Probing the Binding Domain of the *Saccharomyces cerevisiae* α-Mating Factor Receptor with Fluorescent Ligands†

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ABSTRACT: Three analogues of the α-mating factor pheromone of *Saccharomyces cerevisiae* containing the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group were synthesized that had high binding affinity to the receptor and retained biological activity. The fluorescence emission maximum of the NBD group in [K^7(NBD),Nle^{12}]-α-factor was blue shifted by 35 nm compared to buffer when the pheromone bound to its receptor. Fluorescence quenching experiments revealed that the NBD group in [K^7(NBD),Nle^{12}]-α-factor bound to the receptor was shielded from collision with iodide anion when in aqueous buffer. In contrast, the emission maximum of NBD in [K^7(*ah*NBD),Nle^{12}]-α-factor or [Orn^7(NBD),Nle^{12}]-α-factor was not significantly shifted and iodide anion efficiently quenched the fluorescence of these derivatives when they were bound to receptor. The fluorescence investigation suggests that when the α-factor is bound to its receptor, K^7 resides in an environment that has both hydrophobic and hydrophilic groups within a few angstroms of each other.

The α-factor receptor (Ste2p)[1] from *Saccharomyces cerevisiae* belongs to the family of G protein-coupled receptors (GPCRs) that, upon the binding of a ligand, transduce a signal via an associated guanine nucleotide binding protein (G protein) (*1*−*3*). The binding of the tridecapeptide pheromone α-factor to Ste2p initiates a cascade of events that leads to the mating of haploid yeast cells (*4*, *5*). Like all GPCRs, Ste2p is predicted to have seven hydrophobic transmembrane domains connected by intracellular and extracellular loops (*6*, *7*). Recently we synthesized the seven transmembrane domains and found two of them were β-sheets and five of them were mostly α-helix in trifluoroethanol, sodium dodecyl sulfate micelles, and dimyristoylphosphatidylcholine vesicles (*8*).

To understand the signaling process, one must first understand how ligands are recognized by, and are associated with, their receptor. Although a number of molecular biological investigations have suggested regions and residues of the receptor involved in signal transduction and pheromone binding (*9*−*15*), biophysical studies on α-factor−Ste2p interaction have not been carried out. To perform such investigations it is useful to incorporate fluorescent probes into the ligand of interest. Fluorescence techniques, as reviewed recently (*16*), have been successfully used for investigating ligand−GPCR interactions such as β2-adrenergic (*17*), chemokine (*18*), human transferrin (*19*), NK2 (*20*), and GalR1 galanin receptors (*21*). In a prior paper the synthesis of some 7-nitrobenz-2-oxa-1,3-diazol-4-yl- (NBD-) labeled α-factor and **a**-factor analogues having an 8−80-fold decrease in biological activity relative to the native ligands was reported (*22*). Especially relevant to the present study was the synthesis of 6-amino-*N*-NBD-hexanoyl-[Nle^{12}]-α-factor. However, only the biological characterization of this probe was reported and no fluorescence spectroscopy of this analogue in the presence of the α-factor receptor was carried out. Nevertheless, the relatively good biological activity of this analogue and the sensitivity of its fluorescence spectrum to solvent encouraged us to explore the possibility of using extrinsic fluorescent probes to investigate the α-factor−receptor interaction. This work reports the use of the NBD group to probe the environment of the Lys^7 side chain of α-factor (WHWLQL<u>K</u>PGQPMY) when bound to the Ste2p receptor.

MATERIALS AND METHODS

Synthesis of N-α-Fmoc-Protected α-Factor Analogues. N-α-Fmoc-protected α-factor analogues were synthesized by solid-phase peptide synthesis on an Applied Biosystems 433 peptide synthesizer (Applied Biosystems, Foster City, CA) starting with *N*-α-Fmoc-Tyr(OtBu)-Wang resin (0.7 mmol/g resin, Advanced ChemTech, Louisville, KY) as reported previously, except that the last *N*-α-Fmoc group in the peptide was not deprotected (*23*). Fmoc-protected amino acids were purchased from Advanced ChemTech (Louisville, KY) except Fmoc-His(Trt) from Calbiochem−Novabiochem

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[1] Abbreviations: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; MES, 2-(*N*-morpholino)ethanesulfonic acid; Nle, norleucine; Orn, ornithine; *ah*, 6-aminohexanoyl; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; G protein, guanine nucleotide binding protein; GPCRs, G protein-coupled receptors; BSA, bovine serum albumin.

