultant pellet washed twice with ice-cold YM-1+i medium (YM-1 containing 10mM NaN_3, 10 mM KF, and 10mM *p*-tosyl-L-arginine methyl ester) and resuspended to 1.25 x 10^9 cells/ml in YM-1+i. Final assay concentrations of the unlabeled competitors were between 3 x 10^{-5} to 1 x 10^{-9} M as determined from the A_{287} for each peptide and the extinction coefficients calibrated for [Nle^{12}]α-factor from quantitative elemental analysis. The reaction was started by the addition of 100 µl of analog/[^{3}H]α-factor mix (120 µl of appropriate

unlabeled α-factor analog mixed with 120 µl of labeled α-factor 6 x 10^{-8} M, 15.8 Ci/mmole) to 400 µl of cell suspension. After the reaction was started, two 20 µl samples were removed and counted to determine total amount of radioactivity present. Duplicate reactions were started 3 min. after initial reaction for each analog tested. At 30 min. two 200 µl portions of cell suspension were added separately to 2 ml of YM-1+i and filtered over GN-6 Metricel filters (Gelman Sciences, Inc., Ann Arbor, MI) presoaked in 1% bovine serum albumin. The reaction tubes were then rinsed twice with 2 ml ice-cold YM-1+i medium, with each rinse filtered over the same filter. Finally, each filter was rinsed twice with 2 ml YM-1+i medium. The non-specific binding of labeled α-factor in the absence of cells was less than 100 cpm. The filters were counted in 5 ml of Budget-solve counting cocktail (Research Products International Corp., Mt. Prospect, IL). All assays and manipulations were done in siliconized borosilicate tubes or vials. The binding curve for α-factor has been previously done by the infinite dilution experiment by us and others (Raths *et al.,* 1988; Blumer *et al.,* 1988; Jenness *et al.,* 1986) to show that there is no evident cooperativity in the binding between α-factor and its receptor.

Tritiated [Nle^{12}]α-factor was prepared as previously described (Raths *et al.,* 1988). Briefly, α-factor containing dehydroproline (Dhp) in place of proline in positions eight and eleven and norleucine at position twelve in place of methionine was synthesized by standard methods of solid phase peptide synthesis. [Dhp^{8},Dhp^{11},Nle^{12}]α-factor was tritiated by Amersham Corp. by the T-3 method involving reduction by tritium gas. The crude tritiated peptide was purified to homogeneity by HPLC as previously described (Raths *et al.,* 1988). Purity and specific activity were monitored by TLC, HPLC, amino acid analysis, and biological activity. Control reductions with hydrogen gas indicated that [Nle^{12}]α-factor was fully regenerated (Raths *et al.,* 1988).

HPLC Analysis of cyclic analog stability

To determine whether the cyclic analogs maintained the disulfide bonds during incubation with cells in the binding assay, HPLC analysis was done. *S. cerevisiae* 4202-15-3 (2 X 10^5 cells/ml) were incubated with cyclic analogs (5 μg/ml) in YM-1 + i medium (see binding competition assay protocol) at 30°C for 0, 0.5, or 2 hours. The cells were removed by filtration and the medium analyzed by HPLC on a C_{18} column as described above under analysis of peptides.

CHAPTER III

RESULTS

Synthesis

Intrachain disulfide bond formation in synthetic peptides can be achieved by oxidation in solution or while the peptide remains attached to a solid support. Oxidation in solution is nonspecific and requires the use of a very low peptide concentration to minimize interchain dimerization and oligomerization. In contrast, oxidation of cysteines while the peptide chain remains anchored to the resin utilizes solid phase *pseudo-dilution* chemistry and favors specific intrachain disulfide formation (Barany & Merrifield, 1979). Therefore, in the synthesis of all cyclic $Cys^{7,10}$ [Nle^{12}]α-factor analogs **Ia-Ie**, we carried out the oxidation before the peptide was cleaved from the resin. Thallic trifluoroacetate which was found to be an efficient oxidizing agent for disulfide formation (Garcia-Echeverria *et al.,* 1989; Yajima *et al.,* 1988) was used as the oxidizing agent for **Ia-Ie**. In most cases, the crude disulfide product was over 80% pure, and 20-30% yields of cyclic peptides were obtained after purification using reversed-phase HPLC (Figure 2). In addition to analysis in acetonitrile-water systems, the peptides were also homogenous in methanol/water/TFA gradients (data not shown). The cyclic analogs had the following retention times in the methanol gradient (*L*-Ala, 14.2 min; *D*-Ala, 13.3 min; Gly, 13.9 min; *D*-Val, 14.5 min; APA, 13.5 min). The peptides were characterized by amino acid analysis, fast atom bombardment mass spectrometry (Table 1) and 400 MHz NMR (data not shown). The NMR analysis indicated no spurious peaks, and COSY and NOESY spectroscopy provided total confirmation of the structures of these compounds (Naider *et al.,* 1992; Gounarides *et al.,* 1994). The difference of two mass units in the mass spectra

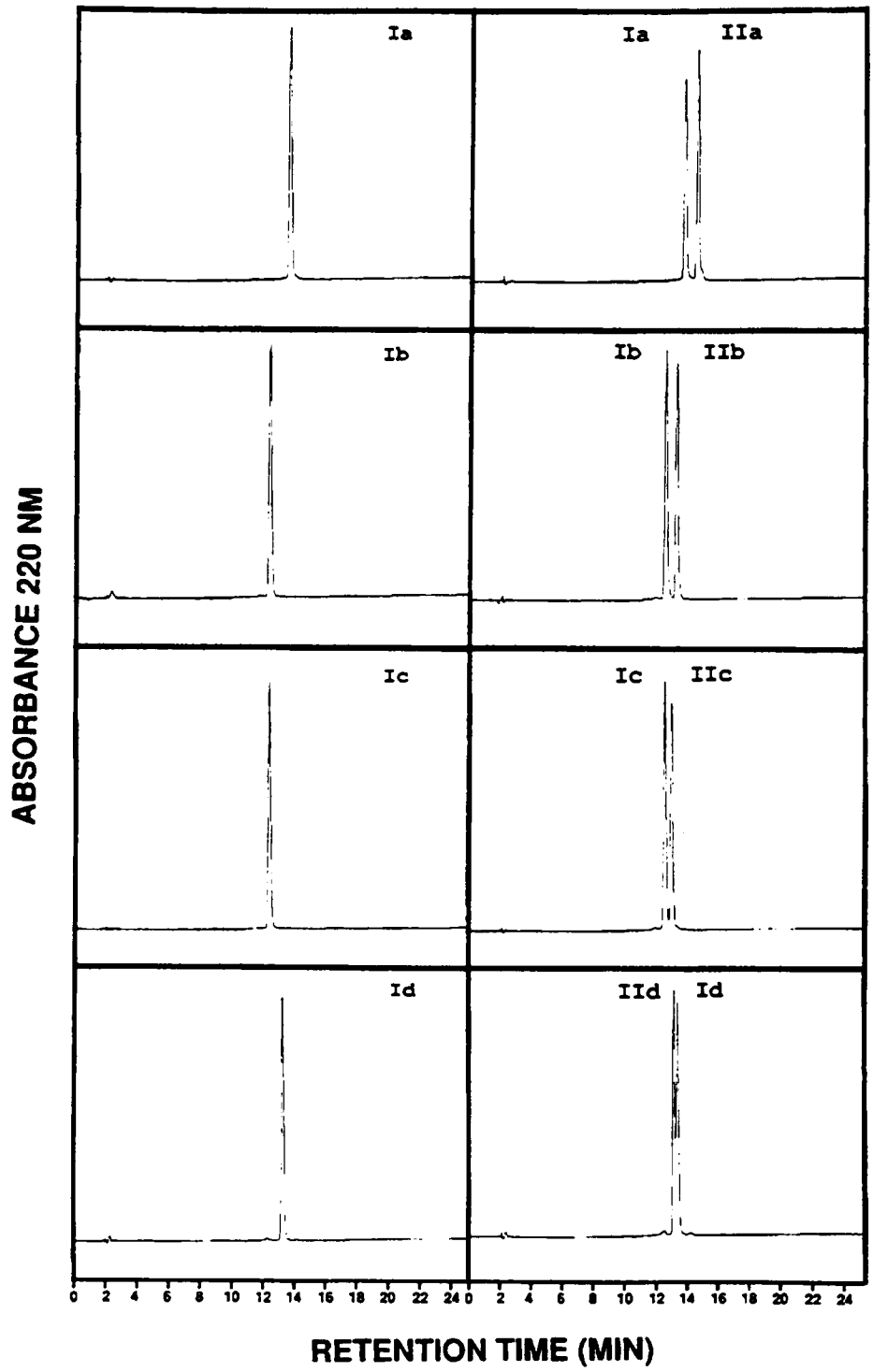


FIGURE 2

HPLC of [Cys^{7},Cys^{10},X^{9},Nle^{12}]α-factors. Refer to Fig. 1 for the structure of the compounds. The panels on the right-hand side of the figure represent coinjections of the cyclic and the linear analogs.

TABLE I

Chemical properties of synthetic cyclic and linear α-factor analogs[a]

	Cyclic D-Val[9] (Ia)	Linear D-Val[9] (IIa)	Cyclic D-Ala[9] (Ib)	Linear D-Ala[9] (IIb)	Cyclic Gly[9] (Ic)	Linear Gly[9] (IIc)	Cyclic L-Ala[9] (Id)	Linear L-Ala[9] (IId)	Cyclic APA[8,9] (Ie)	Linear APA[8,9] (IIe)	Cys(CH_3)[7,10] L-Ala[9] (IIf)	Cys(CH_3)[7,10] D-Ala[9] (IIg)	Ser[7,10] L-Ala[9] (IIh)	Ser[7,10] D-Ala[9] (IIi)
Yield %	25		27		26		20		30		40	48	41	42
							Amino-acid analysis							
Gln	1.00		0.96		0.97		0.98		0.97		1.09	1.08	1.11	1.10
Gly	–		–		1.09		–		–		–	–	–	–
His	0.79		0.84		0.83		0.82		0.84		0.88	0.91	0.94	0.93
Ala	–		1.05		–		1.03		–		1.01	1.00	1.04	1.03
Pro	2.24		2.17		2.21		2.19		1.13		b	b	1.98	1.98
Tyr	1.01		1.00		1.00		1.00		1.00		0.97	0.98	1.00	1.01
Val	1.02		–		–		–		–		–	–	–	–
Leu	2.10		2.06		2.10		2.10		2.08		2.00	1.98	2.06	2.06
Ser	–		–		–		–		–		–	–	1.78	1.78
							FAB-MS ($M+1$)							
Found	1655.9	1657.9	1627.7	1629.7	1613.8	1615.8	1627.7	1629.7	1558.8	1560.8	1657.9	1657.9	1597.9	1597.9
Calculated	1655.8	1657.8	1627.8	1629.8	1613.7	1615.8	1627.8	1629.8	1558.7	1560.7	1658.0	1658.0	1597.8	1597.8

[a] For all peptides, the expected amino acid values for Gln = 1, His = 1, Pro = 2, Tyr = 1, Leu = 2, Nle = 1, Cys = 2 and Trp = 2. Norleucine (Nle) tryptophan, and cysteine were not determined in the amino acid analysis. Gly, Ala, Val, or APA were obtained in those peptides substituted with these amino acids at position 9, or in positions 8 and 9 in the case of APA.

[b] Independent studies determined that Cys(CH_3) and Pro comigrate, therefore proline could not be determined accurately.

of the cyclic peptides and their linear counterparts confirmed the presence of the disulfide bond in the cyclic peptides.

HPLC Profile

During analysis of purified cyclic and linear peptides using reversed-phase HPLC, we observed interesting chromatographic behavior of various isomers. In order to assess these results accurately, we coinjected each cyclic peptide with its linear counterpart and all pairs of peptides were run under identical conditions. Cyclic peptides with *D*-Valine, *D*-Ala, or glycine at position 9 consistently eluted off the column earlier than their linear counterparts (Figure 2). Furthermore, the differences in retention time decreased as the side chain of the position 9 residue changed from $-CH(CH_3)_2$ (*D*-Val) to H (Gly). In contrast, the cyclic peptide with a *L*-alanine at position 9 eluted off the column later than its linear counterpart. It is interesting to note that in both the acetonitrile and methanol gradients cyclic *D*-Ala peptide had a retention time almost identical to that of the cyclic Gly^9 containing homolog. The *L*-Ala^9 isomer was retained by the reversed-phase column in comparison to the Gly^9 and *D*-Ala^9 cyclic peptides. These results probably reflect some conformational change when a *D*-residue or a glycine at position 9 is replaced by a *L*-residue.

Conformational analysis

The cyclic tetrapeptide segments (Cys-Pro-X-Cys) replacing the Lys-Pro-Gly-Gln sequence in α-factor were expected to assume β-turn conformations (Kishore *et al.*, 1988; Garcia-Echeverria *et al.*, 1989; Garcia-Echeverria *et al.*, 1991). In order to verify the conformation of the central region of the cyclic α-factor analogs a detailed two dimensional NMR analysis was carried out on the *cyclo*^{7,10}[Cys^7,*D*-Ala^9,Cys^{10},Nle^{12}]α-

factor in DMSO-d_6/water (80:20). In the NOESY spectrum of this compound a strong cross-peak is observed between the $Pro_{\alpha CH}$ and the *D*-Ala_{NH} (Figure 3). In addition a strong NH-NH crosspeak was observed between the NH protons of residues nine and ten and the Cys NH had a temperature coefficient of -1.2 ppb/K (data not shown). These results support the existence of a Type II β-turn in the disulfide portion of the *cyclo*^{7,10}[Cys^7,*D*-Ala^9,Cys^{10},Nle^{12}]α-factor. Similar investigations on the other peptides examined herein led to the conclusion that the *D*-Val analog preferred a Type II β-turn whereas the *L*-Ala analog favored a Type I β-turn. The Gly analog favored a Type II β-turn in organic solvents but was a mixture of conformations in water. A more detailed presentation of the NMR procedures for all peptides as well as the results of a quantitative NOE analysis to determine specific internuclear distances is reported elsewhere (Gounarides *et al.*, 1994).

Growth Arrest Activity of Cyclic Disulfide α-factor Analogs

To determine the influence of cyclization on α-factor activity we measured the potency of peptides **Ia-e** in a growth arrest assay. *Cyclo*^{7,10}[Cys^7,*D*-Ala^9,Cys^{10},Nle^{12}]α-factor (**Ib**) caused growth arrest at 0.5 μg/disk (Table 2). The Gly^9 homolog (**Ic**) had a similar activity based on a comparison of zone size versus dose, whereas cyclo^{7,10}[Cys^7,*L*-Ala^9,Cys^{10},Nle^{12}]α-factor (**Id**) was 2 to 5-fold less active. The *D*-Val^9 analog (**Ia**) was 20-fold less potent whereas the analog containing 5-aminopentanoic acid (**Ie**) was inactive up to a concentration of 50 μg/disk. The most potent cyclic analogs (**Ib** and **Ic**) were approximately an order of magnitude less active than the [Nle^{12}]α-factor.

Figure 3. NOESY spectrum of the α-CH-NH region of cyclo^{7,10} [Cys^{7}, *D*-Ala^{9}, Cys^{10}, Nle^{12}] α-factor in DMSO-d_6/water. The spectrum was recorded at 25 °C with a peptide concentration of 6 mg/ml.

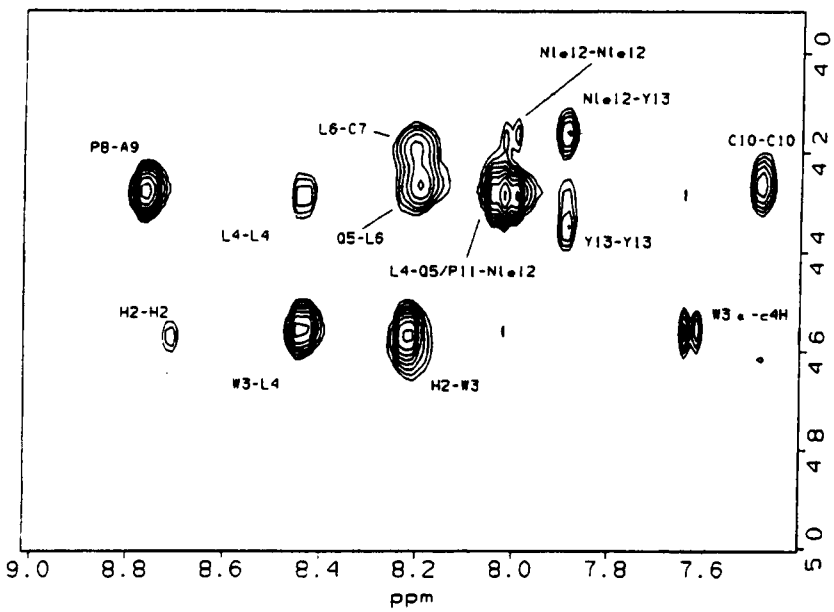

Nle12-Nle12
Nle12-Y13
L6-C7
P8-A9
C10-C10
L4-L4
Q5-L6
Y13-Y13
L4-Q5/P11-Nle12
H2-H2
W3 ε -c4H
W3-L4
H2-W3
9.0
8.8
8.6
8.4
8.2
8.0
7.8
7.6
ppm
4.0
4.2
4.4
4.6
4.8
5.0

TABLE 2

Growth arrest of S. cerevisiae *RC629 by cyclic and linear analogs of α-factor*[a]

	Peptide															
μg/disk	[Nle^{12}] α-factor	Cyclic lactam[b]	Cyclic D-Val^{9} **(Ia)**	Linear D-Val^{9} **(IIa)**	Cyclic D-Ala^{9} **(Ib)**	Linear D-Ala^{9} **(IIb)**	Ser^{7,10} D-Ala^{9} **(IIi)**	Cys(CH$_3$)^{7,10} D-Ala^{9} **(IIg)**	Cyclic Gly^{9} **(Ic)**	Linear Gly^{9} **(IIc)**	Cyclic L-Ala^{9} **(Id)**	Linear L-Ala^{9} **(IId)**	Ser^{7,10} L-Ala^{9} **(IIh)**	Cys(CH$_3$)^{7,10} L-Ala^{9} **(IIf)**	Cyclic APA^{8,9} **(Ie)**	Linear APA^{8,9} **(IIe)**
50	–	–	16	0	–	–	–	–	–	–	–	–	0	0	0	0
30	–	–	13	0	–	–	–	–	–	–	–	–	0	0	0	0
20	36	–	11	0	25	14	–	–	25	18	18	12	0	0	0	0
10	34	23	9	0	20	12	21	12	22	15	14	9	0	0	0	0
5	29	–	0	0	15	10	18	11	17	12	11	0	0	0	0	0
1	23	12	0	0	11	0	11	7	11	0	9	0	0	0	0	0
0.5	21	8	0	0	8	0	8	0	0	0	0	0	0	0	0	0
0.1	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.05	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Results are presented as the diameter of the zone of inhibition in mm and represent the average of at least three determinations. In all cases, the values averaged were within 2 mm of each other. [b] Data taken from ref. 4. Cyclic lactam is cyclo^{7,10}[Nle^{12}]α-factor.

Growth Arrest Activity of Linear α–Factor Analogs

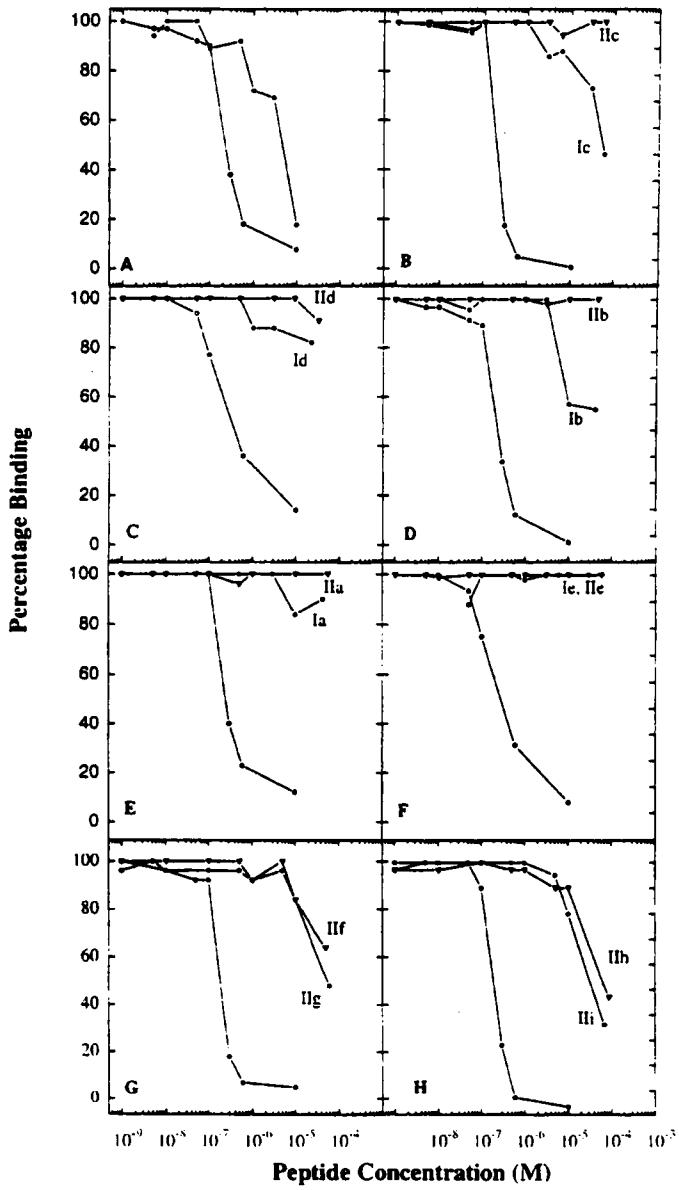
The cyclic disulfides represent major perturbations in the atomic composition of the side chains in the central region of the pheromone. In order to separate the contribution of cyclization from the effect of the changes in the side chain atoms we measured the biological activity of linear analogs containing sulfhydryl groups at positions 7 and 10. Among the linear sulfhydryl-containing peptides (**IIa-e**) the *D*-Ala^9 (**IIb**) and the Gly^9 (**IIc**) were approximately equally active at 5 μg/disk, which is 100-fold less active than [Nle^{12}]α-factor. The linear *L*-Ala^9 analog (**IId**) was half as active as **IIb** and **IIc** whereas the *D*-Val^9 (**IIa**) and the APA^{8,9} (**IIe**) analogs were not active at the highest amount tested. In every case examined the sulfhydryl containing peptides were less active than the corresponding cyclic homolog.

The sulfhydryl group is highly reactive, and it is not possible to definitively determine its chemical state under conditions of the bioassay. We therefore synthesized α-factor analogs containing Ser and Cys(CH_3) at positions 7 and 10 as additional linear controls. [Ser^7,*D*-Ala^9,Ser^{10},Nle^{12}]α-factor (**IIi**) was equally active with the *D*-Ala^9 cyclic disulfide and [Cys(CH_3)^7,*D*-Ala^9,Cys(CH_3)^{10},Nle^{12}]α-factor (**IIg**) was 2-5 fold less active. In contrast [Ser^7,*L*-Ala^9,Ser^{10},Nle^{12}]α-factor (**IIh**) and [Cys(CH_3)^7,*L*-Ala^9,Cys(CH_3)^{10},Nle^{12}]α-factor (**IIf**) were at least 50-fold less active than the *L*-Ala^9 cyclic disulfide.

