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Review

The α-factor mating pheromone of *Saccharomyces cerevisiae*: a model for studying the interaction of peptide hormones and G protein-coupled receptors

Fred Naider[a,b,*,1], Jeffrey M. Becker[c]

[a] *Department of Chemistry, College of Staten Island and Institute for Macromolecular Assemblies of The City University of New York, Staten Island, NY 10314, USA*
[b] *Graduate School and University Center of The City University of New York, New York, NY 10016, USA*
[c] *Department of Microbiology and the UT-ORNL Graduate School of Genome Science and Technology, University of Tennessee, Knoxville, TN 37996, USA*

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Abstract

Mating in *Saccharomyces cerevisiae* is initiated by the secretion of diffusible peptide pheromones that are recognized by G protein-coupled receptors (GPCR). This review summarizes the use of the α-factor (WHWLQLKPGQPMY) – GPCR (Ste2p) interaction as a paradigm to understand the recognition between medium-sized peptide hormones and their cognate receptors. Studies over the past 15 years have indicated that the α-factor is bent around the center of the pheromone and that residues near the amine terminus play a central role in triggering signal transduction. The bend in the center appears not to be rigid and this flexibility is likely necessary for conformational changes that occur as the receptor switches from the inactive to active state. The results of synthetic, biological, biochemical, molecular biological, and biophysical analyses have led to a preliminary model for the structure of the peptide bound to its receptor. Antagonists for Ste2p have changes near the N-terminus of α-factor, and mutated forms of Ste2p were discovered that appear to favor binding of these antagonists relative to agonists. Many features of this yeast recognition system are relevant to and have counterparts in mammalian cells.

Keywords: *Saccharomyces cerevisiae*; Antagonists; G protein-coupled receptors; Mating pheromone

Contents

* Corresponding author. Tel.: +1 718 982 3896; fax: +1 718 982 3910.
E-mail address: naider@postbox.csi.cuny.edu (F. Naider).
[1] Fred Naider is currently the Leonard and Esther Kurtz Term Professor at the College of Staten Island. Much of this review was written while FN was a visiting Professor at the Weizmann Institute of Science.

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1. Introduction

Sequencing of eukaryotic genomes reveals that G protein-coupled receptors (GPCRs) constitute a major family of proteins and that humans manifest numerous examples of these signal transduction proteins [50]. To date more than 1000 GPCRs have been identified, and these proteins recognize neurotransmitters, sensory molecules and chemotactic agents [85]. GPCRs are involved in the control of many aspects of metabolism and play important roles in diverse processes such as pain perception, growth and blood pressure regulation, and viral pathogenesis. The ubiquitous nature of GPCRs together with their highly specific ligand recognition makes them an important target for therapeutic agents [8,78,148]; recent reports indicate that nearly 40% of drugs currently prescribed for human ailments target this family of proteins [60,115].

Despite the widespread occurrence of GPCRs and the fact that they are studied in many laboratories, fundamental information concerning the molecular details of their mechanism of action is lacking. In particular the recognition of many ligands by this class of proteins, conformational changes driving signal transduction, and activation of heterotrimeric G proteins by these molecules is still not well understood, although a number of ideas have been proposed as to the mode of action of these receptors [26,56,62,121,147]. Our research group is utilizing mating in yeast as a paradigm to understand many of the above details, focusing on the recognition and activation of a GPCR by an intermediate length peptide. Yeast pheromones, α-factor and **a**-factor, are peptides of 13 and 12 residues, respectively, recognized by Ste2p and Ste3p their conjugate GPCRs (see Table 1 for explanation of terms used in this review). Although early structure–activity studies on α-factor and assay of cell morphogenesis and agglutination triggered by this pheromone suggested more than one class of receptor for this peptide, overwhelming evidence now indicates that only Ste2p, the product of the *STE2* gene, is involved in recognition of the pheromone [12,18,61,107].

The power of yeast genetics has allowed many laboratories to provide important information concerning the biochemistry of Ste2p and to a lesser extent Ste3p. In this review we will focus on the mating pheromones and discuss research since the mid-1980s on these peptide hormone-like molecules. At this time there is still no complete understanding of the molecular details of the interaction of a medium length (10–15 residue) peptide hormone and its GPCR. Thus, studies on the α-factor and to a lesser extent the **a**-factor, are aimed at understanding the interaction of mid-sized peptides with their cognate GPCRs. The observation, as stated in our last review, that α-factor and the human gonadotropin releasing hormone (GnRH) have sequence similarities suggesting evolutionary relatedness [104] reinforces the relevance of α-factor structure–function studies as having significance to human biology. However, the sequence similarity may not be statistically significant, a traceable evolutionary relationship has not been established, and a recent study using synthetic hybrids of GnRH/α-factor suggested that the structural homology between these two reproductive hormones may not reflect an evolutionary relationship [69].

Although the reader is referred to previous reviews that discuss structure–activity relationships in α-factor [104] and **a**-factor [19] that serve as the predecessors to this communication, we briefly summarize here some major points of the previous reviews. Derivitization of the ε-amine of Lys at position 7 of dodecapeptide analogs α-factor with biotin, a fluorescent moiety, or short chain alkyl groups did not destroy the activity of the analogs. Various substitutions at positions 8 and 9 of α-factor supported the prediction of the involvement of these residues in a β-turn of the pheromone. The synthesis and antagonistic activity of position-1-deleted, position-3-substituted α-factor analogs were reported. For **a**-factor, N-terminal truncation led to progressively less active analogs yet

Table 1
Selected terminology relevant to yeast mating system

Term	Definition
α-factor	Tridecapeptide (WHWLQLKPGQPMY) pheromone secreted by *S. cerevisiae MAT*α cells
a-factor	Farnesylated dodecapeptide pheromone [YIIKGVFWDPAC(farnesyl)OCH_3] secreted by *S. cerevisiae MAT***a** cells
BAR1	Gene encoding the secreted Bar1p protease
Bar1p	Protease secreted by *MAT***a** cells that degrades α-factor between L^6 and K^7
FUS1-lacZ	Gene fusion between *FUS1*, a pheromone-inducible gene, and *lacZ* that serves as a reporter for pheromone biological activity
*MAT***a**	*S. cerevisiae* mating-type **a** cell
*MAT*α	*S. cerevisiae* mating-type α cell
MFα1 and *MFα2*	Genes encoding α-factor
Shmoo	The elongated, pear-shaped *S. cerevisiae* cells formed in response to pheromone
STE2	Gene encoding Ste2p, the G protein-coupled receptor for α-factor
Ste2p	Receptor for α-factor found on *MAT***a** cells
STE3	Gene encoding Ste3p, the G protein-coupled receptor for **a**-factor
Ste3p	Receptor for **a**-factor found on *MAT*α cells

measurable bioactivity was retained until over two-thirds of the peptidyl portion of the pheromone was removed. Analogs lacking the carboxy-terminal carboxylmethyl ester or farnesyl groups were 100- or 1000-fold less active, respectively, than the natural pheromone, and the dodocamer lacking both post-translational modifications exhibited a more than 100,000-fold decrease in biological activity. Finally, specific residues contributed to pheromone activity differentially, and a highly active analog of **a**-factor**,** containing D-Ala substitution for glycine at position 5 was identified. As very little work has been done on **a**-factor since the previous reviews, this review will be highly concentrated on α-factor.

2. Mating in *Saccharomyces cerevisia*: overview

Mating of the *MAT***a** and *MAT*α haploid cells of *Saccharomyces cerevisiae* is initiated by the secretion of diffusible peptide pheromones that are recognized by receptors on the opposite cell type (for reviews see [40,41,47,80,135]). The mating pheromones are absolutely required to trigger the mating cycle; cells that cannot produce these molecules or lack their cognate receptors Ste2p or Ste3p are sterile. α-Factor, a tridecapeptide pheromone synthesized constitutively by *MATα* cells and acting on *MAT***a** cells, was isolated and characterized from medium of *MATα* cell cultures by Stotzler and Duntze [137] and later found to be encoded by two genes *MFα1* and *MFα2* [81]. The WHWLQLKPGQPMY peptide sequence encoded by the genes was chemically synthesized and found to exhibit all the properties of the natural pheromone [28,98]. The **a**-factor of *S. cerevisiae* is a dodecapeptide pheromone (YIIKGVFWDPAC[Farnesyl]-OCH3) [5] in which post-translational modification with a farnesyl isoprenoid and carboxymethyl group is required for full biological activity [94]. The biosynthesis of these mating pheromones has been studied extensively and used as a model for post-translational processing, modification and secretion of mammalian peptide hormones and proteins [15,19,24,36]. This interesting area of investigation will not be covered in this review.

Pheromone challenge of the opposite mating-type cells (e.g. *MAT***a** cells) with α-factor results in a number of biological responses: the cells synthesize cell surface molecules necessary for agglutination with their mating partners, arrest in the G1 phase of the growth cycle to obtain synchrony for mating, form mating projections that are involved in the fusion process, thereby exhibiting a marked change in shape, and activate a number of genes that are necessary for sexual conjugation [41,80,97]. Recently, it was proposed that α-factor induces apoptosis in *MAT***a** cells [129]. Many of these cellular responses have been used to assay the potency of pheromones and their analogs. Originally, the most widely used assay was the formation of pear shaped mating projections known as shmoos [102,141]. However, more recently most laboratories have used a plate assay indicating growth arrest (halo assay; [93]) or measured β-galactosidase activity generated by a *FUS1-lacZ* construct that is a sensitive indicator of pheromone based gene induction [100]. Furthermore, beginning in the late 1980s a number of groups reported direct receptor binding assays using radioactive pheromones [12,74,75,113].

3. α-Factor

3.1. A brief retrospective

In our 1986 review we highlighted studies carried out on analogs of the native α-factor (TrpHisTrpLeuGlnLeuLysProGlyGlnProMetTyr) and of dodecapeptides corresponding to the $desTrp^1$ pheromone that had also been observed in culture supernatants [104]. Interestingly, these earlier studies showed that virtually any replacement in the primary sequence of the tridecapeptide had a very major effect on biological activity, as judged using a shmoo assay. However, much smaller differences were observed in dodecapeptide analogs. It should also be noted that in 1986 few studies had appeared where the receptor binding affinities of different analogs had been measured.

Initial studies on binding of α-factor to Ste2p utilized ^{35}S-labeled pheromone that was prepared biosynthetically. This elegant work [74,75] defined the number of binding sites and the K_d of the receptor for the pheromone. It also provided strong genetic evidence for the existence of the receptor that was confirmed using crosslinking and antibody detection procedures [12]. Despite these successes, the preparation of radioactively labeled α-factor by biosynthetic approaches was tedious and resulted in small quantities of the desired probe. There was a clear need for other routes to the radioactive pheromone.

With the above as background, our studies on the structure–activity relationship (SAR) of α-factor over the last 15 years targeted the understanding of the interaction of this peptide with its receptor Ste2p, the determination of key contacts between it and Ste2p, and the biologically active structure of the tridecapeptide. These studies necessitated a systematic analysis of the influence of different side chains and functional groups on biological activity, receptor affinity and pheromone structure. This elicited parallel biochemical and biophysical studies on the structure of α-factor.

3.2. Development of a binding assay using tritiated α-factor

Based on structure–activity relationship studies by Masui et al. [98,99] and frustrating experiences with producing iodinated α-factor useful for binding studies (however, see below section on position 13 analogs), approaches to the chemical radiolabeling of α-factor by tritiation were explored. The first report of the use of tritiated α-factor in a binding assay utilized a custom synthesized pheromone with a specific activity of 13.2 Ci/mmol [75]. This study pointed out the clear advantages of tritiated peptide providing more accurate values for the binding constant and the number of Ste2p receptors per cell. However, no procedure for preparation of the peptide was provided and the pheromone was found to be labeled at His (∼90%), Trp (∼5%) and Tyr residues. In a later study we developed a reproducible methodology to prepare [^{3}H]α-factor. We showed that catalytic reduction of α-factor containing dehydroproline in positions 8 and/or 11 resulted in a pheromone with biological activity identical to that of α-factor and with specific activities ranging from 10 to 20 Ci/mmol [113]. The radiolabeling (73%) occurred at the Pro residues as expected and could be carried out on a well-characterized precursor peptide. Our probe also contained norleucine substituted for Met^{12}. Norleucine is considered to be isosteric to methionine and is used by many investigators as a sulfur-less methionine analog. We found [Nle^{12}]α-factor to have the same biological activity and receptor affinity as the native pheromone. Most importantly it was stable to oxidation and more stable to autoradiolysis. The above probe has been used extensively by us during the past 15 years and it has been validated in other laboratories. It should be noted, however, that the specific activity obtained is considerably lower than the 110 Ci/mmol theoretically available from the precursor containing two dehydroproline residues. Nevertheless, we found that even with proper storage, at a specific activity of 10 Ci/mmol, autoradiolysis limits the lifetime of the radioactive probe to about one year. Much greater degradation rates are observed as the specific activity is increased.