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Corp. (San Diego, CA). *N*-α-Fmoc-protected α-factor analogues were more than 99% pure as determined by use of two different mobile phases (CH_3CN/H_2O and CH_3OH/H_2O) on analytical reversed-phase HPLC, and the molecular weights were confirmed by electron-spray mass spectrometry (ES-MS).

Synthesis of NBD-Labeled α-Factor Analogues. To 2.5 μmol of *N*-α-Fmoc-protected α-factor analogues in 2 mL of a mixture of $Na_2B_4O_7$ (pH 9.5, 50 mM in water) and DMF at a ratio of 1:1 was added 7.5 μmol of 7-nitrobenz-2-oxa-1,3-diazol-4-yl fluoride (NBD-F, Molecular Probes Inc.). The solution was protected from room light and stirred at 4 °C until the starting peptide disappeared as monitored by analytical reversed-phase HPLC. Piperidine (0.2 mL; 20% in DMF) was added to the reaction medium and stirring was continued for 30 min. The solution was then neutralized to pH 8 by addition of 20% HCl, and the products were purified by reversed-phase HPLC on a semipreparative Waters μBondapak C18 column (19 × 300 mm) with a linear gradient of water (0.025% TFA) and acetonitrile (0.025% TFA), with acetonitrile from 10% to 80% over 90 min. The NBD-labeled peptides were judged to be more than 99% pure by use of an analytical Waters μBondapak C18 column (3.9 × 300 mm) and linear gradients of water (0.025% TFA) and acetonitrile (0.025% TFA), with acetonitrile from 20% to 80% over 30 min or water (0.025% TFA) and methanol (0.025% TFA), with methanol from 40% to 100% over 30 min. The molecular weights were confirmed by electron-spray mass spectrometry.

Membrane Sample Preparation. Saccharomyces cerevisiae strains DK102 (gene encoding Ste2p was deleted) and DK102(pNED) [this strain contained a plasmid encoding Ste2p] were grown, and membranes containing or lacking Ste2p were prepared as described previously (*24*). To 5 mL of MES buffer [20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.1% bovine serum albumin (BSA), and 150 mM KCl, adjusted to pH 5.8 with 1 N KOH] in a glass vial was added 7 μL of membrane suspensions from DK102 or DK102(pNED) (18 mg/mL, stored at −70 °C and thawed in ice) to give a final membrane concentration of 25 μg of protein/mL. The concentration of receptor in the final DK102(pNED) membrane suspension was about 4.5 nM. The mixture was sonicated at 20 °C for 5 min with a Misonix Inc. W-385 sonicator equipped with a cup horn (40% output power).

Spectroscopy. Fluorescence measurements were recorded on a photon-counting Spex Fluoromax-3 (Spex Industries Inc., Edison) with a 1 × 1 cm quartz cuvette. The membrane suspensions (2.4 mL) prepared above were incubated with stirring in the presence or in the absence of 15 nM fluorescent α-factor analogues at 20 °C for 30 min. In independent experiments we determined that the receptor was about 60% saturated with α-factor under these conditions. The emission spectra were scanned between 500 and 560 nm with an excitation wavelength of 475 nm at intervals of 1 nm with 1 s integration time at each wavelength. The bandwidths for both excitation and emission were 5 nm. Spectra were recorded for the corresponding buffers and membrane backgrounds as controls for each emission measurement. These were then subtracted from spectra of the ligand alone, the ligand in the presence of membranes lacking the Ste2p receptor, or the ligand in the presence of membranes containing Ste2p receptor to yield difference spectra corresponding to the emission of free fluorescent α-factor analogues in MES buffer, of pheromones nonspecifically bound to DK102 (membrane without receptor), and of pheromones specifically bound to DK102 (pNED) (membrane with receptor). In the latter case a correction was necessary to account for the amount of ligand bound to receptor and therefore unavailable for nonspecific binding to the membranes. As an example to correct for the nonspecific interaction of [K^7(NBD),Nle^{12}]-α-factor with membranes, we calculated that for 15 nM of the fluorescent analogue, 4.5 nM receptor, and a K_i = 26.8 nM, 90% of the pheromone was not receptor-bound. Thus in calculating the fluorescence spectrum for pheromone bound to the receptor we measured the fluorescence of membranes containing receptor in the presence of 15 nM [K^7(NBD),Nle^{12}]-α-factor and then subtracted the spectrum measured for membranes lacking the α-factor receptor multiplied by a factor of 0.90. Similar calculations and corrections were made with the experimentally determined values of K_i for [K^7(*ah*NBD),-Nle^{12}]-α-factor and [Orn^7(NBD),Nle^{12}]-α-factor. All spectra used for final determinations were averaged from three independent sample preparations.