Competition with binding to the receptor

The ability of the analogs to bind to the α-factor receptor was determined directly using competition binding assays with radioactively labelled [Nle^{12}]α-factor (Figure 4). Linear cysteine-containing analogs (**IIa-IIe**) did not compete with [Nle^{12}]α-factor at any concentration measured. Serine-containing analogs were poor competitors of α-factor

Figure 4. Competition binding of tritiated [Nle^{12}] α-factor by unlabeled [Nle^{12}] α-factor and various analogs. In each panel, the curve (○) represents tritiated [Nle^{12}] α-factor. Panel A represents competition by the known antagonist des-Trp^1[Ala^3] α-factor (●). In all other panels, the competitor is indicated according to the numbering in Figure 1.

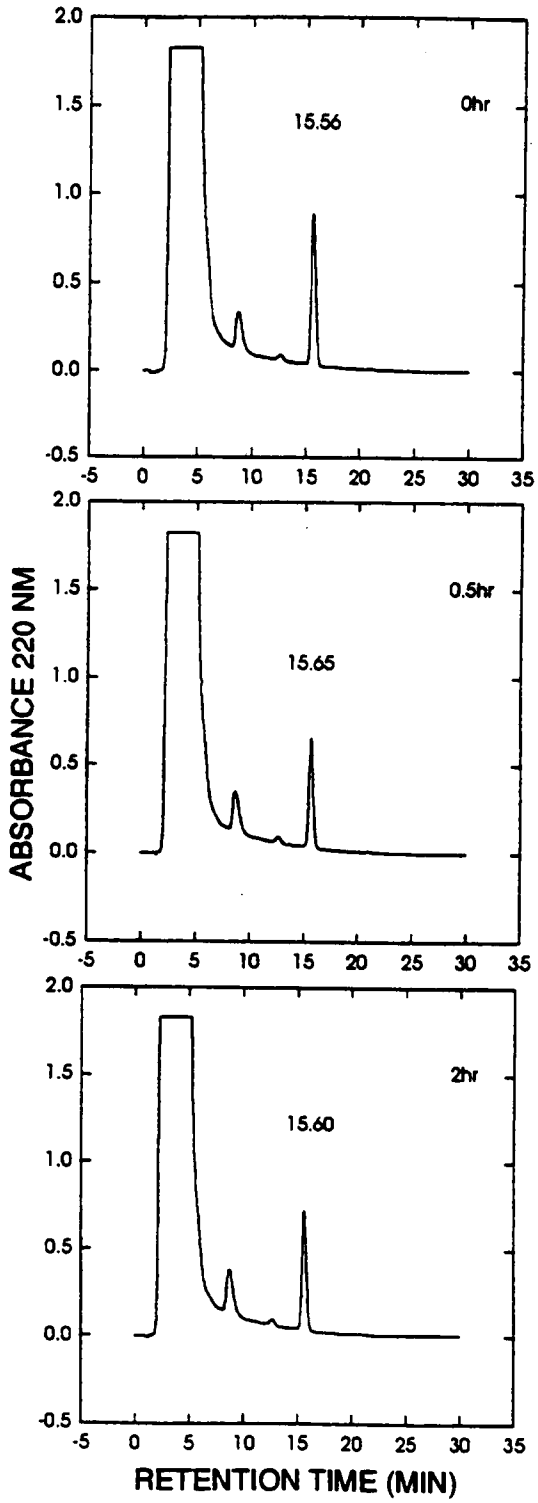

Percentage Binding
Peptide Concentration (M)
A
B
C
D
E
F
G
H
IIc
Ic
IId
Id
IIb
Ib
IIa
Ia
Ie, IIe
IIf
IIg
IIh
IIi

binding exhibiting 200-400-fold lower affinity than [Nle^{12}]α-factor (panel H, Figure 4). In comparison to the serine-containing peptides which caused more than 50% inhibition of binding at 7 to 9 x 10^{-5}M, the Cys(CH_3)-containing analogs did not reach 50% competition at the highest concentration tested (panel G, Figure 4). Cyclic analogs were also poor competitors for receptor binding. The cyclic Gly^9 (**Ic**) and cyclic *D*-Ala^9 (**Ib**) analogs resulted in an approximately 50% reduction in binding when present in several hundred fold excess as compared to the [Nle^{12}]α-factor. In contrast, the cyclic *L*-Ala^9 (**Id**) and *D*-Val^9 (**Ia**) analogs showed less than 20% competition under similar conditions. Neither the linear (**IIe**) nor cyclic APA analog (**Ie**) was a competitor at the highest concentrations tested.

Stability of Cyclic Analogs in the Presence of *S. cerevisiae*

To test the stability of the disulfide-containing peptides under the conditions of the binding competition assay, analogs and cells were incubated for various times and the culture medium was subjected to HPLC analysis. Although high backgrounds from the YM-1 medium obscured the first 10 minutes of the HPLC trace, it is clear from the data in Figure 5 that *cyclo*^{7,10}[Cys^7, *L*-Ala^9, Cys^{10},Nle^{12}]α-factor is not altered by a two hour incubation with cells. Similar results were found with the other cyclic peptides (data not shown).

Figure 5. HPLC of cyclo^{7,10}[Cys^{7}, *L*-Ala^{9}, Cys^{10}, Nle^{12}] α-factor after incubation with *S. cerevisiae* cells for 0, 0.5 or 2.0 h as indicated in the three panels. The retention time of the cyclic peptide is approximately 15.6 min.


ABSORBANCE 220 NM
0hr
15.56
0.5hr
15.65
2hr
15.60
RETENTION TIME (MIN)

CHAPTER IV

DISCUSSION

Previous studies on α-factor provided evidence that the biologically relevant structure of this pheromone involved a Type II β-turn spanning residues 7 to 10 (Jelicks *et al.*, 1988; Shenbagamurthi *et al.*, 1985). These investigations used linear peptides which existed in a distribution of structures in solution. To gain further insights into the bioactivity of the pheromone we have initiated investigations of molecules containing covalent constraints. In this dissertation, the focus is on analogs containing a disulfide bond involving side chains of residues 7 and 10. It is important to note that all bioassays were performed using strains lacking the *BAR1* peptidase that deactivates α-factor via proteolysis at the Leu^{6}-Lys^{7} peptide bond (Ciejek & Thorner, 1979). Therefore, the relative activities and binding constants of compounds reported here are not influenced by the known mechanism of degradation of the pheromone by the target cell. Furthermore, we observed by HPLC analysis that *cyclo*^{7,10}[Cys^{7},*L*-Ala^{9},Cys^{10},Nle^{12}]α-factor was not measurably altered by wild-type *S. cerevisiae* cells (Figure 5). We conclude that it is likely that the disulfide bridge remains intact upon contact with *S. cerevisiae* cells.

The peptides examined in this study reflect a significant perturbation of the primary sequence of α-factor. The replacements involved changing basic and polar residues (Lys and Gln) with sulfur-containing residues or with Ser. Despite these gross changes, many of the analogs (**Ia-Id**, **IIb-IId**, **IIg**, and **IIi**) retained significant potency, exhibiting from one tenth to 1/200th of the activity of α-factor. Indeed, the cyclic disulfide α-factor analogs, with Gly^{9} or *D*-Ala^{9} have identical activity to a cyclic analog containing a lactam bond between the Lys^{7} and Glu^{10} side chains (Xue *et al.*, 1989b). These findings support

the conclusion that the ε-amine of Lys^7 is not necessary for a productive interaction with the receptor (Shenbagamurthi *et al.,* 1983). Finally, we found that the cyclic disulfide containing peptides (**Ia-Id**) had 5- to 10-fold higher biological activity than sulfhydryl-containing homologs (**IIa-IId**).

Peptides **IIa-IId** contain a SH moiety and are potentially reactive under conditions of the bioassay and receptor binding competition. We therefore synthesized four additional linear peptides (**IIf-IIi**) to serve as controls. These analogs contain Ser and $Cys(CH_3)$ residues in place of Cys and represent the most similar non-reactive sidechains that were synthetically accessible. Both of the *L*-Ala variants of these linear pheromones (**IIf, IIh**) had no activity in the growth arrest assay (Table 2). Thus, $cyclo^{7,10}[Cys^7,L\text{-}Ala^9,Cys^{10},Nle^{12}]$α-factor is at least 10-fold and as much as 50-fold more active than any of the linear homologs that we investigated. In contrast to the results with peptides having an *L*-residue in position 9, both of the linear *D*-Ala^9 containing compounds (**IIg, IIi**) were nearly as active as $cyclo^{7,10}[Cys^7,D\text{-}Ala^9,Cys^{10},Nle^{12}]$α-factor (Table 2).

It is important to analyze the above findings with respect to the results of conformational analyses on α-factor and its analogs. As stated above, the disulfide regions of peptides (**Ia-Id**) have been shown by detailed NMR analysis to retain β-turn conformations in both aqueous and organic solvents (20,21). $Cyclo^{7,10}[Cys^7,L\text{-}Ala^9,Cys^{10},Nle^{12}]$α-factor prefers a Type I turn whereas the Gly^9, D-Ala^9, and D-Val^9 homologs assume Type II turns. In addition previous studies have shown that linear peptides containing Gly or a D-residue in position 9 assume transient Type II β-turns whereas those with a L-residue at this position are unstructured (Jelicks *et al.,* 1988; Jelicks *et al.,* 1989; Gounarides *et al.,* 1993). With these conclusions in mind it appears that a critical feature of the bioactive structure of the α-factor is a turn in the center of the peptide. The slightly higher activity of $cyclo^{7,10}[Cys^7,D\text{-}Ala^9,Cys^{10},Nle^{12}]$α-factor in

comparison to the *L*-Ala[9] analog may reflect a small preference for a Type II turn. However, the very low activity of the cyclo[7,10][Cys[7],*D*-Val[9],Cys[10],Nle[12]]α-factor, despite the fact that it assumes a Type II β-turn, suggests that large side chains at the *i*+2 residue of the turn interfere with binding and/or signal transduction.

We also found that inactive peptides result when the Pro-Gly dipeptide is replaced with 5-aminopentanoic acid (APA) in both cyclic and linear analogs (**Ie**, **IIe**). In a β-turn four dihedral angles [ϕ_2,ψ_2,ϕ_3,ψ_3], along with the isomeric state (*cis* or *trans*) of the central peptide bond, determine the exact conformation of the turn. When APA replaces the Pro-X linkage at positions 8 and 9 in the α-factor sequence this peptide bond and all of these dihedral angles are eliminated. Therefore, although the APA replacement retains the atomic spacing between residues 7 and 10 the possibility of a β-turn is eliminated. In the cyclic disulfide containing APA (**Ie**) the bend in the molecule should allow the two ends of the pheromone to achieve conformations that are not very different from those in peptides **Ia-Id**. Therefore, the complete loss of activity found for Pro-Gly dipeptide replacement suggests that the residues in positions 8 and 9 of the native α-factor may be dominant determinants of the active conformation of α-factor and are necessary for a productive receptor-pheromone interaction.

The results of the binding studies reported herein show that several of the disulfide containing α-factors compete with radioactive α-factor for the receptor. Binding of the cyclic pheromones is two to three orders of magnitude poorer than that of the native pheromone. However, the relative order of binding correlates with the activity of the cyclic analogs; the Gly and *D*-Ala peptides show greatest competition followed by the *L*-Ala and *D*-Val analogs, with the cyclic APA demonstrating no competition (Figure 4). Interestingly, none of the linear sulfhydryl analogs competed with the binding of α-factor at the concentrations measured. This is despite the fact that several of these compounds

are agonists (Table 2). Indeed the linear Gly^9 peptide (**IIc**) is more active than the cyclic *D*-Val^9 homolog (**Ia**) yet it is the latter which exhibits a small but reproducible binding competition (Figure 4, Panels B and E). It was possible that the lack of correlation between binding and activity for the linear sulfhydryl-containing peptides reflected their chemical reactivity under conditions used in the binding assay. We found that four non-sulfhydryl-containing linear analogs, [$Cys(CH_3)^7$,X^9,$Cys(CH_3)^{10}$,Nle^{12}]α-factor and [Ser^7,X^9,Ser^{10},Nle^{12}α-factor, where X= *L*-Ala or *D*-Ala, bind to the receptor (Figure 4, Panels G & H) as well or better than the disulfide analogs (Figure 4, Panels B-E). Thus, the chemical reactivity of the linear sulfhydryl-containing pheromones may interfere with their receptor binding.

The very poor affinity of the cyclic Gly and *D*-Ala α-factor analogs for the receptor is somewhat perplexing. The relative activity of these peptides is significantly higher than those of other α-factor analogs that we previously found to have a reasonable affinity for the receptor. For example, des-Trp^1-[Ala^3]α-factor is not active for halo formation at 10 μg/disk whereas it has only about a 10-fold lower affinity for the receptor than [Nle^{12}]α-factor (Raths *et al.*, 1988). In contrast, the active cyclo^{7,10} [$Cys^{7,}$,Gly^9,Cys^{10},Nle^{12}]α-factor and cyclo^{7,10}[$Cys^{7,}$,*D*-Ala^9,Cys^{10},Nle^{12}] α-factor show poor binding to the receptor. It is important to note, however, that many investigators have shown that the binding and bioactivity of peptide hormones can be dissociated. Indeed in an elegant analysis of the β-adrenergic receptor detailed models for the interaction of the hormone, the receptor and the G-proteins have been used to separate binding from receptor activation (Samama *et al.*, 1993). In the present study, this is most evident with [$Cys(CH_3)^7$,X^9,$Cys(CH_3)^{10}$,Nle^{12}]α-factor and [Ser^7,X^9,Ser^{10},Nle^{12}]α-factor where the *L*- homolog and *D*-homolog exhibit nearly equal binding competition curves but where the *L*-analog is completely inactive at the highest concentration tested (Table 2, Figure 4).

Similar conclusions were reached with position two analogs of α–factor (Levin *et al.*, 1993) and in studies on glucagon (Unson *et al.*, 1991) and human chorionic gonadotropin (Willey *et al.*, 1989). It is possible that the cyclic α–factors are in an optimal conformation for triggering biological response. At the same time, the cyclic structure might not have a high affinity for the receptor. Accordingly, fewer molecules would bind at a given concentration. Nevertheless, those molecules that bound might provide an especially strong signal. Such a hypothesis would explain the poor competition with native α–factor and the relatively good biological activity of the cyclic pheromones.

In conclusion, the results reported herein provide direct correlation between the presence of a turn and the bioactivity of the α–factor. The fact that cyclo^{7,10}[Cys^{7},*L*-Ala^{9},Cys^{10},Nle^{12}]α–factor is more active than three linear homologs suggest that a bent conformation of α–factor is required to trigger the biological response. It is still not resolved whether the turn conformation is needed to orient the ends of the peptide for signal transduction, whether productive binding of the pheromone to its receptor involves the atoms of the β-bend itself, or whether some combination of the above is the dominant factor in pheromone activity.

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PART IV

SYNTHESIS OF α-FACTOR ANALOGUES CONTAINING PHOTOACTIVATABLE AND LABELING GROUPS*

*Part IV was published in its entirety as **Y. Jiang, M. Breslav, R.K. Khare, A. McKinney, J. Becker, and F. Naider.** 1995. Synthesis of α-factor analogues containing photoactivatable and labeling groups. *Int. J. Peptide & Protein Res.* **45**,106-115. The research for Part IV was done in collaboration with the laboratory of Dr. Fred Naider (College of Staten Island, Staten Island, NY). Dr. Naider's laboratory was responsible for the synthesis and analysis of the α-factor analogues containing photoactivatable and labeling groups. Angela McKinney was responsible for the biological studies which include the growth arrest assay and the competition binding assay.

CHAPTER I

INTRODUCTION

Recent studies have demonstrated quite elegantly that the mating process in *Saccharomyces cerevisiae* represents a powerful paradigm to understand the mode of action of peptide hormones (Herskowitz, 1988; Herskowitz & Marsh, 1987). Mating between haploid strains of opposite mating type in unicellular yeast is initiated by diffusible pheromones (Sprague & Thorner, 1992; Sprague *et al.,* 1992) which are biosynthesized by one mating type and recognized by the opposite mating type. The peptide pheromones that control mating in yeast are α-factor, a tridecapeptide with the sequence H-Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr-OH (Stotzler *et al.,* 1976), and **a**-factor, a lipopeptide with the structure H-Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys(S-farnesyl)$COCH_3$ (Anderegg *et al.,* 1988). The latter peptide has not been subjected to detailed structure-activity investigations. In contrast, numerous studies have been conducted on the relationships among primary structure, the biological activity, and conformation of α-factor (Naider & Becker, 1986; Gounarides *et al.,* 1993; Eriotou-Bargiota *et al.,* 1992). Ligand binding studies revealed that α-factor binds specifically to its receptor with $K_d \approx 10^{-9}$ M^{-1} and there are approximately 10^4 binding sites per cell (Jenness *et al.,* 1986; Blumer *et al.,* 1988; Raths *et al.,* 1988). The α-factor receptor is encoded by the *STE2* gene and is a 431-residue protein (Burkholder & Hartwell, 1985; Nakayama *et al.,* 1985). Hydropathy plots deduced from the amino acid sequence of the *STE2* gene product lead to the prediction that the protein will form seven transmembrane helices with both intra- and extracellular peptide loops. Recent studies have shown that the 105 residues at the carboxyl terminus of the α-factor receptor are not necessary for activity but are involved in the recovery of the yeast cells from the effects of

the pheromone (Konopka *et al.,* 1988; Reneke *et al.,* 1988) and have begun to identify the transmembrane helices which contribute residues to the active site (Konopka & Jenness, 1991). No biochemical information, however, is available on specific residues which interact with bound α-factor.

In order to discern the binding site of the α-factor with its receptor, a probe having high biological affinity to the α-factor receptor will be needed. To identify such a probe, we undertook the synthesis of a-factor analogs in which photoactivatable and/or labeling groups were incorporated in the pheromone. Photoaffinity labeling (Miller & Kaiser, 1988) has been widely successful for the identification and localization of macromolecular receptors (Shoelson *et al.,* 1993; Servant *et al.,* 1993; Williams & Shoelson, 1993). Although numerous photoactivatable moieties have been utilized to prepare peptides (Chavan *et al.,* 1993; Kauer *et al.,* 1986; Eberly & De Graan, 1985), *p*-benzoylphenylalanine has proved to be especially useful in peptide photoaffinity labels (Miller & Kaiser, 1988) because of (i) its high chemical stability, such that it can be used in conventional solid-phase peptide synthesis; (ii) its low photoactivity with water; (iii) activation by low-energy UV light (300-350 nm); (iv) rapid reaction of the activated intermediate. Molecular tags are necessary to localize the peptide after insertion into the binding site of the receptor. The biotin/avidin system (Wilchek & Bayer, 1990) has found a wide range of applications as labeling group and ^{125}I tracer is also highly useful in a variety of biochemical studies (Shoelson *et al.,* 1993; Basak *et al.,* 1993). In this dissertation, strategies used to synthesize α-factor analogs containing Bpa and either iodinated HPP or biotin are discussed. Using knowledge gained from previous structure-activity analyses and the present investigation, we have identified analogs which have sufficient biological activity and affinity to α-factor receptor to probe the structure of the binding region.

CHAPTER II

MATERIALS AND METHODS

Boc-*O*-(2-bromo-CBZ)-L-tyrosine PAM resin, di-*tert*-butyldicarbonate and most of the protected amino acids were purchased from Bachem Inc. (Torrance, CA). *N-tert*-Boc-3,5-Diiodo-L-tyrosine hydroxysuccinimide ester; 3-[*p*-hydroxyphenyl]propanoic acid, *d*-Biotin and leucine aminopeptidase [(EC 3.4.11.1) 140 units/mg] were from Sigma Chem. Co. All solvents used were of analytical grade and were from either Fisher Scientific, Aldrich Chemical Co., or VWR, and other reagents used were of reagent grade and from Aldrich Chemical Co. Thin layer chromatography plates were RP C_{18} F_{254} reversed-phase precoated glass plates or silica gel 60 F_{254} precoated aluminum sheets, from E. Merck [Darmstadt (EM Science Co.)]. *N*-Acetyl-*p*-benzoyl-D,L-phenylalanine was a gift from Michael Chorev, Jerusalem, Israel. Boc-*p*-benzoyl-D,L-phenylalanine was prepared in our laboratory, 3-(diiodo-4-hydroxyphenyl) propanoic acid hydroxysuccinimide ester was prepared according to a published procedure (Michelot *et al.,* 1980).

Analysis

All melting points were measured by the capillary method and are uncorrected. Homogeneity of all peptides was checked by thin-layer chromatography (TLC) on silica gel and reversed-phase high performance liquid chromatography (HPLC). TLC was carried out using the following eluents: (A) butanol: acetic acid: water (4:1:2); (B) butanol: acetic acid: water: pyridine (15:3:12:10), and the R_f values are reported as R_fA and R_fB, respectively. Detection was by ultraviolet light and chlorine-starch indicator. Reversed-phase HPLC was performed on a Waters system using Waters μ-Bondapak-C_{18}

columns (3.9 x 300 mm, analytical; 19 x 150 or 19 x 300 mm, preparative). Peptides were eluted with a linear gradient of either water (0.025% TFA)/acetonitrile system or water (0.025% TFA)/methanol system with acetonitrile (methanol) from 20 to 80% over 30 min (unless otherwise noted). Detection was at 220, 254, or 279 nm. Amino acid analysis was carried out at the Wistar Institute, Philadelphia, PA and at Brigham and Women's Hospital, Boston, MA. Bpa did not elute from the analyzer as observed by Miller & Kaiser (Miller & Kaiser,1988). Fast atom bombardment mass spectrometry (FAB-MS) was carried out at the University of Tennessee. FAB mass spectra were obtained with a VG ZAB EQ mass spectrometer equipped with a flow FAB ion source of an Ion Tech fast atom gun. Approximately 2 mL of a peptide solution (0.1 nmol/μL) dissolved in acetonitrile/water (2:1), 0.1% trifluoroacetic acid was dispersed on the stainless-steel target in a matrix of monothioglycerol. The accelerating voltage of the mass spectrometer was maintained in 8 kV, whereas 8keV xenon atoms at a discharge current of 1 mA were used to bombard the sample. Monoisotopic masses to 0.1 Da are reported. Elemental analyses were performed by E + R Microanalytical Laboratory, Inc., Corona, New York.

p-Benzoyl-D,L-phenylalanine (Bpa)

A suspension fo acetyl-*D*,*L*-Bpa (1g; 3.215 mmol) in 6 N HCl (12 mL) was refluxed for 4 h, the acid was evaporated under reduced pressure with a water pump, and the residue was dried in vacuum overnight to give 0.97 g (98% yield), m.p. 198-200°C [Lit. 202-204°C (Kauer *et al.*, 1986)]. NMR δ 8.55 (b, 2H, $-NH_2$); 7.56 (m, 9H, ArH); 4.26 (t, 1H, αCH); 3.26 (m, 2H, βCH_2). Elemental analysis: calculated for $C_{16}H_{15}O_3N.1.1HCl$, C 62.11; H5.24; N 4.52; found: C 62.00; H 5.02; N 4.31.