Using [^{3}H, Nle^{12}]α-factor we were able to obtain highly reproducible binding curves with whole cells and membranes. It is important to note that we carried out binding experiments in the presence of general protease inhibitors with strains lacking the protease (*bar1*) that cleaves α-factor [29]. Under these conditions peptide degradation has been shown to be negligible. The K_d value found in our initial study was 2.2×10^{-8} M with $k_{on} = 4.9 \times 10^4$ M^{-1} s^{-1} and $k_{off} = 1.1 \times 10^{-3}$ s^{-1}. In subsequent investigations K_d values between 3×10^{-9} and 1×10^{-7} M were observed with most values being near 10 nM. These K_d values are in excellent agreement with those reported by Sen and Marsh using the same probe [126], and in reasonable agreements with the dissociation constants of Jenness et al. [75] and those of other reports using ^{35}S labeled α-factor [12,27,74,114]. It is important to note that the slow off-rate of α-factor has significant implications in biophysical analysis of the structure of bound α-factor.

3.3. Ala-scanned analogs

The original structure–activity studies on the tridecapeptide pheromone [98,99] used a strain containing the protease encoded by the *BAR1* gene that cleaved the peptide between Leu^6 and Lys^7 [29]. The Bar1p protein is secreted by *MAT***a** cells resulting in the destruction of α-factor to allow recovery of the target cell from growth arrest. Using this Bar1p+ strain the activities reported for the native pheromone were more than three orders of magnitude lower than those found by any other research group. Finally, the original report did not measure receptor affinities. We therefore decided to re-examine the importance of various residues using alanine-scanning and to use *bar1*-deletion strains [22,23] where pheromone degradation did not interfere with measurements of biological activity or receptor affinity.

During the course of our studies we have systematically examined the influence of all residues of α-factor, of the α- and ε-amino groups, and of the carboxyl terminus on pheromone activity and binding. The most comprehensive study involved replacement of each residue by Ala residues. To this end 13 α-factor analogs were synthesized in which L-alanine was substituted at each position of the parent pheromone and a second series of analogs with D-alanine substituted at each residue was synthesized as well [2]. This approach is widely accepted as a method to determine which residues of a peptide contribute to either the binding to the receptor or its activation [9,34,53,119,139]. Similarly, alanine-scanning of receptors has been used to determine interaction between receptors and ligands [4,30,68]. Furthermore, the insertion of D-residues often permits the analysis of confor-

mational contributions to the biology of the peptide ligand. In other studies we extensively probed individual side chains. In some cases these analyses were carried out prior to the alanine-scanning. However, since alanine-scanning provided several general insights into the interaction of the peptide and Ste2p we discuss these studies first. We then discuss the more detailed probing of a specific site, although in some cases these are chronologically out of order. We note that for the reasons discussed above we always use α-factor analogs with Nle^{12} in place of Met. In this review, except if explicitly stated, all analogs of α-factor contain this substitution and will be referred to as α-factor.

The analysis of the Ala-scanned α-factor analogs involved measurement of both biological activities and receptor binding affinities. The biological activities were determined by measuring the efficacy of the analogs in a growth arrest assay. This assay measures growth arrest of *MAT***a** target cells that contain the α-factor receptor and the full signal transduction pathway; pheromone initiation of the pathway results in growth arrest. The diameter of the zone of inhibition is determined versus the amount of the peptide on the disk (Fig. 1(A)). For comparison purposes the results are analyzed using semi-logarithmic plots and relative potencies are reported as the amount of peptide causing a 15 mm halo (Fig. 1(B)). The validity of this approach requires that the various analogs have similar diffusivities into the agar and similar stabilities to chemical or enzymatic degradation. Since we work with strains lacking the Bar1 protease [140], the primary proteolytic event in α-factor degradation is eliminated. In separate experiments we directly measured α-factor and analog degradation using HPLC and found no evidence for pheromone cleavage under the bioassay conditions. We also measured biological potency in solution by a morphogenesis assay and induction of the *FUS1* gene. Similar activity trends were noted in both the solid-agar growth arrest assay and the two assays with cells in suspension. Therefore, we conclude that the halo assay does indeed represent the intrinsic biological potency of the various analogs and forms the basis for almost all of our structure–activity relationship analyses. The data in Table 2 show that the L-Ala-scanned analogs exhibit about a one order of magnitude variation in biological activity. [Ala^1]α-factor is about three-fold more potent than the native structure and [Ala^9]α-factor has the lowest activity being about four-fold lower in potency. Clearly no side chain in this peptide pheromone is absolutely required for activity. The influence of D-Ala replacement on pheromone activity is much greater; four of the 13 analogs have no activity and two have only 5% of the activity of α-factor. Intriguingly [D-Ala^9]α-factor is nearly six-fold more active than the parent pheromone and 25 times as active as its L-Ala^9 diastereomeric isomer. Moreover, of all of the Ala-scanned analogs we analyzed, [D-Ala^9]α-factor was the most active. Binding competition studies of the Ala-scanned series against tritiated α-factor allowed determination of the relative affinities of these peptides. In every case except one, substitution with Ala or D-Ala results in a significant loss in binding affinity.

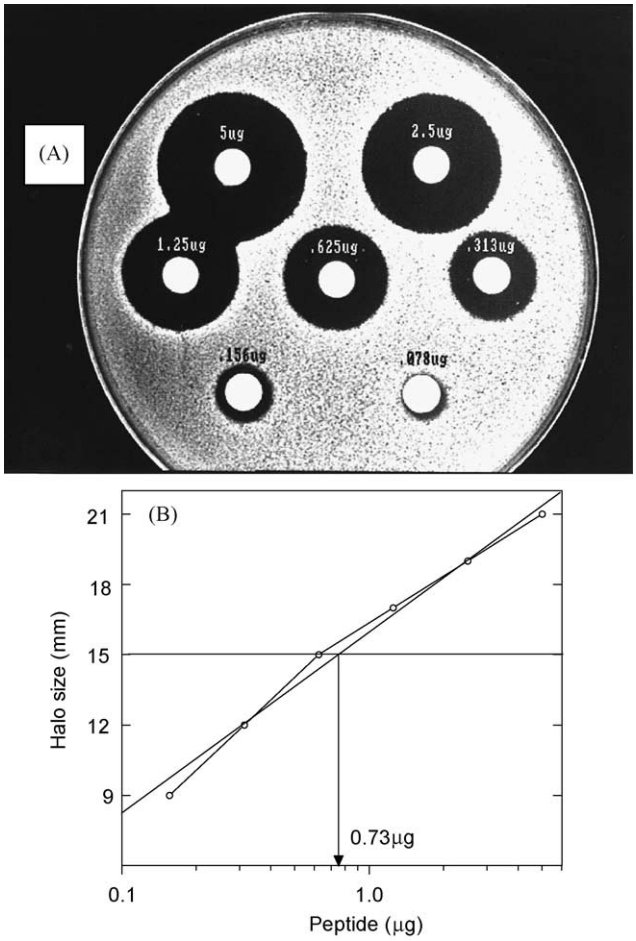


Fig. 1. Halo assay (Panel A) and the semi-logarithmic plot (Panel B) of α-factor biological activity on a lawn of target *MAT***a** cells. The halo assay reflects arrest of growth of the tester strain by increasing amounts of the pheromone. The determination of the amount of peptide causing a zone of inhibition of a given diameter using the semi-logarithmic plot allows comparison of the activity of different pheromones.

In the L-Ala series the loss is between 6- and 500-fold. The greatest loss in binding is observed for residues 9 and 13 with residues near the carboxyl end in general showing greater contributions to binding than residues at the amine terminus, and residues 5, 7 and 8 having the lowest influence on binding. In the corresponding *D* series, residues 7 and 10–13 exhibited the greatest drop in affinity upon substitution with D-Ala, with the greatest drop off exceeding 3000-fold. We believe it is significant that the ratio of *Ki* values for the L-Ala and D-Ala analogs for substitution at residues 1–4 is between 1 and 2 whereas for residues 5, 7, 8, 10, 11, 12 and perhaps 13, it is greater than 10. The high ratios found for the residues in the middle and at the carboxyl terminus indicate that the spatial presentation of these side chains or the overall topology of the N-terminus relative to the C-terminus of α-factor is critical for strong receptor binding.

Only one analog had a receptor affinity equal or greater than that of wild-type α-factor – [D-Ala^9]α-factor. It should be noted that some of the quantitative conclusions concern-

Table 2
Biological activity and binding affinities of alanine-scanned α-factor analogs

Peptide[a]	Biological activity[b]	Activity ratio[c]	*Ki*[d] (μM)	*Ki* ratio[e]
[Nle^{12}]α-factor	0.76	1.00	0.011	1000
Ala^{1}	0.22	3.46	0.79	14
Ala^{2}	2.14	0.36	1.25	9
Ala^{3}	2.14	0.36	0.47	23
Ala^{4}	0.82	0.94	0.31	35
Ala^{5}	0.53	1.44	0.06	175
Ala^{6}	0.92	0.83	2.50	4
Ala^{7}	2.23	0.34	0.23	48
Ala^{8}	0.58	1.32	0.13	88
Ala^{9}	2.80	0.27	3.91	3
Ala^{10}	1.91	0.40	1.17	9
Ala^{11}	1.13	0.68	0.70	16
Ala^{12}	0.42	1.82	1.72	6
Ala^{13}	2.30	0.33	6.09	2
D-Ala^{1}	2.41	0.32	1.41	8
D-Ala^{2}	na[f]	–	1.80	6
D-Ala^{3}	na	–	0.22	52
D-Ala^{4}	na	–	0.34	33
D-Ala^{5}	0.72	1.06	0.69	16
D-Ala^{6}	13.60[g]	0.06	6.41	2
D-Ala^{7}	3.30	0.23	15.23	0.7
D-Ala^{8}	0.93	0.82	3.28	3
D-Ala^{9}	0.13	5.79	0.008	1400
D-Ala^{10}	na	–	17.18	0.6
D-Ala^{11}	2.02	0.38	18.75	0.6
D-Ala^{12}	5.47	0.14	32.0[h]	<0.3
D-Ala^{13}	14.10[g]	0.05	36.0[h]	<0.3

[a] Peptide analogs designated by position of alanine substitution and synthesized with norleucine^{12} in place of native methionine^{12} except for the L- and D-Ala^{12} peptides.

[b] Comparisons of biological activity among analogs is shown by the amount of peptide (μg) required to produce a growth arrest halo of 15 mm interpolated from a first order regression line equation and rounded to the nearest 0.01 (see Fig. 1(B)).

[c] The ratio of biological activity represents the amount of [Nle^{12}]α-factor required to produce a 15 mm halo divided by the amount of peptide analog required to produce a 15 mm halo.

[d] The dissociation constant for displacement of [^{3}H-Nle^{12}]α-factor from the receptor. See reference [2] for method of calculation.

[e] The *Ki* ratio represents the *Ki* of [Nle^{12}]α-factor divided by the *Ki* of the analog (×1000).

[f] No activity.

[g] The analogs D-Ala^{6} and D-Ala^{13} did not produce a halo of 15 mm or greater at any concentration tested; the biological activity reported for these two analogs was extrapolated.