Collisional Quenching Experiments. The above membrane suspensions (2.4 mL) were incubated in a 3.4 mL cuvette in the presence or absence of 15 nM fluorescent α-factor analogues at 20 °C for 30 min. Fluorescence collisional quenching experiments with iodide were performed by adding increasing amounts of a 4 M KI or KCl solution in MES buffer to a final concentration of 65.6 mM. The fluorescent intensities were calculated by integration within the emission range from 500 to 560 nm except that the fluorescent intensities of [K^7(NBD),Nle^{12}]-α-factor in membrane suspensions were calculated at a wavelength of 510 nm because of the large blue shift of emission peaks from nonspecific membrane binding to specific receptor binding. The fluorescent intensity of α-factor analogues in MES buffer minus the intensities of buffer at appropriate KI and KCl concentrations were used to calculate the quenching constant of fluorescent α-factor analogues free in MES buffer. As stated above, the receptor-bound ligands of [K^7(NBD),Nle^{12}]-α-factor, [Orn^7(NBD),Nle^{12}]-α-factor, and [K^7(*ah*NBD),-Nle^{12}]-α-factors were estimated to be 1.5, 3.0, and 2.3 nM, respectively, on the basis of measured K_i values in Table 1. Thus, the fluorescent intensities of α-factor analogues specifically bound to receptor were obtained by subtracting 90% (for [K^7(NBD),Nle^{12}]-α-factor), 80% (for [Orn^7(NBD),-Nle^{12}]-α-factor), and 85%(for [K^7(*ah*NBD),Nle^{12}]-α-factor), respectively, of the nonspecific intensities (in the presence of DK102) from the total intensities [in the presence of DK102 (pNED)]. The quenching constants of fluorescent α-factor analogues specifically bound to the receptor were calculated with the Stern−Volmer equation:

$$F_0/F = 1 + K_{sv}[I^-] \quad (1)$$

where F_0/F is the ratio of fluorescence intensities in the presence of KCl and KI (*25*). The Stern−Volmer quenching constant K_{sv} was determined from the slope of F_0/F as a function of the iodide concentration [I^-].

Growth Arrest (Halo) and Binding Competition Assays. Strain LM102 (*9*, *13*) was used for both assays. In the growth

Table 1: Structures, Biological Actitivies, and Binding Affinities of Fluorescent α-Factor Analogues

α-factor analogues[a]	structures	relative activity[b]	binding affinity[c] K_i (nM)
[Nle^{12}]	H_2NWHWLQLKPGQPNleYOH	100	8.2 ± 1.6
[K^7(NBD),Nle^{12}]	H_2NWHWLQLK(ϵ-NBD)PGQPNleYOH	5	26.8 ± 4.5
[K^7(*ah*NBD),Nle^{12}]	H_2NWHWLQLK(ϵ-*ah*NBD)PGQPNleYOH	58	11.8 ± 2.6
[Orn^7(NBD),Nle^{12}]	H_2NWHWLQLOrn(δ-NBD)PGQPNleYOH	28	5.7 ± 2.1

[a] Nle = norleucine, an isosteric replacement for the wild-type methionyl residue that results in an equally active pheromone; NBD = 7-nitrobenz-2-oxa-1,3-diazol-4-yl; *ah* = 6-aminohexanoyl. [b] Percent activity of [Nle^{12}]-α-factor. Determined by activity of peptide in a growth arrest assay. The value for [Nle^{12}]-α-factor that caused a 15 mm halo was 0.48 μg. [c] Determined as described under Materials and Methods.