N-tert-Butyloxycarbonyl-*p*-benzoyl-D-L-phenylalanine (Boc-Bpa)

To a stirred solution of HCl-*D,L*-Bpa (918 mg, 3 mmol) dissolved in 6 mL of 1 N NaOH and 15 mL dioxane was added a solution of 0.8 mL of di-*tert*-butyldicarbonate (Boc_2O, 3.6 mmol) in 5 mL di-oxane drop by drop, the solution was maintained at pH 9-10 by adding 1 N NaOH. After 5 h, dioxane was removed under reduced pressure and the aqueous solution was extracted three times with petroleum ether. The aqueous layer was separated and acidified with 1 N H_2SO_4 to pH 2.5-3. The solution was extracted with ethyl acetate, and the organic layer was washed with water and dried (Na_2SO_4) overnight. The solvent was evaporated (approximately 5 mL) and petroleum ether was added drop by drop until turbid. The mixture was placed in the refrigerator overnight, the resulting white crystal were filtered, to give Boc-*D,L*-Bpa 807 mg (73% yield), m.p. 122-125°C. NMR δ 7.5 (m, 9H, ArH); 7.19 (d, 1H, -NH-); 4.11 (t, 1H, αCH). Elemental analysis; calculated for $C_{21}H_{23}O_5N$, C 68.27; H 6.28; N 3.79, found: C 68.17; H 6.58; N 3.80.

3-(Diiodo-4-hydroxyphenyl)propanoic acid

To a solution of 98% 4-hydroxyphenylpropanoic acid (4 g; 24 mmol) dissolved in 30% ammonia solution (480 mL) was added an aqueous solution of **KI** (20 g) and I_2 (9 g) in 50 mL of water drop by drop during 1 h. The reaction was monitored with reversed-phase TLC using a water/acetonitrile (40/60) developing system. The resulting light yellow mixture was stirred at room temperature for an additional hour, and the solution was evaporated under reduced pressure using a water aspirator to remove ammonia, then acidificated to pH 4 with concentrated HCl (12 N). The resulting precipitate was filtered and washed with HCl and water. The precipitate was dissolved in ethyl ether and dicyclohexylamine was added to form the precipitate of its DCHA salt. After washing with ethyl ether, the precipitate was dissolved in a mixture of 1 N H_2SO_4

(40 mL) and ethyl acetate in separatory funnel, the organic layer was washed with water, the solvent was evaporated to dryness yielding a pale yellow solid which was recrystallized from toluene. The product: 6.21 g (yield 62%), was more than 99% homogeneous as determined by HPLC (K' = 9.1, at a gradient from 90% water to 75% acetonitrile over 30 min), m.p. 162-164°C [Lit. 165-167°C (36)]. NMR δ 7.59 (s, 2H ArH); 2.66 (t, 2H, βCH_2); 2.47 (t, 2H, αCH_2). Elemental analysis: calculated for $C_9H_8O_3I_2$, C 25.86; H 1.93; I 60.72, found: C 26.06; H 1.81; I 60.86.

Using the same procedure as above except that 5 g of $\mathbf{I_2}$ was used in the reaction, we obtained 3-(monoiodo-4-hydroxyphenyl)propanoic acid containing diiodinated product. This material (77% monoiodo product; 23% diiodo product) was used to prepare the 3-(monoiodo-4-hydroxphenyl) propanoic acid *N*-hydroxysuccinimide ester.

3-(Diiodo-4-hydroxyphenyl)propanoic acid *N*-hydroxy-succinimide ester

1,3-Diisopropylcarbodiimide (2.4 mmol) in cold dry dioxane (5 mL) was poured slowly into a mixture of 3-(diiodo-4-hydroxphenyl)propanoic acid (836 mg, 2 mmol) and *N*-hydroxysuccinimide (230 mg, 2 mmol) in dry dioxane (15 mL), the mixture was maintained at 0°C for 1 h, and then was stirred for 14 h at room temperature. *N,N'*-diisopropyl urea was discarded, and the dioxane solution was evaporated in vacuum; the residue was then recrystallized from isopropanol, yielding 800 mg product (yield 78%). The product was homogeneous on thin layer of silica gel 60 F_{254} in toluene/ethyl acetate (1:1 v/v), R_f = 0.65. NMR δ 7.68 (s, 2H, ArH); 2.97 (t, 2H, βCH_2); 2.88 (t, 6H, αCH_2 & OSu -OC-CH_2-CH_2-CO-).

Using the same procedure as above, we obtained 3-(monoiodo-4-hydroxyphenyl)propanoic acid *N*-hydroxysuccinimide ester containing diiodinated product. This material was used directly in the synthesis of monoiodinated α-factor analogs.

Synthesis of peptides containing Bpa

Peptides containing Bpa were synthesized manually by solid-phase methodology using the procedures described previously (Xue *et al.*, 1989) starting with *N*-α-Boc-Tyr (2-Br-CBZ) OCH_2-PAM-resin (0.5 mmol/g of resin). The Boc group was used for *N*-α-protection except for α-Fmoc-Lys(Boc); formyl and tosyl were used as the side chain protecting groups for Trp (For), His (Tos) and Arg (Tos), respectively. The Boc group was deprotected with 40% trifluoroacetic acid/2% dimethyl sulfide/CH_2Cl_2 except for Gln, where the Boc group was deprotected with 4 N HCl in dioxane. Coupling was carried out twice for each residue using diisopropylcarbodiimide in CH_2Cl_2 for the first coupling, and diisopropylcarbodiimide/HOBt in CH_2Cl_2/DFM for the second coupling before His (Tos) was incorporated. Boc-Gln was coupled via its HOBt ester preformed in DMF at 0°C. When Bpa was coupled, the SPPS vessel was permanently wrapped with aluminum foil to protect the resin from light. Before HF cleavage, the resin was treated with HOBt in DMF to remove the tosyl group of His, with TFA in CH_2Cl_2 to remove the Boc group, and with piperidine in DMF to remove the formyl group. However, when the Fmoc was used as protecting group on the peptide, the resin could not be treated with piperidine prior to HF. One gram of the dried resin was mixed with 1.5 mL of anisole in a Kel-F HF reaction vessel, and HF was condensed at -78°C under reduced pressure to a total volume of 15 mL. Cleavage proceeded for 1 h at 0-2°C. After evaporation of HF, the residue was washed with precooled ethyl ether three times and then was extracted with glacial acetic acid. The crude product was purified using HPLC on a Waters μ-Bondapak column (19 x 300 mm) eluted with a linear gradient from 100% water (0.025% TFA) to 60% acetonitrile (0.025% TFA) over 60 min. The yield of peptides **I-V** (Table 1) was 49, 46, 46, 60 and 26% respectively. All peptides were over 99% homogeneous as judged by analytical HPLC in both an acetonitrile gradient and a methanol gradient.

Table 1. Sequence of α-factor analogs

Peptide	Sequence of amino acids
I	H-Bpa-His-Trp-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
II	H-Trp-His-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
III	H-Trp-His-D-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Try-OH
IV	H-His-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
V	H-Trp-His-Bpa-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Nle-Tyr-OH
VI	(I_2-HPP)-Trp-His-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
VII	(I-HPP)-Trp-His-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
VIII	(I-HPP)-Trp-His-D-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
IX	(I_2-HPP)-Trp-His-D-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
X	(I_2-HPP)-Lys-Trp-His-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
XI	H-Lys(ε-I_2-HPP)-Trp-His-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
XII	(I_2-HPP)-His-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
XIII	H-Lys(ε-I_2-HPP)-His-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
XIV	(I_2-Tyr)-His-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH
XV	(Biot)-Trp-His-Bpa-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Nle-Tyr-OH
XVI	H-Lys(ε-Biot)-Trp-His-Bpa-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Nle-Tyr-OH
XVII	(Biot-AVA)-Trp-His-Bpa-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Nle-Tyr-OH
XVIII	H-Lys(ε-Biot-AVA)-Trp-His-Bpa-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Nle-TyrOH

These peptides are present in the form of their TFA salts; I-HPP represents monoiodo-HPP; 1_2-HPP represents diiodo-HPP. HPP = 3-(4-hydroxyphenyl) propanoic acid; AVA = 5-aminovaleric acid; Biot = biotinyl.

Synthesis of iodinated peptides

To 3 mL of a solution of the corresponding deprotected peptide (8 μmol) dissolved in a DMF/(0.1 M, pH 8.0) borate buffer (1:1) solvent cocktail was added the acylating reagent (Bolton Hunter reagent or Boc-diiodo-Tyr-OSu, 16 mmol) dissolved in 1 mL of DMF, and the mixture was stirred for 3 h at 0°C. At this time HPLC analysis indicated that the acylation was over 90% complete and 1 mL of 1 M Gly in borate buffer was used to terminate the reaction. 1 mL of piperidine was added into the mixture to remove the Fmoc group on Lys when necessary. The Boc group was removed using 8 mL of 40% TFA/DCM/DMS, after evaporating the solvent, when Boc-diiodo-Tyr-Osu was used as the acylating agent. After evaporating all solvents to dryness in a SpeedVac, the residue was dissolved in water and acetonitrile (50/50, v/v), and purified by reversed-phase HPLC on a Waters μ-Bondapak C_{18} column (19 x 150 mm) using a linear gradient from 95% water (0.025% TFA) to 65% acetonitrile (0.025% TFA) over 60 min at flow rate of 6 mL/min. The yield was 29-55%. In certain cases the low yields of purified peptide reflect either a mixture of mono-or di-iodoacylating agent which was used in the synthesis or the necessity for several purifications. All products were over 99% homogeneous as judged by HPLC analysis in both acetonitrile and methanol gradients.

Synthesis of biotinylated peptides

After the synthesis of the corresponding protected peptide assembled on PAM resins, the Boc group was removed with 50% TFA/2% DMS/CH_2Cl_2 for 30 min, and peptides **XV** and **XVII** (Table 1) were prepared by reacting the Boc-deprotected peptide with the preformed symmetrical anhydride of biotin in DCM for 3 h at room temperature. The Kaiser test showed that the reaction was incomplete, and a second overnight coupling for peptide **XV** and a third coupling for peptide **XVII**, using the symmetrical anhydride of biotin in the presence of 0.5 mL of 0.4 M DIEA in DCM as catalyst, were necessary to

drive the reaction to completion. Peptides **XVI** and **XVIII** (Table 1) were synthesized by coupling the appropriate Boc-deprotected peptide with biotin using BOP and DIEA as the coupling agent in DMF for 2 h. Under these conditions a negative Kaiser test was attained. All biotinylated peptides were treated with HOBt in DMV (0.6 eq.) for 2 h to remove the tosyl group. Piperidine (20-30%) in DMF was used to deprotect the Fmoc group for peptides **XVI** and **XVIII**. The completely dried resin was subjected to HF cleavage by mixing (200 mg) with 0.5 mL anisol and 7 mL HF, and stirred at 0-5°C for 1 h. HF was evaporated, the residue was extracted with 30% HOAc, and the crude product was purified on a semipreparative μ-Bondapak C_{18} column (19 x 300 mm) with a linear gradient of water and acetonitrile containing 0.025% TFA with water from 100 to 30% over 60 min for peptide **XV**; 80 to 40% for peptide **XVII**; 100 to 40% for peptides **XVI** and **XVIII**, detected at 279 nm. The yield was 8-30%. All products were over 99% homogeneous as judged by analytical HPLC.

Leucine aminopeptidase degradation

To peptide (0.5 mg) suspended in 20 mL Tris buffer (pH 8) was added a commercial suspension of leucine aminopeptidase (20 μL), and the solution was incubated for 1.5 h at 36°C. The mixture was diluted with Tris buffer, filtered and analyzed by HPLC.

Biological activity assay

The biological activity of a-factor analogs was tested by growth arrest (halo assay). YEPD plates were overlayered with 4 mL of *S. cerevisiae RC 629* (*MATa* cells (2.5×10^5 cells per mL)] in Nobel agar (1.1%). Filter disks (sterile blanks from Difco), 8 mm in diameter, were placed on the overlay, and 10 μL portions of peptide solutions at various concentrations were spotted on the disks. The plates were incubated at 30°C for 24 h and

then observed for clear zones (halos) around the disks. The data were expressed as the diameter of the halo, including the diameter of the disk.

Competition assay

Competition of bound [^{3}H] α-factor by unlabeled α-factor analogs was measured as described previously (Raths *et al.,* 1988) with the exception that the *S. cerevisiae* strain used in the assay was *S. cerevisiae RC 629.* Typically, the binding competition was started by the addition of 50 μL of [^{3}H] α-factor (9.3×10^{-7} M, 7.57 Ci/mmol) to 400 μL of the cell suspension (1.25×10^9 cell/mL) and 50 μL of the appropriate unlabeled analogue at increasing concentrations.

CHAPTER III

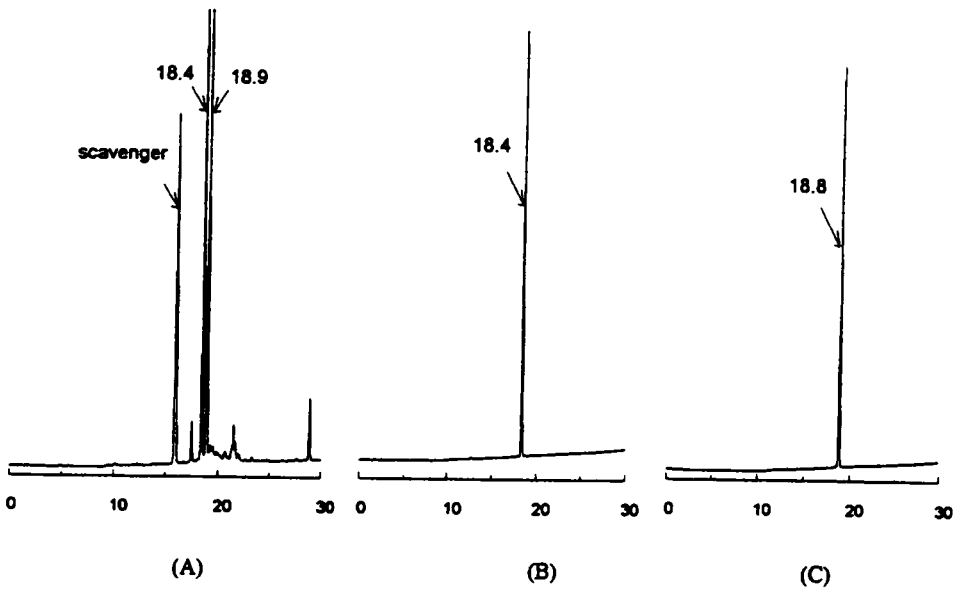
RESULTS AND DISCUSSION

Synthesis and resolution of *D*- or *L*-Bpa peptides

Previous investigations of structure-activity relationships pointed out that the histidine in position 2 and tyrosine in position 13 were extremely important for α-factor activity. However, Trp^1 could be removed from the pheromone or Trp^3 could be substituted by certain hydrophobic residues with only slight reduction in activity (Masui *et al.*, 1979; Shenbagamurthi *et al.*, 1983). Based on these results, α-factor analogs were synthesized in which Trp in position 1 or position 3 was replaced by the photolabile amino acid *L*-Bpa or *D*-Bpa. (*D/L*)Bpa was smoothly incorporated into the peptide using DIPC as coupling agent directly or via the 1-hydroxybenzotriazole active ester of *N*-protected Bpa utilizing standard solid-phase methodologies. The peptide containing Bpa was protected from light. However, no other special precautions were found to be necessary in handling either Bpa-derivatives or Bpa-containing peptides. Previous studies showed that α-factor and [Nle^{12}]α-factor had equal biological activity (Eriotou-Bargiota *et al.*, 1992). We replicated Met^{12} with the isosteric Nle to obtain analogs less prone to oxidative degradation. The synthesis of peptides was performed utilizing diisopropylcarbodiimide/hydroxybenzotriazole accelerated active esters as the reactive species from position 3 to position 12. Since tosyl-histidine used in our synthesis was labile to HOBt (Gutte & Merrifield, 1971), the accelerator was omitted for incorporation of residues 1 and 2.

Separation of the H-Trp-His-D-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH and the H-Trp-His-L-Bpa-Leu-Gln-Leu-Arg-Pro-Gly-Gln-Pro-Nle-Tyr-OH peptides was accomplished using reversed-phase semipreparative HPLC (Figure 1). The faster

Figure 1. HPLC of the resolution of WH(*D/L* Bpa)LQLRPGQP(Nle)Y. (A) Crude product containg *D/L*-Bpa as cleaved from the resin; (B) purified WH(*L*-Bpa)-LQLRPGQP(Nle)Y; (C) purified WH(*D*-Bpa)LQLRPGQP(Nle)Y. A linear gradient from 100% water (0.025% TFA) to 100% acetonitrile (0.025% TFA) over 30 min was used. Chromatography was performed on a μ-Bondapak C_{18} column (3.9 x300 mm).

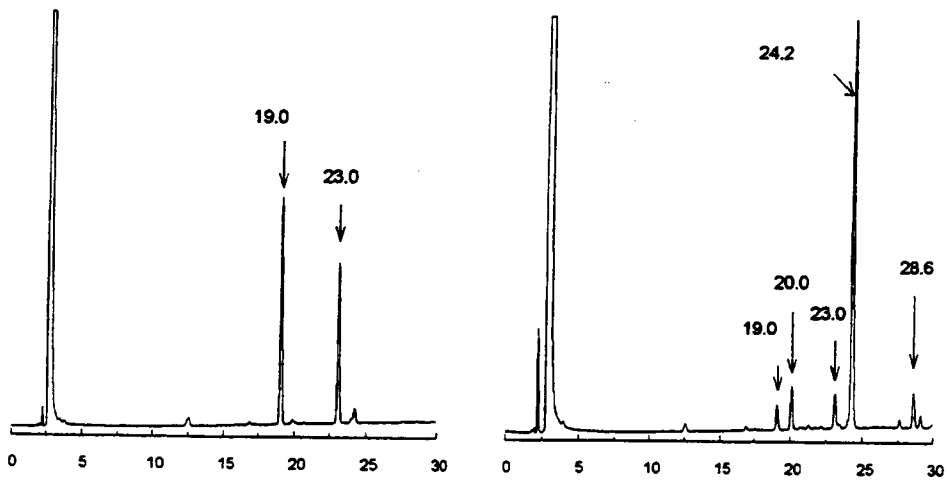

18.4
18.9
scavenger
18.4
18.8
0
10
20
30
0
10
20
30
0
10
20
30
(A)
(B)
(C)
Retention Time (min)

eluting fraction was confirmed as the *L*-Bpa containing peptide by coinjecting with standard *L*-Bpa peptide which had been synthesized using *L*-Bpa (data not shown). The successful resolution of the diastereomeric peptide allowed both products to be obtained from one chain assembly. However, in certain synthesis only the *L*-Bpa peptide was prepared.

Synthesis of iodinated peptide containing Bpa

Since 3-[diiodo-4-hydroxyphenyl]propanoic acid does not survive anhydrous hydrogen fluoride (Naider *et al.,* 1990), it was necessary to acylate peptides following cleavage from the resin. Despite the success achieved in direct acylation of α-factor, a mixture of α- and ε-acylated Bpa containing α-factor analogs was obtained at various pHs, using different organic solvent, and varying the ratio of buffer to organic phase (Naider *et al.,* 1990). In contrast to the ε-amino group of lysine, the guanidino group of arginine is an unusually basic group, forming stable salts with strong and weak acids alike. Furthermore, some of its acyl derivatives are unstable (Bodansky *et al.,* 1976). Finally, substitution of Lys^7 by Arg does not affect biological activity of α-factor (Samokhin *et al.,* 1979). Therefore, we decided to replace lysine with arginine to minimize interference by the ε-amino group in position 7 during acylation of the *N*-terminal amino group with Bolton Hunter reagent. 3-[Diiodo-4-hydroxyphenyl]propanoic acid hydroxysucciminide ester is sensitive to both basic and acidic medium. Above pH 10 and below pH 6, significant hydrolysis of this reagent was observed. Acylation of peptides was carried out in the mixture of organic solvent and borate buffer (pH 7.5-8.5) in which *N*-terminal amino groups would be in the free amine state. The acylation of peptide **II** (Table 1) is given as a typical reaction. Initially large peaks at 19.0 min, corresponding to unreacted peptide **II**, and at 23.0 min, corresponding to diiodo Bolton Hunter reagent, were evident in the HPLC chromatogram (Figure 2A).

Figure 2. HPLC profile of acylation of peptide **II** with Bolton Hunter reagent. Chromatography was performed on a μ-Bondapak C_{18} column (3.9 x 300 mm) using a linear gradient of water (0.025% TFA)/acetonitrile (0.025% TFA) with water from 90 to 25 % over 30 min. (A) Reaction mixture immediately after addition of Bolton Hunter; (B) reaction mixture after 3 h.


19.0
23.0
0
5
10
15
20
25
30
24.2
20.0
19.0
23.0
28.6
0
5
10
15
20
25
30
(A)
(B)
Retention Time (min)

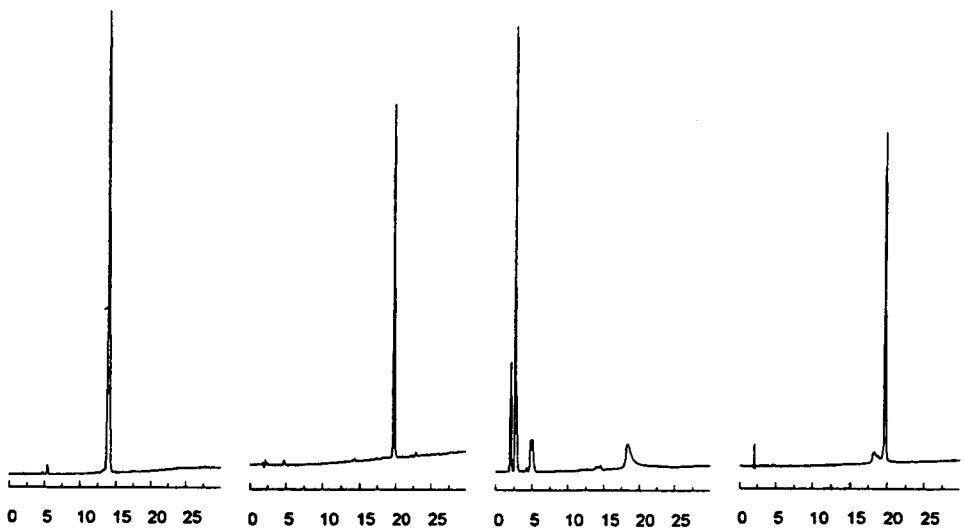
With time, both of these peaks decreased in intensity while a major peak appeared at 24.2 min which was subsequently shown to be monoacylated peptide (peptide **VI**, Table 1). The reaction was over 90% complete in 3 h and the peak at 20.0 min was attributed to diiodo-HPP by comparison with the unreacted reagent. The small peak at 28.6 min may be some diacylated product in which the Arg side chain is probably acylated.

