STRUCTURE-ACTIVITY RELATIONSHIPS OF THE YEAST α-FACTOR

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I. INTRODUCTION

Interactions between cells constitute the basis for the development of higher forms of life. At the outset of conception, fertilization of an ovum by a spermatozoan triggers the complex process which, if successful, ultimately results in the birth of an organism. Throughout the developmental sequence, cell-cell interactions are involved in both growth and cellular differentiation. In addition to these "normal" interactions, recognition on the cellular level is fundamental to the immune response and thus an integral part of the defense system which protects us from disease. Finally, incorrect recognition by various cell types can lead to a host of problems including autoimmune dysfunction and the uncontrolled cellular growth typified by different forms of cancer.

The question of how two cells recognize and communicate with each other is central to biology. It is now known that the cell surface contains macromolecular constituents which are involved in information transmission. These receptor systems recognize intercellular signals and are involved in triggering a cascade of events in response to signal binding. The effectiveness and elegance of such pheromonal and hormonal messengers belies the complex molecular events which follow binding of these molecules and which precede the ultimate response of the target cell. These events are presently an area of intensive research in the field of molecular biology, and numerous laboratories are investigating both binding of peptide and steroid hormones and the mechanisms involved in such information transmission.

Despite the effort concentrated on the study of mammalian peptide hormones, a number of difficulties have hampered progress in this area. Among these difficulties are the tendency for nonspecific binding to membranes and the inability to genetically manipulate the target cell. The latter shortcoming has removed an important tool from the arsenal of the hormone biologist and eliminated a critical control experiment. Given these difficulties, cell biologists have sought simpler systems with which to investigate cellular development and hormone

action. Among these, the sexual conjugation of the yeast *Saccharomyces cerevisiae* has many advantages.

The unicellular eukaryote *S. cerevisiae* is easy to culture in the laboratory and has been the subject of a host of biochemical and genetic investigations. In fact, this yeast is considered by many to be the "*Escherichia coli*" of eukaryotic cellular and molecular biology. Most importantly, sexual conjugation of *S. cerevisiae* is controlled, in part, by diffusable molecules known as mating factors. These mating factors are peptides and have been the subject of scrutiny in biological, genetic, biochemical, and, to some extent, spectroscopic studies. During the last 10 years a number of reviews have been written on the genetics and biochemistry of sexual conjugation in *S. cerevisiae*.[1-7] Given this proliferation of biologically oriented reviews no detailed discussion of the genetics of α-factor mediated sexual conjugation will be presented. Rather the purpose of this review is to discuss progress in understanding the relationship between the structure of the α-factor and its biological activity. Toward this end the authors will review strategies used to synthesize and purify the α-factor and discuss recent studies of the structure of this peptide in solution.

II. VALUE OF STRUCTURE-FUNCTION STUDIES

It is obvious that the function of a peptide is coded by its primary structure and that peptide derivatives can be used to define structural domains necessary for biological activity. Utilizing structural analogues it should be possible to dissect various steps in the biological response to a peptide pheromone. This approach can be used to understand the mode of action of α-factor as a complement to the genetic approaches that have dominated the field to date. Peptide analogues have been used to distinguish different receptors involved in the recognition of biologically active molecules such as the α-factor. Such analogues or antagonists can also be employed to select for receptor mutants, and to study binding to receptor and pheromone processing. Therefore, structure-function studies are necessary and important components of the overall understanding of the mode of action of α-factor and other peptide pheromones.

III. THE LIFE CYCLE OF *S. CEREVISIAE*

The yeast *Saccharomyces* has been a companion of humans since antiquity and recorded as such since the days of the ancient Canaanites. References in the Old Testament to wine, beer, and bread and the forbading of leaven during the holiday of Passover are clearly indicative of the role of this eukaryote in early civilizations. Considering the current excitement with biotechnology and its economic potentials, it is sobering to realize that some of the earliest industries developed by mankind involved the exploitation of this single-celled microorganism.

Cells of *S. cerevisiae* appear in nature either as haploids (one complement of DNA) or diploids (two complements of DNA). Either of these cell types can propagate by asexual reproduction involving vegetative growth, mitosis, and separation into mother and daughter cells by budding (Figure 1). During the 1950s a number of laboratories observed that yeast haploids could undergo sexual fusion to form a zygote which then buds and results in the propagation of a diploid line. The formation of diploids required the presence in the culture medium of haploids of opposite mating types termed α and *a* cells.[8] Recent studies have shown that the characteristic mating type specific features of the *a* and α yeast cells are under the control of a region on chromosome III termed the mating-type locus (*MAT*). *MATa* and *MAT*α cells mate efficiently with each other but are unable to mate with cells of the same mating type. *MAT*a/α diploids do not mate. The genetic control of events leading to and following mating has been the subject of detailed investigation[5,9] and will not be discussed

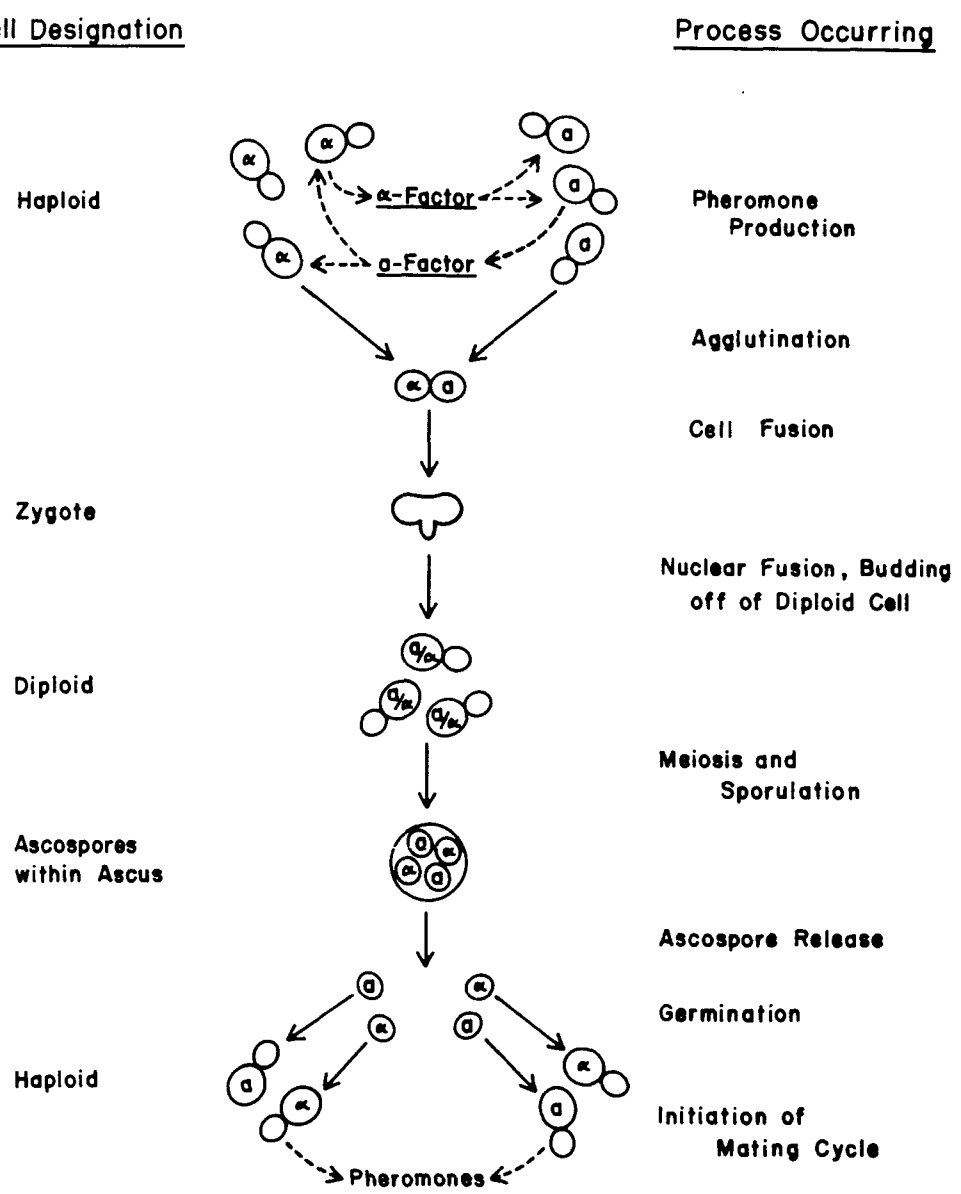

FIGURE 1. Life cycle of *Saccharomyces cerevisiae*.

further. More pertinent to this review was the observation by Levi[10] that *MATa* cells were affected by the presence of *MAT*α cells and underwent morphological changes which did not require cell-cell contact. This was the first evidence that sexual conjugation in *S. cerevisiae* was under the control of diffusable factors.