[h] The analogs D-Ala^{12} and D-Ala^{13} were not able to demonstrate 50% binding competition at the highest concentration tested.

ing the data in Table 2 vary when different strains are used as the test organism [83]. Nevertheless, the overall trends are maintained. Careful analysis of the binding and activity profiles allows us to point out a number of general aspects of the biochemistry of α-factor and to make several inferences concerning its interaction with Ste2p.

3.3.1. There is a lack of correlation between receptor affinity and biological efficacy for some α-factor analogs

Substitution of Ala at position 1 results in a pheromone that is more active than α-factor but binds with a 70-fold lower affinity. This lack of correlation of biological activity and receptor affinity has been noted by us in many studies. The results indicate that the indole side chain in position 1 contributes significantly to the free energy of binding. At the same time it is possible that this side chain may attenuate receptor activation. The currently accepted model of G protein activation is a dynamic equilibrium between the active and inactivated state with agonist binding favoring the active conformation [76,121]. Within the scope of this model, binding of a strong agonist such as α-factor should result in a productive interaction. The fact that the poorly binding [L-Ala^{1}]α-factor is highly active suggests that the bulky Trp side chain may actually slow down receptor isomerization and thereby play a regulatory role in the signaling pathway. An important implication of these findings is that potent agonists that could have valuable clinical utility may be missed when screens involve only receptor binding assays.

3.3.2. Residues near the amine terminus play an important role in receptor activation

As noted above, several of the D-Ala series analogs, specifically [D-Ala^{2}]α-factor, [D-Ala^{3}]α-factor and [D-Ala^{4}]α-factor had no measurable biological activity. Nevertheless, these peptides bound relatively strongly to Ste2p and antagonized the biological activity of α-factor in halo, shmoo and gene induction assays. In previous studies we reported that desTrp^{1}[Ala^{3}]α-factor was a potent antagonist and that most of the antagonists we discovered involved changes in residues near the amine terminus (see below for a detailed discussion of Ste2p antagonists and synergists). Based on these findings it seems clear that residues near the amine terminus play an important role in triggering cell signaling through Ste2p or in stabilizing the activated state of this receptor. It should be noted that the D-Ala series antagonists represented the first full-length antagonists for this receptor.

3.3.3. Residues near the carboxyl terminus play an important role in receptor binding

As previously described the residues at the carboxyl terminus of α-factor strongly influence the binding. Replacement of these residues by either L- or D-Ala caused decreases in affinity of up to 3000-fold. Moreover, these carboxy-terminal residues are not sufficient to cause signaling as evidenced by the total absence of agonist activity in [D-Ala^{3}]α-factor. Removal of the carboxyl terminal residues results in pheromones with drastically reduced receptor affinity and, in some cases, also with complete loss of affinity. In developing our model of α-factor function we view the carboxyl terminal residues as playing an important role in binding to Ste2p and a secondary role in GPCR receptor activation.

3.3.4. Central residues of α-factor function to orient the signaling and binding domains of the pheromone

The center of α-factor contains a Pro-Gly sequence. This sequence has been associated with a high probability of β-turns, in particular Type II β-turns [25,143]. Previous studies

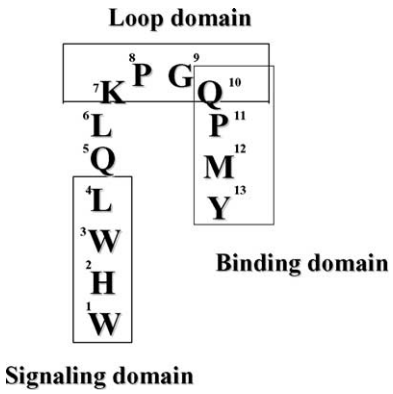


Fig. 2. Cartoon representing functional segmentation of α-factor. The figure indicates that the pheromone may be divided into signaling, loop (conformational hinge) and binding domains. This is to indicate that the residues in these domains contribute most prominently to the designated functions. This does not exclude these same residues from also participating in other functions of the peptide. Data taken from reference [2].

on desTrp^1-α-factor analogs indicated that a turn at this position was involved in the biological activity of the pheromone [131]. The data from the Ala-scanning analysis of α-factor support and extend this conclusion. The [L-Ala^9]α-factor, which is expected to destabilize a Type II β-turn, has the lowest activity and receptor affinity of those analogs where L-Ala replaced one of the four residues (KPGQ) expected to be involved in a turn around Pro-Gly. Moreover when these same residues are replaced by D-Ala, only the [D-Ala^9] isomer has high activity and affinity. The high activity of the [L-Ala^8]α-factor and [D-Ala^8]α-factor provide definitive proof that this side chain is not necessary for activity or effective binding. However, the large drop off in receptor affinities for the [D-Ala^7], [D-Ala^8] and [D-Ala^{10}]α-factors suggest that this region of the peptide may serve to create the proper overall conformation for the molecule. Again in our hypothetical working model this region does not interact strongly with the receptor but serves as a bend region to orient the signaling and binding domains (Fig. 2). In summary the Ala-scan series indicates that even in a relatively small peptide different and somewhat distinct functions are associated with various regions of the signaling molecule. This attribute is probably general to peptide hormones and has been specifically observed with glucagon [129], LHRH [45], and with the biologically active 1–34 fragment of parathyroid hormone [108].

3.4. Position 1 α-factor analogs

The findings of the Ala-scan analysis have been expanded in detail by testing additional analogs at several positions of α-factor. Binding analyses showed that removal of the first residue resulted in about a one order of magnitude drop in affinity [113]. This corresponds to a contribution of about 1.3 Kcal/mol by the first residue to the binding energy. The desTrp^1-α-factor remains a reasonable agonist and is 10–20-fold less effective in the halo assay [113]. A similar drop in activity was reported using a morphogenesis assay [156]. In general replacement of the indole side chain of the first residue with other aromatic side chains or β-cyclohexylalanine (Cha) resulted in potent agonists with affinities only 3–6-fold lower than that of the parent pheromone [156]. It appears that hydrophobicity is as important as aromaticity because the [Cha^1]α-factor had the same binding affinity as [Phe^1]α-factor and both of these analogs were more potent agonists in the halo assay than α-factor. As found with [Ala^1]α-factor, both [Gly^1]α-factor and [Leu^1]α-factor exhibited a greater than 20-fold decrease in receptor affinity but were still more effective agonists compared to α-factor. Thus the lack of correlation of efficacy and affinity is seen for a number of position 1 analogs. Others have also observed this phenomenon with peptide hormones and the lack of correlation between receptor occupancy and activation has been discussed in detail [76,121]. In contrast to the above, placement of a negatively ([Glu^1]α-factor) or positively ([Lys^1]α-factor) charged side chain at position 1 was highly detrimental to agonist activity resulting in a more than 10-fold drop in growth arrest activity, low efficacy in the gene induction assay and most pertinent almost no ability to promote sexual conjugation as judged by a mating restoration assay [156]. The Glu and Lys analogs had more than 100-fold and 25-fold decreases, respectively, in binding affinity. Overall the results suggest that the position 1 side chain occupies a hydrophobic pocket in the receptor active site. The pocket is not selective for aromatic over alicyclic rings but appears to have a size limitation, as iodophenylalanine is a less potent agonist than rings with smaller substituents. Although this pocket can accommodate small hydrophobic side chains such as those of Ala and Leu, these do not make optimum contacts and have increased K_d values. In contrast both negatively and positively charged side chains result in interactions that reduce binding affinity and disfavor receptor activation. Recent receptor mutagenesis studies, combined with the use of some of these α-factor analogs and photocrosslinking analysis, provide information as to the location of this pocket in the α-factor receptor (see below).

3.5. α-*N*-acylation or *C*-amidation of α-factor

The drop in receptor affinity observed with desTrp^1-α-factor could result from the loss of interaction of Ste2p with the indole side chain, the elimination of interactions with the α-NH_3^+ or the first carbonyl of the pheromone, or a combination of these phenomena. Previous studies had provided ambiguous data on the importance of the α-amine, although it was clear that derivatization of the carboxyl terminus of α-factor led to an inactive pheromone [99,104,130]. The importance of the α-amine was probed by synthesizing α-*N*-acetyl derivatives of α-factor, [Cha^1]α-factor and [Cpa^1]α-factor [156], where Cpa is parachlorophenylalanine. Comparison of the biological activity and receptor affinity of these compounds showed that α-acetylation resulted in a 20–40-fold

decrease in receptor affinity but had almost no effect on receptor activation. In contrast to the effect of derivatization of the amine terminus, amidation of the carboxyl terminus leads to a sharp drop off in both agonist activity and receptor affinity. These results have significant implications both for the binding interactions and the design of biochemical probes. The need for a positively charged α-amine for efficient binding together with the poor affinity of [Glu^1]α-factor suggests that there may be a negatively charged group in this region of the receptor binding site. Moreover, placing fluorescent or affinity tags at either the amine or carboxyl terminus would likely not be efficient because this would significantly reduce receptor affinity. We have noted both of these constraints in probing the binding site using fluorescent α-factor analogs (see below).

3.6. Position 2 α-factor analogs

Earlier studies had shown that α-factor affinity for Ste2p was almost one order of magnitude lower at pH 8.0 than at 6.0 [13]. This led to the suggestion that the protonation of His^2 of α-factor might increase binding to the pheromone. To study the importance of the His side chain on α-factor potency and receptor affinity we synthesized a series of derivatives in which the imidazole ring was replaced with thiophene, 1-methyl imidazole, 3-methyl imidazole and 3-pyridyl groups [86]. We noticed some interesting chemistry during the preparation of these compounds. For example, during the synthesis of 1-methylhistidine and 3-methylhistidine analogs of α-factor we observed significant heterogeneity in crude products as judged by HPLC. During the synthesis of [β-DL-thienylalanyl^2]α-factor, starting with the racemic amino acid, we observed that equal mixtures of the D- or L-containing peptide diastereomers were well separated by the reversed-phase column. Based on this we presumed that the closely moving side products found in the chromatogram of the methylhistidine-containing analogs were the result of racemization during the diisopropylcarbodiimide mediated coupling step used in the synthesis [86]. Others have noted racemization during the carbodiimide activated addition of methylhistidines to growing chains [136]. Fortunately the power of HPLC is highly suited for the separation of peptide diastereomers and we isolated both the *L* and *D* isomers from all synthesis.

Bioassays indicated that the substitution of carbon and sulfur atoms for the nitrogens of the imidazole of histidine still yielded agonists as judged by growth arrest and morphogenesis assays. Potency was lowered from 5- to 100-fold depending on the strain and the assay used. Similar results were observed with the L-analogs containing 1- or 3-methylhistidine or β-pyridylalanine in place of histidine. In all cases receptor affinity dropped by 15–30-fold. Similar to findings in the Ala-scan experiments, all analogs containing D-amino acid replacements at position 2 were antagonists. Interestingly both [L-Ala^2] and [D-Ala^2]α-factors had similar receptor affinities (Table 2) approximately 100-fold lower than that of α-factor. The results show that neither the nitrogens nor the five-membered ring in the side chain of α-factor are necessary for activity or receptor binding. Accordingly, protonation of His^2 of α-factor can not be essential for binding. However, it is clear that optimum receptor affinity is achieved only with His in position 2 among the many analogs tested, and this may indicate that protonation of histidine increases interaction of the pheromone with Ste2p or favors the biologically active conformation of α-factor. Only the determination of a high-resolution structure of the peptide bound to Ste2p will resolve this issue.

3.7. Position 13 α-factor analogs: design of iodinatable α-factor

An important objective of studies on GPCRs is learning how they recognize their ligand and how this recognition results in information transfer. Analogs of pheromones are useful from this perspective as they can be designed with chemically or photochemically active groups that promote crosslinking to the receptor. Such crosslinking can be used to characterize the contact points between the ligand and the receptor. In order to conduct the biochemical analysis of peptides crosslinked into receptor binding sites, tags must be inserted into the molecules. Radioisotopes are commonly used for such purposes. Unfortunately the specific activity achievable via tritiation does not provide the sensitivity required for the identification of crosslink sites. In addition the ^{35}S α-factors available biosynthetically are difficult to obtain in quantities necessary for the above analyses and are not compatible with the preparation of peptide analogs suitable for crosslinking. Insertion of ^{125}I into α-factor could result in specific activities exceeding 2000 Ci/mmol and would provide a suitable reagent for the crosslinking studies.