In the above reaction the Arg^7 analog was used to minimize acylation of the position 7 side chain. It is clear from Figure 2B that very little side product is found during the reaction. In order to verify that the major product (24.2 min) was the *N*-terminal monoacylated pheromone, the Sakaguchi reaction (Heesing & Hoppe, 1976) was used to determine if the guanidino groups had been modified. The positive results that we obtained revealed the integrity of the guanidino group of the arginine residue after acylation by Bolton Hunter reagent. Incubation of peptide **VI** with leucine aminopeptidase resulted in no significant reaction in contrast to peptide **II** which was completed degraded under identical conditions (Figure 3). In addition, FAB MS gave protonated molecular ions that were in agreement with values calculated for the protonated monoacylated α-factor analogue. These results supported the conclusion that peptide **VI** is α-acylated. Similar studies were used to support the structure of peptide **VII-XIII** (Table 1) which were prepared by the same synthetic method. *N*α-Boc-diiodo-Tyr-OSu was used as an acylating agent to synthesize peptide **XIV** from peptide **IV**.

Synthesis of biotinylated photoactive a-factor

In contrast to iodinated HPP, biotin has been shown to survive anhydrous HF (Lobl *et al.*, 1988). Therefore, it can be attached to the peptide prior to HF cleavage by assembling the desired peptide on the resin, and then reacting the appropriate amino group either with the symmetric anhydride of biotin or with biotin using BOP and DIEA as activating agent. Acylation on the resin with the *p*-nitrophenyl ester of biotin to give

Figure 3. Leucine aminopeptidase digestion of free and α-amino-acylated peptides. (A and B) peptides **II** and **VI**, respectively. (C) Reaction mixture of peptide **II** after 1.5 h incubation with leucine aminopeptidase at 36 °C. (D) Reaction mixture of peptide VI after 1.5 h incubation with leucine aminopeptidase at 36 °C. A linear gradient from 80% water (0.025% TFA) to 80% acetonitrile (0.025% TFA) over 30 min was used in the HPLC analysis.


0 5 10 15 20 25
0 5 10 15 20 25
0 5 10 15 20 25
0 5 10 15 20 25
(A)
(B)
(C)
(D)
Retention Time (min)

peptide **XV** proceeded very slowly; almost no coupling took place after 22 h. The reaction was 50% complete in 3 h using biotin symmetric anhydride which was preformed by mixing biotin and DCC in DCM. A second acylation was carried out overnight with the same symmetrical anhydride and DIEA as catalyst, and the Kaiser test showed that the coupling was now complete. A third coupling was needed for acylation to give peptide **XVII** using the symmetric anhydride method mentioned above. However, when BOP was used as activating agent (with addition of DIEA) for the acylation of the same peptide with biotin, the reaction was complete in 2 h. All peptide pheromones including Bpa-, iodinated HPP-, or biotin-containing peptides were over 98% homogeneous and were characterized by amino acid analysis and fast atom bombardment mass-spectrometry (Table 2).

Biological affinities

The biological activity of all α-factor analogs containing Bpa and iodinated HPP or biotin was measured by a growth arrest assay on *S. cerevisiae RC 629* strain (Table 3). We noted that the α-factor analogue in which tryptophan in position 3 was replaced by *L*-Bpa (peptide **II**) was approximately one-tenth as active as native α-factor. Incorporation of *D*-Bpa in position 3 (peptide **III**), however, resulted in a peptide with 200-fold lower activity. A previous investigation showed that amino terminus extended analogs of α-factor, with Ala; Glu-Ala; Ala-glu-Ala; Glu-Ala-Glu-Ala, retained significant activities; the 14-peptide was equally active with α-factor, whereas the 17-peptide was 5-10-fold less active. This result suggested that the α-factor receptor has a binding site that can readily accommodate additional residues at the amino terminus of the tridecapeptide pheromone (Tallon *et al.,* 1987). On the basis of this assumption, analogs containing Bpa^3 in which iodinated HPP or biotin was attached to the amino terminal group were designed. These analogs (peptides **VI-IX** and **XV**) were more than 100-fold less active

Table 2. Chemical and physical properties of synthetic α-factor analogs I-XVIII

Peptide	K'[a]		RfA	RfB	FAB-MB[b]	[α][c]	Amino acid analysis[d]								
	(A)	(B)			$(M+H)^+$		Arg	Gly	Gln	His	Leu	Lys	Nle	Pro	Tyr
I	5.89	11.04	0.41	0.58	1759.0 (1758.9)	-41.5	1.00	1.02	2.29	0.90	1.89	--	1.02	1.96	0.92
II	5.55	10.86	0.26	0.59	1758.9 (1758.9)	-33.0	0.98	1.02	2.28	0.87	1.90	--	1.03	1.97	0.93
III	5.94	11.30	0.26	0.59	1758.9 (1758.9)	-30.0	0.95	1.01	2.14	0.85	1.98	--	1.05	1.94	0.96
IV	4.55	10.02	0.33	0.56	1573.3 (1572.8)	-24.0	0.98	1.01	2.15	0.94	1.96	--	1.05	1.96	0.95
V	4.66	10.89	0.41	0.62	1730.8 (1730.9)	-52.0	--	1.06	1.89	0.89	1.91	1.05	ND	2.13	1.05
VI	8.26	13.49	0.35	0.64	2159.4 (2158.8)	-12.4	1.00	1.03	2.23	0.91	2.01	--	1.07	2.01	0.95
VII	7.64	12.97	0.35	0.64	2033.6 (2032.9)	-30.0	0.94	10.04	2.19	0.95	2.02	--	1.11	2.02	1.00
VIII[e]	7.71	13.07	0.38	0.65	2033.6 (2032.9)	-34.0	ND	ND	ND	ND	ND	--	ND	ND	ND
IX[e]	8.27	13.72	0.38	0.65	2159.5 (2158.8)	-26.0	ND	ND	ND	ND	ND	--	ND	ND	ND
X	6.60	12.04	0.37	0.62	2286.6 (2286.9)	-28.3	0.96	1.01	2.21	0.94	2.00	0.98	1.06	2.01	0.96
XI	6.81	12.27	0.38	0.66	2286.6 (2286.9)	-16.2	1.00	1.06	2.19	0.91	2.03	0.93	1.06	2.00	0.94
XII	7.38	12.50	.035	0.63	1972.6 (1972.7)	-15.8	0.98	1.00	2.16	0.96	1.96	--	0.97	1.78	1.70
XIII	6.58	6.58	0.31	0.59	2100.6 (2100.8)	- 6.0	0.90	1.00	2.18	0.98	1.99	0.96	1.05	1.98	0.95
XIV	5.93	8.94	0.35	0.60	1987.6 (1987.7)	+ 4.0	0.89	0.97	2.01	0.86	1.83	--	0.97	1.78	1.70
XV	5.62	12.38	0.43	0.66	1957.0 (1957.2)	-49.2	--	1.04	1.87	.095	1.95	1.07	ND	2.10	1.02
XVI	5.00	11.40	0.40	0.61	2085.0 (2085.4)	-29.6	--	0.99	2.15	0.96	1.94	1.98	1.05	1.95	0.97
XVII	5.50	12.38	0.44	0.68	2056.0 (2056.4)	-45.5	--	1.07	1.86	0.94	1.91	1.05	ND	2.16	1.01
XVIII	4.85	11.51	0.37	0.61	2184.8 (2184.6)	- 9.6	--	1.07	1.89	0.95	1.90	2.04	ND	2.14	1.02

[a] K' is defined as $(V_p - V_f)/V_f$, where V_p = the elution volume for the peptide and V_f = the breakthrough volume. K'-values (A) were determined on a μ-Bondapak C_{18} column using a 20-80% acetonitrile gradient (0.025% trifluoroacetic acid) over 30 min, and (B) were determined using a 20-80% methanol gradient (0.025% trifluoroacetic acid) over 30 min.

[b] Values in parentheses are calculated ones.

[c] The optical rotation was determined at 26°C (*c* 0.1), acetic acid was used as solvent for peptides I-IV and peptides VI-XIV; 30% acetic acid was used as solvent for peptide X and peptides XV-XVIII.

[d] ND = not determined.

[e] Peptides XIII and IX were synthesized by acylating peptide III. Since the amino acid analysis of peptide III was correct, amino acid analysis was not carried out on peptides XIII and IX.

Table 3. Growth arrest of *S. cerevisiae RC629* by α-factor analogs

Peptide conc.	Zone of inhibition (mm)[a]																	
(μg/disk)	α-Factor	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
50	--[b]	--	--	--	0	--	12	14	0	0	10	9	0	15	17	--	--	--	--
20	--	--	22	10	0	--	10	11	0	0	9	8	0	13	16	--	--	--	--
10	24	22	20	9	0	25	8	9	0	0	8	0	0	12	15	10	20	0	16
5	22	21	19	0	0	23	0	0	0	0	0	0	0	10	13	9	17	0	14
2	20	--	15	0	0	--	--	--	--	--	0	0	0	8	10	--	--	--	--
1	18	16	12	0	0	20	--	--	--	--	0	0	0	0	8	0	12	0	0
0.5	16	15	0	0	0	15	--	--	--	--	0	0	0	0	0	0	0	0	0
0.2	14	--	0	0	0	--	--	--	--	--	0	0	0	0	0	--	--	--	--
0.1	10	0	0	0	0	0	--	--	--	--	0	0	0	0	0	0	0	0	0
0.05	9	0	--	--	0	0	--	--	--	--	0	0	--	--	--	0	0	0	0

[a]The zone of inhibition includes the diameter of the filter paper (ca. 8 mm) on which the peptide was spotted. The results represent the average of at least three independent assays, and the halo sizes from these assay were within 2 mm of each other.
[b] -- represents concentration not tested

than native α-factor. There was no difference between mono- and diiodination as judged by the activities of peptides **VI** and **VII** in spite of the large size of iodine. The conjugation between iodinated HPP or biotin and the *N*-terminal amino group of α-factor analogs by using lysine or 5-amino valeric acid as spacers (peptides **X**, **XI**, and **XVII**) resulted in peptides with low activities. However, peptide **XVI** (lysine as spacer) and peptide **XVIII** (ε-AVA-lysine as spacer) were only two- and ten-fold less active, respectively, compared with [Bpa^3, Nle^{12}] α-factor. Comparison of these analogs suggests that the activities of *N*-terminal modified derivatives are affected by both the size and nature of the spacer and by the specific tag incorporated into the pheromone. The replacement of Trp^1 with Tyr yielded a peptide with extremely low activity (Masui *et al.*, 1979). We reasoned that increasing the hydrophobicity and bulk of this residue might achieve higher biological activities. Interestingly, peptide **XIV** in which Trp^1 was replaced by diiodo-Tyr showed almost the same activity as [Bpa^3, Arg^7, Nle^{12}] α-factor. The finding that peptide **XIV** (having α-amine) was active, whereas peptide **XII** (having the same structure as peptide **XIV** except no α-amine) had no activity at 50 μg per disk in the growth arrest assay indicated that the α-amine is important for the activity. This conclusion appears to contradict previous conclusions based on results with des Trp^1[α-Dns-His^2-Cha^3] α-factor, which exhibited significant activity (Shenbagamurthi *et al.*, 1983). However, it is possible that $N(CH_3)_2$ group on naphthalene ring of dansyl fulfills a charge requirement which is normally satisfied by the α-amine on unmodified pheromones.

A binding competition assay was carried out in order to detect directly whether these analogs interacted with the α-factor receptor (Table 4). Peptides **XIII**, **XIV** and **XVIII** exhibited significant competition with IC_{50} (the concentration needed to reduce binding by 50%) values of 9×10^{-6}, 7×10^{-6} and 3×10^{-5}M, respectively. In this assay

Table 4. Binding affinity of photoactivatable α-factor analogs

Peptide	IC_{50} (M)[a]
α-Factor	3.5×10^{-7}
Peptide XIII	9×10^{-6}
Peptide XIV	7×10^{-6}
Peptide XVIII	3×10^{-5}

[a]IC_{50} is the concentration of competitor which inhibits binding of [^{3}H] α-factor by 50%.

the IC_{50} found for α-factor was 3.5×10^{-7} M. The 20-85-fold decrease in binding affinity found for the modified analogs was consistent with 20-100-fold lower activity in the growth arrest assay in *RC 629* strain, which was also used in the binding assay. The IC_{50} values of peptides **XV** and **XVII** were more than 5×10^{-5} M. Peptides **XVI** exhibited lower affinity to the receptor (IC_{50} value more than 5×10^{-5}M), although its activity was higher.

In conclusion, the results outlined in this dissertation demonstrate the successful synthesis of a family of analogs which contain both a photoactivatable group (Bpa) and affinity (biotin) or potential radioactive (^{125}**I**) tags. Specific members of this family exhibit high biological activity and receptor affinity. We are presently attempting to exchange the iodine of peptides **XIII** and **XIV** with ^{125}**I** and crosslink these probes into the *STE2* gene product.

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PART V

DETERMINATION OF THE α-FACTOR BINDING DOMAIN UTILIZING PHOTOAFFINITY LABELING

CHAPTER I

INTRODUCTION

Affinity labeling is a method which involves the specific and covalent crosslinking of a ligand to its receptor. Peptide hormone receptors, which are present in low abundance in the plasma membrane, can be identified using this method; identification is the first step to elucidation of the binding domain. The information obtained from this kind of study can increase our understanding of the molecular interactions involved in hormone-receptor binding. The two biochemical methods used to achieve a covalent hormone-receptor complex are chemical crosslinking and photoaffinity labeling. Chemical crosslinking is comprised of adding an exogenous crosslinker to a pre-existing hormone receptor complex in order to achieve a covalent linkage between the hormone and its receptor. Photoaffinity labeling utilizes photolabile peptides generated either from the use of a heterobifunctional cross-linking agent which contains in one molecule both a chemical and photochemical reactive group (Kauer *et al* ., 1986) or from the synthesis of a peptide analog containing a photoreactive group. A covalent linkage between the photolabile peptide hormone and its receptor is achieved when this complex is irradiated with ultraviolet light (Pilch & Czech, 1984; Brunner, 1993).

Chemical crosslinking of hormones with their receptors entails bifunctional reagents, such as glutaraldehyde, disuccinimidyl suberate (DSS), and dimethyl suberimidate (DMS), which react with amino groups. This is a relatively straightforward procedure which requires the addition of a crosslinker to a reaction mix containing radioactively labeled hormone bound to its receptor. The crosslinked, radioactively-labeled hormone-receptor complex is analyzed using electrophoresis and autoradiography. The utility of this technique has been demonstrated by the identification of many hormone

receptors, including insulin and epidermal growth factor receptors. To have successful chemical crosslinking, the spacing between the amino groups on both the hormone and the receptor must be in the appropriate geometric orientation. Having the correct geometric spacing for the functional group of every hormone and its receptor is very unlikely and therefore this technique cannot be used in all circumstances. An additional disadvantage of this technique is the nonspecific crosslinking between proteins in the reaction mixture in addition to crosslinking of the hormone to its receptor. In order to circumvent the problems associated with chemical crosslinking, photoaffinity labeling can be used (Pilch & Czech, 1984).

Photoaffinity labeling achieves covalently linkage of a photolabile peptide hormone with its receptor using photolysis. There are a number of different photoactivatable groups which can be used; they include the azides, diazirines, and the diazo photolabile precursors, 4'-(trifluoromethyl-diazirinyl)-phenylalanine and 4'-benzoyl-phenylalanine (BPA). A photoactivatable group should be small so that it does not sterically hinder binding of the ligand to the receptor, it should be stable in the dark , and finally, it should be activated at a specific wavelength. As mentioned earlier, photoaffinity crosslinking reagents can be either chemically reacted with the peptide hormone or synthetically incorporated into the peptide hormone. The incorporation of a photoactivatable amino acid into a peptide instead of the attachment of a photolabile group to the peptide is preferred since analogs similar to the native peptide can be made (Brunner, 1993); but regardless of the approach chosen it is important that the photolabile peptide bind to the receptor and be biologically active (Brunner, 1993; Bayley & Knowles, 1977).

After binding of the photoactivatable peptide to its specific receptor, photoactivation of the analog is expected to crosslink it into the receptor binding pocket. This procedure should generate a few distinct crosslinked adducts which can be isolated,

purified and then analyzed to reveal the binding domain within the receptor (Brunner, 1993; Pilch & Czech, 1984). As with chemical crosslinking, photoaffinity labeling has been widely used for the identification of hormone receptors and for obtaining information about the receptor binding domain. The number of peptide hormone receptors which have been studied using this technique is comprehensive and the discussion of each is beyond the scope of this dissertation. To demonstrate the validity of this technique, photoaffinity labeling studies of the insulin receptor, the glucagon receptor, the substance P receptor and the gonadotropin-releasing hormone receptor will be discussed and additional studies mentioned.

The insulin receptor in rat was identified by Yip and colleagues (1978) using a photolabile radioactive insulin derivative. This photoreactive derivative of insulin was incubated with plasma membranes prepared form rat adipocytes and then irradiated with ultraviolet light. After SDS-polyacrylamide gel electrophoresis and autoradiography, a protein having a molecular weight of 130,000 was specifically labeled by the photolabile radioactive insulin which suggested that this protein was the insulin receptor. Photoactivatable insulin was also used to determine the bivalency (capable of binding two molecules of insulin) of the insulin receptor (Yip & Jack, 1992). Residues within the insulin receptor involved in insulin binding have been revealed using photoactivatable insulin analogs. From these studies, Gly^{390} (Fabry *et al* ., 1992) and residues 704-718 (Kurose *et al* ., 1994) within the α-subunit of the receptor were found to contribute to the insulin binding domain.

^{125}-I glucagon was specifically linked to its receptor, a protein with a molecular weight of 53,000, using a photoreactive heterobifunctional cross-linking reagent. Photoaffinity labeling was used when various bifunctional chemical crosslinkers were unsuccessful in crosslinking glucagon to its receptor in rat liver plasma membranes (Johnson *et al* ., 1981).

The Substance P receptor is another hormone receptor identified using a photoactivatable analog of substance P (SP). In this case, SP was synthesized with p-benzoylphenylalanine (BPA) at position 8 of the peptide and subsequently iodinated. This radioactive photoactivatable analog of SP was able to compete with unlabeled SP for binding on rat submaxillary gland membranes and was found to be biologically active *in vivo*. Two photolabeled proteins (46 kDa and 53 kDa) were identified (Boyd *et al* ., 1991) from rat submaxillary membranes. Use of another photoreactive SP analogue in which p-azidophenylalanine, instead of BPA, was substituted for residue 8 also demonstrated specific photolabeling of a 46 kDa protein in a rat brain membrane preparation (Dam *et al* ., 1987). The use of these two site-specific, high affinity photoactivatable analogs have helped to identify residues within the SP receptor involved in peptide binding. The analogues employed had BPA incorporated in either position 3 or 8 of SP and it was established that Lys^3 of SP interacts with the amino terminal extracellular domain (residues 1-21) and that Phe^8 of SP interacts with the second extracellular loop (residues 173-183) of the SP receptor (Li *et al* ., 1995).

A high affinity photoreactive radio-iodinated analog of gonadotropin releasing hormone (GnRH) was used to label and characterize the pituitary GnRH receptors in rat, rabbit, mouse, sheep, and cow and the gonadal GnRH receptor in the rat ovary (Iwashita & Catt, 1985). The photoactivatable group (2-nitro-4(5)azidophenyl-sulfonyl[2,4(5)-NAPS] was incorporated at different amino acid residues of GnRH to generate a series of GnRH photoreactive analogs. This series of analogs demonstrated the importance of placing the photoaffinity labeling group in a position which would allow the ligand to retain a binding affinity close to that of the natural ligand. The active photoactivatable GnRH analog labeled a 59 and 57 kDa protein in rat and a 58 and 56 kDa protein in bovine pituitary membrane preparations. This peptide also labeled pituitary GnRH

receptors in the solubilized state and therefore appears to be a suitable ligand for the isolation and further characterization of the receptor (Nikolics, *et al* ., 1988).

The above mentioned hormone receptors comprise only a fraction of the hormone receptors that have been studied using photoaffinity labeling. Additional hormone receptors which have been identified and information obtained regarding the binding domain using photoaffinity labeling include the lutropin receptor (Kundu *et al* ., 1996), the somatostatin receptor (Brown *et al* ., 1990), the thyroid hormone receptor (Horowitz *et al* ., 1988) and the parathyroid hormone receptor (Adams *et al* ., 1995).

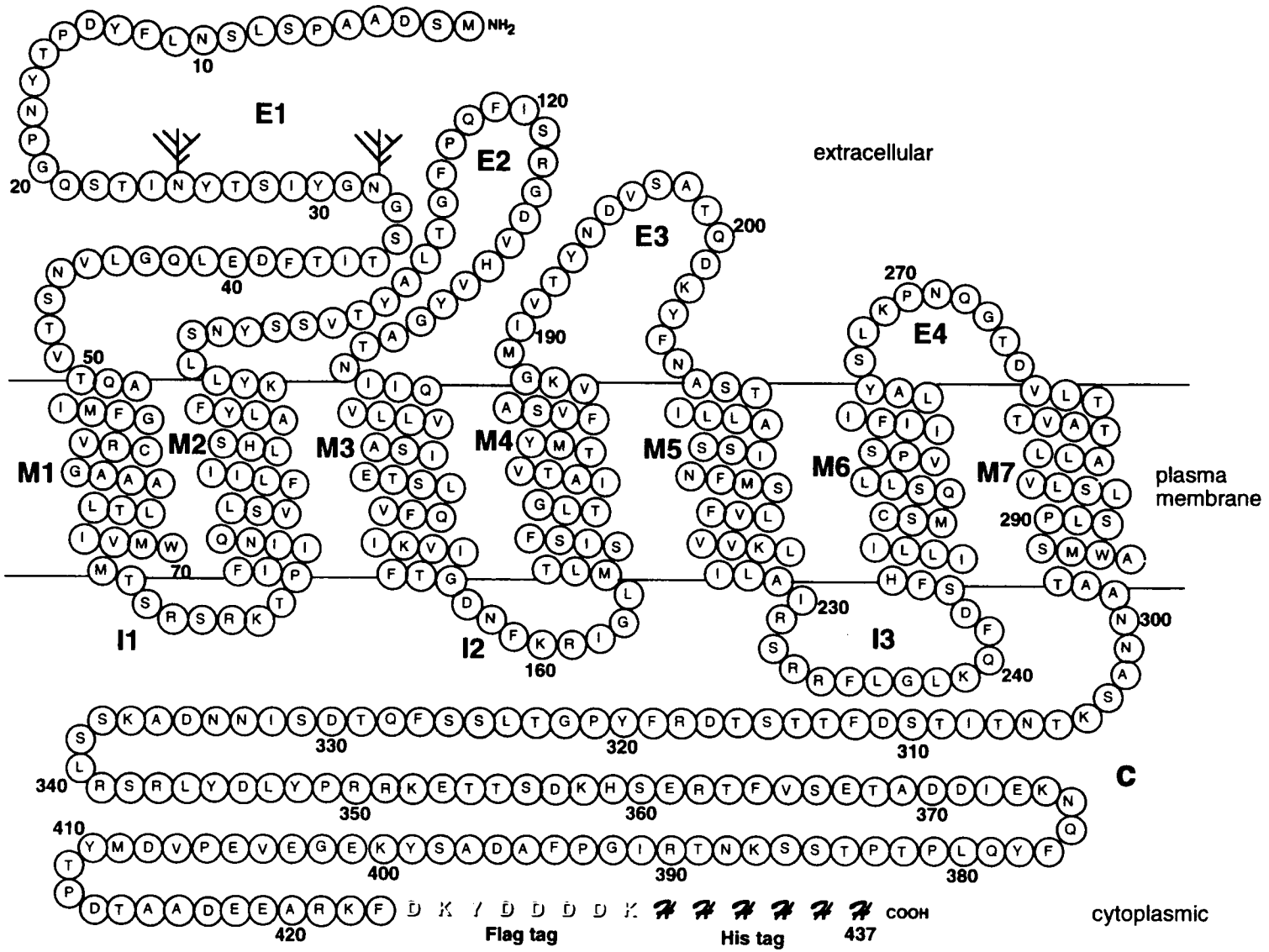
The technique of photoaffinity labeling is not limited to peptide hormones and their receptors. This technique has been used to photolabel in solution purified preparations of calmodulin (Kauer *et al* ., 1986) and to determine the partial binding domain within calmodulin (O'Neil *et al* ., 1989). Regions of the p85 SH2 domain involved in phosphoprotein binding were mapped using photoaffinity labeling (Williams & Shoelson, 1993). cAMP-dependent protein kinase was labeled using photoactivatable cAMP-dependent protein kinase substrate and it was determined that Gly^{125} and Met^{127} (Miller & Kaiser, 1988) of the kinase was labeled. Additionally, phosphatidylinositol 4-kinase from the yeast *Saccharomyces cerevisiae* and the acetylcholine receptor from *Torpedo californica* were labeled using photoactivatable substrates (Nickels & Carman, 1993; Tine & Raftery, 1993).