arrest assay, media plates were overlaid with 4 mL of cell suspension (2.5×10^5 cells/mL in Nobel agar). Filter disks (sterile blanks from Difco), 8 mm in diameter, were impregnated with 10 μL portions of peptide solutions at various concentrations and placed onto the overlay. The plates were incubated at 30 °C for 24−36 h and then observed for clear zones (halos) around the disks. All assays were carried out at least three times with no more than a 2 mm variation in halo size at a particular amount applied for each peptide. The size of the halo produced was plotted vs the amount of peptide tested, and the amount of peptide required to produce a 15 mm halo was determined from a first-order regression analysis of the plot. In the binding competition assay for [^{3}H]-α-factor, cells were washed and resuspended in ice-cold YM-1 medium (*23*). The binding assay was started by addition of [^{3}H]-α-factor and various concentration of nonlabeled peptide to a cell suspension so that the final concentration of radioactive peptide was 6×10^{-9} M (20 Ci/mmol). After a 30 min incubation, triplicate samples were filtered and washed over glass fiber filtermats in the standard cell harvester (Skatron Instruments, Sterling, VA) and placed in scintillation vials for counting. Binding data were analyzed with GraphPad Prism Program (Software) and K_i values were calculated as $K_i = IC_{50}/(1 + [\text{labeled ligand}]/K_d \text{ of labeled ligand})$.

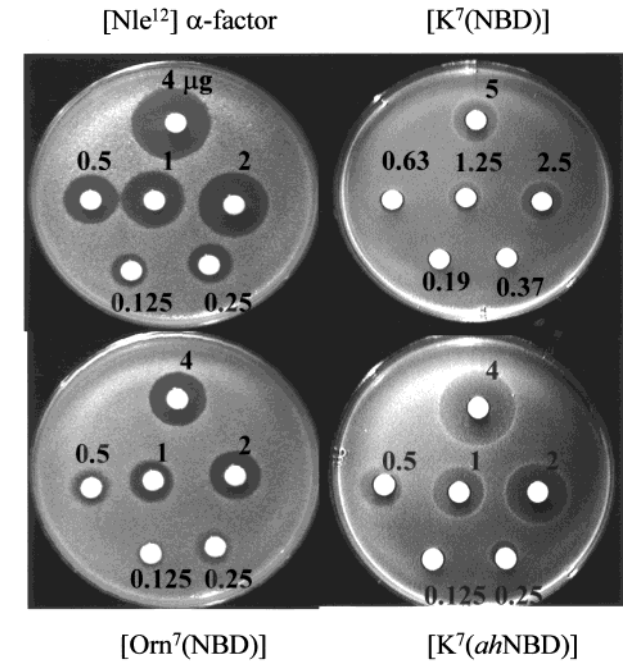
RESULTS

Synthesis of Fluorescent Ligands. We have designed and prepared three fluorescently labeled α-factor analogues shown in Table 1. Each analogue contains the NBD group as the fluorescent probe. The NBD was chosen as a fluorescent probe because (1) it has a relatively small molecular volume and was predicted to have minimal perturbation on the affinity of the α-factor analogues for their receptor; (2) it has favorable absorbance and fluorescence properties, and (3) its fluorescence is strongly sensitive to the environment. The three fluorescent peptides differ in length of the spacer arm between the fluorescent probe to the backbone of the peptides. The nitrogen attached to NBD is separated from the backbone at position 7 by four methylenes in [K^7(NBD),Nle^{12}]-α-factor, by three methylenes in [Orn^7(NBD),Nle^{12}]-α-factor, and by nine methylenes, a nitrogen, and a carbonyl group in [K^7(*ah*NBD),Nle^{12}]-α-factor. Thus we could study the environment of the probe at varying distances from the peptide backbone and presumably learn about aspects of a region of the pheromone binding pocket.