Attempts to iodinate α-factor were originally reported in the 1970s [49,91]. These studies indicated low activity and low recoveries of iodinated α-factor and did not discuss chemical characterization of the radioactive products. Moreover, the chloramine T method was reported to result in complete inactivation of the pheromone. Subsequently, treatment of native α-factor (Met^{12}) with chloramine T was shown by HPLC to completely degrade this peptide [132]. None of the aforementioned iodinated materials was used in a receptor binding assay. Furthermore, in the original report by Masui et al., replacement of Tyr^{13} in α-factor with Phe resulted in a 10^4 loss in biological activity [99] and our Ala-scan study suggested that the Tyr side chain was extremely important for binding.

Despite these examples we and others decided to reevaluate structural modifications of the position 13 side chain [88,132]. Our working hypothesis was that the OH of the phenolic side chain could be replaced with other electronegative groups. Accordingly we synthesized a series of analogs in which Tyr was replaced by Phe, *m*F-Phe, *p*F-Phe, *p*NH_2-Phe and *p*NO_2-Phe. Surprisingly we found that all of these analogs were excellent agonists with potencies nearly equiv-

alent to that of α-factor in both the halo and gene induction assay, and binding constants nearly equal to (Phe, *p*F-Phe) or within a factor of 6 of that of α-factor [88]. These studies taken together with the lack of binding observed for [Ser^{13}] and [Ala^{13}]α-factors [2] suggest that productive binding to Ste2p by α-factor requires an aromatic ring in position 13 and that the phenolic oxygen atom is not essential. Moreover the fact that Ser and Ala residues in position 13 still lead to signaling suggests that the aromatic ring is not necessary for the conformational change(s) leading to the activated receptor. Interestingly another report indicated that [Phe^{13}]α-factor had a receptor affinity of 4.05×10^{-6} M [125]. This is nearly 20-fold lower than our binding constant for the same peptide.

Having learned that Tyr^{13} could be replaced, an iodinatable pheromone could be generated by putting Tyr in other positions of the α-factor backbone. We found that iodotyrosine could be substituted for either Trp^1 or Trp^3 and that 100 nM affinities could be obtained. Indeed [Tyr^1(^{125}I), Phe^{13}]α-factor was prepared using Iodogen at high (>1000 Ci/mmol) specific activity. This probe was readily purified to homogeneity by HPLC, exhibited saturation binding with a K_d = 81 nM (Fig. 3A) and was specifically competed from the receptor by α-factor and an α-factor antagonist (Fig. 3B; [88]). Binding was receptor dependent and replacement by α-factor indicated a *Ki* = 7.7 nM, in good agreement with the reported affinity of the α-factor for Ste2p. Another group inserted Tyr at the amino terminus of the tridecapeptide to give a tetradecapeptide [Y^0, Phe^{13}]α-factor that was iodinated by the chloramine T method. The [Y^0(^{125}I), Phe^{13}]α-factor so generated had a $K_d = 6.4 \times 10^{-8}$ M, [132]). The iodinatable products can be generated in a few hours from stable precursors and can be rapidly purified by HPLC. We note however, that we have experienced difficulty in inserting iodine when Trp is at position 3 of α-factor (see below), and we have not been successful with the chloramine T procedure. Moreover in order to remove problems with background we use low protein binding filters and 1% BSA in the binding cocktail.

3.8. α-*Factor antagonists*

The first analog found that interfered with α-factor induced growth arrest or change in cell shape of *S. cerevisiae* was desTrp^1[Ala^3]α-factor [104]. Interestingly the corresponding Phe^3 dodecapeptide was also an antagonist whereas the Cha^3 dodecapeptide was not. This suggested that a minimal hydrophobicity near the amine terminus was critical for the induction of a receptor-mediated response. A systematic analysis of the effect of removing residues from the termini on activity and receptor affinity indicated that loss of one residue from either terminus (desTrp^1 α-factor; desTyr^{13} α-factor) led to weak agonists [48]. Interestingly, removal of two residues from either terminus (desTrp^1, desHis^2 α-factor; desMet^{12}, desTyr^{13} α-factor resulted in completely inactive peptides. However, desTrp^1, desHis^2 α-factor was a strong antagonist whereas desMet^{12}, desTyr^{13} α-factor did not bind at the pheromone binding site of the receptor. Remarkably,

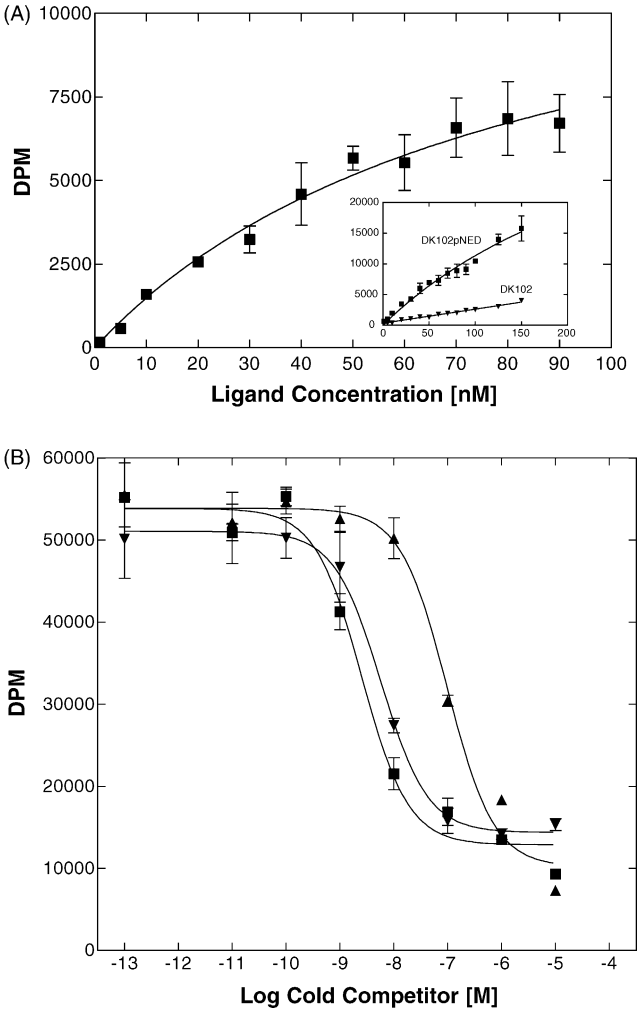


Fig. 3. Binding of iodinated α-factor to *S. cerevisiae* plotted as disintegrations per minute (DPM) versus ligand concentration. Saturation [Panel A] and competition binding assays with [Tyr^1(^{125}I), Phe^{13}]α-factor [Panel B] are represented. The inset represents the total counts bound to cells with receptor (DK102pNED1, ■) and total counts bound to cells without receptor (DK102, ▲) [Panel A]. Competition for binding of [Tyr^1(^{125}I), Phe^{13}]α-factor to Ste2p receptor was performed using α-factor (■), Tyr^1(^{127}I$_2$), Phe^{13} (▲), and Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Nle-Tyr an antagonist (▼) as competitors (Panel B). Data is taken from reference [88].

when mixtures of α-factor and desMet^{12}, desTyr^{13} α-factor were tested in a cell morphogenesis assay the inactive (1–11 analog) peptide potentiated the activity of α-factor. This synergistic activity was not due to influence on peptide degradation nor was it due to a direct interaction with the α-factor. To our knowledge there is no other example of a synergistic effect of an inactive peptide hormone; the molecular mechanism of this phenomenon is not known.

In later studies antagonists and synergists were also found for tridecapeptide analogs. In particular analogs with D-residues in position 2, 3 or 4 were all found to be antagonists [2,86] whereas [D-Ala^{10}] was a synergist [2]. Antagonism and synergism could be demonstrated both on agar plates and in solution assays (Fig. 4; [2]). These results again point to the

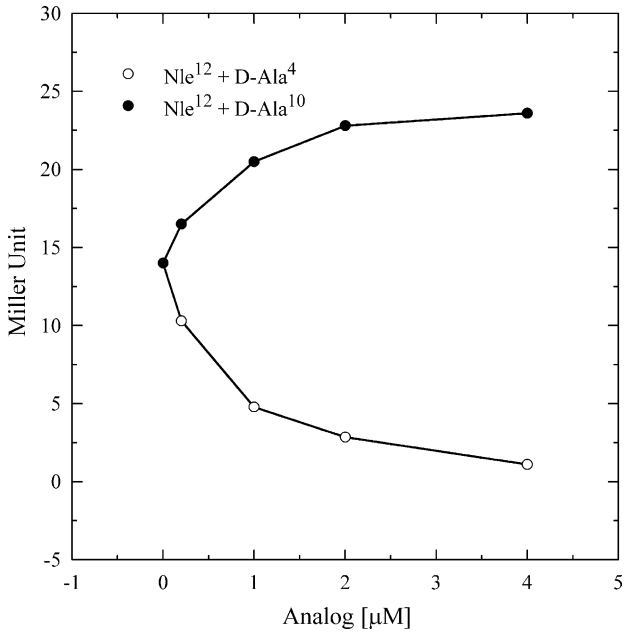


Fig. 4. Antagonist and synergist activity of α-factor analogs in the presence of α-factor. The α-factor induction of *FUS1* gene activity, as judged using a β-galactosidase assay expressed in Miller units, was measured in the presence of an antagonist [D-Ala^4]α-factor or a synergist [D-Ala^{10}]α-factor. Data taken from reference [2].

importance of the pheromone termini for binding (carboxyl end) and signaling (amino end) and show that the correct spatial configuration at certain residues is critical for productive interactions. The requirement of the correct conformation of the central region of the pheromones was also demonstrated in studies of peptidomimetic α-factors where analogs containing the Freidinger γ-lactam were agonists or antagonists depending on the terminal residues (see below).

In addition to the intrinsic interest in antagonists because they function as pharmacologically active agents, these molecules have been useful tools for biochemists and geneticists interested in the α-factor/Ste2p interaction. Studies using desTrp^1[Ala^3]α-factor showed that receptor with bound agonist or antagonist or with no ligand bound had different sensitivities to proteolysis [17]. This provided experimental support that Ste2p conformed to models hypothesizing various isomeric states for GPCRs at different stages in the activation pathway. Antagonists have been used to characterize mutant receptors that were isolated in screens designed to find Ste2p with constitutive activity [134] or chimeras of *S. cerevisiae* and *S. kluyveri* Ste2ps [127]. In studies on chimeric receptors derived from *S. cerevisiae* and *S. kluyveri* it was noted that an α-factor antagonist behaved like an agonist. It was suggested that the antagonist desTrp^1[Ala^3]α-factor behaved as a kinetic antagonist with wild-type Ste2p. In such a case receptor activation is energetically favorable but a high free energy of activation prevents equilibrium of the ligand/receptor complex from being obtained [128]. Our studies have also shown that characterization of receptor mutants using antagonists often provides insights not readily apparent from bioassays or binding studies using agonists. For example, we found that Y266A mutant of Ste2p preferentially bound antagonist as compared to wild-type receptor [84]. This creates the possibility that the mutant receptor could be used to screen for antagonists. Since many pharmaceuticals are receptor antagonists, knowledge of factors that stabilize an antagonist favored state of a GPCR would be of considerable interest.

3.9. Conformationally constrained analogs: the hunt for the bound structure of α-factor

The elucidation of the bound structure of a peptide ligand to its GPCR is a key issue in understanding how the ligand can activate its signal transduction pathway. Just as understanding the transition state of an enzyme substrate gives insight into the mechanism of enzymatic catalysis, revealing the atomic-level structure of a bound ligand would provide insight into the mode of GPCR activation. Unfortunately, little information exists concerning the structure of peptides bound to their cognate GPCRs. This is due to the absence of crystal structures on these membrane bound receptors and the high molecular weight of receptor-membrane complexes which prevents high-resolution NMR analysis. Given this situation one approach to the structure of α-factor bound to Ste2p would be transferred nuclear Overhauser spectroscopy [20,31-33,59]. This technique has been used to study the bound structure of some peptides and G proteins [7,82] and of acetylcholine and its receptor [11]. However, this procedure requires that the off-rate of the ligand be fast in comparison to the T_1 relaxation rate. As noted above α-factor has a very slow off-rate preventing such studies at this time, although recent work in our laboratory suggests that certain α-factor analogs or derivatives might be suitable for such studies (Naider and Becker, unpublished results).