Later experiments showed that spent culture medium from *MAT*α cells could lead to a variety of responses by *MATa* cells. These responses occur at various times after challenge by the culture medium and are in order of appearance (1) increased agglutinability; (2) cell cycle arrest in G1 phase; (3) blockage of the initiation of nuclear DNA synthesis; (4) changes in biosynthesis of cell wall components; and (5) pronounced morphological changes. The latter response termed "shmooing" has provided the most widely exploited assay for mating factor activity. Purification of the activity from culture supernatants of *MAT*α haploids in

the early 1970s[8,11-14] revealed that the mating pheromone which affected *a*-haploids was a tridecapeptide with the following structure:

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

This molecule was termed α-factor and is the primary subject of this review. During the determination of the structure of α-factor, peptides devoid of the N-terminal Trp and/or containing methionine sulfoxide in place of methionine were also recovered from the culture medium.[12,15] It is highly likely that these additional peptides are artifacts of the purification procedure.

Culture supernatants from *MATa* haploids elicit responses from *MATα* cells which are similar to those triggered in *a*-cells by the α-factor. Significantly less is known about the corresponding *a*-factor although it also appears to be a low molecular weight peptide (see below). Most importantly the α-factor appears to contain the information for all of the activities associated with mating which are found in α-culture supernatants. Since it is a small molecule which is amenable to chemical modification and derivatization, it affords the biochemist an ideal opportunity for studying the relationship between primary structure and biological activity.

IV. SYNTHESIS OF α-FACTOR BY SOLUTION PHASE PROCEDURES

The α-factor has been synthesized using both solution phase[16-18] and solid phase[19] procedures. In addition, both the desTrp^1-dodecapeptide α-factor[16,19,20] and analogues of the tridecapeptide and dodecapeptide pheromone have been prepared.[18,20-22] The strategy employed for solution phase synthesis involved the stepwise synthesis of fragments and coupling at the Pro^{11}-Met^{12} linkage and the Pro^8-Gly^9 linkage,[16,20] or at the Gly^9-Gln^{10} linkage.[17] The majority of the α-factor analogues were synthesized by the Japanese group. Unfortunately, no yields were reported for these syntheses. However, it should be noted that despite a careful choice of protecting groups and the use of scavengers during acidolysis, extensive purification was required to obtain homogeneous product. Another solution phase synthesis of α-factor reported an 11% overall yield but no details were given on purification procedures.[17] The solution phase synthesis of the desTrp^1-dodecapeptide was accomplished in 13% overall yield and a one step high performance liquid chromatography (HPLC) purification was sufficient to give homogeneous product.[20] Significantly, during the latter synthesis, fragments were built up using repetitive excess mixed anhydride coupling with no purification of intermediate fragments. This method minimizes losses and accelerates the pace at which final product can be obtained.

V. SYNTHESIS OF α-FACTOR BY SOLID-PHASE PROCEDURES

The only solid phase synthesis of α-factor reported in the literature resulted in very low overall yields (1 to 2%).[19] Such yields reflect improper application of the Merrifield technique and probably were due to the use of benzyloxycarbonyl protecting group on the epsilon amine of lysine, the failure to protect tryptophan, and the absence of the proper scavengers in most acidolysis steps. Recently, we have attempted the solid phase synthesis of α-factor using a classical Merrifield resin. The 2-chlorobenzyloxycarbonyl group was used to protect the lysine side chain, formyl protection was used for Trp, 2,6-dichlorobenzyl for Tyr, toluenesulfonyl for His, dimethyl sulfide was used as a scavenger during acidolysis, and cleavage was accomplished by the low-high procedure.[23] An overall yield of 25 to 30% was achieved and the product was greater than 98% pure on HPLC. Even higher yields should be obtainable using a phenylacetomidomethyl (PAM) resin.

Obviously, when attempting to prepare a variety of analogues for structure-activity studies the solid phase method is advantageous. Our results indicate that this method can be efficiently applied to obtain 100 mg quantities of pure α-factor if care is taken in the procedure. The authors note that the solid phase procedure is now being routinely utilized in numerous laboratories. The simplicity and elegance of this method permits biochemists, unskilled in the art of organic synthesis to prepare peptides for biological evaluation. In contrast, solution phase peptide synthesis is complicated by poor fragment solubility, and decreasing coupling efficiencies with increased peptide chain length; it is the province of highly skilled synthetic chemists. Despite the apparent advantages of the solid phase procedure, its misuse can lead to impure peptides and misleading biological results. Proper side chain protection, correct choice of scavengers and cleavage conditions, and rigorous purification are essential to obtain peptides which will give unambiguous biochemical results.

VI. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF α-FACTOR

The efficient synthesis of α-factor analogues requires procedures to purify the pheromone. Early synthetic strategies employed methods of purification developed for isolation of the pheromone from fermentation broths. Specifically, various combinations of ion exchange chromatography, gel filtration chromatography, and partition chromatography have been reported.[11,16,19] Such procedures are laborious and lengthen the time necessary for analogue preparation.

In contrast to the above strategies, HPLC has been applied to the purification of α-factor in a one-step procedure.[18,24] Crude peptide is directly applied to a C_{18}-reversed phase column and eluted with a methanol:water:trifluoroacetic acid mobile phase. In most cases a step gradient is used to remove impurities and homogeneous product is obtained on a 50 to 300 mg scale. Studies have shown that the trifluoroacetic acid is a critical component of the mobile phase in that it markedly influences the K′ values of peptides. Moreover, it behaves as a lipophilic ion pair and is readily removed from the final product by lyophilization. In our laboratories we have prepared α-factor and approximately 30 analogues using this methodology. The synthetic peptides have high biological activity and are obtained, after lyophilization, as fluffy white trifluoroacetate salts. In contrast to reports on the insolubilty of α-factor,[6,11] we find that such salts are readily dissolved in aqueous buffers and alcohols and remain stable in aqueous solution for months. Moreover, freeze-dried α-factor obtained in this manner is stable in the solid state for several years.

The mobility of various α-factor analogues correlates well with the hydrophobicity of the peptide. In particular, position 3, 6, 7, and 9 analogues exhibit K′ values which directly reflect the length of the aliphatic side chains. We have also observed that diastereomeric α-factor analogues are well separated on C_{18}-reversed-phase chromatography suggesting that this method should be useful in monitoring racemization during peptide synthesis. Several other laboratories have reported that reversed-phase chromatography distinguishes diastereomeric peptides.[25,26] The resolving power of this technique is illustrated in the base line separation found during gradient elution of desTrp^1,Cha^3, L-X^9- and desTrp^1,Cha^3,D-X^9-dodecapeptides (Figure 2).

VII. STRUCTURE-ACTIVITY RELATIONSHIPS FOR THE α-FACTOR

Most of the studies on the relationship between the structure of α-factor and its function have come from our laboratory and from the laboratory of Masui. These laboratories have concentrated their efforts on the dodecapeptide and tridecapeptide pheromones, respectively. In reviewing this work it is important to note that most structure-activity relationships refer to the ability of the pheromone to induce changes in cell shape (''shmoos''). The absolute