Biological Activity and Receptor Affinity of the Fluorescent α-Factor Analogues. Biological activity of the fluorescent α-factors was assessed by determining the amount of pheromone causing a clear zone of growth arrest of 15 mm. Increasing amounts of pheromone caused an increase in growth arrest as measured by halo size (Figure 1, top panel). These data were plotted and linearized by regression analysis, and the amount of peptide causing a halo size of 15 mm was determined from the regression line. The relative activity of peptide was determined from the following formula: (amount of α-factor for a 15 mm halo/amount of peptide analogue for a 15 mm halo) × 100. The three fluorescent peptides were from 20-fold to 2-fold less active than native α-factor (Table 1). Binding affinities of three fluorescent peptides for α-factor receptor were determined by measuring competition binding of the analogues against [^{3}H]-α-factor in a whole-cell binding assay (Figure 1, bottom panel). All competition curves were parallel and K_i values were determined as described under Materials and Methods. The three fluorescent α-factor analogues had affinities for the receptor ranging from slightly better than that of α-factor [Orn^7(NBD) analogue] to about 3-fold poorer binding [Lys^7(NBD) analogue] (Table 1). A lack of correlation between biological activity and receptor affinity has been previously observed for α-factor analogues (*23*). Most important for the present study, the high binding affinities of the fluorescent α-factors made it possible to investigate the environment of the ligand side chain bound to the receptor at nanomolar concentrations of both the receptor and the ligand.

Fluorescence Properties of NBD Ligands. The excitation and emission maxima of NBD ligands in MES buffer, pH 5.8, are 478 ± 2 nm and 545 ± 2 nm, respectively. The molar extinction coefficient (ϵ) of [K^7(NBD),Nle^{12}]-α-factor at 20 °C in methanol is 56 800 ± 1900 M^{-1} cm^{-1} at 469 nm. The quantum yield of NBD is dependent upon the polarity of the environment, with a higher quantum yield in a low-polarity environment (*26*). This property has been utilized to study the environment of the ribosome−nascent chain−membrane complexes with NBD incorporated into the signal sequence (*27*). To evaluate the utility of NBD as a probe for the polarity of the medium surrounding the α-factor, the emission of [K^7(NBD),Nle^{12}]-α-factor was examined in solvents of different polarities (Figure 2). For [K^7(NBD),Nle^{12}]-α-factor, the emission maximum shifted from 545 to 530 nm and the emission intensity increased 25-fold when going from aqueous (MES buffer) to a less polar solvent (e.g., 90% dioxane/H_2O). Similar results were found with the other fluorescent derivatives of the pheromone (data not shown). When similar studies were conducted with analogues containing fluorescein groups, very little sensitivity to solvent was noticed (data not shown). Thus NBD was chosen as the probe for studying the interaction of α-factor with its receptor.

Interactions of the Fluorescent Ligands with α-Factor Receptor in Membranes. Figure 3 shows a typical fluores-



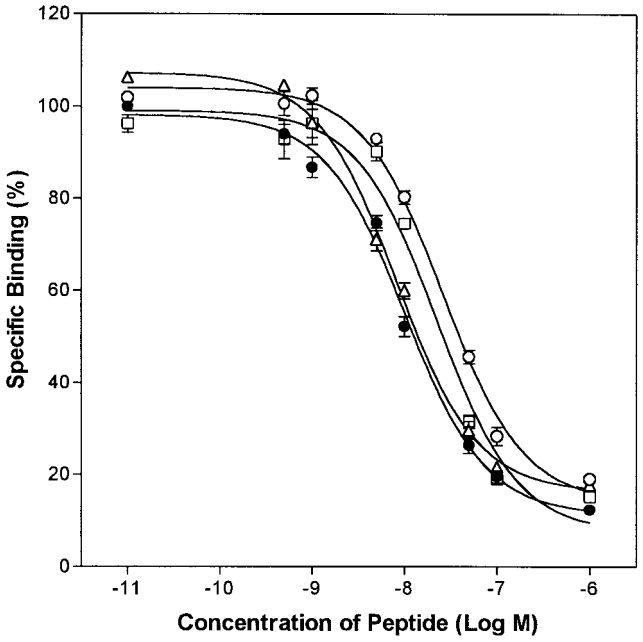


FIGURE 1: Comparison of α-factor receptor activation and binding by fluorescent α-factor analogues. (Top panel) Dose−response growth arrest assay. Yeast strain LM102 carrying wild-type STE2 was spread on solid medium and then filter disks containing the indicated amounts of peptides were applied. Halo sizes were plotted and amount of peptide for a 15 mm halo was compared in Table 1. (Bottom panel) Competition analysis of fluorescent analogues to α-factor receptor in LM102 strain. Binding of [^{3}H]-α-factor in the presence of various concentrations of competitor [Nle^{12}]-α-factor (●), [K^7(NBD),Nle^{12}]-α-factor (○), [Orn^7(NBD),Nle^{12}]-α-factor (△), or [K^7(*ah*NBD),Nle^{12}]-α-factor (□) was determined.