Indirect information concerning the biologically active structure of α-factor can be obtained by comparing the structures of active and inactive analogs in solution and by then using this information to design conformationally constrained probes. Early attempts to analyze the structure of α-factor in solution gave contradictory results. CD studies on native α-factor (Met^{12}) in buffer indicated a disordered molecule; addition of lipid resulted in a significant change that was interpreted as an increase in secondary structure [64,65]. Other CD studies in trifluoroethanol and Tris buffer on desTrp^1-α-factors gave similar results with active and inactive pheromones exhibiting different CD patterns [131]. Nevertheless, none of the CD patterns were suggestive of an ordered pheromone. A significant observation in the early literature that remained unnoted was the conclusion based on fluorescence analyses that the C terminal carboxylate group of α-factor was close to the N-terminal domain [64]. This supports current models for the structure of the bound pheromone (see below).

An NMR study in solution suggested that the active conformation of α-factor had a helical domain at the amine terminus and had β-turns in the center and at the carboxyl

terminus of the tridecapeptide [64]. Later work using transferred NOE analysis on membrane bound α-factor concluded that the amine terminus bound strongly to membranes and was a compact helix, while the center of the peptide was extended and the carboxyl terminus was free outside the membrane [144,145]. These studies claimed correlation of NMR structures with biological activity. Unfortunately this group was using incorrect biological activity data that concluded that residues Pro^{11}-Tyr^{13} were not essential for activity [98]. Furthermore all of these early studies used 1D NMR approaches that did not have sufficient resolution to make unequivocal assignments. Our group has carried out detailed NMR studies, using 2D NMR procedures including NOESY and ROESY approaches, on active and inactive dodecapeptide and tridecapeptide α-factors [57,72,73,106]. Studies were conducted in organic solvents, water, and in the presence of vesicles. We base our conclusions primarily on NOE data and on amide proton exchange rates. In all media examined, α-factor and its analogs are primarily disordered, flexible peptides. The studies show these peptides bind strongly with lipid with the amine terminus interacting preferentially with the bilayer and thereby more highly immobilized. Nevertheless, no evidence has been seen in any study for a helical structure at the N-terminus either in the solution or lipid-bound state. A typical NOESY spectrum for an active compound exhibited a very strong $(Pro_{\alpha CH})^8$-$(X_{NH})^9$ crosspeak (Fig. 5) that was weak or absent in inactive compounds [57,73]. These results indicate that a transient Type II β-turn involving residues Lys^7-Gln^{10} is present in the solution structure of active agonists. Inactive pheromones lack this structural feature, and it is mostly absent in weak agonists. The presence of a turn around the central residues of α-factor is a major feature of our working hypothesis for the biologically active structure of this peptide, presumably its receptor-bound state.

The Ala-scanning analysis and previous studies on dodecapeptides indicated that a Type II β-turn might be present in the biologically active structure of the pheromone [1,131]. If this were true, then constraining the structure of the center of α-factor would be expected to lead to active analogs, possibly with increased receptor affinity. In parallel with the conformational analyses we set out to synthesize analogs of α-factor having constraints in the center of the peptide. Two strategies were used. The first involved covalent bond formation between the side chains of residues that were at the termini of the putative turn region. The second involved insertion of unnatural amino acid residues or peptidomimetics that would be expected to rigidify the central region of α-factor and thereby favor a turn.

The first conformationally constrained analog of α-factor to be synthesized contained a covalent link between the side chains of Lys^7 and Gln^{10} ($cyclo^{7,10}$α-factor) and was found to be an agonist with one-fourth to one-twentieth of the activity of the linear pheromone and a 20–40-fold lower receptor affinity [151]. These results provided conclusive evidence that an analog of α-factor forced into a turn-like structure

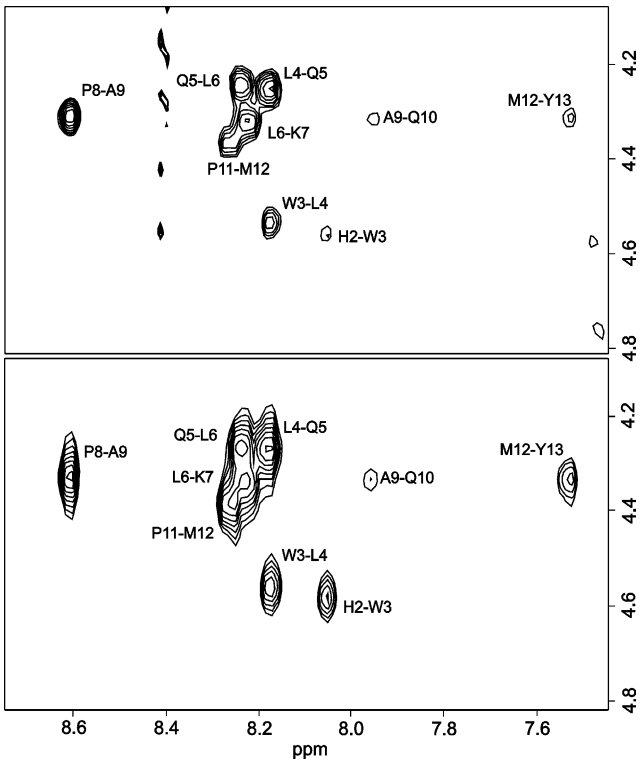


Fig. 5. Nuclear Overhauser spectra of [D-Ala^9]α-factor in water. The top panel represents the 400 ms NOESY spectrum. The bottom panel the 250 ms ROESY spectrum. Crosspeaks in the αCH-NH region are indicated by a single letter notation for the amino acid residues. The strong P^8-A^9 crosspeak indicates a Type II β-turn. Data taken from reference [57].

around the central residues could both bind to the receptor and trigger activity. This was de facto evidence that the biologically active pheromone was likely bent when bound to the receptor. The cyclic lactam constraining the center of $cyclo^{7,10}$α-factor was an 18-membered ring and retained considerable flexibility. We therefore carried out a systematic analysis of the effect of composition and ring size in the cyclic α-factors by preparing different analogs containing from 14 to 18 atoms in the lactam moiety [154]. The biological activites and receptor affinities of this series of compounds showed that lactams with Glu at position 10 exhibited higher affinity than similar analogs with Asp at this position. As with studies on other analogs (see above) our results pointed again to a clear dissociation between binding and biological activity. For example whereas [$cycloDap^7$, Glu^{10}]α-factor, [$cycloDab^7$, Glu^{10}]α-factor and $cyclo^{7,10}$α-factor (where Dap = 2,3-diaminopropanoic acid and Dab = 2,4-diaminobutanoic acid) had basically the same potency in the halo assay, receptor affinities differed by nearly 15-fold. A similar absence of a correlation between binding affinity was observed in a control series of linear compounds. This study provided additional proof that the presence of a turn in the center of these α-factor analogs was compatible with receptor recognition and triggering, although none of these cyclic peptides had binding affinities within a factor of ten of the linear analogs or of wild-type α-factor. This suggested

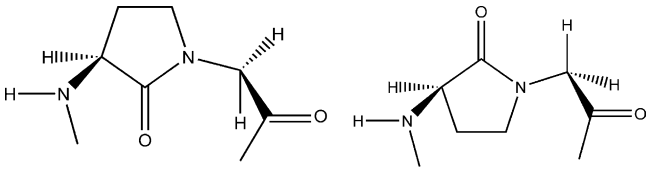
that the cyclic lactams did not result in the correct conformation of the center of the molecule, that specific interactions of the side chains of residues 7 and 10 also were important for binding affinity, or that the constraints prevented optimal interactions between the N- and C-termini of α-factor and Ste2p.

In separate studies, we carefully analyzed the conformations assumed by the cyclic regions of the constrained α-factors. For these studies model tetrapeptides corresponding to the central region of these α-factor analogs were synthesized and analyzed. The synthesis of the cyclic lactams was accomplished using on resin cyclization [112,151]. Interestingly, during preparation of the model compounds we noted a side product that reflected intermolecular cyclization [112]. The cyclic peptides were subjected to a comprehensive analysis using CD, NMR and molecular modeling. The results indicated that these cyclic peptides adopt a variety of conformations that can be related to Type II β-turns, and various γ-turns but never to Type I β-turns [95]. Most importantly similar analyses on the corresponding α-factor analogs showed that these structural features are maintained in the tridecapeptide [6]. Thus, in solution these pheromones assume turn structures around the Lys-Pro-Gly-Gln sequence but the turns are different in the various cyclic analogs, and there does not appear to be an absolute correlation between a given turn structure and either bioactivity or receptor affinity. For example although both cyclo^{7,10}[Lys, Glu]α-factor and cyclo^{7,10}[Dab, Glu]α-factor have virtually the same biological activities, the first analog assumes a γ′-turn around Pro^{8} whereas the latter assumes a nearly perfect Type II β-turn [6,95]. Thus it appears that different conformations in the center of α-factor can result in productive interactions with Ste2p. Perhaps one advantage of having different conformations of a peptide ligand is to provide flexibility in the bend region. Such elasticity would allow the pheromone to "breathe" in the binding pocket and act as a "triggering mechanism". This model would envisage the movement of the pheromone when bound to the receptor initiating further conformational change in the receptor to activate the signal transduction pathway.

The results with the lactam-constrained α-factor analogs supported the presence of a turn in the central region of this peptide and gave evidence that a bent molecule could both activate and bind to the receptor. Nevertheless, the biophysical analyses indicated that the rigidified portion of many of the pheromones had significant conformational flexibility. Moreover, the biological activities of the constrained analogs were never as high as those of the linear homologs. To further probe the influence of conformational rigidification, analogs containing cyclic disulfides in the central region of α-factor were designed and synthesized. The logic for these compounds was the observation by several groups that cyclic tetrapeptides with the sequence -Cys-Pro-X-Cys- form β-turn conformations stabilized by 4 → 1 hydrogen bonds [3,54,77]. Most significant was the report that Ac-Cys-Pro-D-Val-Cys-NH_2 formed a Type II β-turn in solution [54]. We reasoned that incorporation of these 14-membered cyclic constraints in the middle of α-factor would allow us to test the importance of the Type II β-turn in α-factor activity.

The synthesis of cyclic disulfide-constrained analogs of α-factor such as cyclo^{7,10}[Cys^{7}, X^{9}, Cys^{10}]α-factor (X = Gly, L-Ala, D-Ala, D-Val) was carried out using on-resin disulfide formation with thallic trifluoroacetate as the oxidizing agent [3,153]. Overall yields of 20–30% of the purified cyclic product were obtained. Bioassays indicated that similar to cyclo^{7,10}-α-factor, cyclo^{7,10}[Cys^{7}, D-Ala^{9}, Cys^{10}]α-factor was about 10-fold less active than α-factor [152]. The corresponding L-Ala^{9} disulfide constrained peptide was about 2–5-fold less potent than its D-Ala^{9} homolog. Significantly, conformational analysis in water and DMSO/water (80:20) showed that the D-Ala analog assumed a Type II β-turn whereas the L-Ala analog assumed a mixture of Type I/III turns under identical conditions [58,105]. This suggested that a Type II β-turn in the center of α-factor was slightly more efficient in activating the receptor. Furthermore, insertion of 5-aminopentanoic acid in place of the Pro-X moiety in the above disulfide constrained α-factor resulted in a completely inactive pheromone. This suggested that a specific conformation in the center of the peptide and not the 14-membered cyclic ring was essential for bioactivity, although the contribution from the Pro side chain may also be a contributing factor (see below). It is most important that for the cysteine-containing α-factor analogs in all cases the cyclic peptide was more active than the homologous linear variant. Moreover, in the case of the L-Ala^{9}-peptides all linear control molecules had no activity, whereas the cyclic disulfide was nearly as active as cyclo^{7,10}[Cys^{7}, D-Ala^{9}, Cys^{10}]α-factor. The affinity of the cyclic disulfide containing α-factors were at least an order of magnitude lower than that of α-factor. However, again the linear peptides were usually much poorer binders than their cyclic counterparts. These results strongly suggest that a turn in the center of the molecule is essential for binding to the receptor and agonist efficacy, although a Type II β-turn is clearly not required.