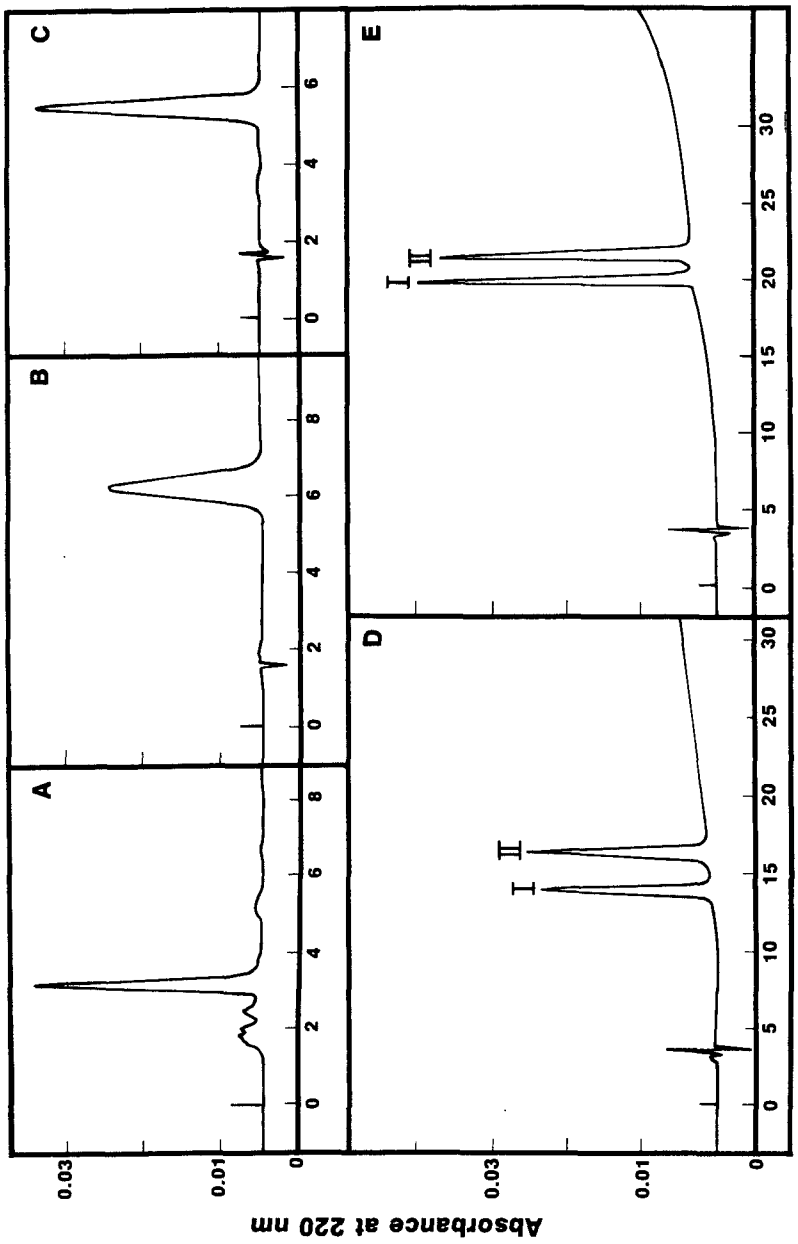

FIGURE 2. HPLC of desTrp¹,Cha³,X⁹-α-factors on a reversed-phase C_{18} column. Panel A — crude desTrp¹,Cha³,D-Leu⁷-α-factor before purification. Mobile phase $CH_3OH:H_2O:CF_3COOH$ (600:400:0.25). Panels B,C — desTrp¹,Cha³,D-Leu⁹-α-factor after purification. Panel B — mobile phase — $CH_3OH:H_2O:CF_3COOH$ (500:500:0.25). Panel C — mobile phase — $CH_3CN:H_2O:CF_3COOH$ (280:720:0.25). Panel D — Gradient elution of a mixture of (I)-desTrp¹,Cha³,L-Ala⁹- and (II)-desTrp¹,Cha³,D-Ala⁹-dodecapeptides. Gradient from 21 to 36% CH_3CN. Panel E — Gradient elution of mixture of (I)-desTrp¹,Cha³,L-Leu⁷- and (II)-desTrp¹,Cha³,D-Leu⁷-dodecapeptides. Gradient from 21 to 36% CH_3CN. (Reprinted with permission from Biological Activity and Conformational Isomerism in Position 9 Analogs of the desTrp¹,Cha³-α-Factor from *S. cerevisiae*. *Biochemistry*, 24, 7070, 1985.)

activity of α-factor and its analogues, in terms of effect of peptide concentration on cellular activities, varies according to the assay procedure used, the *MATa* target cell tested, the temperature and pH of assay, and the number of yeast cells measured. These factors must be taken into account in comparing the activity of α-factor analogues among various literature reports. For example, the Japanese group reported that their synthetic or biologically produced α-factor induced shmoos at a concentration of 6 pg/mℓ.[16] This activity is three orders of magnitude higher than that reported by any other group working with α-factor. Although the activity of α-factor is dependent on the factors mentioned above, none of these appears to explain the high activities of the peptides produced by the Japanese group. In fact, a very careful analysis of α-factor activity under conditions which eliminated pheromone degradation reported that biologically produced pheromone induced half maximal shmoo formation of *S. cerevisiae* 381G at $1.4 \times 10^{-8} M$ (~ 20 ng/mℓ).[27] Due to these discrepancies, when evaluating the results of the Japanese group, we will assume only that they are internally consistent and will comment on the reported activities relative to their own standards.

There is conclusive evidence that synthetic α-factor is identical to and represents the total activity of biosynthesized or "natural" α-factor. The primary sequence of α-factor has been verified by comparison to the nucleotide sequence of the gene coding for α-factor.[28] No posttranslational modifications of the α-factor sequence have been reported and the measured chemical and physical properties of totally synthetic and biosynthesized α-factor are identical.[87] Differences in activity of natural and synthetic α-factor reflect different assay procedures and conditions idiosyncratic to the particular laboratory in which the biological activity is measured.

VIII. RELATIONSHIP BETWEEN PHERMONE LENGTH AND ACTIVITY

The tripdecapeptide α-factor and the dodecapeptide lacking the N-terminal tryptophan (des-Trp^1-α-factor) were reported to have equal activity in the shmoo assay.[15,16,19] In contrast, using the minimum concentration of pheromone causing change in morphology, Shenbagamurthi et al.[18] reported that the tridecapeptide was 16 times more potent than the homologous dodecapeptide. Nevertheless, both the 13- and 12-peptides have activities in the nanogram per milliliter range. In comparison, removal of any other residue markedly lowers the activity of the resulting peptide; the des-Tyr^{13}-α-factor results in activity lowered by 10^3 and removal of His from the 12-peptide, des(Trp^{1}His^2)-α-factor, lowers activity by 10^8.[16] A variety of fragments containing partial sequences of the peptide contain less than one millionth of the activity of the native structures.[16] Moreover, the Trp^1-Leu^6 and Lys^7-Tyr^{13} segments have virtually no activity[29,30,87] This latter finding is highly significant since *a*-cells of *S. cerevisiae* contain a surface-associated peptidase which hydrolyzes α-factor by cleavage of the Leu^6-Lys^7 peptide bond.[29,31] The inactivity of the resulting fragments suggests that intact α-factor is responsible for all biological responses tested and that degradation of α-factor allows *a*-cells to recover from the cellular control promulgated by the pheromone.

In contrast to the low activity of partial sequences of the α-factor, synthetic oligopeptides lacking pheromone activity were found to stimulate α-factor potency. Specifically, whereas $8 \times 10^{-5} M$ of the N-terminal pentapeptide, His-Trp-Leu-Gln-Leu had no effect on cell cycle arrest, this concentration of the pentapeptide increased growth inhibition by α-factor from 1.2 to 3.9 hr.[32] Similar results were found with Trp-Leu-Gln-Leu, but the Pro-Gly-Gln-Pro-OMe, a tetrapeptide mimicking the sequence near the C-terminus of α-factor, had no effect. The increased growth inhibition in the presence of approximately a 1000-fold excess of the N-terminal tetra- or pentapeptide was proposed to result either from saturation of nonspecific α-factor binding sites on the yeast cell surface leading to higher effective α-factor concentrations or from interference with processes that might inactivate the native pheromone. Recently, using an HPLC assay, we found that neither the tetra- nor pentapeptide

when present in tenfold molar excess inhibit hydrolysis of α-factor by *MATa* cells.[87] Thus it is unlikely that the results observed by Stotzler et al.,[32] reflect an influence of the peptide fragments on α-factor degradation.

IX. ACTIVITY OF α-FACTOR DERIVATIVES

Derivatives of peptide hormones can prove especially valuable in both purification and isolation of receptors and in attempts to localize receptors on the cell surface. A prerequisite for such biochemical studies is knowledge of the activity of covalently modified peptides. Most derivatives of the α-factor were reported to have markedly reduced activity. Early studies indicated that the intact pheromone with an oxidized sulfur atom maintained a high percentage of its activity.[15] In contrast esterification of tyrosine resulted in a 1000-fold drop in activity[6] and conversion of the carboxyl terminus to a carboxamide resulted in a 10^4 loss in potency.[21] These findings are in agreement with a study where α-factor was attached to poly(ethylene glycol) as a carboxyl terminal protecting group.[18] The peptide-polymer conjugate had no measurable activity. One can conclude, therefore, that modification of the tyrosine carboxyl drastically reduces activity.

Two groups have reported on the activity of α-factor iodinated on tyrosine.[31,33] Although claims were made for retention of high biological activity, a number of discrepancies appear in these studies. Manness and Edelman[33] claimed that both α-factor and the iodinated pheromone comigrate, but present no proof for completeness of the iodination reaction. Furthermore, they reported that iodination by the chloramine-T procedure resulted in complete inactivation. Lipke[34] reported that chloramine T iodinated α-factor comigrated on TLC with unmodified pheromone, but was unable to demonstrate a cell-specific binding. In contrast, Finkelstein and Strausberg[31] used the chloramine-T procedure to prepare iodinated α-factor in yields of 8 to 30% and reported a product with 20 to 25% of the original pheromone activity. Based on these results it is not clear whether the iodinated pheromone is active and prudence would suggest other routes for preparing radiolabeled pheromone.

Derivatization of the α- or ε-amino groups of α-factor affords the biochemist direct routes for insertion of fluorescent groups or attachment of α-factor to affinity chromatography supports. Again literature reports on the importance of these amino groups for biological activity are contradictory. After reaction of α-factor with fluorescein isothiocyanate the fluorescent derivative was reported to have the same biological activity as the natural pheromone.[30] In contrast, *N*-acetylation of α-factor using acetic anhydride resulted in an inactive pheromone.[34] Thorner[7] has reported that fluorescein isothiocyanate (FITC)-α-factor is not biologically active. The problem with all of these studies is that the modified pheromone was not chemically characterized and that modification was carried out under conditions which are expected to derivatize more than one functional group on the α-factor. desTrp^1 α-factor modified on the ε-amine with a dansyl or biotinyl group and a number of acylated lysine derivatives of desTrp^1,Cha^3,-α-factor were reported to maintain significant biological activity (Table 1).[18,35] All of these acylated lysine analogues were synthesized by strategies which insured reaction only at this position. Furthermore, the derivatives were purified to homogeneity on HPLC and were characterized using FT/NMR and amino acid analysis. These studies conclusively show that the ε-amine of lysine in the dodecapeptide α-factor can be acylated without abolition of activity.