Since the technique has been proven successful, it can be applied to determine the α-factor binding domain within its receptor, the *STE2* gene product. The protein (Ste2p) encoded by this gene contains 431 amino acids and hydropathy analysis suggests that this protein is a seven transmembrane domain G-protein coupled receptor (Figure 1). An earlier approach to determine the binding domain of Ste2p utilized radioactively labeled α-factor (Blumer *et al* ., 1988). The radiolabeled α-factor was chemically crosslinked into the receptor using membrane proteins isolated from a yeast strain which overproduced

the receptor. The membrane preparations retained active Ste2p and the majority of the radioactively labeled α-factor bound to the membranes. However, in this study, only 1% of the label was incorporated into the receptor (a 47.8 kDa species) and immuno-precipitation of the labeled receptor was unsuccessful, precluding the isolation of the pheromone-receptor complex and the subsequent identification of the binding domain. These studies also reported a 33 kDa labeled protein when cross-linking was performed using membranes of cells that expressed a C-terminal truncated Ste2p. This finding demonstrated that the ligand binding domain of Ste2p is localized to the amino-terminal two thirds of the protein which consists of the transmembrane domains connected by short extracellular and intracellular loops. Additional evidence for this region of Ste2p comprising the ligand binding portion of the protein came from single substitution mutations of the receptor (Marsh, 1992) and from chimeric α-factor receptors generated from *S.cerevisiae* and *S.kluyveri* (Sen & Marsh, 1995).

Photoaffinity labeling was used in the research presented herein to determine the α-factor binding domain within its receptor. A variety of α-factor analogs containing p-benzoylphenylalanine (BPA) were synthesized and tested for their biological activity as well as for their ability to bind to Ste2p. The results of those studies were discussed in Part IV of this dissertation and indicated which analogues could be used as probes for labeling the α-factor receptor. Photoaffinity labeling experiments were attempted using both membrane proteins and affinity-purified Ste2p isolated from a strain which overexpresses a FLAG tagged (FT), HIS tagged (HT) Ste2p (refer to Figure 1). The results of the photoaffinity labeling experiments indicated a protein was labeled that migrated at a molecular weight of 52 kDa (calculated molecular weight of Ste2p.FT.HT is 48.5 kDa). This protein was verified to be Ste2p using anti-FLAG antibodies. These

Figure 1. Model of Ste2p.FT.HT. Ste2p is a 431 amino acid seven transmembrane domain receptor. Ste2p encoded by pG3.STE2.FT.HT (Figure 2) has 437 amino acids. Amino acids 424 - 431 (WTEDNNNL) of Ste2p were removed and a FLAG tag (DKYDDDDK) and HIS tag (HHHHHH) were added at the carboxyl terminus of Ste2p to generate Ste2p.FT.HT.


NH2
E1
E2
E3
E4
extracellular
plasma membrane
cytoplasmic
M1
M2
M3
M4
M5
M6
M7
I1
I2
I3
C
COOH
Flag tag
His tag
10
20
30
40
50
70
120
160
190
200
230
240
270
290
300
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320
330
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360
370
380
390
400
410
420
437

observations suggest that a photoactivatable α-factor analog is able to covalently label the α-factor receptor and may be suitable for isolating and characterizing the receptor binding site(s).

CHAPTER II

MATERIALS AND METHODS

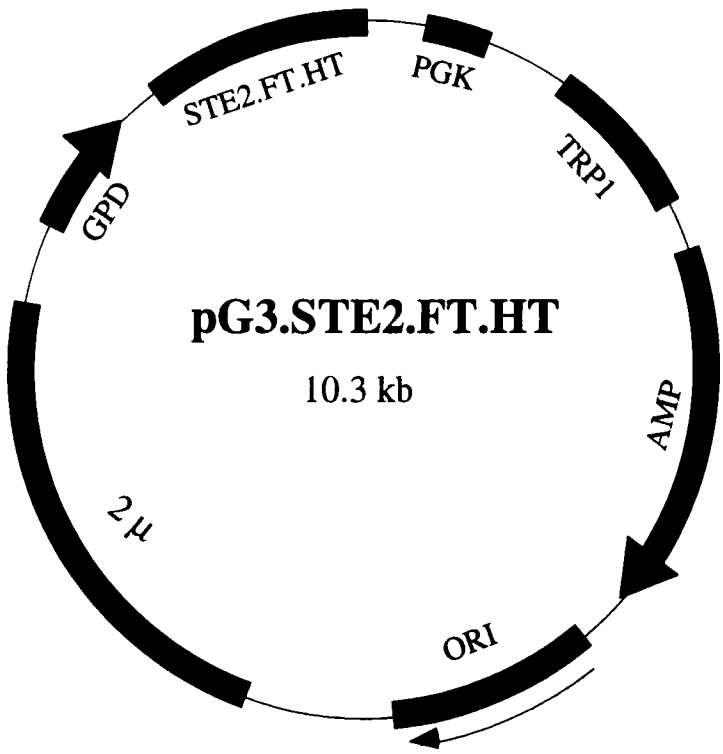
Saccharomyces cerevisiae Strains Used

The relevant genotype and sources of the strains used in this chapter are: *S. cerevisiae* RC629 (*MATa sst1-2*, supersensitive to α-factor because of a mutation in the *BAR1* protease [allelic to *SST1*] which hydrolyzes α-factor) used in growth arrest assays provided by R. Chan (University of Cincinnati); *S. cerevisiae* 4202-15-3 (*MATa cry1 bar1-1 ade2-1 his 4-580 lys2 tyr1 SUP4-3*) used in binding assay provided by D. Jenness (University of Massachusetts, Amherst); *S. cerevisiae* DK102 (*MATa ura3-52 Lys 2-801am ade2-101Δc trp1-ΔG3 his3-Δ200 leu2-Δ1 ste2Δ::HIS3, sst1-Δ5*) and *S. cerevisiae* DK102 transformed with the pG3.STE2.FT.HT plasmid (Figure 2) used in photoaffinity labeling experiments provided by R. Stevens (Berkeley, CA). The pG3.STE2.FT.HT plasmid has both a FLAG tag (FT) and a HIS tag (HT) introduced at the 3'-end of *STE2*; the structure of Ste2p encoded by this plasmid is shown in Figure 1.

Media Used for Growth of Organisms

The following media was used for growth of yeast strains in this study: YM-1 medium [yeast extract (Difco), 5 g/l; peptone (Difco), 10 g/l; yeast nitrogen base without amino acids (Difco), 6.7 g/l; adenine sulfate (ICN), 0.01 g/l; uracil, 0.01 g/l; succinic acid, 10 g/ml; sodium hydroxide, 6 g/l; and glucose, 10 g/ml, at a final pH of 5.8]. MLT medium [yeast nitrogen base without amino acids (Difco), 6.7g/l; casamino acids (Difco), 10g/l; glucose, 20g/l; adenine sulfate (ICN), 0.058 g/l; arginine, 0.026 g/l; asparagine, 0.058 g/l; aspartic acid, 0.14 g/l; glutamic acid, 0.14 g/l; histidine, 0.028 g/l; isoleucine,

Figure 2. The pG3.STE2.FT.HT. expression plasmid. The expression plasmid was constructed from the progenitor plasmid pG3 (Schena, 1991) in the laboratory of R. Stevens (Berkeley, CA). pG3.STE2.FT.HT contains the following yeast genes: the glyceraldehyde 3-phosphate dehydrogenase promoter (GPD); the phosphoglycerate kinase terminator (PGK); the tryptophan gene (*TRP1*) for selection of *Saccharomyces cerevisiae* transformants containing the plasmid; the *STE2* gene containing sequence for both the FLAG tag (FT) and HIS tag (HT) at the 3' end of the gene; and the 2 micron origin (2μ) of replication. The bacterial origin of replication (ORI) and ampicillin gene (AMP) derived from pUC18 is also contained within the expression plasmid


STE2.FT.HT
PGK
TRP1
AMP
ORI
2μ
GPD
pG3.STE2.FT.HT
10.3 kb

0.058 g/l; leucine, 0.083 g/l; lysine, 0.042 g/l; methionine, 0.028 g/l; phenylalanine, 0.69 g/l; serine, 0.52 g/l; threonine, 0.28 g/l; tyrosine, 0.042 g/l; valine, 0.21 g/l; and uracil, 0.028 g/l] and YEPD medium [yeast extract (Difco), 10g/l; peptone (Difco), 20g/l and glucose 20g/l]. To prepare agar plates of each medium, 20 g/l of agar (Difco) was added to the above listed components.

Growth Arrest (Halo Assay)

The biological activity of α-factor analogs was tested by growth arrest (halo assay). Agar plates were overlayered with 4 mL of either *S. cerevisiae* RC629, DK102(Δ*STE2*), or DK102(pG3.STE2.FT.HT) cells (2.5 x 10^5 cells per mL)] in Nobel agar (1.1%). Filter disks (sterile blanks from Difco), 8 mm in diameter, were placed on the overlay, and 10 μL portions of peptide solutions at various concentrations were spotted on the disks. The plates were incubated at 30°C for 24 h and then observed for clear zones (halos) around the disks. The data were expressed as the diameter of the halo, including the diameter of the disk.

Binding Competition Assay

Competition of bound tritiated [Nle^{12}]α-factor by unlabeled α-factor analogs was measured by the following protocol. *S. cerevisiae* strain 4202-15-3 (*MATa cry1 bar1-1 ade2-1 his 4-580 lys2 tyr1 SUP4-3*) and DK102 (*MATa ura3-52 Lys 2-801am ade2-101Δc trp1-ΔG3 his3-Δ200 leu2-Δ1 ste2Δ::HIS3 sst1-Δ5*) were grown in YM-1 medium and DK102(pG3.STE2.FT.HT), henceforth referred to as DK102(pSte2p.FT.HT) was grown in MLT medium overnight at 30°C with shaking at 120 rpm to 1 x 10^7 cells/ml. Cells were harvested by centrifugation (6000 x g for 10 min. at 4°C), and the resultant pellets washed twice with ice-cold YM-1+i medium (YM-1 medium containing 10mM

NaN_3, 10 mM KF, and 10mM *p*-tosyl-L-arginine methyl ester) and resuspended to 1.25 x 10^9 cells/ml in YM-1+i medium. Final assay concentrations of the unlabeled competitors were between 1 x 10^{-5} to 1 x 10^{-9} M as determined from the A_{287} for each peptide and the extinction coefficients calibrated for [Nle^{12}]α-factor from quantitative elemental analysis. The reaction was started by the addition of 100 µl of analog/[^{3}H]α-factor mix (120 µl of appropriate unlabeled α-factor analog mixed with 120 µl of labeled α-factor at 6 x 10^{-8} M, 15.8 Ci/mmole) to 400 µl of cell suspension. After the reaction was started, two 20 µl samples were removed and counted to determine total amount of radioactivity present. Duplicate reactions were started 3 min. after initial reaction for each analog tested. At 30 min. two 200 µl portions of cell suspension were added separately to 2 ml of YM-1+i medium and filtered over GN-6 Metricel filters (Gelman Sciences, Inc., Ann Arbor, MI) presoaked in 1% bovine serum albumin. The reaction tubes were then rinsed twice with 2 ml ice-cold YM-1+i medium, with each rinse filtered over the same filter. Finally, each filter was rinsed twice with 2 ml YM-1+i medium. The non-specific binding of labeled α-factor in the absence of cells was less than 100 cpm. The filters were counted in 5 ml of Budget-solve counting cocktail (Research Products International Corp., Mt. Prospect, IL). All assays and manipulations were done in siliconized borosilicate tubes or vials. The binding curve for α-factor has been previously done by the infinite dilution experiment by us and others (Raths *et al.*, 1988; Blumer *et al.*, 1988; Jenness *et al.*, 1986) to show that there is no evident cooperativity in the binding between α-factor and its receptor. Tritiated [Nle^{12}]α-factor was prepared as previously described (Raths *et al* ., 1988).

Synthesis of α-factor analogs containing photoactivatable and labeling groups

α-factor analogs containing photoactivatable and labeling groups were synthesized in the laboratory of Dr. Fred Naider at the College of Staten Island and were provided for biological analysis and photoaffinity labeling studies. The synthesis and biological assays performed are discussed in Part IV of this dissertation which represent published data from our laboratory (Jiang *et al* ., 1995). In this study, the following analogs were used: (ε-Av-dBiotin)K^{+1},BPA^3, Nle^{12} α-factor referred to as Bio-BPA α-factor; BPA^3, Nle^{12} α-factor referred to as BPA α-factor; and [Nle^{12}]α-factor (see Figure 3 for structures of analogs).

Membrane Protein Preparation

DK102 (ΔSte2p) was grown in YEPD medium and DK102(pSte2p.FT.HT) was grown in MLT medium (to select for maintenance of the plasmid), to a density of 1 x 10^7 cells/ml. Cells were harvested by centrifugation (6000 x g for 10 min. at 4°C), and the resultant pellets washed once with sterile distilled water and resuspended in lysis buffer at pH 6.0 (10mM PIPES, pH 6.0, 1mM $MgCl_2$, 0.1mM EDTA, 10% glycerol, 17.4 μg/ml phenylmethylmethylsulfonyl fluoride (PMSF), 15.6 μg/ml benzamidine, 7 μg/ml tosyl-phenylalanine chloromethyl ketone, 3.5 μg/ml pepstatin A) or lysis buffer at pH 7.9 (50mM Tris pH 7.9, 75mM NaCl, 1mM EDTA, 52 μg/ml phenylmethylmethylsulfonyl fluoride (PMSF), 3 μg/ml pepstatin A, 15 μg/ml aprotinin, 30 μg/ml soybean trypsin inhibitor, 3 μg/ml leupeptin, 30 μg/m bacitracin, 30 μg/ml benzamidine) at a ratio of 1:4 (w/v). Cells were lysed using glass beads (0.45-0.50 mm, B. Braun Biotech, Germany) and either one of two methods depending on the volume of cell suspension processed. For less than 30 ml of cell suspension, glass conical tubes were used to which the cell suspension and an equal volume of glass beads were added. Lysis occured by vigorously

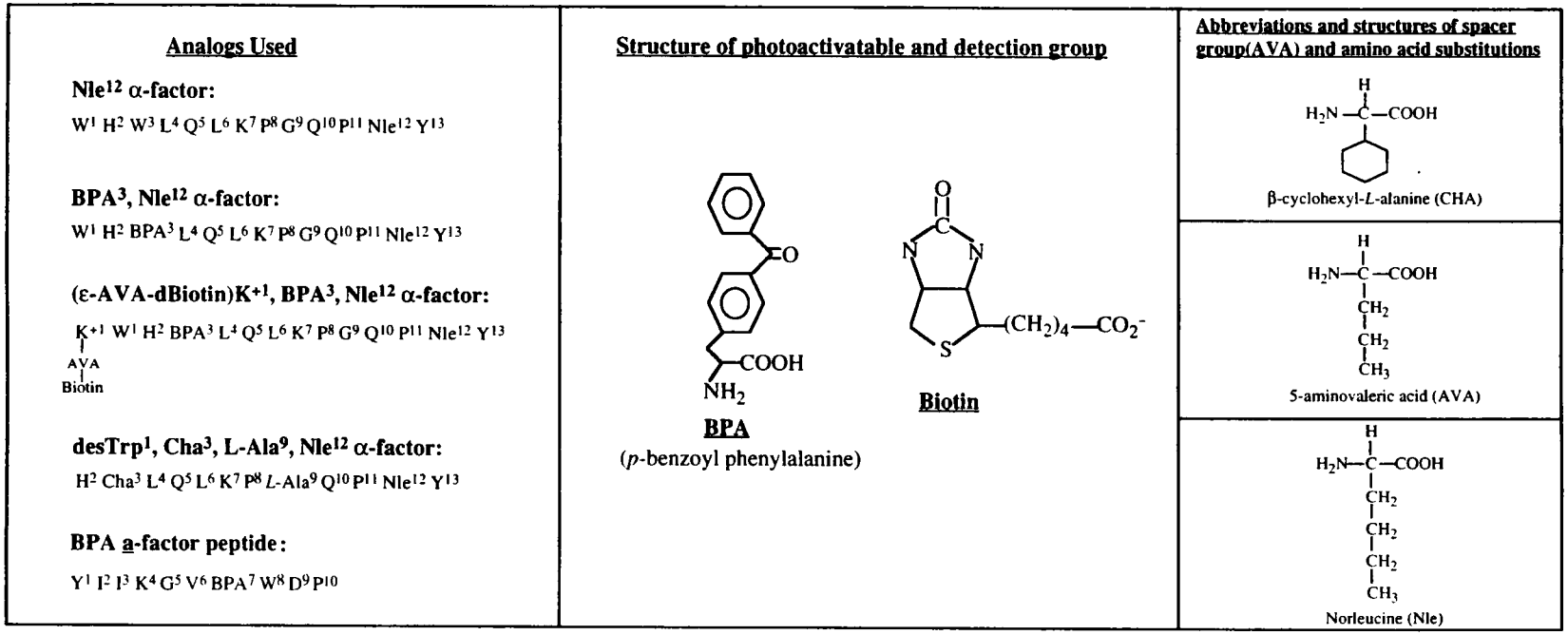


Figure 3. Structures of compounds used in this study.

vortexing the glass bead/cell suspension for 1 minute intervals, with placement on ice for 1 minute between each interval to allow the cell suspension to cool. This process was repeated 20 times and microscopic inspection of the cells verified lysis had occurred. For larger volumes of cell suspension (≥ 30 ml), the Cell Homogenizer and glass disruption chambers from B. Braun Biotech (Germany) were used. Disruption chambers, containing the cell suspension and glass beads (1/3rd volume of chamber), were placed in the homogenizer for 1 minute to lyse the cells followed by incubation on ice for 1 minute to allow the cells to cool. This process was repeated 20 times and was performed in the cold room (4 °C). After cell lysis, as determined by microscopic inspection, cellular debris was collected by centrifugation for 15 minutes at 10,000 x g. The resultant supernatant fraction was subjected to centrifugation at 100,000 x g for 90 minutes to collect membrane proteins. Membrane protein pellets were resuspended in lysis buffer, protein concentration determined (Bio-Rad Protein Assay Concentrate), and aliquots stored at -70 °C until use.

Photoaffinity Labeling Experiments of Yeast Membrane Proteins

Photoaffinity labeling reactions (400µl) containing yeast membranes (400µg) prepared from DK102(Δ*STE2*) or DK102(pSte2p.FT.HT) and Bio-BPA α-factor (10-50µM) in YM-1+i medium were incubated at room temperature for 30 minutes (protected from light). The reaction mixture was then exposed to ultraviolet light using a pair of Rayonet 350-nm reactor bulbs (Southern New England Ultraviolet Co., Middletown, CT) for 30 minutes. After photolysis, membranes were diluted 2-fold with YM-1+i medium and collected by centrifugation (15,000xg). To the membrane pellet, 200µl of SDS-PAGE sample buffer (15% sucrose, 2.5% SDS, 50mM DTT, 50mM Na_2CO_3) plus 200µl of 2% nonyl-glucoside was added resulting in a protein concentration of 1µg/µl.

Samples were heated at 37°C for 15 minutes and 10 μg of protein was loaded per lane on a 1.5mm thick SDS-Polyacrylamide minigel having a 4% stacking (4% acrylamide,125mM Tris, pH 6.8, 0.1% SDS) and 10% separating gel (10% acrylamide, 0.75M Tris, pH 8.85; 0.1% sodium dodecyl sulfate). The acrylamide used was National Diagnostics' ProtoGel™ 37.5:1 acrylamide to bisacrylamide solution (Atlanta, Georgia). Separation conditions were 35mAmps for 1.5hr in the presence of 1X Running Buffer (50mM Tris, 190mM glycine, and 0.1% SDS). The SDS-PAGE system used is based on the recipe published by Fling & Gregerson (1986) which uses a high molar concentration of Tris in the separating gel and running buffer.