cence emission spectra of 15 nM [K^7(NBD),Nle^{12}]-α-factor in aqueous buffer (spectrum 1) and in the presence of DK102 (spectrum 2) or DK102(pNED) membranes(spectrum 3). The intensity of NBD fluorescence emission increased about 62% and the emission maximum shifted from 545 to 539 nm when the environment of [K^7(NBD),Nle^{12}]-α-factor was changed from aqueous MES buffer to DK102 membranes, indicating that the fluorophore (NBD group) of [K^7(NBD),Nle^{12}]-α-

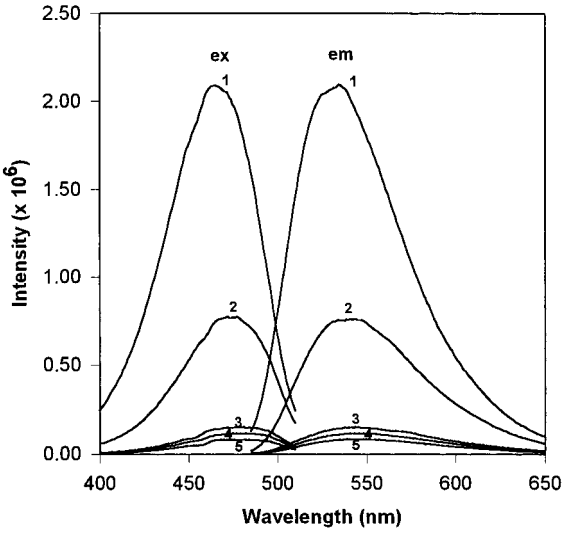


FIGURE 2: Excitation and emission spectra of [K^7(NBD),Nle^{12}]-α-factor (1 μM) in media of different polarity: 90% dioxane in water (v/v) (1); 50% dioxane in water (2); 10% dioxane in water (3); 5% dioxane in water (4); and MES buffer, pH 5.8 (5).

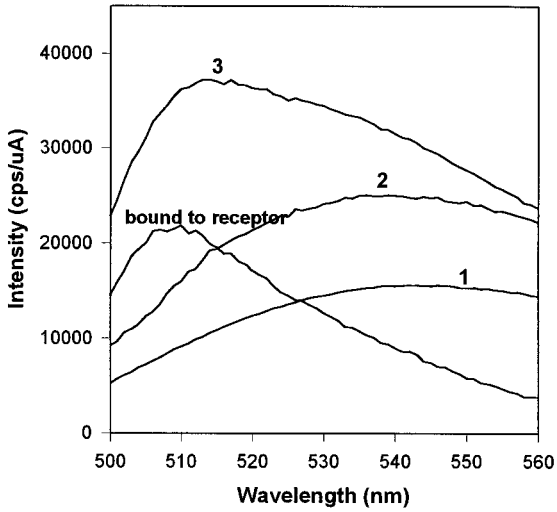


FIGURE 3: Fluorescence emission spectra of [K^7(NBD),Nle^{12}]-α-factor free in MES buffer (1), in the presence of DK102 membranes (no receptor) (2), and in the presence of DK102(pNED) membranes (3). The spectrum labeled bound to receptor represents a spectrum calculated by multiplying spectrum 2 by 0.9 and subtracting it from spectrum 3. The concentration of ligand was 15 nM. The concentration of membranes was 25 μg/mL, while the concentration of receptor was about 4.5 nM. The concentration of ligand bound to receptor was estimated to be 1.5 nM on the basis of the K_i value of 26.8 nM. All spectra were corrected for the corresponding background spectra as stated under Materials and Methods.