Recently, we have synthesized *N*-ε-FITC Lys^7-α-factor and found it to maintain high biological activity. The ε-amino group of both dodecapeptide and tridecapeptides can therefore be used as an attachment point for a broad variety of haptens and chromophores. Although the evidence for modification of the α-amine is based on only one derivative, desTrp^1,(αDns)His^2,Cha^3-α-factor, it appears that a free α-amine is also not essential for activity. This conclusion is supported by biosynthetic studies on α-factor (see below) and

Table 1
BIOLOGICAL ACTIVITIES OF $N(\epsilon)$ ACYLATED α-FACTOR ANALOGUES

Peptide	Activity (ng/mℓ)
des-Trp[1]-Cha[3]-α-factor	250[a]
des-Trp[1],Cha[3],Lys[7](Bio)-α-factor[c]	3,300[a]
des-Trp[1],Lys[7](Dns-Gly)-α-factor[c]	800[a]
des-Trp[1],Cha[3],Lys[7](Ac)-α-factor[c]	1,000[a]
des-Trp[1],Cha[3],Lys[7](Bu)-α-factor[c]	800[b]
des-Trp[1],Cha[3],Lys[7](Oc)-α-factor[c]	800[b]
des-Trp[1],Cha[3],Lys[7](La)-α-factor[c]	6,000[b]
des-Trp[1],Cha[3],Lys[7](St)-α-factor[c]	> 50,000[b]

[a] Taken from Reference 18.
[b] Taken from Reference 35.
[c] Abbreviations used: Ac, acetyl; Bio, biotinyl; Bu, butanoyl; Dns, dansyl; La, lauryl; Oc, octanoyl; St, stearyl.

a report that α-factor containing an additional Trp at the N-terminus and Arg in place of Lys[7] retained high activity.[36] However, given the dearth of experimental studies, additional derviatives of the α-amine terminus must be examined before generalizations can be made.

X. AMINO ACID SUBSTITUTIONS IN THE TRIDECAPEPTIDE α-FACTOR

Masui et al.[21] reported on the morphogenic activities of some 30 analogues of the tridecapeptide α-factor. Substitutions were made in positions 1, 2, 3, 6, 7, 8, 11, 12, and 13 of the peptide backbone. In every case examined the α-factor analogue was less active than the parent pheromone and in more than two thirds of the derivatives, activity decreased a minimum of 10,000-fold. The histidine in position 2 was found to be extremely important for activity. Substitution of this residue by basic (Lys), hydrophobic (Phe,Leu), or enantiomeric (D-His) amino acids resulted in at least a 100,000-fold loss in activity. In contrast to the importance of His[2], replacement of Trp[1] or Trp[3] by certain hydrophobic residues resulted in only a 10- to 100-fold decrease in activity. Specifically, replacement of Trp[1] by Phe led to only an order of magnitude drop in potency. To some extent this result is predictable in view of the high activity reported for the desTrp[1]-α-factor. However, it is important to emphasize that replacement of Trp[1] by Tyr resulted in a 2500-fold decrease in activity and replacement by Lys leads to a pheromone which was virtually inactive. Thus, despite the activity of dodecapeptide-α-factors, only certain residues are well tolerated at position 1.

Replacement of Trp[3] with Ala,Leu,Phe, or Tyr resulted in similar reductions (~ 50-fold) in potency. However, insertion of basic, neutral, or acidic residues at position 3 resulted in at least a 10^4 loss of activity. It appears that a minimum hydrophobicity at position 3, and perhaps at the amine terminus of the peptide, is required to have a highly active pheromone. Studies on the dodecapeptide pheromone suggest that position 3 may be extremely critical for triggering projection formation and increased agglutination in *MATa* cells.

Of the other α-factor analogues prepared by the Japanese group only the Nle[12]-α-factor retained significant potency. The biological activity of Nle[12]-α-factor is quite reasonable in view of the high activity of the tridecapeptide and dodecapeptide with methionine sulfoxide in place of methionine.[12] Apparently, a nucleophilic sulfur atom is not required at position 12 and Nle is isosteric with Met.

In addition to amino acid replacements Masui et al.[21] also evaluated the insertion of D

residues at various positions. Analogues with D-Leu[6] or D-Lys[7] had very little activity. However, these analogues were highly stable against degradation by *MATa* cells and retained their low activities for long periods after incubation with yeast cultures. A position 7 analogue prepared by Samokhin et al.[17] (Arg[7]-α-factor) had activity identical to the pheromone with the native sequence. This supports the data that the ϵ-NH_2 group of Lys[7] is not essential for activity. Although one could argue that a positive charge is required in the position 7 side chain, studies on α-factor derivatives and dodecapeptide analogues (see below) suggest that the position 7 side chain is not critical for pheromone activity.

XI. AMINO ACID SUBSTITUTIONS IN THE DESTRP[1]-DODECAPEPTIDE α-FACTOR

Normally, the dodecapeptide α-factor would be considered as an analogue of the naturally occurring pheromone. As stated above, virtually all analogues of the dodecapeptide have been prepared and characterized by our laboratory, whereas analogues of tridecapeptide-α-factor were studied primarily by the Japanese. In view of the large difference in activity reported by the Japanese for their α-factor in comparison to all other literature reports (see above), we have decided to discuss the dodecapeptide α-factor for as separate pheromone. However, to be consistent in nomenclature, we will consider the tridecapeptide α-factor as the parent compound.

At present 16 analogues of the dodecapeptide α-factor (desTrp[1]-α-factor) have been synthesized in our laboratory. Amino acid replacements in the dodecapeptide were made in positions 3, 6, 7, 8, 9, and 12. In addition, Masui et al.[21] synthesized two diastereomeric desTrp[1]-α-factors containing D residues at positions 6 and 7. Both derivatives had less than one ten thousandth of the activity of parent peptide. In contrast to the findings with tridecapeptides, the dodecapeptide analogues examined by us showed a much smaller variation in morphogenic potency. In fact, for most active analogues, the lowest concentration causing morphogenesis was within one order of magnitude of the parent desTrp[1]-dodecapeptide (Table 2). In addition, several analogues of the dodecapeptide were actually superior pheromones compared with the parent sequence. Specifically, the desTrp[1],Cha[3]-α-factor caused shmoo formation at approximately one third the concentration of desTrp[1]-α-factor. Thus, replacing Trp[3] by Cha (β-cyclohexylalanine) in the desTrp[1]-dodecapeptide is well accepted by the receptor.

Our results suggest that position 3 is especially important for the morphogenic response. Both the desTrp[1],Ala[3]- and desTrp[1],Phe[3]-analogues were devoid of activity. This shows that in the dodecapeptide the nature of the position 3 side chain is critical in inducing morphological changes in *MATa* cells. It is especially interesting that the Ala[3]- and Phe[3]-analogues of the tridecapeptide α-factor were among the most potent variants of the tridecapeptide.[21] Clearly, this points to different structure-activity relationships in the dodecapeptide and tridecapeptide pheromones. Moreover, the amine terminus of α-factor is hydrophobic and Cha is more hydrophobic than Phe and Trp as judged by retention times on reversed-phase HPLC columns.[88] It is possible, therefore, that a minimum overall hydrophobicity at the amine terminus is required before pheromone activity is manifested. However, benzene and cyclohexane have rather similar solubility properties and we believe that specific steric interactions of the position 3 side chain with the α-factor receptor are responsible for the observed differences.

The high biological activity found for the desTrp[1],Cha[3]-α-factor was important for conformational analysis of the pheromone. Tryptophan absorbs in both the near and far ultraviolet and often complicates interpretation of circular dichroism spectra of short peptides. Furthermore, tryptophan has aromatic proton resonances which often overlap amide NH protons. Thus, removal of Trp was expected to simplify both the circular dichroism (CD) and ^1H

Table 2
BIOLOGICAL ACTIVITIES OF DES-TRP^1-α-FACTOR ANALOGUES

Peptide	Morphogenesis (ng/mℓ)	Agglutination (ng/mℓ)
des-Trp^1-α-factor	800[a]	8[d]
des-Trp^1,Cha^3-α-factor	250[a]	35[d]
des-Trp^1,Ala^3-α-factor	$> 5 \times 10^{6}$[b]	$> 5 \times 10^{5}$
des-Trp^1,Phe^3-α-factor	$> 5 \times 10^{6}$[a]	$> 5 \times 10^{5}$
des-Trp^1,Cha^3,Ala^6-α-factor	$>5 \times 10^{6}$[d]	$> 5 \times 10^{5}$[d]
des-Trp^1,Cha^3,Val^6-α-factor	2,650[d]	12,000[d]
des-Trp^1,Cha^3,Ile^6-α-factor	1,500[d]	5,200[d]
des-Trp^1,Cha^3,Nle^6-α-factor	270[d]	1,200[d]
des-Trp^1,Cha^3,D-Leu^6-α-factor	$> 5 \times 10^{6}$[d]	$> 5 \times 10^{5}$[d]
des-Trp^1,Cha^3,Orn^7-α-factor	2,900[a]	90[c]
des-Trp^1,Cha^3,Nle^7-α-factor	2,700[c]	1,500[c]
des-Trp^1,Cha^3,Nle^{12}-α-factor	250[a]	—

[a] Taken from Reference 18.
[b] Taken from Reference 20.
[c] Taken from Reference 48.
[d] Taken from Reference 22.

nmr spectra of α-factor. Based on these considerations we decided to use the desTrp^1,Cha^3-α-factor as the parent compound for further structure-activity studies on the dodecapeptide pheromone.