Separated proteins were electrophoretically transferred to a nylon membrane (Immobilon-P from Millipore; Bedford, MA) using the Hoefer Tank Transfer System (San Francisco, Ca) and modified Towbin Transfer Buffer (25mM Tris; 200mM glycine; 15% methanol; 0.01% SDS) for 1.5hr at 400mAmps. Following transfer, the nylon membrane was incubated with blocking solution (5% non-fat dry milk) for 1hr at room temperature to block all nonspecific binding sites and then probed with avidin-Horseradish Peroxidase (HRP; Molecular Probes, Eugene, Oregon) diluted in Tris-buffered saline containing Tween 20 (TTBS; 20 mM Tris, 500 mM NaCl, and 0.1 % Tween 20) for 3hr at room temperature. Dilutions of avidin-HRP (1mg/ml) used in this study were between 1:20,000 and 1:100,000. The avidin-HRP is able to recognize Bio-BPA α-factor which has been covalently crosslinked into membrane proteins (See Figure 4). Detection of the biotin-avidin-HRP complex was achieved using the enhanced chemiluminescence (ECL) detection system from Amersham. The ECL reaction is based on the oxidation of luminol by HRP in the presence of chemical enhancers, such as phenol. An equal volume of Reagent 1 and 2 from the Detection kit was incubated on the blot for 1 minute (with shaking) at room temperature and the light produced by this

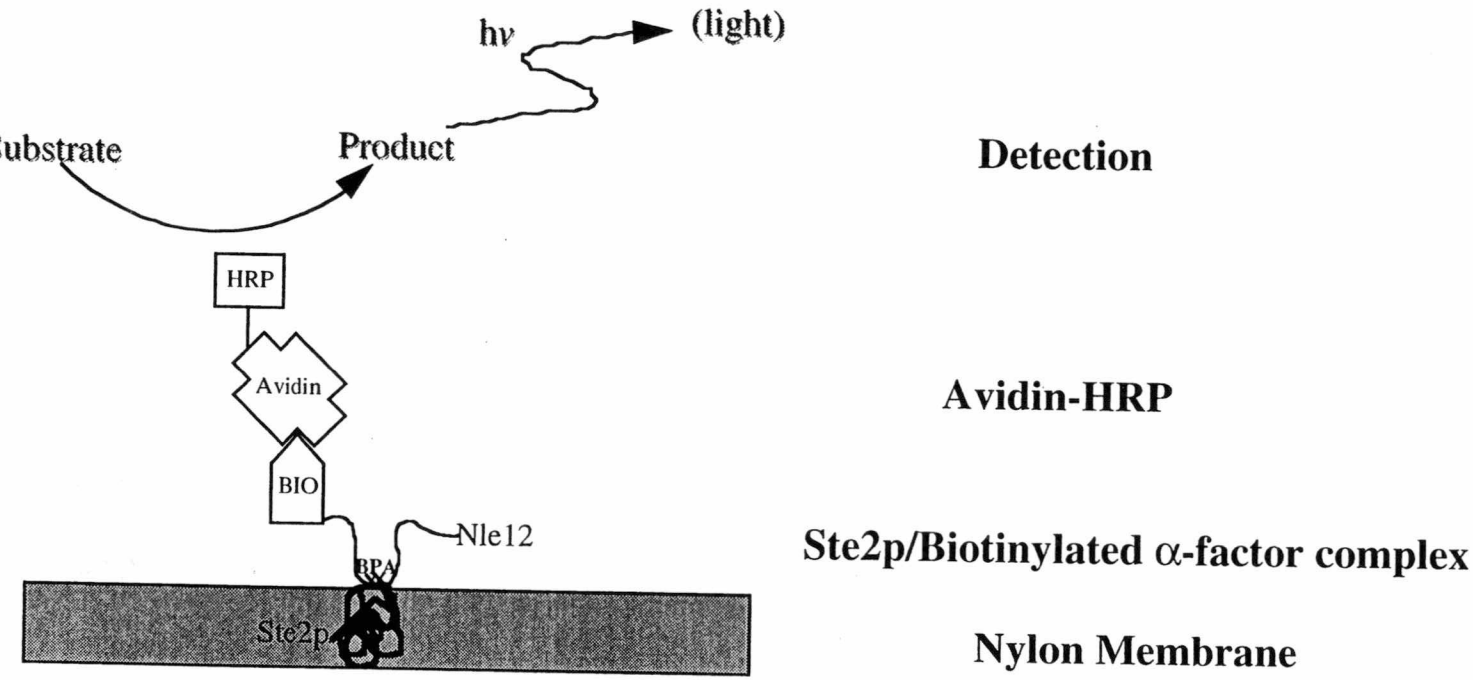


Figure 4. Model to explain the detection of the Ste2p/Biotinylated α-factor complex.

reaction was detected by exposure of the blot to autoradiography film, thereby allowing visualization of the results.

Photoaffinity Labeling Time Course of Yeast Membrane Proteins

Photoaffinity labeling reactions (400µl) containing yeast membranes (400µg) prepared from DK102(ΔSte2p) or DK102(pSte2p.FT.HT) and Bio-BPA α-factor (50µM) in YM-1+i medium were incubated at room temperature for 30 minutes (protected from light). The reaction mixture was then exposed to ultraviolet light (350nm) and 100µl aliquots removed at 0, 5, 15, and 30 minutes following photolysis. All manipulations after photolysis were identical to those mentioned in the photoaffinity labeling experiment using membrane proteins.

Saturation of non-specific binding sites using membrane proteins

Yeast membranes (100µg) prepared from DK102(ΔSte2p) and DK102(pSte2p.FT.HT) were incubated with desTrp^{1},Cha^{3},*L*-Ala^{9}, Nle^{12} α-factor (142µM) in YM-1+i at room temperature for 45 minutes. Bio-BPA α-factor (20µM) was then added to the reaction mixture and incubated at room temperature for 30 minutes (protected from light) followed by exposure to ultraviolet light (350nm) for 15 minutes. All manipulations after photolysis are identical to those mentioned in the photoaffinity labeling experiment using membrane proteins.

Purification of Ste2p.FT.HT using Immobilized Metal Affinity Chromatography

Frozen DK102(pSte2p.FT.HT) cells were received from the laboratory of Ray Stevens, Berkeley, CA. Cells were grown in MLT medium in a 200 liter fermentor to achieve a high cell density. Cells were harvested and frozen in 50 g portions at -80 °C.

Frozen cells were thawed on ice and 1M Tris pH 7.9 was added to a final concentration of 50mM, 5M NaCl was added to a final concentration of 75mM, and 0.5M EDTA was added to a final concentration of 1mM. In addition, each of the following protease inhibitors were added: PMSF (17.4mg/ml) diluted to 52 μg/ml; pepstatin (1mg/ml) diluted to 3 μg/ml; aprotinin (5mg/ml) diluted to 15 μg/ml; soybean trypsin inhibitor (10mg/ml) diluted to 30 μg/ml; leupeptin (1mg/ml) diluted to 3 μg/ml; bacitracin (10mg/ml) diluted to 30 μg/ml; and benzamidine (10mg/ml) diluted to 30 μg/ml (protease inhibitor cocktail). The final volume was then adjusted to 70 ml with sterile distilled water.

Cells were lysed using glass beads and the Braun Scientific Cell Homogenizer as previously described (Membrane Preparation Procedure). After cell lysis, cellular debris was collected by centrifugation for 15 minutes at 10,000 x g. The resultant supernatant was subjected to centrifugation at 100,000 x g for 90 minutes to collect membrane proteins. The membrane protein pellet (s) was resuspended in 50 ml of lysis buffer at pH 7.9 and then 2 volumes (100 ml) of solubilization buffer [1.5% n-dodecyl-β-D-maltoside (DBM) from Anatrace (Maumee, OH), 22.5% glycerol, 225mM NaCl, 75mm Tris, pH 7.9, and protease inhibitor cocktail]. This mixture was stirred for 1 hour at 4 °C to solubilize membranes and then subjected to centrifugation (100,000xg) to remove unsolubilized membranes. Before loading the supernatant (solubilized membrane proteins) on a 2ml chelating sepharose column (Pharmacia Biotech) charged with 200mM copper sulfate, the column was equilibrated with a buffer containing 0.5M NaCl, 0.075% DBM, 50mM Tris, pH7.9, 5mM imidazole and protease inhibitor cocktail. After loading the solubilized membrane proteins, the column was washed with Buffer A (5mM imidazole, 500mM NaCl, 7.5% glycerol, 50mM Tris pH 7.9, 0.075% DBM, and protease inhibitor cocktail) for 10 column volumes, Buffer B (5mM imidazole, 250mM

NaCl, 7.5% glycerol, 50mM Tris, pH 7.9, 0.075% DBM, and protease inhibitor cocktail) for 10 column volumes and then Buffer C (40mM imidazole, 250mM NaCl, 7.5% glycerol, 50mM Tris, pH 7.9, 0.075% DBM, and protease inhibitor cocktail) for 20 column volumes. The Ste2p.FT.HT protein was removed from the copper column using Elution Buffer (300mM imidazole [competes for binding to the copper column with the HIS tag], 250mM NaCl, 7.5% glycerol, 50mM Tris, pH 7.9, 0.075% DBM, and protease inhibitor cocktail). Estimation of protein concentration at different stages of the purification was performed using Bio-Rad Protein Assay Concentrate. Proteins recovered from the copper column were separated on an 10% SDS-Polyacrylamide gel as described earlier and either stained with coomassie blue dye to visualize proteins or transferred to a nylon membrane and probed with anti-FLAG M2 antibodies (Kodak, New Haven, CT) to detect FLAG tagged Ste2p. For the antibody blot, anti-FLAG M2 antibodies (1mg/ml) were diluted 1:1000 in TTBS and incubated overnight at room temperature. The blot was then incubated with secondary antibody, anti-mouse HRP(Pierce), diluted 1:3000 in TTBS and incubated for 3 hours at room temperature. Detection of Ste2p.FT.HT was visualized by exposure of the blot to autoradiography film after incubation with ECL detection reagents.

Photoaffinity labeling of partially purified Ste2p.FT.HT

Partially purified Ste2p.FT.HT was provided by the laboratory of Ray Stevens (Berkeley, CA). Photoaffinity labeling reactions (40µl) containing partially purified Ste2p.FT.HT (~0.12µg) and Bio-BPA α-factor (0-100µM) in YM-1+i medium were incubated for 1 hour at room temperature (protected from light). The reaction mixture was then exposed to ultraviolet light (350nm) for 30 minutes at 4 °C. After photolysis, the reaction mixture was diluted 2-fold with SDS-PAGE sample buffer (15% sucrose,

2.5% SDS, 50mM DTT, 50mM Na_2CO_3) and heated at 37°C for 15 minutes. 20µl of each reaction was subjected to SDS-Polyacrylamide gel electrophoresis and separated proteins were electrophoretically transferred to a nylon membrane. Conditions for SDS-Polyacrylamide electrophoresis and electrophoretic transfer of separated proteins to nylon membrane were identical to those previously described (Photoaffinity Labeling of Membrane Proteins). The nylon membrane was probed with either Avidin-HRP to detect Bio-BPA α-factor or with anti-FLAG M2 antibodies to detect FLAG tagged Ste2p as described earlier. Results were visualized by exposure of the blots to autoradiography film after incubation with ECL detection reagents.

For competition experiments, photoaffinity labeling reactions (50µl) containing affinity purified Ste2p.FT.HT (~0.12µg), Bio-BPA α-factor (50µM), and BPA α-factor (650µM-2600µM), Nle^{12} α-factor (1200µM-3000µM), or BPA **a**-factor peptide (230µM-930µM) were incubated at room temperature for 1 hour (protected from light) and then exposed to ultraviolet light (350nm) for 30 minutes. All manipulations following photolysis were identical to those mentioned above. Densitometric analysis of results were done using Image Quant (Molecular Dynamics; Sunnyvale, CA).

CHAPTER III

RESULTS

Biological activity of Ste2p.FT.HT

To determine if the addition of the FLAG and HIS tag at the carboxyl-terminus of Ste2p would disrupt receptor function, growth arrest assays were performed using the strain DK102(pSte2p.FT.HT). As a positive control, the strain RC629 expressing wild type receptor was also tested. The analogs used in this study include [Nle^{12}]α-factor, BPA α-factor, and Bio-BPA α-factor which were all found to be biologically active to various degrees using both strains (Table 1). [Nle^{12}]α-factor was the most active biologically with an endpoint of 0.1μg/disk for both RC629 and DK102(pSte2p.FT.HT), although the zones of inhibition obtained for DK102(pSte2p.FT.HT) were larger than those obtained using RC629 suggesting that a lower endpoint may have been obtained for DK102(pSte2p.FT.HT) if a lower concentration of [Nle^{12}]α-factor had been tested. Endpoints obtained for BPA α-factor and Bio-BPA α-factor were 0.2μg/disk and 1μg/disk, respectively for both RC629 and DK102(pSte2p.FT.HT), indicating similar activities for each analog regardless of the strain used. Comparing activities of BPA α-factor and Bio-BPA α-factor to [Nle^{12}]α-factor using RC629 and DK102(pSte2p.FT.HT), the following observations were made. Using RC629, BPA α-factor was two-fold and Bio-BPA α-factor was five-fold less active than [Nle^{12}]α-factor. For DK102(pSte2p.FT.HT), BPA α-factor was ten-fold and Bio-BPA α-factor was one hundred-fold less active than [Nle^{12}]α-factor. All analogs tested were biologically active and the addition of a FLAG tag and a HIS tag at the carboxyl terminus of Ste2p did not

Table 1

Growth arrest of *S. cerevisiae* RC629 and DK102(pG3.STE2.FT.HT) by α-factor analogs

Peptide Conc. (μg/disk)	Zone of inhibition (mm)[a]					
	Nle[12] α-factor		BPA α-factor		Bio-BPA α-factor	
	RC629	DK102(Ste2p)[b]	RC629	DK102(Ste2p)[b]	RC629	DK102(Ste2p)[b]
10	22	31	20	23	16	14
5	20	29	19	21	15	12
2	17	27	17	17	12	11
1	16	25	14	16	10	8
0.5	14	22	12	13	NI	NI
0.2	9	20	9	9	NI	NI
0.1	8	15	NI[c]	NI	NI	NI

[a]The zone of inhibition includes the diameter of the filter paper (ca. 8mm) on which the peptide was spotted. The results represent the average of two independent assays, and the halo size from these assays were within 2mm of each other.

[b] DK102(pSte2p.FT.HT) = DK102(Ste2p)

[c] NI = no zone of inhibition observed

disrupt receptor function. DK102 which is deleted for *STE2* did not respond to [Nle^{12}]α-factor (Results not shown).

Competition with binding to Ste2p.FT.HT

A binding competition assay was done to determine if [Nle^{12}]α-factor would compete with tritiated α-factor for binding to Ste2p.FT.HT. In this assay tritiated α-factor bound to Ste2p.FT.HT with an IC_{50} of $1x10^{-6}$M for α-factor. This value is 30-fold higher than the IC_{50} obtained using *S. cerevisiae* 4202-15-3, the strain normally used for binding assays (Table 2), and indicates a lower binding affinity of α-factor for Ste2p.FT.HT as compared to Ste2p. These results provide evidence that α-factor can bind to the modified receptor, although less avidly than to the wild-type receptor. BPA α-factor and Bio-BPA α-factor gave IC_{50} values of $9x10^{-6}$M and $3x10^{-5}$M, respectively, using *S. cerevisiae* RC629 (Part IV of dissertation). Competition binding assays were not done using DK102(pSte2p.FT.HT) for either BPA α-factor or Bio-BPA α-factor. But in accordance with the biological data obtained using these analogs, both analogs were expected to bind to Ste2p with a lower binding affinity than that of [Nle^{12}] α-factor. BPA α-factor and Bio-BPA α-factor are the most active photoactivatable analogs available in our laboratory and were therefore used for photoaffinity labeling studies.

Photoaffinity Labeling Experiments of Yeast Membrane Proteins

In an attempt to photolabel Ste2p, membrane proteins isolated from DK102 (ΔSte2p) and DK102(pSte2p.FT.HT) were incubated with Bio-BPA α-factor (10μM-50μM), and then exposed to ultraviolet light (350nm) to initiate crosslinking of the photoligand to Ste2p.FT.HT. To analyze the photolyzed membranes, SDS-PAGE and

Table 2

Binding affinity of Nle^{12} α-factor

Strain	IC_{50} (M)[a]
S. cerevisiae 4202-15-3	3×10^{-8}
DK102(pSte2p.FT.HT)	1×10^{-6}

[a]IC_{50} is the concentration of competitor which inhibits binding of [^{3}H] α-factor by 50%.

Western blotting were used with incorporation of biotinylated α-factor into membrane proteins monitored by Avidin-HRP (Figure 5). Covalent crosslinking of biotinylated α-factor to membrane proteins isolated from cells expressing Ste2p.FT.HT (lanes 6-8) and from those with a deleted Ste2p (lanes 2-4) was observed at all concentrations of ligand tested. These results indicate that non-receptor proteins are being labeled by Bio-BPA α-factor during photolysis, but does not eliminate the possibility that Ste2p.FT.HT is also being labeled and its detection hindered by the high degree of non-specific labeling observed for both membrane proteins isolated from DK102(ΔSte2p) and DK102(pSte2p.FT.HT). Detection of biotinylated proteins in lanes 1 and 5 (no Bio-BPA α-factor added) represent endogenous biotinylated proteins present in yeast membrane proteins. These native biotinylated proteins migrate differently than Ste2p.FT.HT and therefore would not prohibit the detection of labeled Ste2p.FT.HT in lanes containing Bio-BPA α-factor. Using anti-FLAG antibodies, it was verified that the endogenous biotinylated protein which migrates very close to Ste2p.FT.HT would not mask the detection of Bio-BPA α-factor covalently crosslinked into Ste2p.FT.HT (data not shown). In our SDS-polyacrylamide system, Ste2p.FT.HT migrates with a mobility representative of a 52kDa protein, however the calculated molecular weight of Ste2p.FT.HT is 48.5 kDa.

One factor contributing to the non-specific crosslinking observed may have been the length of ultraviolet light exposure time (30 minutes). If the photolysis time previously used was adding to the non-specific labeling of non-receptor proteins, then a reduction in the exposure time should decrease the non-specific crosslinking observed. To address this concern, a time course experiment using exposure times of 0, 5, 15, and 30 minutes was performed (Figure 6). The results of the timecourse were similar for both membrane proteins isolated from cells expressing Ste2p.FT.HT (lanes 6-8) and from

Figure 5. Photoaffinity labeling experiment using membrane proteins. Membrane proteins isolated from DK102(ΔSte2p) (lanes 1-4) or DK102(pSte2p.FT.HT) (lanes 5-8) were incubated with Bio-BPA α-factor at 10μM (lanes 2 and 6), 20μM (lanes 3 and 7), or 50μM (lanes 4 and 8). Lane 1 and 5 contain no Bio-BPA α-factor. All samples were irradiated at 350nm for 30 minutes, diluted as described under "Materials and Methods", separated by SDS-PAGE, transferred to a nylon membrane, and probed with Avidin-HRP. Detection of cross-linked Bio-BPA α-factor to membrane proteins was visualized by exposure of the blot to autoradiography film after incubation with ECL detection reagents.

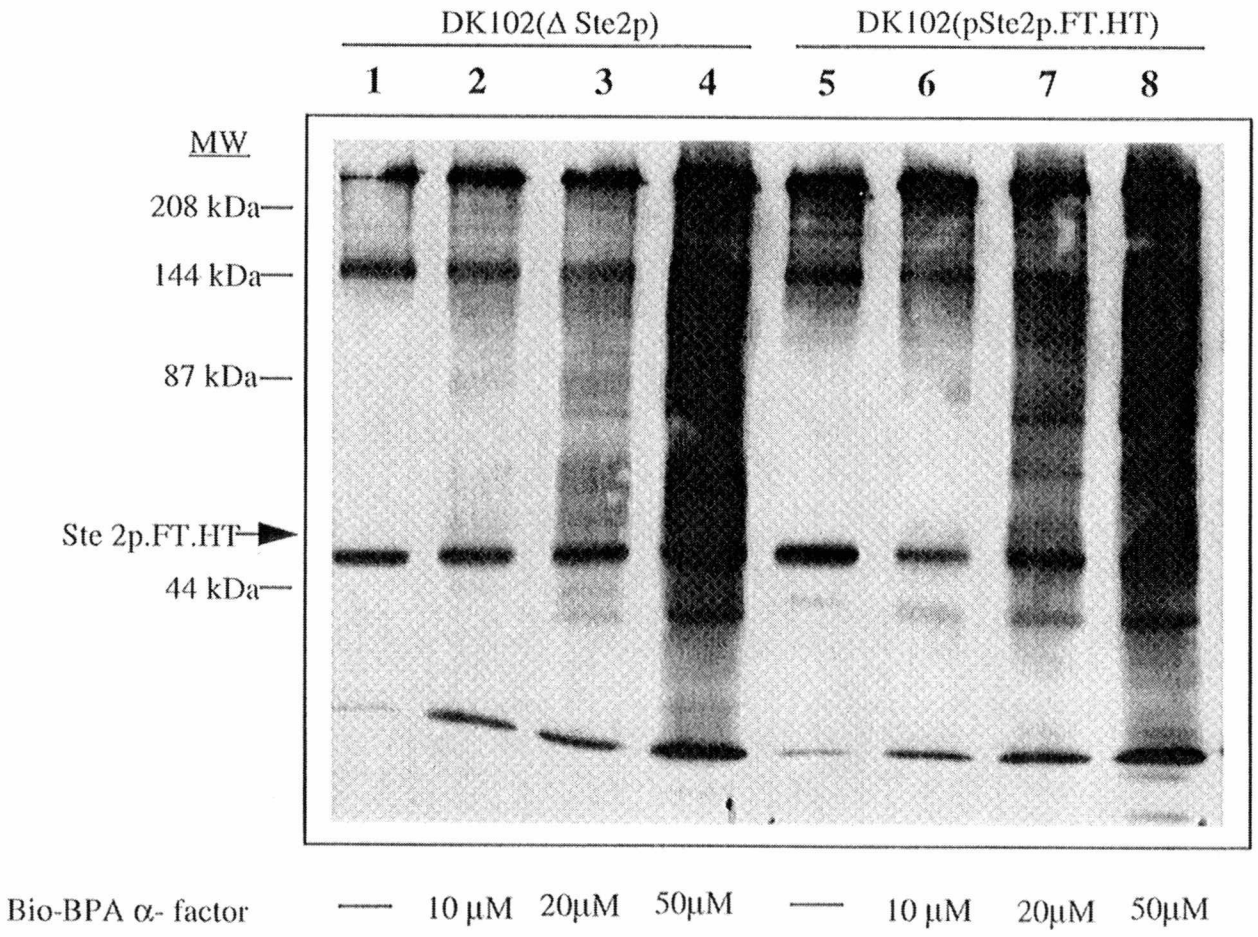
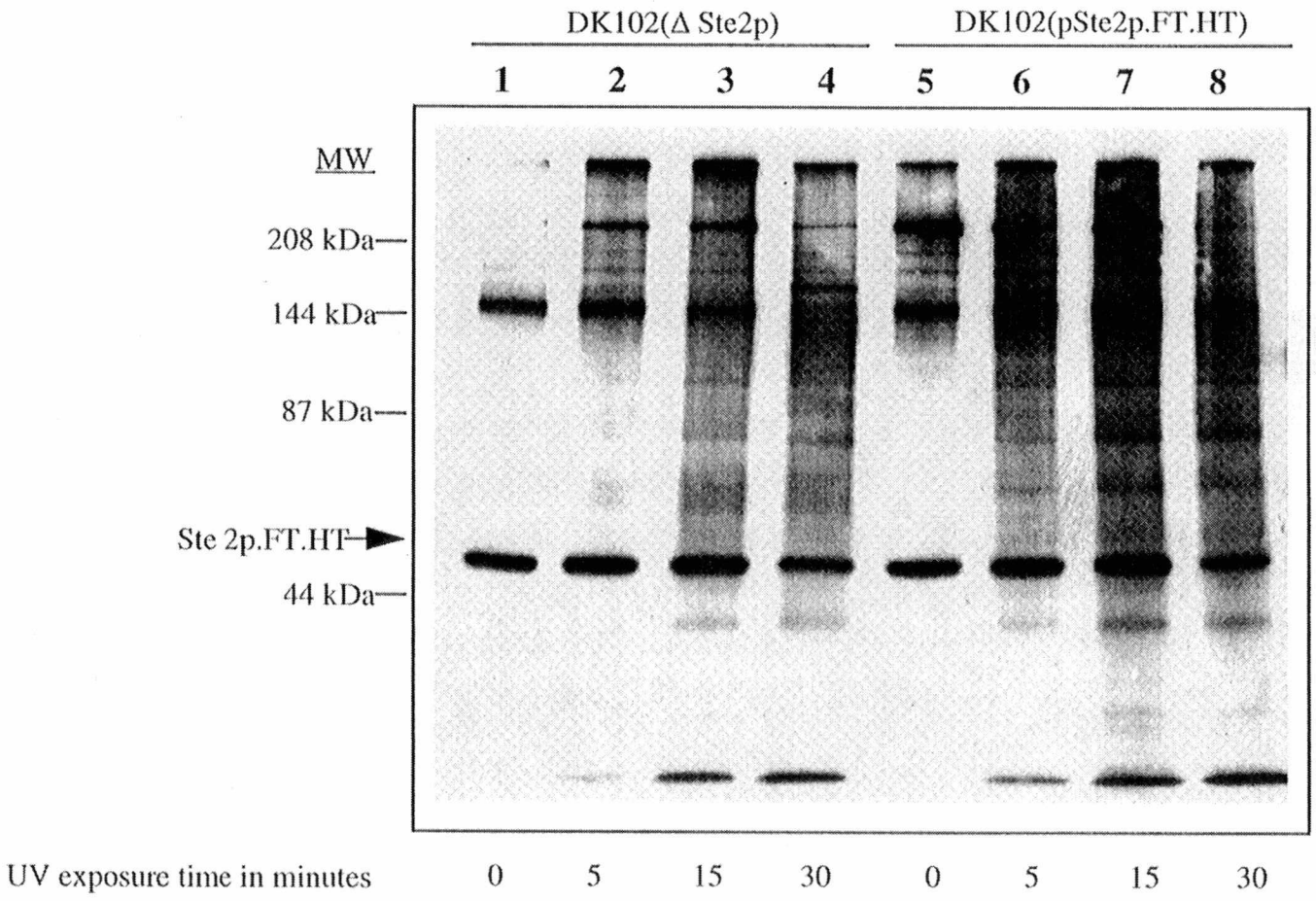

DK102(Δ Ste2p)
DK102(pSte2p.FT.HT)
1 2 3 4 5 6 7 8
MW
208 kDa
144 kDa
87 kDa
Ste 2p.FT.HT
44 kDa
Bio-BPA α- factor
—
10 μM 20μM 50μM
—
10 μM 20μM 50μM

Figure 6. Photoaffinity labeling time course experiment using membrane proteins. Membrane proteins isolated from DK102(ΔSte2p) (lanes 1-4) or DK102(pSte2p.FT.HT) (lanes 5-8) were incubated with Bio-BPA α-factor at 50μM and either not irradiated (lane 1 and 5), or irradiated at 350nm for 5 (lanes 2 and 6), 15 (lanes 3 and 7), or 30 (lanes 4 and 8) minutes. Reaction mixtures were diluted as described under "Materials and Methods", separated by SDS-PAGE, transferred to a nylon membrane, and probed with Avidin-HRP. Detection of cross-linked Bio-BPA α-factor to membrane proteins was visualized by exposure of the blot to autoradiography film after incubation with ECL detection reagents.