The formation of covalent bonds between the side chains of residues 7 and 10 of α-factor greatly alters the chemical nature and spatial orientation of these atomic groupings. Analysis of the Ala-scanned series showed that substitution of L-Ala or D-Ala at either of these positions resulted in significant drops in bioactivity and marked decreases in affinity. Thus in constraining the center of α-factor by cyclization it is possible that the low receptor affinities were simply due to loss of productive interactions between the position 7 and 10 side chains and Ste2p. Many other approaches to conformational rigidification for enhancing the bioactivity and receptor affinity of peptide hormones have been explored [70,117]. In particular peptidomimetics that replace the central residues of β-turns have been developed and inserted in place of the *i* + 1 and *i* + 2 positions [55,66,89,103]. We decided to insert a γ-lactam [3-(*S* or *R*)-amino-2-oxo-1pyrrolidinoacetamido] dipeptide developed by Freidinger et al. [52] in place of Pro^{8}Gly^{9} of α-factor and explore its influence on bioactivity and receptor affinity. This moiety (Fig. 6) was reported to



(S)-γ-lactam
3-(S)-amino-2-oxo-1-pirrolidineacetamido

(R)-γ-lactam
3-(R)-amino-2-oxo-1-pyrrolidineacetamido

Fig. 6. Structure of Freidinger's γ-lactams.

favor a Type II′ β-turn structure and was relatively easy to incorporate into the solid phase synthesis of α-factor. In the same study we also investigated the influence of replacing Gly[9] with D-*N*-methylalanine or sarcosine. Theoretical calculations concluded that substitution of Pro-D-Me-Ala into a peptide strongly stabilizes a β-turn [21].

The α-factor peptidomimetics containing the above moieties proved to be exciting compounds. The (***R***)-γ-lactam α-factor was about as active as α-factor and bound with only four-fold lower affinity [157]. The [N-Me-D-Ala[8]]α-factor was actually slightly more active than α-factor in both the halo and gene induction assays and bound with nearly twice the affinity to Ste2p. Careful inspection of the stereochemistry of the (***R***)-γ-lactam indicates that the configuration of the five-membered ring (the proline replacement) is the same as that of a D-amino acid residue. Since replacement of Pro[8] with D-Ala or D-Pro leads to a 300-fold or 125-fold drop in affinity, respectively, the high affinity of the (***R***)-γ-lactam α-factor is actually remarkable. Apparently the conformational restriction imposed by this peptidomimetic can compensate for an unfavorable configuration at this position in the peptide backbone.

A detailed conformational analysis [157] using NMR, CD and molecular dynamics simulations concluded that the (***R***)-γ-lactam α-factor is a flexible molecule in solution with a propensity to assume the following dihedral angles in the center of the peptide [$\varphi_2 = 110^\circ$, $\psi_2 = 135^\circ$, $\varphi_3 = 97^\circ$, $\psi_3 = -50^\circ$]. The first two angles deviate significantly from a Type II β-turn whereas the latter are consistent with those of a γ-turn. This finding is consistent with results from an X ray analysis of the (***R***)-γ-lactam Pro-Leu-Gly analog [51]. Thus, it is likely that the peptidomimetic (***R***)-γ-lactam α-factor assumes a turn around residues 7–10 that favors γ-turn angles around the *i* + 2 residue (normally Gly[9]). These results are for the only example of an α-factor mimetic that has activities and affinities equal to those of the parent tridecapeptide. They provide convincing evidence that the molecule assumes a bent structure when bound to the receptor. Moreover, the totality of information available suggests that the receptor probably preferentially binds this structure from a mélange of α-factor conformers and that a certain amount of flexibility in the pheromone may be necessary to achieve optimum binding, affinity, and triggering of the signal transduction pathway as suggested previously in this review. From this perspective it is noteworthy that out of the hundreds of analogs of α-factor that we have synthesized none are superactive or have affinity very much greater than the parent hormone. We attribute this to the fact that during the uncountable generations of *S. cerevisiae* existence the α-factor and its receptor have evolved to highly optimal complementarity.

3.10. Position 7 analogs – fluorescent probes of the binding pocket

Studies with analogs of the desTrp[1]-α-factor provided information concerning the relationship between an ε-amino group in the Lys[7] side chain and biological activity [104]. Modification with fluorescent groups, biotinylation and acylation with a variety of groups indicated that marked modification could be accommodated at Lys[7] and replacement of Lys[7] in desTrp[1] α-factor by norleucine provided definitive proof that the ε-amino moiety was not necessary for activity [104]. However, none of the early studies determined the receptor affinity of the dodecapeptide α-factors. After development of the receptor binding assay we observed that desTrp[1][Cha[3], Lys[7](Ac)]α-factor bound with a 20-fold lower affinity than the unacylated homolog [113]. As we wanted to select α-factor derivatives for probing side chain environments in the Ste2p binding site and for use in detecting and isolating receptor fragments generated by photocrosslinking experiments (see below), detailed information on receptor affinities of the derivatized tridecapeptide was critical.

The studies on the lactam-constrained α-factors provided evidence that in the tridecapeptide α-factor a protonatable ε-amine was also not essential for activity. A control peptide in these studies [Lys[7](Ac)]α-factor had nearly the same activity as α-factor [151]. This result was confirmed by the Ala-scanning analysis [2]. Indeed [L-Ala[7]]α-factor had the fourth highest affinity of the peptides in the L-Ala-scanned series. In a series of linear α-factor analogs containing Dap, Dab, Orn or Lys at position 7 (where Dap is diaminopropionic acid, Dab is diaminobutyric acid, and Orn is ornithine), growth arrest activity nearly equal to α-factor was found [154]. Thus the amine group could be 2–5 bonds from the peptide backbone without affecting biological activity. Nevertheless, these peptides varied by a factor of 60 in their receptor affinity, with the lowest affinity observed for [Dab[7], Glu[10]]α-factor.

Given the apparent tolerance of the position 7 side chain to modification, we decided to study the environment of this side chain when α-factor was bound to Ste2p. Our approach involved the incorporation of an environmentally sensitive fluorescent probe at this position of the pheromone and the systematic variation of the distance of the probe from the peptide backbone [38,39]. For these investigations the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group was chosen as the probe because of its high fluorescent yield and its capacity to report on the hydrophobicity/hydrophilicity of its environment. The number of bonds separating the NBD from the C_α of residue 7 varied from 2 to 12 (Table 3) corresponding to a distance from about 3.5 Å to nearly 18 Å if

Table 3
Fluorescent α-factor analogs as probes of the Ste2p binding site

α-Factor analog[a]	Bonds to NBD[b]	*Ki* (nM)[c]	λmax[d] (nm)	*K*sv[e]
α-Factor		8.0	NF[f]	NF[f]
[Dap^7(NBD)]	2	47	539	3.9
[Dab^7(NBD)]	3	43	510	0.2
[Orn^7(NBD)]	4	6	540	6.4
[Lys^7(NBD)]	5	27	510	0.2
[Lys^7(*ah*NBD)]	8	33	539	8.6
[Dap^7(β-Ala-NBD)]	12	12	540	9.0
[Dap^3(NBD), R^7]	2	23	520	1.6
[Lys^3(NBD), R^7]	5	40	538	6.2
[Lys^4(NBD), R^7]	5	560	N.D.[g]	N.D.[g]
desTrp^{1}desHis^2[K^7(NBD)]	5	124	N.D.[g]	N.D.[g]

[a] NBD is nitrobenzoxadiazole.
[b] Number of covalent bonds between the α-carbon of the amino acid and the nitrogen bound to the NBD ring.
[c] Determined by binding competition with tritiated α-factor.
[d] Emission maximum excitation was at 475 nm.
[e] Stern-volmer quenching constant.
[f] Not fluorescent.
[g] Binding was not high enough to determine the λ_{max} of *K*sv values (taken from references [38,39]).

the side chain is extended. Importantly, all of the fluorescent position 7 analogs had high receptor affinities varying from slightly better than α-factor to about six-fold lower than this pheromone. This suggested that all of these peptides bound very similarly to the receptor and that the fluorescence of the side chain fluorophore could tell us about its environment. The λ_{max} values of the six peptides examined fell into two groups. The first group had a λ_{max} of 540 nm, which is close to that expected for NBD in aqueous buffer. The second group had a λ_{max} of 510 nm that was greatly shifted to the blue. Quenching analysis showed that those peptides with a blue shifted λ_{max} were protected from quenching by iodide. Those that were not shifted were quenched to different degrees with the longer side chains showing quenching equivalent to that of the probe free in solution. These results allowed us to conclude that the position 7 side chain was pointed away from the transmembrane domains and was in a region of variable polarity that was composed of residues contributed by the extracellular loops of Ste2p. We have incorporated this observation in our working model of the receptor-bound α-factor.

Following up on the position 7 side chain, we have also inserted the NBD probe at the α-amine, and the side chains of positions 1, 3, 4, 6, 12 and 13 [39]. Only the position 3 side chain modifications had both fluorescent properties and receptor affinities suitable for binding environment analysis. We also made fluorescent α-factor antagonists hoping to examine whether the antagonists and agonists bound similarly to the receptor. However, the affinity of the antagonists was about 120 nM, just on the limit of sensitivity for our fluorescence analysis, and we could not come to definitive conclusions. Recently, in collaboration with Mark Dumont (U. of Rochester) we have developed a whole cell receptor binding assay using [Lys^7(NBD)]α-factor (Dumont and Naider, unpublished results). The results are extremely encouraging because a non-radioactivity based binding assay for the α-factor receptor may soon be available for selecting interesting receptor mutants and analyzing ligand–receptor interactions.

3.11. Position 10 α-factor analogs – pinpointing receptor–pheromone interactions using α-factor analogs

Saccharomyces kluyveri is a budding yeast and like its *S. cerevisiae* cousin encodes mating pheromones and cognate GPCRs. The *S. kluyveri* α-factor (Sk-α-f) is also a tridecapeptide (TrpHisTrpLeuSerPheSerLysGlyGluProMetTyr) with eight residues identical to those of the *S. cerevisiae* α-factor [46,120]. The receptors for these pheromones are 50% identical [96]. The two tridecapeptides show a strong preference for their own receptors [101,127]. A series of studies using constructed chimeric receptors and both pheromones indicated that a significant element of the respective α-factor recognition by *S. cerevisiae* and *S. kluyveri* receptors resides in positions 47–49 of the respective GPCRs [126,127]. Scrutiny of the sequence of these regions indicates that residues KKI in the *S. kluveryi* Ste2p receptor are STV in the *S. cerevisiae* Ste2p. Based on this observation Marsh and co-workers suggested that this region of Ste2p must interact with one of the five non-conserved residues of α-factor [128]. Given that Sk-α-factor and the *S. cerevisiae* α-factor have Glu and Gln, respectively, in position 10, we hypothesized that it was this pheromone side chain that interacted with the 47–49 region of the receptor. We tested this possibility by synthesizing a series of position 10 analogs in which the natural Gln was replaced by Asn, Ser, Lys, Orn, Glu and Asp [83]. Following an approach of concomitant receptor and peptide ligand mutagenesis suggested by others [90,124,138], we carried out a site-directed mutagenesis of Ste2p in parallel.