The peptidase which degrades α-factor in yeast culture catalyzes hydrolysis of the Leu^6-Lys^7 peptide bond.[29,87] It was of interest therefore to examine the biological activity and susceptibility to hydrolysis of pheromones with replacements at these positions. As seen in Table 2, substitution of Ala for Leu^6 resulted in an inactive pheromone. Activity returns as the size of the position 6 side chain is increased from a methyl to an isopropyl group, although the desTrp^1,Cha^3,Val^6-pheromone is still ten times less active than the des-Trp^1,Cha^3,Leu^6-dodecapeptide. Morphogenesis activity of analogues with four carbons in the position 6 side chain is dependent on the chirality and the position of branching. The Ile^6-analogue is sixfold less active than the parent compound whereas the Nle^6-pheromone has equal activity. In contrast, the desTrp^1,Cha^3,D-Leu^6-α-factor has no activity up to 500 μg/mℓ. Thus it appears that a minimum hydrophobicity or steric bulkiness is required at the position 6 side chain and β-branching lowers pheromone potency in the shmoo assay. Interestingly, there is no simple correlation between pheromone potency and degradation of the pheromone (see below).

As mentioned earlier our studies on derviatives of the desTrp^1,Cha^3-α-factor suggested that a free ε-amine is not neccessary for activity. Examination of analogues of the dodecapeptide α-factor support this conclusion. We found that both the desTrp^1,Cha^3,Orn^7-, and the desTrp^1,Cha^3,Nle^7-α-factor were effective pheromones which had one tenth the activity of their parent compound. Since the Nle^7-analogue lacks a nitrogen atom in the side chain the activity of this pheromone is unequivocal evidence that the ε-NH_2 of lysine is not essential.

Proton NMR analysis on the α-factor prompted Higashijima et al.[37] to suggest that the pheromone contains 3-β-turns. We decided to study the Pro^8-Gly^9 sequence and have prepared analogues containing AIB^8, L-Ala^9, D-Ala^9, L-Leu^9 in the desTrp^1,Cha^3-dodecapeptide. For the position 9 analogues an interesting picture is beginning to evolve. As seen in Figure 3, comparison of the morphogenic activity of the L-Ala^9- and D-Ala^9-pheromones shows that the D-Ala^9-analogue has high activity whereas the L-Ala^9-mating factor analogue is at least 200-fold less active. A similar trend was observed for the L-Leu^9 and D-Leu^9 dode-

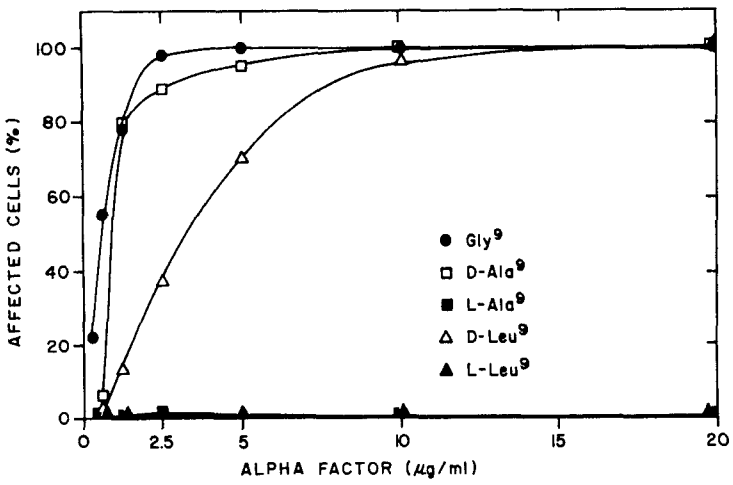

FIGURE 3. Morphogenic activity of desTrp[1],Cha[3],X[9]-α-factors. The X[9] residue is indicated in the figure.

capeptides although the D-Leu[9] containing pheromone is two to five times less active than the Gly[9]- or D-Ala[9]-peptides. Thus a Pro[8]-Gly[9] or Pro[8]-*D*-X[9] sequence is well tolerated whereas a Pro-L-X[9] sequence severely reduces pheromone potency. A Pro-Gly sequence is very common in Type II-β-turns[38,39] and theoretical considerations[40] suggest that a Type II turn can tolerate D residues but not L-residues at the i + 2 position. Our structure-activity studies, therefore, indicated that a Type II β-turn involving Pro[8]-Gly[9] may be an important feature of the biologically active conformation of α-factor (also see below).

Replacement of Met[12] with the isosteric Nle did not affect pheromone potency in the desTrp[1],Cha[3]-dodecapeptide. The Nle[12] analogue of the tridecapeptide was among the most active pheromones reported by Masui et al.[21] Despite the lack of influence on pheromone potency replacement of Met by Nle was observed to markedly affect the degradation rate of the α-factor (Reference 21 and see Section XVI). At the present time we can offer no complete argument to explain why this substitution at position 12 would greatly affect the scission of the peptide bond between residues six and seven. Perhaps the secondary structure of the peptide juxtaposes position 12 atoms with atoms of residues six and/or seven. In any case, different structure activity relationships are indicated for pheromone potency and for the peptidase which processes the α-factor.

XII. STRUCTURE-ACTIVITY RELATIONSHIPS FOR AGGLUTINATION

In addition to eliciting changes in cell shape, α-factor also induces the synthesis of surface glycoproteins which increase the sexual agglutinability of *MATa* haploids, but no structure-activity relationships have been reported for induction of sexual agglutination using tridecapeptide analogues.[41-47] A number of laboratories have reported that the concentration of α-factor necessary to induce increased agglutination is significantly lower than that necessary to induce projection formation. In the most carefully controlled study reported to date, half-maximal projection formation was induced at 1.4×10^{-8} *M* α-factor with *S. cerevisiae* 381G.[27] Using the same strain the half-maximal response for agglutination occurred at 1×10^{-10} *M* pheromone. Similarly, with strain X2180-1A we found that morphogenesis required 140-fold higher concentration of α-factor than that concentration needed to induce agglutinability.[48] Given these differences we thought it would be valuable to examine structure-activity relationships for agglutination induction using our dodecapeptide analogues.

As seen in Table 2 all analogues that we tested which were active in the shmoo assay were also capable of inducing increased agglutinability. In most cases the dodecapeptide α-factor analogues were better inducers of agglutination than they were inducers of shmoo formation. However, several position 6 analogues were three-to-fivefold better morphogens than inducers of agglutinability. The ratio of the concentration of pheromone inducing shmoo formation to that inducing a half maximal increase in agglutinability varied by a factor of 500 for the dodecapeptide α-factor. Although some of the analogues tested are rather poor pheromones and their ability to induce mating between *MATa* and *MAT*α strains has not been determined, the above results reflect different structure-activity relationships for α-factor in inducing morphogenesis as compared to agglutination. The position 6 side chain appears to be especially important in the agglutination response. Activity decreased more than 300-fold when the "natural" leucine[6] was replaced by valine in the desTrp[1],Cha[3]-α-factor. Even substitution with Nle[6], a change which did not affect morphogenesis activity, caused a 35-fold decrease in pheromone potency as judged by the agglutination assay. Although additional work is necessary, we believe the different structure-activity relationships provide evidence for more than one class of α-factor receptor.[48]

XIII. α-FACTOR ANTAGONISTS

Important tools in the arsenal of the hormone biochemist are molecules which bind to the hormone receptor but do not trigger a biological response. Such molecules, commonly called antagonists, allow the hormonologist to dissect hormone activity into a variety of stages such as binding to receptor, triggering, release of secondary messenger, etc., and thereby aid in building models of activity. The observation of α-factor analogues devoid of pheromone activity permitted us to examine the possibility that some of these were α-factor antagonists.

Both desTrp[1],Phe[3]- and desTrp[1],Ala[3]-α-factor prevented the induction of morphogenesis and increased agglutinability by the natural α-factor and the desTrp[1],Cha[3]-dodecapeptide.[18,48] Competition was observed at relatively low concentrations, and the desTrp[1],Phe[3]-pheromone eliminated induction of agglutination when present in the growth medium at a concentration equimolar to that of the desTrp[1],Cha[3]-α-factor. Competition with the tridecapeptide α-factor required a significantly higher concentration of the antagonist. In contrast to the position 3 analogues, two inactive position 6 analogues, desTrp[1],Cha[3],Ala[6]- and desTrp[1],Cha[3],D-Leu[6]-α-factor did not compete with the desTrp[1],Cha[3]-dodecapeptide at concentration ratios as high as 500:1. These competition studies confirm the conclusion that the Ala[3]- and Phe[3]-analogues of the dodecapeptide are inactive and rule out the possibility that they have low activity which would be manifested at very high concentration.