factor in the membrane state was in a slightly less polar environment than that free in aqueous buffer. Large differences in fluorescence intensity and the fluorescence emission maximum of [K^7(NBD),Nle^{12}]-α-factor were found for this pheromone analogue bound to DK102 (pNED) as compared to DK102 membranes (Figure 3). When spectrum 2 multiplied by 0.9 (see Materials and Methods) is subtracted from spectrum 3, the difference shows the emission spectrum of [K^7(NBD),Nle^{12}]-α-factor specifically bound to the receptor. Comparing the specific binding of [K^7(NBD),Nle^{12}]-α-factor to its receptor with the nonspecific binding to yeast membranes shows that the emission maximum is shifted from 539 to 510 nm. The emission maximum for the NBD group

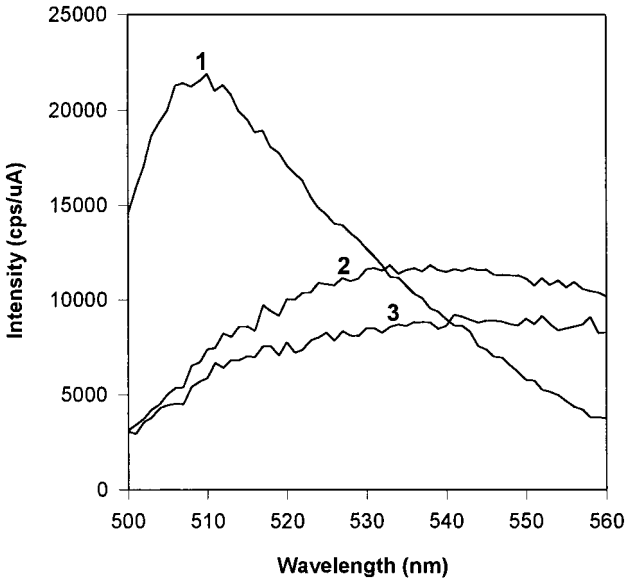


FIGURE 4: Emission of [K^7(NBD),Nle^{12}]-α-factor (1), [Orn^7(NBD),-Nle^{12}]-α-factor (2), and [K^7(*ah*NBD),Nle^{12}]-α-factor (3) specifically bound to the receptor. The specific binding was determined as described in the caption to Figure 3 except that the spectra for binding of 15 nM pheromone to DK102 membranes were multiplied by 80% and 85%, respectively, for [Orn^7(NBD),Nle^{12}]- and [K^7-(*ah*NBD),Nle^{12}]-α-factors prior to subtracting from the spectra measured in the presence of DK102(pNED) membranes.

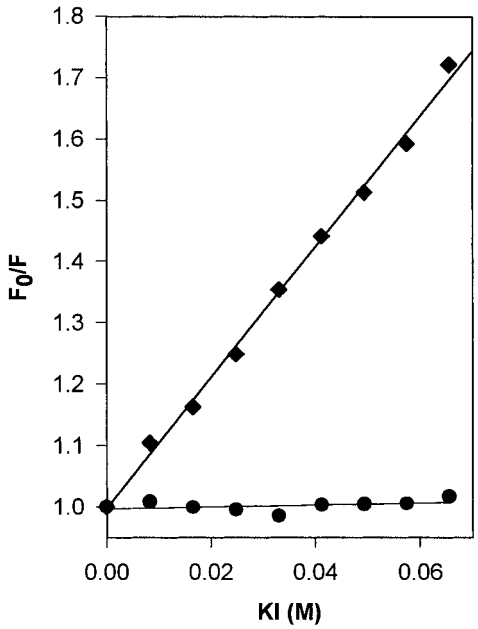


FIGURE 5: Stern−Volmer plots of F_0/F vs iodide concentration for quenching of the fluorescence of receptor-bound [K^7(NBD),-Nle^{12}]-α-factor by iodide. (◆) Free ligand in solution; (●) ligand bound to receptor.

in bound [K^7(NBD),Nle^{12}] α-factor is at an unusually low wavelength. This observation indicates that the NBD fluorophore at the side chain of [K^7(NBD),Nle^{12}]-α-factor bound to the receptor is in a significantly less polar environment than when it is nonspecifically bound to DK102 membranes, free in aqueous buffer, or even free in 90% dioxane 10% water (Figure 2).