Study of the above series of compounds showed that the structural requirement of the position 10 side chain of α-factor for high affinity binding to its receptor was very restrictive [83]. Changing Gln to Glu resulted in a 30-fold decrease in affinity. Moreover, changing Gln to Asn resulted in a 300-fold decrease in affinity, and the *Ki* for [Asp^{10}]α-factor was so low it could not be measured. Analysis of the data indicated that the binding of α-factor at this position could be related to the van der Waals' volume of the position 10 side chain. In all cases analogs with side chains having volumes close to that of Gln exhibited the highest binding. As found previously there was not a tight correlation with activity; [Glu^{10}]α-factor was actually a better agonist in a halo assay than α-factor and was nearly as good in a gene induction assay despite the 30-fold decrease in receptor affinity. Evaluation of position 10 analogs using Ste2p mutants in which S47 and T48 were mutated to K or E gave support for the interaction between these residues of the receptor and the position 10 side chain. For example the binding affinity of [Lys^{10}]α-factor was nearly twice as strong in the

EE receptor (Ste2p with mutations S47E and T48E) compared to wild-type, whereas it did not bind to the KK receptor (S47K, T48K receptor). Similarly the [Asp^{10}]α-factor and the Sk-α-factor ([Glu^{10}]) bound best to the KK receptor and did not bind at all to the EE receptor. Studies in the presence and absence of salt supported an ionic interaction at these positions of the mutant receptors and the α-factor analogs.

The results of these studies provide evidence that the side chain of the tenth residue of α-factor is in close proximity with the side chains of residues 47 and/or 48 of Ste2p when the pheromone is bound to the receptor. Interestingly, the Ala-scanning analysis also suggests an important role for the stereochemistry of the position 10 side chain. When D-Ala replaced Gln^{10} the resulting peptide had no biological activity and a greater than 1600-fold decrease in affinity. However, this peptide, which was shown to be a synergist (see above), bound relatively strongly to an F55V mutant receptor that was isolated in a study that used a screen for receptors responsive to α-factor antagonists [1]. This is further support for interactions between the carboxyl terminus of the pheromone and side chains of receptor residues near the extracellular interface of the first transmembrane domain and the receptor N-terminus.

3.12. Photoactivateable α-factor analogs: crosslinking α-factor into Ste2p

Perhaps the ultimate indication of contacts between atoms on a ligand and the associated receptor is the formation of a covalent bond between these partners. To achieve this end affinity labeling approaches involving the placement of chemically reactive groups in the side chains or end groups of peptides were developed. Although some success was realized with chemical groups, this approach is often limited by the requirement for nucleophilic side chains to react with the affinity label. The power of affinity labeling was raised significantly by the incorporation of photoactivateable groups into biomolecules. The active species could be generated when desired and many of the reactive intermediates could insert into C–H bonds, which are ubiquitous in amino acid side chains. This strategy has been applied for a number of peptide hormone – receptor studies [10,14,42,109,110], and we are using it to map contacts between α-factor and its binding site in Ste2p. One caveat concerning this approach is that the activateable moiety must not affect the binding to the receptor and must react specifically when bound to the receptor.

For these studies we synthesized a series of α-factor analogs in which *p*-benzoyl-L-phenylalanine (Bpa) was scanned throughout the backbone [63]. Bpa has been shown to have desireable photochemical properties for use as a probe of biological systems [146], although recent reports suggest that it may have a preference to react with Met residues [116]. In particular, replacement of residues 1, 3, 5, 7, 8 and 13 resulted in less than a 30-fold decrease in receptor affinity (Fig. 7). Perhaps the most surprising finding was the tolerance of this group at position 13 (see above). Based on these results we prepared [Bpa^1, (^{125}I)Tyr^3, Arg^7, Phe^{13}]α-factor and crosslinked it to yeast membranes. Western blots indicated incorporation of radioactivity into a protein with the molecular weight expected for Ste2p, and competition by α-factor showed crosslinking was at the pheromone-binding site (Fig. 8). It should be noted that in other investigations [^{35}S]α-factor [12] and [^{125}I]α-factor [132] were chemically crosslinked into Ste2p. However, no attempt was made to determine the site of the linkage to the receptor.

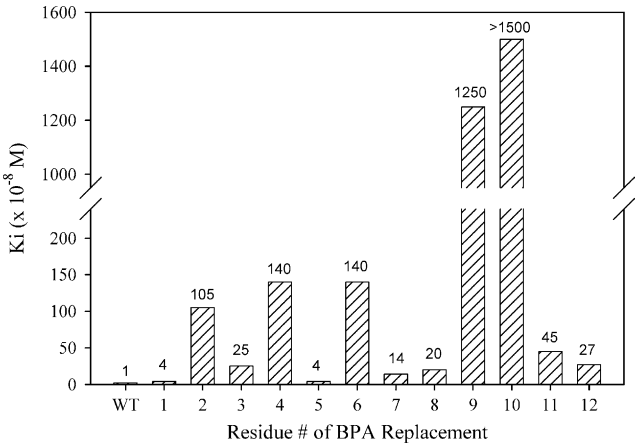


Fig. 7. Binding affinity of Bpa-scanned α-factor analogs. The number above each bar represents the *Ki* value ($\times 10^8$) of that analog. Data taken from reference [63].

Biochemical fragmentation of the [Bpa^1, Tyr^3(^{125}I), Arg^7, Phe^{13}]α-factor-crosslinked-Ste2p complex using CNBr, trypsin and BNPS-skatole reagent gave distinct fragmentation patterns. Using these analyses, [Bpa^1, Lys^7(ε-biotinyl-β-alanyl)]α-factor and an epitope tagged Ste2p (Ste2p-T7; [44]) the region of crosslinking was localized to residues 251–294 of the receptor corresponding to a portion of Ste2p comprising the sixth and seventh transmembrane domains and the connecting third extracellular loop. Thus, it seems highly likely that the side chain of position 1 of α-factor binds in this domain of Ste2p. Given our belief that the α-factor likely does not penetrate deeply into the GPCR, it is reasonable that position 1 side chain is near the extracellular face of the TM6 and/or TM7.

3.13. The activity and binding of alanine-scanned α-factor analogs support an interaction between Tyr 266 of Ste2p and residues near the amine terminus of α-factor

Many laboratories working on GPCRs are interested in TM5 and TM6, because these TMs surround the third intracellular loop of the receptor, which is known to interact with the heterotrimeric G proteins. Therefore, it is highly likely that subsequent to ligand binding, conformational changes mediated through TM5 and TM6 change the interaction of I3 with $G_{\alpha\beta\gamma}$ leading to downfield signal transduction. During site-directed mutagenesis of the extracellular face of TM6

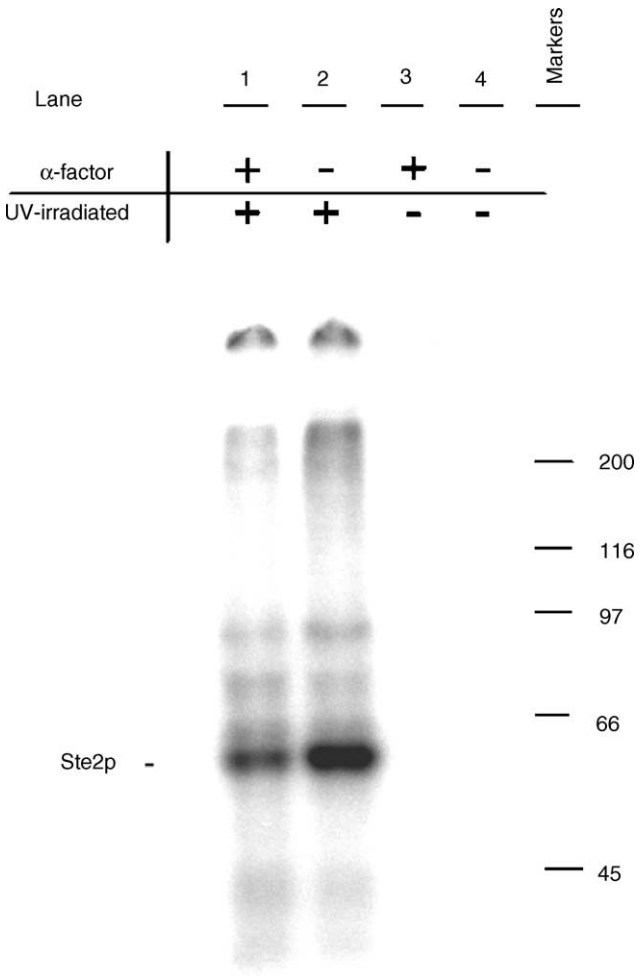


Fig. 8. Autoradiogram of SDS-PAGE analysis of photocrosslinking of α-factor into Ste2p. Membranes containing Ste2p were incubated with [Bpa[1], Tyr[3](125I), Arg[7], Phe[13]]α-factor (Lanes 1–4). Control samples either were not irradiated (Lanes 3 and 4) or contained α-factor as a competitor (Lanes 1 and 3). Lane 1, UV irradiated plus 100-fold excess nonradiolabeled α-factor; lane 2, UV irradiated; lane 3, 100-fold excess nonradiolabeled α-factor without UV irradiation; lane 4, without excess nonradiolabeled α-factor and without UV irradiation; lane 5, molecular weight markers. Data taken from reference [63].

(F262-P270) we observed that only the Y266A mutant was deficient in signaling [84]. A similar phenotype was reported for the Y266C receptor [43,133]. Although deficient in signaling, the Y266A mutant bound α-factor with high affinity (IC_{50} = 47 nM). Aromatic amino acids such as Phe and Trp were acceptable as Y266 replacements but hydrophilic (Ser, Lys) and alicyclic (Leu) side chains were not. Trypsin digestion patterns indicated that the Y266A mutant receptor did not undergo transition to the activated form of Ste2p upon ligand binding. The reason for this deficiency is unknown. It seems apparent that aromatic residues near the exofacial surface of the transmembrane regions of Ste2p are critical for stabilizing the activated conformation of this GPCR.

Studies with the Ala-scan series indicated that the binding relative to α-factor by Y266A receptor for Ala replacements in positions 1–4 of the pheromone was better compared to that of the wild-type receptor. In contrast, binding with respect to α-factor by Ste2p(Y266A) of Ala-α-factor analogs where substitution was at residues 5–13 was severely impaired. These results indicate that there is likely a direct interaction between Y266 and residues near the amine terminus of α-factor. Similar conclusions were reached by looking at binding affinities for antagonists and synergists (see above). Recently, we determined that Ste2p(N205A) exhibited a similar pattern of binding with single and double alanine analogs of α-factor (Becker and Lee, unpublished results). These results suggest that N205 and Y266 are in close proximity and are important residues for signal transduction. The contact between Y266 and the amine terminal residues of α-factor would be consistent with the crosslink found for the side chain of residue 1 and the 251–294 region of Ste2p.

3.14. A model for α-factor bound to Ste2p

Using results from studies with α-factor analogs and derivatives, photoaffinity labeling analysis and site-directed mutagenesis, a model for α-factor bound to Ste2p was proposed. The model (Fig. 9) places α-factor bent around the Pro-Gly center of the peptide with the Lys side chain facing away from the transmembrane domains and interacting with a binding pocket formed by the extracellular loops. The Gln^{10} side chain is proximal to residues 47 and 48 near the exofacial side of TM1, whereas the Trp^1 side chain is near a pocket formed by TM6-E3-TM7. It is likely that Trp^1 and Trp^3 interact with an aromatic group near the interface of the transmembrane domains and the outside surface of the membrane. The bent shape of α-factor could place Tyr^{13} in the general region of the two Trp residues. This might allow another interaction with another aromatic group in the receptor that lies in the same plane. Studies have implicated Tyr266 (TM6) and Phe204 (TM5) as such residues ([87]; Becker and Lee, unpublished results). Using this model of bound α-factor and results from mutagenesis, Cys scanning and Cys accessibility studies, the Konopka lab has proposed a 3D model that explains certain aspects of the activation of the receptor [87]. This model shows clusters of receptor residues that upon mutation lead to a dominant negative phenotype. As more α-factor–Ste2p contacts become available from the photocrosslinking studies, the pheromone can be docked into the binding site and a higher resolution, biologically relevant 3D structure can be proposed.