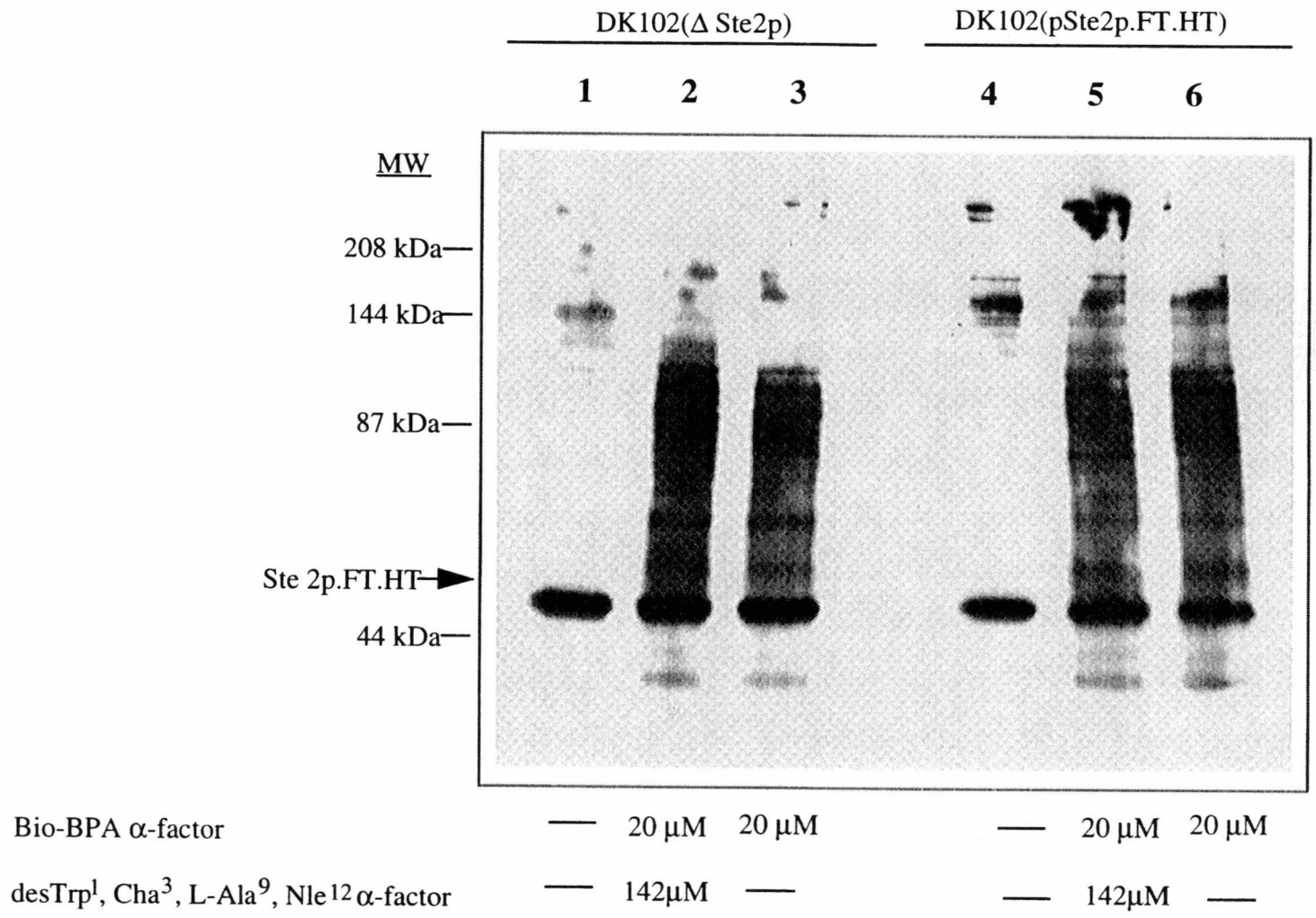

DK102(Δ Ste2p)
DK102(pSte2p.FT.HT)
1 2 3 4 5 6 7 8
MW
208 kDa
144 kDa
87 kDa
Ste 2p.FT.HT
44 kDa
UV exposure time in minutes
0 5 15 30 0 5 15 30

those with a deleted Ste2p (lanes 2-4) which showed an increase in the amount of Bio-BPA α-factor detected as the exposure time was increased from 5 to 15 minutes. The 15 and 30 minute exposure gave similar results, suggesting that 15 minutes was sufficient for crosslinking to occur (lanes 3-4 and 7-8). However, even after a 5 minute UV exposure, the non-specific labeling of membrane proteins appeared similar in membranes isolated from cells without [DK102(ΔSte2p)] and with [DK102(pSte2p.FT.HT)] receptor (lanes 2 and 6). Lanes 1 and 5 represent membrane proteins incubated with Bio-BPA α-factor without exposure to UV light and indicate that in order to get labeling of membrane proteins with the photoactivatable ligand, UV light is required. Biotinylated proteins observed in lanes 1 and 5 where no Bio-BPA α-factor was added represent yeast membrane proteins which are biotinylated.

In an attempt to eliminate the non-specific crosslinking seen with the receptor deficient and receptor containing strain, the α-factor analog, desTrp^{1}, Cha^{3}, *L*-Ala^{9}, Nle^{12} α-factor was incubated with membrane proteins (Figure 7). This peptide does not bind to the receptor (Raths *et al* ., 1988) and should block all non-specific binding sites, thereby allowing visualization of specific binding of Bio-BPA α-factor to Ste2p. The addition of desTrp^{1}, Cha^{3}, *L*-Ala^{9}, Nle^{12} α-factor (lanes 2 and 5) did not decrease the non-specific crosslinking of Bio-BPA α-factor to membrane proteins isolated from DK102(ΔSte2p) and DK102(pSte2p.FT.HT) when compared to lanes 3 and 6 which were incubated with only Bio-BPA α-factor.

In conclusion, photoaffinity labeling of Ste2p.FT.HT was not observable due to the nonspecific labeling of non-receptor proteins some of which co-migrate with Ste2p.FT.HT (52 kDa) seen in both membrane proteins isolated from DK102(pSte2p.FT.HT) and DK102(ΔSte2p). Due to the nonspecificity observed using

Figure 7. Photoaffinity labeling experiment using desTrp^{1},Cha^{3}, *L*-Ala^{9} Nle^{12} α-factor and membrane proteins. Membrane proteins isolated from DK102(ΔSte2p) (lanes 1-4) or DK102(pSte2p.FT.HT) (lanes 5-8) were incubated without (lane 1 and 5) or with Bio-BPA α-factor at 20μM(lanes 2-3 and 5-6) alone or in the presence of desTrp^{1},Cha^{3}, *L*-Ala^{9}, Nle^{12}α-factor at 142 μM (lanes 2 and 5). Reaction mixtures were irradiated at 350nm for 15 minutes, diluted as described under "Materials and Methods", separated by SDS-PAGE, transferred to a nylon membrane, and probed with Avidin-HRP. Detection of cross-linked Bio-BPA α-factor to membrane proteins was visualized by exposure of the blot to autoradiography film after incubation with ECL detection reagents.


DK102(Δ Ste2p)
DK102(pSte2p.FT.HT)
1 2 3 4 5 6
MW
208 kDa
144 kDa
87 kDa
Ste 2p.FT.HT
44 kDa
Bio-BPA α-factor — 20 μM 20 μM — 20 μM 20 μM
desTrp[1], Cha[3], L-Ala[9], Nle[12] α-factor — 142μM — — 142μM —

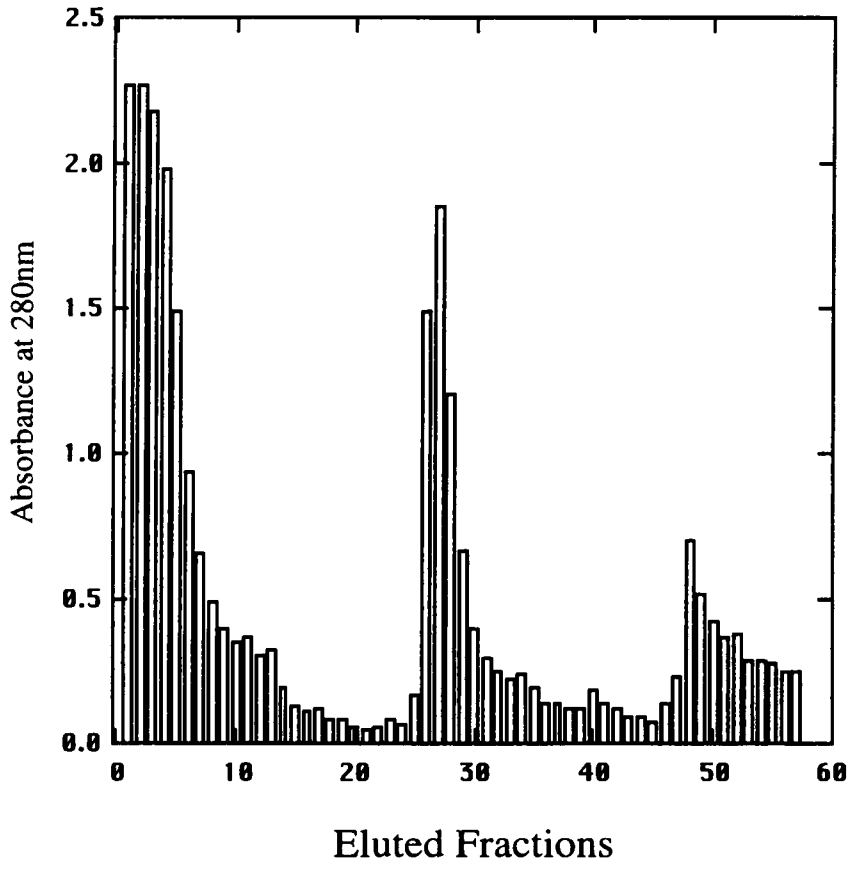
membrane proteins, photoaffinity labeling was attempted using partially purified Ste2p.FT.HT.

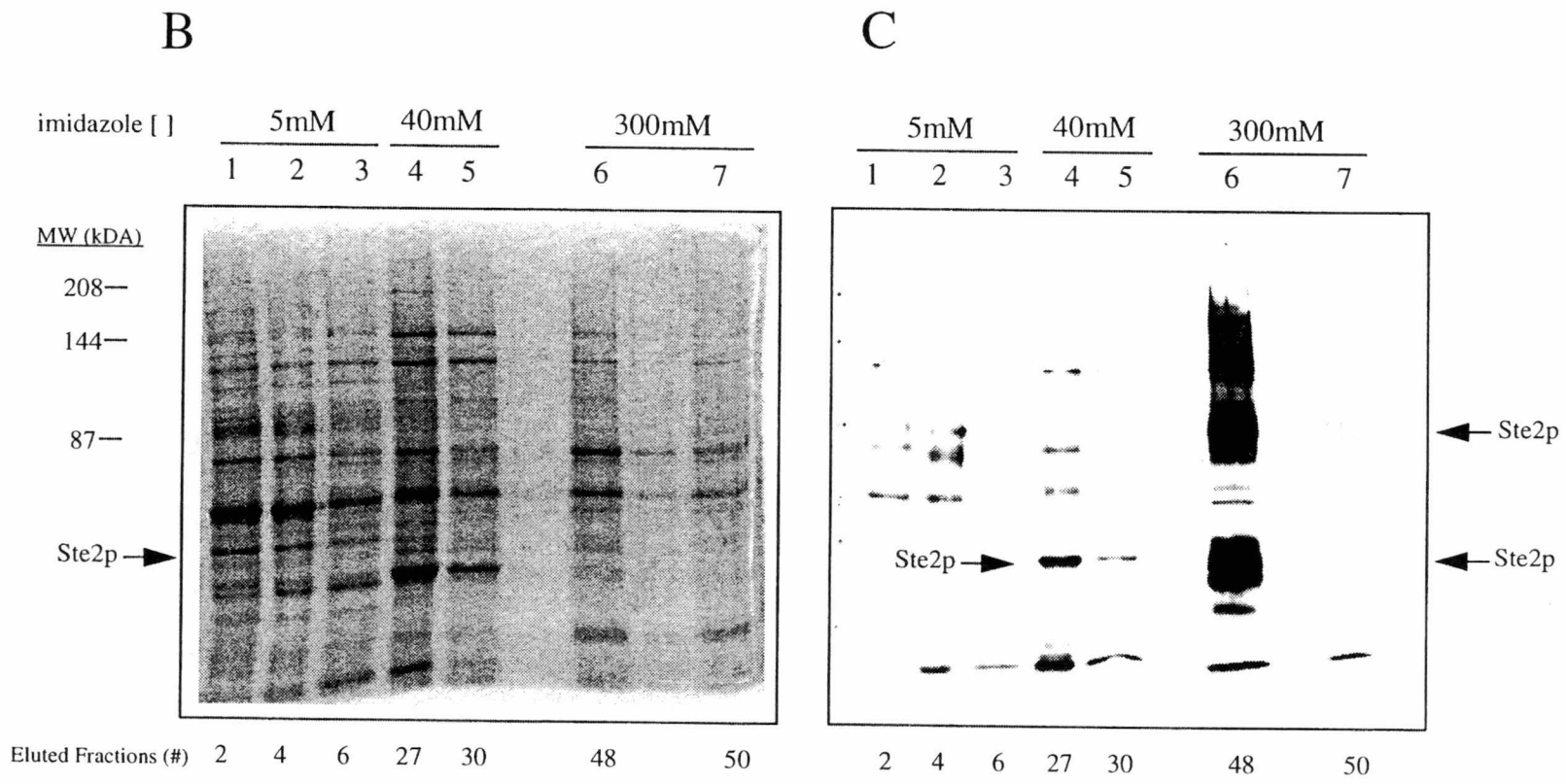
Purification of Ste2p.FT.HT.

Ste2p.FT.HT. was purified using immobilized metal affinity chromatography which takes advantage of the high degree of affinity of histidine for metals such as copper, nickel, and iron. To purify Ste2p.FT.HT, solubilized membrane proteins were chromatographed over a copper column. Proteins which bound to the column with a low affinity were washed from the column using buffers containing either 5mM or 40mM imidazole. Proteins containing a HIS tag bound to the column with a high affinity and were eluted with 300mM imidazole which competed for binding with the tandem histidines of the genetically engineered Ste2p.FT.HT. Proteins recovered from the copper column were separated on a 10% SDS-Polyacrylamide gel and either stained with coomassie blue dye to visualize proteins or transferred to a nylon membrane and probed with anti-FLAG antibodies to detect Ste2p.FT.HT as described in Materials and Methods. Figure 8A represents the elution profile of solubilized membrane proteins, containing Ste2p.FT.HT, chromatographed over a copper column. As seen in the Coomassie blue stained gel (Figure 8B) fractions recovered from the copper column using 5mM (lane 1-3), 40mM (lane 4-5) and 300mM imidazole (lane 6-7) contain an abundance of proteins. The gel transferred to a nylon membrane and probed with anti-FLAG antibodies (Figure 8C) indicate the presence of Ste2p.FT.HT in lane 4-6, but no Ste2p.FT.HT present in lanes 1-3 and 7. The amount of Ste2p.FT.HT in lane 4 and 5 is reduced compared to lane 6, indicating the majority of Ste2p.FT.HT was eluted using 300mM imidazole and that Ste2p containing the HIS tag bound specifically to the copper column. The higher molecular weight species found in lane 6 may represent a dimer of Ste2p.FT.HT and the

Figure 8. Purification of Ste2p.FT.HT using immobilized metal affinity chromatography. A) Elution profile of solubilized membrane proteins chromatographed over a copper sepharose column. Fractions 1-24 were eluted using 5mM imidazole, fractions 25-44 using 40mM imidazole, and fractions 45-58 using 300mM imidazole. B) Coomassie Blue stained 10% SDS-polyacrylamide gel and (C) immunoblot analysis using anti-FLAG antibodies of the following eluted column fractions: Lane 1(Fraction 2); Lane 2 (Fraction 4); Lane 3 (Fraction 6); Lane 4 (Fraction 27); Lane 5 (Fraction 30); Lane 6 (Fraction 48); and Lane 7 (Fraction 50).

A.


2.5
2.0
1.5
1.0
0.5
0.0
Absorbance at 280nm
0
10
20
30
40
50
60
Eluted Fractions


B
C
imidazole [ ]
5mM
40mM
300mM
1 2 3 4 5 6 7
MW (kDA)
208—
144—
87—
Ste2p
Ste2p
Ste2p
Ste2p
Eluted Fractions (#) 2 4 6 27 30 48 50
2 4 6 27 30 48 50

smear above this species an excess of protein loaded on the gel. These results indicate that the protein fraction recovered from the copper column using 300mM imidazole is enriched for Ste2p.FT.HT and also contains additional proteins as determined by the coomassie stained gel (Figure 8B, lane 6). Partial purification of Ste2p.FT.HT was achieved, although the quantity of protein recovered was not sufficient for photoaffinity labeling reactions. The protein concentration obtained for fraction number 48 (Figure 8A) which was eluted using 300mM imidazole was 0.18mg/ml. Due to this recovery problem, partially purified Ste2p.FT.HT used in this work was received from R. Stevens (Berkeley, CA).

Photoaffinity labeling using partially purified Ste2p.FT.HT

As with the protein purified in our laboratory, the protein received was enriched for Ste2p.FT.HT and contained additional proteins which co-eluted with Ste2p.FT.HT as observed from the coomassie stained gel in Figure 9. However, the preparation received from Berkeley appeared to be more highly enriched in Ste2p.FT.HT than the preparation prepared at Tennessee.

The partially purified protein was incubated with Bio-BPA α-factor at either 10μM, 20μM, or 50μM and then photolyzed to initiate crosslinking. The reaction mixture was resolved on an SDS-Polyacrylamide gel. Separated proteins were transferred to a nylon membrane and either probed with Avidin-HRP to detect biotinylated α-factor or with anti-FLAG antibody to detect Ste2p. The results indicated a band recognized by Avidin-HRP at all concentrations tested (Figure 10B, lanes 2-5; band in lane 2 was very faint and therefore did not reproduce well) which migrated at a molecular weight of 52 kDA (calculated molecular weight of Ste2p.FT.HT is 48.5 kDa). Confirmation that the presumed crosslinked species was Ste2p.FT.HT was obtained by

Figure 9. Coomassie stained gel of partially purified Ste2p.FT.HT received from the laboratory of R. Stevens. Lane 1, Molecular weight markers (BioRad). Lane 2, partially purified Ste2p.FT.HT.

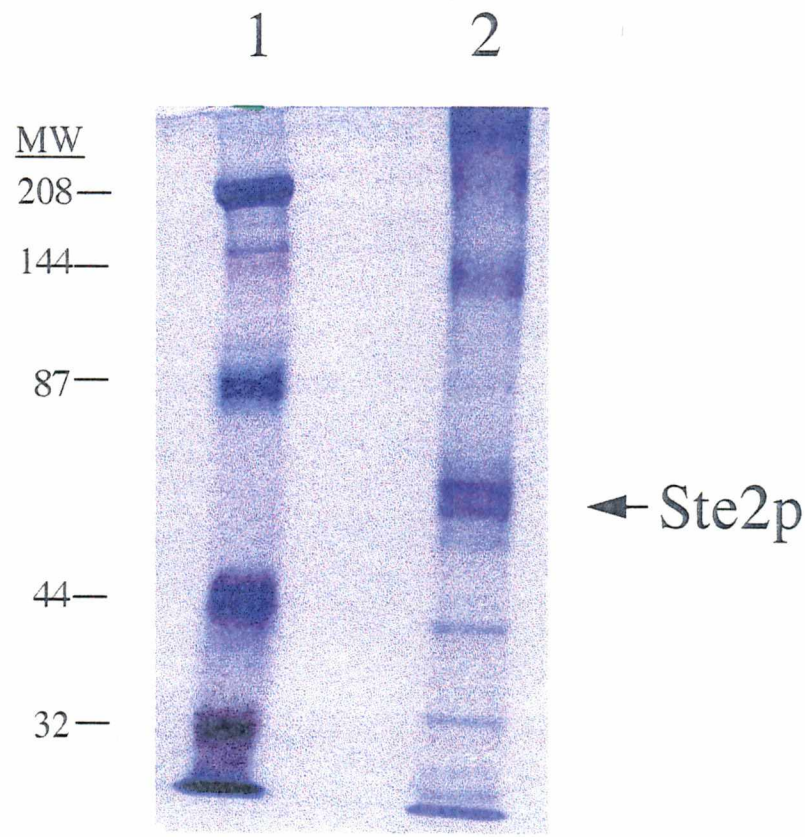

1
2
MW
208
144
87
44
32
Ste2p

Figure 10. Photoaffinity labeling experiment using partially purified Ste2p.FT.HT. Partially purified Ste2p.FT.HT was incubated without (lane 1) or with Bio-BPA α-factor at 12.5μM (lane 2), 25 μM (lane 3), 50μM (lane 4), or 100 μM (lane 5). Reactions were irradiated at 350nm for 30 minutes, separated by SDS-polyacrylamide gel electrophoresis, transferred to a nylon membrane, and either probed with anti-FLAG antibodies (A) to detect Ste2p.FT.HT or Avidin-HRP (B) to detect cross-linked Bio-BPA α-factor to partially purified Ste2p.FT.HT. Exposure of the blots to autoradiography film after incubation with ECL detection reagents allowed visualization of the results.