The failure of certain inactive analogues to compete suggest that only certain modified α-factors can bind to the α-factor receptor without triggering a response. A corollary of this conclusion is that, as previously mentioned, the position 3 residue appears to be critical for the α-factor response. Finally, the competition results give additional evidence that the pleiotropic responses of *S. cerevisiae* to α-factor reflect more than one class of α-factor receptor. Specifically, competition studies have shown that one can prevent induction of agglutination under conditions where shmoo formation still occurs even though agglutination is usually induced by concentrations of α-factor several orders of magnitude less than those required for shmoo formation.[48] Thus one pheromone is capable of eliciting projection formation without inducing increased agglutinability, or of inducing agglutinability without affecting morphology. Studies on α-factor degradation preclude differential proteolysis of the pheromone as the explanation for these data.

To date the desTrp[1],Ala[3]- and desTrp[1],Phe[3]-dodecapeptides are the only known antagonists of α-factor activity. We use them routinely in assessing the specificity of binding to the receptor. For example, in studying binding of fluorescent or chromogenic pheromones to

yeast cells, it is important to relate activity to cell-associated fluorescence. Competition with the above antagonists is extremely valuable in discerning whether the pheromone is truly interacting with the receptor.

XIV. NATURE'S STRUCTURAL ANALOGUES

Two α-factor structural genes, designated *MFα1* and *MFα2*, have been identified by cloning.[28,49] The *MFα1* gene was identified in cells overproducing α-factors due to the presence of a high copy number plasmid containing *MFα1*.[28] Synthetic oligonucleotide probes predicted to be homologous to α-factor coding sequences were used to identify the *MFα2* gene.[49] The roles of *MFα1* and *MFα2* were investigated further by Kurjan,[50] who showed that at least one of the genes must be functional for mating by *MAT*α cells.

The *MFα1* gene contains four identical copies of the α-factor sequence, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr, whereas the *MFα2* precursor contains two non-identical α-factor copies: one copy of the above sequence and another copy in which residue five is Asn (in place of Gln) and residue seven is Arg (in place of Lys). The two α-factor sequences result in a pheromone with equal biological activity.[89] No biologically isolated α-factor with Asn in position 5 and Arg in position 7 has been reported, although Singh et al.,[49] and Kurjan[50] reported that the *MFα2* gene is functional. This α-factor species would constitute only 17% of the pheromone molecules if both genes were equally expressed and processed. Furthermore, it appears that the *MFα1* message may be present at much higher levels in *MAT*α cells than the *MFα2* message.[50] Finally, our studies indicate that the normal α-factor sequence and the Asn^5, Arg^7 modificant almost comigrate during gradient HPLC.[87] It is possible, therefore, that the Asn^5,Arg^7-α-factor is secreted but has not been detected.

The finding of multiple copies of the same, or nearly the same, oligopeptide in a precursor to the mature α-factor is not unique to *S. cerevisiae*. Mammalian precursors to melanocortin and dynorphin contain multiple copies destined for secretion.[51,52] The mammalian enkephalin precursor contains six methionyl enkephalin sequences and one leucyl enkephalin sequence.[53] Why cells produce multiple copies of a pheromone from one precursor molecule is an unanswered question. Two hypotheses offered to explain this phenomenon are that multiple copies allow more efficient secretion,[28] and/or that redundant copies free the duplicated sequence to mutate to acquire different functional and/or structural domains.[54]

Other *Saccharomyces* species also contain α-factor structural genes with tandemly arrayed spacers and peptide pheromone coding regions.[55] *S. oviformis* appears to have five repeats, whereas *S. norbensis* contains four repeats, and *S. carlsbergensis* and *S. diastaticus* have only three repeats. All of these species are taxonomically related as evidenced by their ability to interbreed. The structures of the pheromones of these different species have not been ascertained. Based on the structure-activity relationships in the tridecapeptide α-factor of *S. cerevisiae* (see above), we would not expect major variations in the size and amino acid sequence of pheromones from interbreeding species.

S. kluyveri will not conjugate with *S. cerevisiae*.[56] This lack of interbreeding is reflected in the structure of the *S. kluyveri* α-pheromone: Trp-His-Trp-Leu-Ser-Phe-Ser-Lys-Gly-Glu-Pro-Met-Tyr.[57] Significant changes are found in the *S. kluyveri* pheromone at residues 5, 6, 7, 8, and 10 in which Gln^5,Leu^6,Lys^7,Pro^8, and Gln^{10} of *S. cerevisiae* have been replaced in *S. kluyveri* by Ser^5,Phe^6,Ser^7,Lys^8, and Glu^{10}. Despite these major differences the *S. kluyveri* pheromone is required at only 10- to 100-fold higher concentrations than *S. cerevisiae* α-factor to induce agglutinability of *S. cerevisiae MATa* cells. McCollough and Herskowitz[56] also noted some physiological cross reactions of the unpurified pheromones from *S. cerevisiae* and *S. kluyveri*.

Not only do peptide pheromones of similar structure exist in other *Saccharomyces* species, but the α-factor, and perhaps the *a*-factor, is structurally similar to peptide pheromones and

hormones of other organisms. The findings that prokaryotes, unicellular eukaryotes, and invertebrates produce peptides with structural or conformational similarity to mammalian hormones has led to much speculation about the evolutionary origins of hormones.[58-60] Alpha-factor is structurally related to the mammalian GnRH, gonadotropin-releasing hromone or gonadoliberin, also known as LHRH, luteinizing hormone releasing hormone.[61] The structure of GnRH is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 which is homologous to residues 2, 3, 6, 7, 8, and 9 of α-factor. In binding assays Loumaye et al.[60] demonstrated that α-factor was a low-affinity ligand, 9000-fold less active than native GnRH, for the rat pituitary GnRH receptor, and that α-factor was 10,000-fold less effective than GnRH for release of luteinizing hormone from rat pituitary cells. Whether this apparent functional relationship denotes some evolutinary relationship is not known. Nevertheless, these data do indicate again that the study of the α-factor and its receptor should yield important information relevant to our understanding or peptide hormones of mammalian cells.

XV. BIOSYNTHESIS OF α-FACTOR

Many mammalian peptides are biosynthesized in the form of large polypeptides from which the biologically active message is ultimately processed.[62] As stated above, two laboratories have cloned genes which code for the α-factor.[28,49] These genes termed *MFα1* and *MFα2* code for polypeptides containing 165 amino acid residues and 120 amino acid residues, respectively. The N-terminus of both α-factor precursor polypeptides is decidedly hydrophobic and mimics signal sequences described in a variety of secretory proteins.[51,52] In addition, three potential glycosylation sites, Asn-X-Thr[63], are observed on the amino end of the polypeptides from *MFα1* and *MFα2*.[28,49] The amino acid residues flanking the α-factor-like sequences in the precursor start with a Lys-Arg sequence in both genes, a site readily cleaved by trypsin-like proteases. Such sites have been found in a number of polypeptide hormone precursors such as proinsulin[62] and proenkephalin.[64] To this point the similarity between the α-factor biosynthetic genes and those described for mammalian peptide hormones allows one to conclude that the pheromones are synthesized as prepropoly-α-factor. Indeed, a high molecular weight α-factor precursor was found to be synthesized in vivo.[65] It has a molecular weight of 28,000 and is *N*-glycosylated. The unglycosylated molecule has an Mr = 18,000.

Several differences exist between the processing of most mammalian peptide hormones and α-factor. In α-factor, cleavage at the Lys-Arg sequence does not generate the native α-factor structure as is the case with enkephalin and the neuropeptides. Instead, the biosynthetic product is cleaved by proteinase yscF leaving precursors flanked on the carboxyl terminus by Lys-Arg and on the amine terminus by tetra- or hexapeptide sequences with a Glu-Ala or Asp-Ala repeat. The flanking Lys-Arg is removed by carboxypeptidase yscα and the amine terminus is removed by a dipeptidylaminopeptidase activity to generate the active pheromone.[66—68] The dipeptidylaminopeptidase activity is the product of the *STE*13 gene and mutants deficient in this protease produce a series of incompletely processed forms of α-factor. Recent studies indicated that the prepropoly-α-factor is multiply glycosylated and defined several points of processing that occur during the secretion of the pheromone by yeast cells.[28,29]

The presence of incompletely processed forms of α-factor provides additional insights into structure-activity relationships for this pheromone. Heptadecapeptides with either Glu-Ala-Glu-Ala or Asp-Ala-Glu-Ala on the amine terminus of α-factor were reported to be at least two orders of magnitude less active than the native pheromone.[67] It is not clear whether the decrease in activity is due to the length of the pheromone or the addition of two basic residues to the hydrophobic end of the α-factor. The finding that α-factor with an extra Ala residue on the amino terminus has specific biological activity equal to the native pheromone

suggests an extended active site for the α-factor and proves that a free amino group on Trp^1 is not required for activity. This conclusion is supported by studies on synthetic α-factor containing an additional Trp at the amine terminus.[36]

To summarize, the α-factor appears to be biosynthesized and secreted from yeast cells by routes which are quite similar to those for many mammalian secretory peptides and proteins. Differences exhibited by the yeast system may be explained in terms of evolutionary divergence or specific factors which make this route advantageous to the unicellular eukaryote. Most importantly study of α-factor biosynthesis has provided additional insights into the mechanism of protein secretion in *Saccharomyces* and structure-activity relationships in the pheromone.