Given the above model and what is known about the activity and receptor affinity of truncated pheromones, we can suggest a pathway for binding and activation of Ste2p by α-factor. It seems likely that the first step in binding to the active site involves residues near the carboxyl terminus. As mentioned above, removal of Met^{12} and Tyr^{13} resulted in an inactive peptide that did not bind to Ste2p. In contrast removal of Trp^1 and His^2 lead to an inactive peptide, which bound strongly to the receptor. The α-factor diffuses to the active site, either directly, or by first binding to the membrane lipid [37,122,125], and then binds via contacts involving Met^{12}-

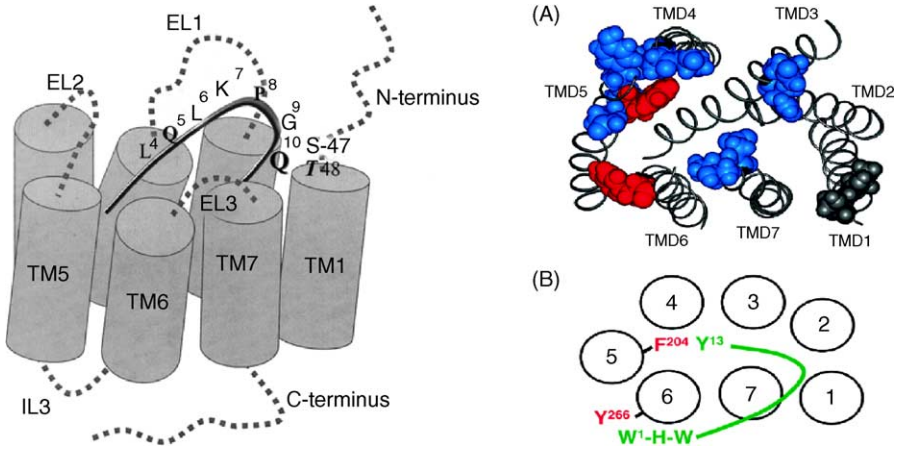


Fig. 9. Schematic representation of the α-factor binding to its receptor. The model at the left is taken from reference [83]. Only residues 4–10 (Leu-Gln) of α-factor are shown for clarity. The model at the right is taken from reference [87]. Panel A of this model shows a 3D model of the transmembrane domains with F204 and Y266 shown in red. Panel B indicates putative interactions of aromatic residues of α-factor with F204 and Y266.

Tyr^{13} and other residues at the carboxyl terminus. The peptide is not structured at this point and is sampling conformational space. When the appropriate bend at the Pro-Gly center is formed the amine terminus interacts with the binding pocket. This provides considerable additional energy and stabilizes the active conformation of α-factor. Signaling then begins, perhaps by rotation of the TMs of Ste2p or piston-like TM movements that lead to biochemical changes in G proteins. Evidence from studies on other agonists indicates that binding is a stepwise process [16,71,67,142] and that it may be aided by spontaneous movements of transmembrane domains of receptors that favor interactions with various functional groups on the ligand.

3.15. Biological approaches to structure–function relationships

In addition to chemically synthesized analogs of α-factor used in various structure–function analyses and for probing Ste2p, the α-factor/Ste2p paradigm was the basis for developing a novel biological method to screen for agonists and antagonists of GPCRs. In this approach *S. cerevisiae* strains were "engineered" to respond to α-factor biosynthesized and secreted by the same cell. This so-called autocrine assay was manipulated so that both agonists and antagonists could be detected [92]. The power of this approach is that random and semi-random libraries of α-factor can be produced which represent millions of α-factor sequences. Obviously this diversity cannot be matched by chemical synthesis. Using this method a number of interesting insights into structure–activity relationships were revealed. Perhaps most interesting among these was the discovery of putative strong agonists such as TrpHisTrpLeu**ArgLeuGlyAla**GlyGlnProMetTyr in which the center of the peptide has been drastically changed, and strong antagonists such as **CysArgGlyPro**GlnLeuLysProGlyGlnProMetTyr in which each of the first four residues are non-native. These findings indicate that the activation and/or binding of peptides to Ste2p can be accomplished by a multiplicity of peptide sequences and that the biological screen might reveal unanticipated lead structures. The authors of the study provided a detailed analysis of SAR for different positions of the α-factor. We will not analyze these results because, for the most part, they come to the same conclusions as those discussed above in this review.

We note, however, that several concerns were raised that should be considered in applying the biological screen. For example in a parallel paracrine assay in which the activity of the library peptides on a second tester strain were evaluated, certain strong agonists had virtually no activity. In addition although in many cases chemically synthesized peptides corroborated the screen, in several cases the chemically synthesized peptide did not show the expected properties. The differences observed can be due to the processing of the prepro-α-factor precursor, differences in transport rates out of the cell and differences in diffusion in the agar test medium. The chemically synthesized peptides were only 50–70% pure and were not characterized. Furthermore, no receptor binding assays were carried out in these studies so the target can only be inferred. Despite the size of the libraries produced, some interesting compounds appeared to have been missed. For example the study concluded that Trp^1 is very important for activating Ste2p and cited the inactivity of [Ser^1] and [Glu^1]α-factor. However, we found that [Ala^1]α-factor, [Gly^1]α-factor and [Leu^1]α-factor are all excellent agonists (see above) and none of these agonists were revealed by the screen.

Nevertheless, this is a powerful methodology and it is being used to identify novel ligands for mammalian G protein-coupled receptors heterologously expressed in *S. cerevisiae*. Agonists for an orphan GPCR, the human formyl peptide receptor like-1 (FPRL-1) receptor, were identified and found to activate FPRL-1 in human embryonic kidney cells [79]. Recently, the same approach was used to identify allosteric peptide agonists of the human chemokine receptor CXCR4

expressed in yeast [118]. Thus, the pheromone response pathway in yeast has great potential in revealing agonists and antagonists for human GPCRs.

3.16. Glycosylated α-factors

The genes for α-factor biosynthesis MFα1 and MFα2 code for the expression of pre-pro-polypeptide precursors that have multiple copies of the pheromones. Although these precursors contain a signal peptide sequence for secretion through the Golgi complex and cleavage sites for various peptidases, there are no apparent glycosylation sites within the sequence of the tripeptide pheromone. Despite this fact a recent report has appeared on the chemo-enzymatic synthesis of glycosylated α-factor [123]. Analogs prepared contain *N*-acetyl-glucosamine (Glc-Nac) on positions 5 and/or 10 and a sialo complex type oligosaccharide on the Gln side chains. Interestingly, the [Gln(Glc-NAc)^5]α-factor and [Asn(Glc-NAc)^5]α-factors exhibited very high biological activities with the former pheromone reported to be 2.1-fold higher in activity than the native pheromone. No data was presented on the receptor affinities of the peptides evaluated. The presence of complex oligosaccharides at these positions, especially those terminated with sialic acid, greatly lowered agonist potency. As stated throughout this review it is very unusual to find α-factor analogs with higher activities than the native pheromone. Nevertheless, the effect of glycosylation appears to be a curiosity that has little bearing on the biological activity of this tridecapeptide or its interaction with its GPCR.

3.17. A non-peptide ligand for Ste2p

About ten years ago it was shown that the coumarin drug novobiocin activated the mating response in *S. cerevisiae* through its interaction with Ste2p [111]. This novel finding was used recently to show that certain receptor mutants (F294C and Y266C) responded to novobiocin as an alternate agonist [87]. The search for non-peptidyl ligands for GPCRs activated by peptides is an extremely important activity in the pharmaceutical industry owing to the poor drug properties of many peptides compared to non-peptidyl substitutes. Thus, the Ste2p-α-factor system again manifests desirable characteristics as a model system for studying peptide ligand–GPCR interaction. Further work on the binding of novobiocin to Ste2p should contribute to the understanding of the binding of peptidomimetics to GPCRs.

4. a-Factor

In contrast to structure activity studies on α-factor, which have greatly expanded since our last review there has not been tremendous progress on **a**-factor SAR since we last reviewed this literature. One of the reasons for this distinction is that a receptor binding assay for **a**-factor has still not been developed. Since the relationship between activity and binding is not always tightly correlated and since **a**-factor analogs often differ markedly in their hydrophobicity, the plate assays used to assay this pheromone may not a reliable indicator of the ligand–receptor interaction.

At the beginning of the review we summarized information from structure–activity relationship analyses that were published up to 1994. Following our discovery that [D-Ala^5] **a**-factor had higher biological activity than the native structure we examined a number of modifications of the central region of the pheromone. Incorporation of (***R***)-3-amino-2-oxo-1-pyrrolidinoacetic acid (a conformation restricting γ-lactam) in place of Lys^{4}Gly^5 in **a**-factor resulted in a pheromone with 32-fold higher activity than the parent pheromone [155]. In contrast replacement of Gly^{5}Val^6 by the corresponding (***S***)-γ-lactam led to a 30–60-fold decrease in activity compared to **a**-factor. Moreover, replacement of Lys^4 by Pro and Lys^4, Gly^5 by Pro-D-Ala resulted in biological activities nearly equal to those of **a**-factor whereas diastereomers of the former analogs were at least 50-fold less active [149]. These observations led us to conclude that a turn around Lys-Gly was likely present in the biologically active structure of **a**-factor. Although this conclusion is made in the absence of receptor binding data it is interesting in light of the much stronger evidence that the bound structure of α-factor is bent.

It was previously surmised that the role of isoprenoid addition was solely to target the attached polypeptide to membranes by increasing its hydrophobicity. However, in analyses of **a**-factor analogs containing subtle modification of the farnesyl moiety we concluded that modest changes in the isoprenoid can significantly affect the biological activity of the pheromone and that there was not a correlation between membrane partitioning and biological potency. These results were not consistent with a simple hydrophobic role for the isoprenoid [35]. We suggested that farnesyl moieties in peptide pheromones do not simply localize these signal molecules into membranes but also play an active role in their presentation to their target proteins [150]. However, a study on **a**-factor analogs containing geometrical isomers of the farnesyl moiety showed virtually identical biological activities for these stereoisomers, a finding that would not be consistent with a tight ligand–receptor binding model [150]. Until a reliable receptor binding assay is developed for **a**-factor it will be difficult to analyze these results at the level of the pheromone–receptor interaction.

5. Conclusions

Our studies on the structure–activity relationships of α-factor have been focused on the understanding of the interaction of this tridecapeptide pheromone with its G protein-coupled receptor Ste2p, including the determination of key contacts between ligand and Ste2p, and the biologically active structure of the tridecapeptide. These studies necessitated

a systematic analysis of the influence of different side chains and functional groups on biological activity, receptor affinity, and pheromone structure requiring parallel biochemical and biophysical studies on the structure of α-factor. The following conclusions delineating domains of the pheromone were made: (a) residues near the amine terminus play an important role in receptor activation; (b) residues near the carboxyl terminus play an important role in receptor binding; (c) central residues of α-factor allow flexion of the pheromone perhaps to orient the signaling and binding domains of the pheromone in the receptor. Such separation of domains in a medium peptide ligand may be indicative of similar domain structure in other peptide hormones. Furthermore, the flexibility in the bend region would permit the pheromone to "breathe" in the binding pocket and act as a "triggering mechanism". This model would envisage coordinated movements of the receptor-bound pheromone and its receptor on the pathway to activation of signal transduction.

We have shown that characterization of receptor mutants using antagonists provides insights not readily apparent from bioassays or binding studies using agonists. For example, we found that the Y266A mutant of Ste2p preferentially bound antagonist as compared to wild-type receptor. This creates the possibility that the mutant receptor could be used to screen for antagonists. Since many pharmaceuticals are receptor antagonists, knowledge of factors that stabilize an antagonist favored state of a GPCR would be of considerable interest. The α-factor–Ste2p system also exhibited a dissociation of biological activity and receptor affinity. An important implication of this finding is that potent agonists that could have valuable clinical utility may be missed when screens involve only receptor binding assays.

Overall we and others have developed a solid working model for α-factor bound to Ste2p. The image created serves as a template upon which other experiments are being planned to determine the structure of α-factor bound to Ste2p and the mechanism(s) by which this ligand–GPCR interaction initiates signal transduction. We trust that this model system will continue to serve as an excellent basis for elucidating molecular events that are involved in signal transduction by G protein-coupled receptors.

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