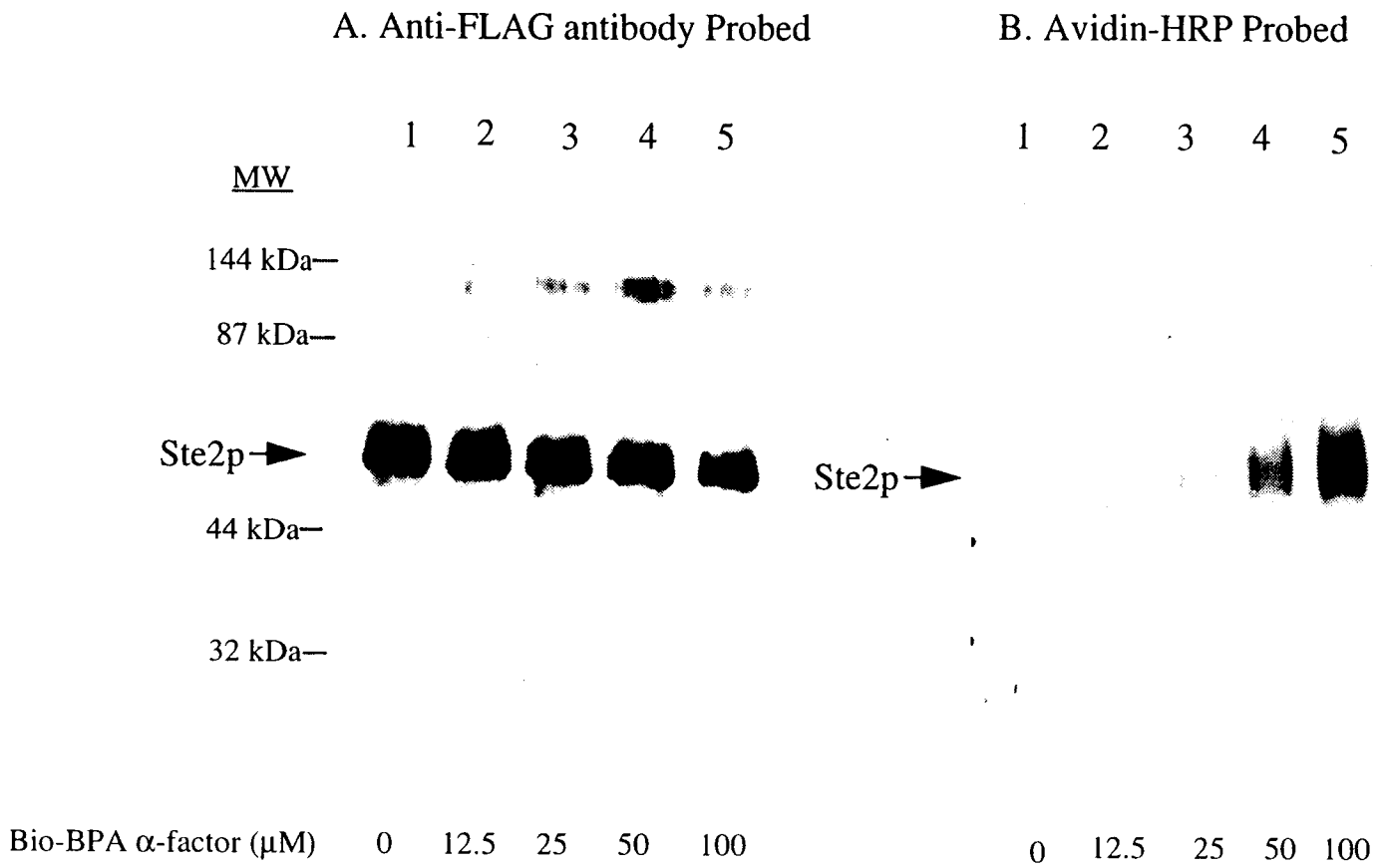
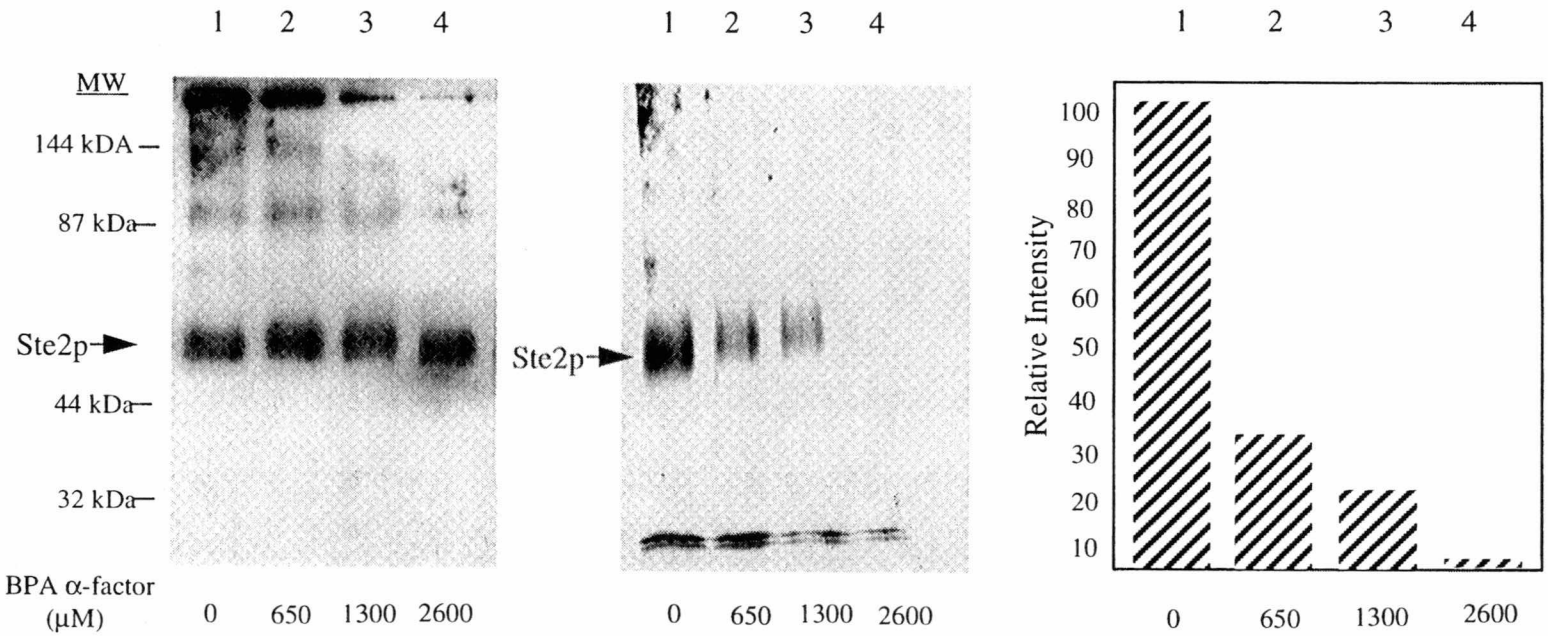

A. Anti-FLAG antibody Probed
B. Avidin-HRP Probed
1 2 3 4 5
1 2 3 4 5
MW
144 kDa—
87 kDa—
Ste2p
44 kDa—
32 kDa—
Ste2p
Bio-BPA α-factor (μM) 0 12.5 25 50 100
0 12.5 25 50 100

Figure 11. Competition photoaffinity labeling experiment using partially purified Ste2p.FT.HT. Partially purified Ste2p.FT.HT was incubated with Bio-BPA α-factor at 50μM either without (lane 1) or with the competitor, BPA α-factor, at 650μM (lane 2), 1300μM (lane 3) or 2600μM (lane 4). Reactions were irradiated at 350nm for 30 minutes, separated by SDS-polyacrylamide gel electrophoresis, transferred to a nylon membrane, and either probed with anti-FLAG antibodies (A) to detect Ste2p.FT.HT or Avidin-HRP (B) to detect cross-linked Bio-BPA α-factor to partially purified Ste2p.FT.HT. Exposure of the blots to autoradiography film after incubation with ECL detection reagents allowed visualization of the results. C) Densitometric analysis of Panel B.


A. Anti-FLAG antibody Probed
B. Avidin-HRP Probed
C. Densitometric Analysis
1 2 3 4
MW
144 kDA
87 kDa
Ste2p
44 kDa
32 kDa
BPA α-factor (μM)
0 650 1300 2600
Relative Intensity
100
90
80
70
60
50
40
30
20
10

the use of anti-Flag antibodies (Figure 10A, lanes 2-5). Avidin-HRP does not bind nonspecifically to Ste2p.FT.HT as demonstrated in lane 1 (Figure 10B) which contains Ste2p.FT.HT but no Bio-BPA α-factor. These observations suggest that Ste2p has been labeled by Bio-BPA α-factor and is detectable using Avidin-HRP. The photoaffinity labeled complex was specifically competed by incubation with an excess of non-biotinylated BPA α-factor (650μM-2600μM) as shown in lanes 2-4 of Figure 11B and in Figure 11C (Densitometric analysis of results). A peptide having no affinity to Ste2p but containing the photoactivatable BPA group (**a**-factor peptide; see Figure 3) showed no competition for crosslinking by Bio-BPA α-factor when used at concentrations up to 930μM (data not shown). These results suggest that the Ste2p.FT.HT binding site was specifically labeled by Bio-BPA α-factor. Confirmation that the crosslinked species was Ste2p.FT.HT was obtained by the use of anti-Flag antibodies (Figure 11A, lanes 1-4). However, no competition was observed by incubation with non-photoreactive α-factor ([Nle^{12}]α-factor) at a concentration up to 3000μM (data not shown).

CHAPTER IV

DISCUSSION

The availability of a biologically-active, photoactivatable α-factor analog which can be covalently crosslinked into Ste2p provides a means for investigating the molecular interactions involved in binding of α-factor to its receptor and for determination of the α-factor binding domain within its receptor. The identification of specific amino acids comprising the α-factor binding domain of Ste2p should aid in the rational design of novel analogs and provide insight into the mechanism of hormone-receptor mediated signal transduction.

For photoaffinity labeling studies presented in this dissertation, the *Saccharomyces cerevisiae* strain DK102(Δ*STE2*) transformed with the plasmid pG3.STE2.FT.HT was used. This plasmid encodes a FLAG tagged, HIS tagged Ste2p enabling the protein to be purified using metal affinity chromatography and detected by anti-FLAG antibodies during immunoblot analysis. The addition of the FLAG and HIS tag at the carboxyl-terminus of Ste2p did not greatly distort receptor function as indicated by the growth arrest assay (Table 1) in which both photoactivatable (Bio-BPA α-factor and BPA α-factor) and non-photoactivatable ([Nle^{12}] α-factor) α-factor analogs were biologically active.

Not only was the modified receptor (Ste2p.FT.HT) biologically active, it showed increased sensitivity to [Nle^{12}]α-factor as determined by the larger zones of growth inhibition obtained in the growth arrest assay when compared to the zones obtained using RC629 (wild type Ste2p). This observation is consistent with data obtained from *S. cerevisiae* strains which showed an increased sensitivity to α-factor when the C-

termininal domain of the receptor was mutated (Konopka *et al.,* 1988; Kurjan, 1992). The C-terminal region of the receptor (134 residues) contain a combined total of 34 phosphorylatable serine and threonine residues, thereby suggesting that this receptor is regulated by phosphorylation. An investigation into which residues where important for receptor regulation, identified Ser^{398}, Thr^{411}, Thr^{414}, and Thr^{425} as important for normal α-factor sensitivity (Chen and Konopka, 1996). When any of these four residues were mutated to the nonphosphorylatable alanine residue, an increased sensitivity to α-factor was obtained as determined by the growth arrest assay. In our study, amino acids 424-431 (WTEDNNNL) of Ste2p were removed and a FLAG tag (DKDDDDK) and HIS tag (HHHHHH) added at the carboxyl-terminus of Ste2p to generate Ste2p.FT.HT. The removal of Thr^{425} of Ste2p likely contributed to the increased sensitivity of strain DK102(pSte2p.FT.HT) to [Nle^{12}]α-factor observed in the growth arrest assay.

Although it was demonstrated in the growth arrest assay that strain DK102(pSte2p.FT.HT) was more sensitive to [Nle^{12}]α-factor than that of strain RC629, the binding affinity of [Nle^{12}]α-factor for DK102(pSte2p.FT.HT) did not increase. This observation is consistent with binding studies performed by Chen and Konopka (1996) who used a *S. cerevisiae* strain containing Ste2p with a mutated C-terminal domain. In those studies, the binding affinity of α-factor for the mutated receptor did not increase. We may conclude that the increased sensitivity of strain DK102(pSte2p.FT.HT) to [Nle12]α-factor, observed in the growth arrest assay, was not due to an increase in receptor affinity or receptor number. The results obtained in our laboratory and by others (Chen and Konopka, 1996) suggest phosphorylation in the C-terminal region of the receptor plays a role in regulating the function of Ste2p. It is possible that when phosphorylation in this region of the receptor is hindered, desensitization of Ste2p to

α-factor is impaired thereby producing larger zones of growth inhibition (Dietzel and Kurjan, 1987). The β-adrenergic receptor and the muscarinic cholinergic receptor are two G-protein-coupled receptors which are regulated by phosphorylation (Dohlman *et al.*, 1991; Lefkowitz, 1993; Richardson *et al.*, 1993) and it has been shown that activated receptors are desensitized by phosphorylated C-terminal residues.

In our studies, [Nle^{12}]α-factor bound to strain DK102(pSte2p.FT.HT) 30-fold less efficiently than what is normally observed using the strain 4202-15-3 (Table 2). This finding may be attributable to the different growth conditions used for the two strains in the binding assay. Normally, the medium for growth of cells used in the binding assay is YM-1, but MLT medium had to be used for growth of DK102(pSte2p.FT.HT) in order to maintain the plasmid (pG3.STE2.FT.HT). In our laboratory, strain 4202-15-3 is normally used in the binding assay and all assay conditions have been optimized for this strain. Therefore, it would be advantageous to optimize binding conditions for DK102 expressing wild type receptor (Ste2p) and use those conditions for binding assays of DK102(pSte2p.FT.HT). The binding affinity obtained for DK102(pSte2p.FT.HT) would then be compared to the binding value obtained for DK102(Ste2p) and this comparison would reflect a more accurate determination of the binding affinity of [Nle^{12}]α-factor for DK102(pSte2p.FT.HT).

BPA α-factor and Bio-BPA α-factor were 10- and 100-fold less active than [Nle^{12}] α-factor, respectively, as determined by the growth arrest assay using strain DK102(pSte2p.FT.HT). As mentioned earlier, these were the most active photoactivatable analogs available in our laboratory and were therefore used for photoaffinity labeling studies. Membrane proteins isolated from DK102(ΔSte2p) and DK102(pSte2p.FT.HT) were incubated with Bio-BPA α-factor and photolyzed. In those experiments, photoaffinity labeling of Ste2p.FT.HT was not observable due to the

nonspecific labeling of non-receptor proteins, some of which co-migrate with Ste2p.FT.HT as seen in both membrane proteins isolated from DK102(pSte2p.FT.HT) and DK102(ΔSte2p) (Figures 5-7). A possible reason for labeling of non-receptor proteins may be due to the high concentration of Bio-BPA α-factor (10-50μM) used in the photoaffinity labeling experiments. Previous studies performed by Blumer and colleagues (1988) attempted to determine the binding domain of Ste2p using chemical crosslinking. In that study, ^{35}S labeled α-factor (10nM) was chemically crosslinked into Ste2p using membrane proteins. A comparison of the concentration of Bio-BPA α-factor used in photoaffinity labeling studies and ^{35}S-α-factor used in chemical crosslinking studies indicate that there is at least a 1000-fold excess of photoactivatable α-factor used in our labeling experiments. The concentration of Bio-BPA α-factor used in photoaffinity labeling studies was required in order to visualize Bio-BPA α-factor which was covalently crosslinked into receptor proteins. As an alternative to Bio-BPA α-factor, a radioactively labeled photoactivatable α-factor analog which binds specifically to Ste2p could be used for photoaffinity labeling studies. This may allow a reduction in the concentration of photoactivatable α-factor needed for photoaffinity labeling studies which would reduce the nonspecificity observed.

It is also possible that the BPA moiety is contributing to the nonspecificity observed due to its hydrophobic nature. BPA was chosen due to its chemical stability and high degree of selectivity for C-H bonds, but for our purposes it may be worth the effort to synthesize additional photoactivatable α-factor analogs containing other photoactivatable groups (i.e., azido group) in an attempt to obtain specific photolabeling of Ste2p using membrane proteins.

To circumvent the photoaffinity labeling of non-receptor proteins obtained using membrane proteins, partially purified Ste2p.FT.HT was used for photoaffinity labeling

studies. In this study, Bio-BPA α-factor was covalently crosslinked into partially purified Ste2p.FT.HT as determined by Avidin-HRP and anti-FLAG antibodies (Figure 10). During photoaffinity labeling, it is possible for two non-specific events to occur. The first is labeling of non-receptor proteins and the second labeling of sites on the receptor which do not comprise the binding domain. For this reason, it is important to determine specificity of labeling by demonstrating that formation of the ligand-receptor complex detected can be inhibited in a concentration dependent manner by unlabeled ligand. Specificity of labeling of Ste2p.FT.HT was analyzed by displacement of Bio-BPA α-factor with increasing concentrations of unlabeled BPA α-factor. It was shown that as the concentration of BPA α-factor increased, the amount of Bio-BPA α-factor detected decreased, suggesting that the labeled species was Ste2p.FT.HT and that it was labeled in a site-specific manner (Figure 11).

To verify that BPA α-factor and not the BPA group of this analog was competing with Bio-BPA α-factor for binding to Ste2p.FT.HT, a peptide having no affinity for Ste2p, but containing the photoactivatable BPA group (**a**-factor peptide) was used in photoaffinity competition experiments. This peptide was unable to compete with Bio-BPA α-factor for binding to Ste2p.FT.HT, further suggesting that Ste2p.FT.HT was specifically labeled by Bio-BPA α-factor and that BPA α-factor binds the same site on Ste2p.FT.HT as Bio-BPA α-factor.

The ability of BPA α-factor to compete for binding with Bio-BPA α-factor to Ste2p.FT.HT entailed the use of two α-factor peptides which were covalently crosslinked into Ste2p.FT.HT. To confirm that the binding site labeled by Bio-BPA α-factor constitutes the α-factor binding domain, [Nle^{12}]α-factor was used in photoaffinity labeling competition experiments. The use of this non-photoreactive [Nle^{12}]α-factor represented a non-covalent interaction of α-factor with Ste2p. No competition was

observed by incubation with non-photoreactive [Nle^{12}] α-factor when used at an 100-fold excess of Bio-BPA α-factor. This observation was unexpected as it is known that [Nle^{12}]α-factor binds Ste2p in a site-specific manner. One possible reason for no competition being observed may be due to use of an insufficient amount of competing [Nle^{12}] α-factor. In other studies where hormone receptors have been identified using photoaffinity labeling, such as the insulin receptor (Kurose *et al.*, 1994) and the lutropin receptor (Kundu et al., 1996), a 1000-fold excess of unlabeled ligand was used to show specificity of labeling. To observe competition during photoaffinity labeling studies using [Nle^{12}] α-factor, it may be necessary to increase the concentration of this analog. Due to the concentration of Bio-BPA α-factor used in these labeling experiments (20μM), it was not feasible to go above a 100-fold excess of [Nle^{12}] α-factor due to insolubility of this peptide at concentrations above 2000μM. The concentration problem encountered using [Nle^{12}]α-factor and Bio-BPA α-factor emphasize the need for a radioactively labeled BPA α-factor analog which is biologically active and binds to the receptor in a site-specific manner. As mentioned earlier, if such an analog were available, a lower concentration of photoactivatable α-factor could be used in photoaffinity labeling experiments allowing use of a 1000-fold excess of competitor.

While further studies are necessary, the results of the photoaffinity labeling experiments suggest that Ste2p.FT.HT has been labeled by Bio-BPA α-factor. However, whether the actual binding site was labeled remains to be shown. In order to verify that Ste2p.FT.HT has been specifically labeled and to determine the region of the receptor which has been labeled, the following experiments are required. The first step would be digestion of the Ste2p.FT.HT/Bio-BPA α-factor complex either enzymatically (trypsin) or chemically (cyanogen bromide). This digestion should result in Ste2p.FT.HT labeled by Bio-BPA α-factor cleaved into fragments of various sizes, some

of which would have Bio-BPA α-factor covalently attached. The digested product would then be subjected to SDS-polyacrylamide electrophoresis, transferred to a nylon membrane and the Ste2p.FT.HT fragment(s) labeled by Bio-BPA α-factor detected by Avidin-HRP. The labeled fragment(s) would be recovered from the nylon membrane and amino acid analysis performed to determine the region(s) of the receptor comprising the α-factor binding domain. Characterization of the binding domain would increase our understanding of the molecular interactions involved in α-factor/Ste2p binding and the information obtained might be applicable to other hormone-receptor systems.

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APPENDIX

LIST OF ABBREVIATIONS

Ac	acetyl
APA	5-aminopentanoic acid
AVA	5-aminovaleric acid
BPA	*p*-benzoyl phenylalanine
Boc	*tert*-butoxycarbonyl
BOP	benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate
BrZ	2-bromobenzoylcarbonyl
Cha	β-cyclohexyl-*L*-alanine
COSY	correlated spectroscopy
CBZ	benzyloxycarbonyl
Dab	*L*-2,4-diaminobutyric acid
DCC	dicyclohexylcarbodiimide
DCHA	dicyclohexylamine
DCM	methylene chloride
DCU	dicyclohexyl urea
DIEA	diisopropylethylamine
DIPC	diiosopropylcarbodiimide
Dhp	dehydroproline
DMF	*N,N*-dimethylformamide
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
Dns	dansyl
Dpr	*L*-2,3-diaminopropionic acid
FAB-MS	fast atom bombardment mass spectrometry
Fmoc	9-fluorenylmethoxycarbonyl
For	formyl
HOAc	acetic acid
HOBt	1-hydroxybenztriazole
HOSu	1-hydroxysuccinimide
HPLC	high performance liquid chromatography
HPP	3-(4-hydroxyphenyl)propanoic acid

Me	methyl
Nle	norleucine
NMR	nuclear magnetic resonance
NOESY	2D nuclear Overhauser spectroscopy
Obzl	benzyl ester
OFm	9-fluorenylmethyl
PAM	(phenylacetamido)methyl
PE	petroleum ether
Ph	phenyl
SPPS	solid phase peptide synthesis
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
Tos	toluenesulfonyl

VITA

Angela L. McKinney was born in Fort Wayne, Indiana on June 2, 1967. She attended NorthSide High School in Fort Wayne and then Taylor University in Upland, Indiana where she earned a Bachelor of Arts degree in Biology/Pre-Medicine in May of 1989. She then entered the Biotechnology Program at the University of Tennessee, Knoxville and received a Master's degree in May of 1992. After entering the Graduate Program in Cellular, Molecular, and Developmental Biology and performing her dissertation research in the laboratory of Dr. Jeffrey Becker, she received a Doctor of Philosophy degree in Life Science in 1996. She will be teaching Biology at Messiah College in Grantham, Pennsylvania.