XVI. DEGRADATION OF α-FACTOR

When challenged with α-factor, in the absence of *MAT*α cells, the *MATa* haploid is arrested at the G1 phase of its reproductive cycle and exhibits aberrant morphologies. After a period of time, which depends on cell number, pheromone concentration, and culture conditions, the yeast cells recover from the effects of α-factor and resume normal growth. Under certain conditions, recovery from pheromone action is accompanied by disappearance of α-factor from the growth medium.[70] A number of laboratories have shown that this disappearance is due to proteolysis of α-factor rather than uptake of the pheromone by the cell.[29,31,33] The inactivation of α-factor due to an *a*-cell associated process was first termed "Barrier function".[71] Mutants lacking this function are called *bar*1 mutants and are supersensitive to the pheromone in that they respond to extremely low concentrations and have impaired abilities to recover from mating factor-induced arrest.[72,73] Two complementation groups supersensitive to α-factor were identified by Chan and Otte.[72,73] One of the two groups of supersensitive strains is unable to degrade α-factor and this complementation group (*sst*1) is allelic with the *bar*1 mutants. Recently, Moore[74] has shown that when continually challenged with fresh α-factor *a*-haploids become desensitized to the pheromone. That is they grow normally in the presence of the pheromone. The molecular basis for this desensitization is at present unknown, but a similar desensitization has been observed frequently for mammalian peptide hormones.[75] In summary, yeast cells can recover from the effects of α-factor by more than one mechanism. This fact must be taken into consideration when evaluating studies on the response of *S. cerevisiae* to this sex pheromone.

The activity which hydrolyzes the α-factor appears to be a specific peptidase which cleaves the pheromone at the Leu^6-Lys^7 linkage.[29] This endopeptidase is found in the culture medium and its secretion is stimulated by α-factor.[31] Manney[76] has also reported on a secreted activity which inteferes with the ability of the -pheromone to inhibit the growth of *MATa* cells. This activity was reported to be under control of the *BAR*1 gene, stable to boiling, and inactivated by pronase. Although no definitive evidence was presented, it was suggested that the barrier function may not be a protease.[76] Recently, two proteins under control of *SST*1 and (*BAR*1) and thought to be responsible for the barrier function were reported to be glycoproteins with molecular weights of 200,000 and 400,000.[77] Both glycoproteins were stable to heat shock for 5 min at 100°C. However, the hydrolytic activity of these proteins was not examined. At present, it is not clear whether the barrier function has an associated peptidase activity nor is it clear exactly how the barrier function inactivates the α-factor. Clarification of this uncertainty awaits the correlation of defined chemical inactivation assays with studies measuring decreased *a*-cell response. Such correlations will be aided by the isolation and complete purification of the barrier function, and the development of a direct assay of the α-factor hydrolase.

Most of the studies on the degradation of α-factor by *MATa* cells have applied either indirect bioassays or paper electrophoresis. A direct and rapid HPLC assay has recently

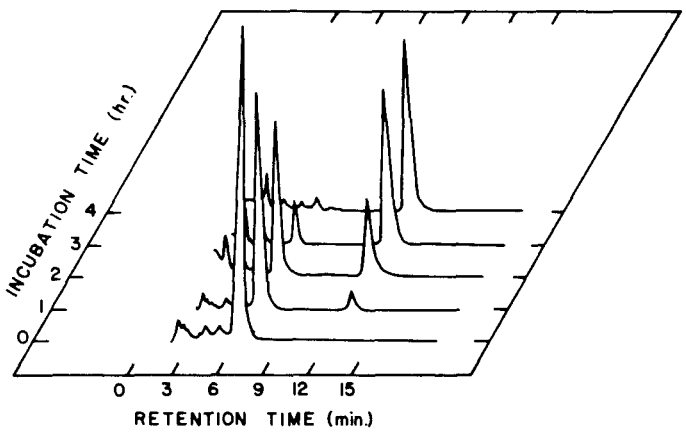

FIGURE 4. Degradation of α-factor by *S. cerevisiae* 2180 *MATa* cells. The α-factor (20 μg/mℓ) was incubated with *S. cerevisiae MATa* (2 × 10^6 cells per milliliter) at 30° for various time periods. The supernatant was separated from cells and chromatographed on a reversed-phase HPLC column with acetonitrile:water:trifluoroacetic acid (30:70:0.025) as the mobile phase.

been developed in our laboratory. Samples from a mixture of whole cells and pheromone can be separated from the cells by centrifugation and injected onto a reversed phase column. Separation between the intact pheromone and the degradation products is excellent (Figure 4). We have isolated the late-moving peak derived from both the tridecapeptide and the desTrp^1,Cha^3-α-factor and found that it corresponds to the N-terminal hexapeptide or N-terminal pentatpeptide. Our HPLC studies were quite reproducible, and we have achieved recoveries of ~ 90% of the peptides from the reversed phase column. This procedure confirmed that the α-factor protease was associated with whole cells and was secreted into the culture medium. In contrast to the findings of Manney,[76] the secreted activity we examined was inactivated by heating at 85°C for 15 min. Most important was that using the HPLC assay and our dodecapeptide analogues we were able to examine relative breakdown rates of various pheromones.

As seen from the data in Table 3 the peptidase is highly specific for α-factor. Changes at positions 3, 6, 9, and even 12 markedly affect the rate at which the pheromone is processed. In particular, the desTrp^1,Cha^3,Nle^{12}-, desTrp^1,Cha^3,D-Ala^9-, and desTrp^1,Cha^3,Ala^6-α-factors were not degraded by *S. cerevisiae* 2180 *MATa* during a 6 hr incubation. Under identical conditions the desTrp^1,Cha^3-dodecapeptide is almost completely cleaved.[87] The Nle^{12}-α-factor[21] and the *N*-Trp-Arg^7-α-factor[36] were also highly resistant to cleavage by *MATa* cells. These results show that substitutions far from the point of cleavage markedly influence activity of the enzyme. In addition both the desTrp^1,Cha^3,D-Ala^9-α-factor which is highly active and the desTrp^1,Cha^3,Ala^6-α-factor which is not active are resistant to the peptidase while the highly active desTrp^1,Cha^3-dodecapeptide and the inactive desTrp^1,Phe^3-dodecapeptide are both hydrolyzed. Thus different structure-activity relationships exist for the endopeptidase and pheromone activity, and α-factor-like activity obviously does not require proteolytic degradation of the pheromone. At present, the physiological relevance of the barrier function remains unknown.

XVII. CONFORMATIONAL ANALYSIS ON THE α-FACTOR

An axiom of molecular biology is that effector molecules bind to their receptor in a unique three-dimensional manner. Modern biochemistry has expended much effort tyring to relate

Table 3
HYDROLYSIS RATES OF α-FACTOR AND ITS ANALOGUES BY *MATa*-ASSOCIATED PEPTIDASE ACTIVITY

Peptide	$T_{1/2}$ (min)[a]
α-factor	70
desTrp^1-α-factor	70
des,Trp^1,Ala^3-α-factor	> 720
des,Trp^1,Cha^3-α-facotr	105
desTrp^1,Cha^3,Ala^6-α-factor	> 720[b]
desTrp^1,Cha^3,Ile^6-α-factor	> 720[b]
desTrp^1,Cha^3,Nle^6-α-factor	240
desTrp^1,Cha^3,Val^6-α-factor	> 720[b]
desTrp^1,Cha^3,D-Leu^6-α-factor	> 720[b]
desTrp^1,Cha^3,Ala^9-α-factor	300
desTrp^1,Cha^3,D-Ala^9-α-factor	> 720
desTrp^1,Cha^3,Nle^{12}-α-factor	> 720

[a] Whole cells (2×10^6 cells per milliliter) were incubated with the peptide (10 μg/mℓ) in yeast nitrogen base at 30°C. At various times portions were removed and examined using HPLC. The $T_{1/2}$ was determined by plotting the area under the peak corresponding to the pheromone vs. time on a semi-log graph.

[b] Not detectably degraded after a 6 hr incubation.

peptide conformation to function. Great successes have been achieved with a variety of peptide hormones, most notably oxytocin, vasopressin, and somatostatin. Indeed, structural analysis on this latter hormone resulted in the prediction of potential analogues with high activity and prolonged duration of action.[78]

The conformation of a linear peptide in solution may not reflect the active 2 and 3° structure assumed at its receptor. Nevertheless, studies of linear peptides in aqueous and organic media and more recently in the presence of lipid have provided great insights into the conformational tendencies of these molecules. A deficiency encountered with mammalian systems is the inability of the molecular biologist to readily modify the receptor. In contrast, the ease of manipulation of yeast allows the geneticist to isolate receptorless mutants (*ste2*) which serve as ideal controls, to clone the receptor on multicopy plasmids allowing high production, and in principle to conduct site specific mutagenesis which can result in predictable changes in the receptor primary sequence. Thus the yeast α-factor system in comparison to mammalian hormone-receptor experimental systems, is characterized by unmatched flexibility which permits modification of both the pheromone and the target receptor.

Chou-Fasman analysis of the α-factor suggests that this molecule has a high tendency to assume a helix near the N-terminus.[38,79,80] The Pro^8-Gly^9 sequence in the center of the peptide is both a helix and β-sheet breaker, but has a high tendency to assume a β-turn. Conformational analysis of the α-factor is still in its infancy. To date only preliminary CD, NMR, and fluorescence studies have been reported.

The CD pattern for the native tridecapeptide in Tris-HCl buffer, pH = 7.2, is typical of a random coil peptide. In the presence of lipid or small unilamellar vesicles of phosphatidylcholine-phosphatidyl-serine, a drastic change in the CD pattern occurs.[81] The changes observed are consistent with increased structure and in particular the band near 220 nm is associable with both α-helixes and β-structures. The apparent transition found for the α-

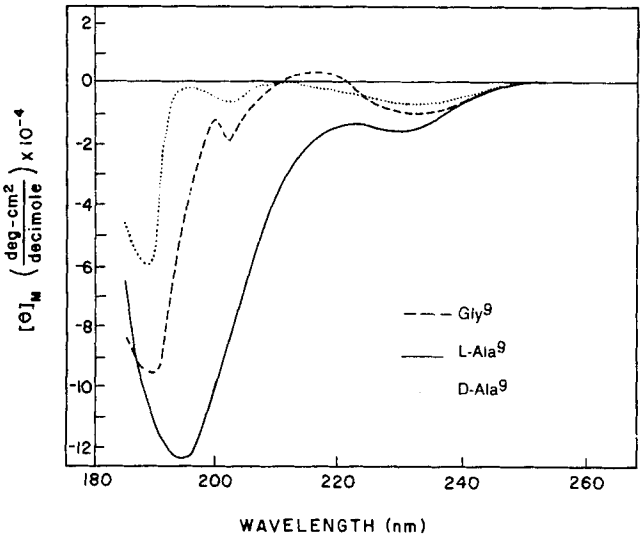

FIGURE 5. CD of the desTrp[1],Cha[3],X[9]-α-factor in trifluoroethanol peptide concentration ~1 mg/mℓ. The X residue is indicated on the figure.

factor in the presence of lipid was not noted for a biologically inactive α-factor analogue. Thus, it is reasonable to assume that the structure present in lipid is related to the biologically active form of the pheromone. Shifts in the fluorescence spectrum in the presence of vesicles indicate a strong hydrophobic interaction of the lipid and tryptophan residues. CD studies at pH 4 show that the α-factor is partially ordered.[82]

Recently, we have examined position 9 analogues of the desTrp[1],Cha[3]-dodecapeptide. As seen in Figure 5 the desTrp[1],Cha[3]- and the desTrp[1],Cha[3],D-Ala[9]-α-factor exhibit one class of CD patterns in trifluoroethanol (TFE) whereas the L-Ala[9] homologue has a different pattern. Similar results were observed in Tris buffer and using the desTrp[1],Cha[3],D-Leu[9]- and desTrp[1],Cha[3],L-Leu[9]-dodecapeptides.[87] None of the CD curves observed for the dodecapeptides is indicative of a structured peptide. However, the data suggests that the Gly[9] and D-X-α-factors assume similar distributions of ϕ, ψ angles whereas the L-X[9] peptides assume a different array of structures. Given our findings that the *D*-X[9] analogues have high biological activity whereas the L-X[9] analogues are almost inactive, we conclude that the Pro[8]-Gly[9] sequence is an important determinant of the biologically active structure for the peptide. The allowed replacement of Gly by a D-residue is consistent with a Type II-β-turn at this position.[40]

A number of NMR studies on α-factor and its analogues have appeared in the literature. Assignments for a α-factor in water[37,82] and for the desTrp[1],Cha[3]-α-factor in dimethylsulfoxide (DMSO[83]) were reported. The latter study used specifically α-deuterated α-factor analogues and is unequivocal. The former study used α-factor fragments and comparisons of active and inactive pheromones to make assignments. To date the most detailed NMR study has used a combination pH profiles of chemical shifts, comparison of coupling constants, measurement of temperature coefficients of N-H protons, and studies of relaxation rates in the presence of Gd(III).[82] The study concluded that the N-terminus from Trp[1]-Leu[6] is α-helical and that β-turns are present in the central and C terminal domains. Although such a structure may contribute to the conformation of the peptide it is clearly not indicated by the CD studies and does not predominate in aqueous solution. Furthermore, the $J_{NH\text{-}\alpha CH}$ coupling constants reported are more consistent with a distribution of structures than one predominant conformation. Nevertheless, this first attempt has provided some insights into

the relationship between the conformation of α-factor and its activity. In particular the NMR results seem to support the importance of a β-turn at the Pro^8-Gly^9 residues.

Recently using a combination of 2D-COSY and 2D-NOESY experiments 90% of all the protons in the $desTrp^1$,Cha^3-α-factor were assigned in DMSO-d_6.[90] In addition, the NOESY experiment indicated a random structure for the dodecapeptide in this organic solvent. Given the conformational equilibria which exists in linear peptides 2D-NMR should prove especially valuable in unraveling the structure of the α-factor in solution.

XVIII. STUDIES ON THE α-FACTOR

MATa haploids produce a pheromone which is involved in sexual conjugation that appears to parallel the α-factor.[4,7,84] The *a*-factor was reported to be purified to homogeneity and appeared to be undecapeptides with the following structures.[85,86]

Tyr-Ile-Ile-Lys-Gly-Leu-Phe-Trp-Ala-Asx-Pro (1)

Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Ala-Asx-Pro (2)

Compared to the α-factor very little is known about structure activity relationships in the *a*-factor. Despite the fact that the above structures were published in 1981 and have been quoted in review articles[1,7] and symposia, the sequences have not been verified.

Based on the above reports we synthesized the four undecapeptides represented by Structures 1 and 2. None of these alone or in various combinations elicited *a*-factor responses from *MAT*α haploids. A different structure for *a*-factor was deduced from cloning of the *a*-factor gene.[91] According to the nucleotide sequence a potential structure for *a*-factor is

Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala (3)

This peptide is identical to that of Betz et al.[84] except that the three carboxyl residues are rearranged. Again, synthesis of this sequence resulted in an inactive pheromone.[87] We note that the cloned nucleotide sequence for the presumptive *a*-factor gene contains flanking residues on both the carboxyl and amino termini of the undecapeptide. In addition, Betz and co-workers[84] suggested that the *a*-factor might be modified on the side chain of aspartic acid. It is clear, however, that the above undecapeptides do not represent the *a*-mating factor activity. The determination of the structure of *a*-factor is dependent on the synthesis of a compound possessing the biological activity associated with *a*-factor.

XIX. PROSPECTUS

As described in this communication, structure-activity studies on the yeast α-factor have provided insights into the amino acid residues critical for biological function and the multiplicity of receptor systems involved in the recognition of this pheromone. Discovery of α-factor antagonists allows the separation of pheromone binding and signal transmission to the intracellular sites responsive to the mating factor. Such antagonists should be extremely useful in future efforts to examine the interaction of the α-factor and its receptor at the molecular level. Clearly, the structure-activity relationships provide information on those sites of the pheromone which are amenable to chemical modification and which can be used for insertion of radioactive, fluorescent, or photoactivatable groups.

Preliminary studies suggest that the α-factor assumes a specific secondary structure in the yeast membrane. Future efforts must concentrate on elucidating this conformation on the molecular level. Once the active conformation is defined it can be used as a basis for

preparing stereochemically restricted analogues which should have significantly higher biological activities. Indeed, if different receptors exist for the mating factor it may be possible to distinguish conformational domains on the peptide which trigger individual biological responses to this pheromone.

The ultimate goal of studies on hormonal activity is the definition of each step in the pathway between hormone biosynthesis and target cellular effect. It is clear that major progress has already been made towards understanding the biosynthesis of α-factor, and that the molecular events involved in pheromone binding will soon be unraveled. Less success has been achieved in understanding the transmission of information from the α-factor receptor to the pleiotropic intracellular targets that it activates. Application of molecular genetics and properly chosen α-factor analogues provide a powerful approach to solving this fundamental problem. We stand at the portal to an exciting future. Kadema!

ACKNOWLEDGMENTS

We gratefully acknowledge the collaboration of the following students, postdocs, and colleagues without whose assistance our investigations on α-factor could never have been undertaken: Robert Baffi, Charlotte Boney, Michelle Broido, Linda Hughes, Shabbir Khan, Bijoy Kundu, Peter Lipke, Glen Merkel, Cynthia Pousman, Susan Raths, and Alvin Steinfeld. We are particularly indebted to Dr. Ponniah Shenbagamurthi and Ms. Susan Raths for carefully reading this review. Support for our studies on α-factor came from the National Insitutes of Health GM22086 and GM22087 and the PSC-BHE grants of C.U.N.Y. We thank the following individuals for providing their results prior to publication: Anthony Brake, Wolfgang Duntze, Susan Moore, Gennady Samokhin, Argun Singh, George Sprague, Takaharu Tanaka, and Jeremy Thorner.

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