In contrast to the results with [K^7(NBD),Nle^{12}]-α-factor, the emission spectra of [Orn^7(NBD),Nle^{12}]- and [K^7(*ah*NBD),-Nle^{12}]-α-factor analogues bound to receptor exhibited maxima of 540−542 nm and fluorescence intensities of 54% and 42%, respectively, that of [K^7(NBD),Nle^{12}]-α-factor (Figure 4). Most importantly, when the three fluorescent analogues were examined in the presence of DK102(pNED) and a 500-fold excess of [Nle^{12}]-α-factor, nearly identical spectra were obtained (data not shown). This latter control suggests that the differences between the three pheromones are due to their specific interactions with the receptor and not to different interactions with the membrane lipids or other membrane proteins.

Collisional Fluorescence Quenching Experiments. Collisional quenching of fluorescence was used to probe the solvent accessibility of receptor-bound ligand. The fluorescence of [K^7(NBD),Nle^{12}]-, [Orn^7(NBD),Nle^{12}]-, and [K^7(*ah*NBD),Nle^{12}]-α-factors free in solution was efficiently quenched by addition of increasing amounts of potassium iodide to solutions of these peptides in MES buffer (Figure 5, Table 2). To correct for ionic strength effects, control solutions were prepared with KCl in place of iodide. As seen for [K^7(NBD),Nle^{12}]-α-factor, a linear Stern−Volmer plot was obtained, indicative of collisional quenching. The slope of F_0/F as a function of iodide concentration (Stern−Volmer constant, K_{sv}), is a measure of the degree of exposure of the NBD group to the solvent. As seen in Table 2, the three fluorescent pheromones have nearly identical K_{sv} values when

Table 2: Stern−Volmer Constants of Quenching of Fluorescent α-Factor Ligands by Iodide[a]

ligands	K_{sv} free (M^{-1})	K_{sv} receptor-bound (M^{-1})
[K^7(NBD),Nle^{12}]-α-factor	10.7 ± 0.2	0.2 ± 0.2
[Orn^7(NBD),Nle^{12}]-α-factor	10.3 ± 0.3	6.4 ± 1.1
[K^7(*ah*NBD),Nle^{12}]-α-factor	10.6 ± 0.6	9.0 ± 1.2

[a] K_{sv} free and bound values are average values of three independent experiments.

measured in aqueous buffer. However, in the presence of receptor the NBD group in [K^7(NBD),Nle^{12}]-α-factor had a $K_{sv} = 0.2$, indicating that it was not accessible to the iodide anion. In contrast, [Orn^7(NBD),Nle^{12}]- and [K^7(*ah*NBD),-Nle^{12}]-α-factors in the presence of receptor were quenched by iodide with Stern−Volmer constants either slightly lower than, or comparable to, those of ligands free in solution, respectively. These data suggest that the NBD group on [K^7(*ah*NBD),Nle^{12}]-α-factor was fully exposed to the solvent, whereas the NBD group on [Orn^7(NBD),Nle^{12}]-α-factor may have been about 20% less accessible to the reagent. The results are consistent with the conclusion that the NBD group in [K^7(NBD),Nle^{12}]-α-factor bound to the receptor resides in a highly hydrophobic environment that excludes iodide anion, while NBD groups in [Orn^7(NBD),-Nle^{12}]- and [K^7(*ah*NBD),Nle^{12}]-α-factors bound to the receptor were in more polar environments that are in contact with the aqueous solvent.

DISCUSSION

Previous studies on α-factor analogues showed that while the position 7 side chain was not necessary for biological activity, it influenced binding affinity (*23*, *28*, *29*). Thus, replacement of Lys^7 with Ala resulted in a 3-fold decrease in activity and a 20-fold decrease in binding affinity (*23*). Interestingly, in a study of position 7 analogues where the

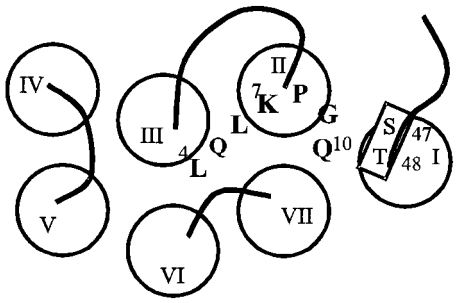


FIGURE 6: Schematic representation of the ligand binding of α-factor to its receptor as viewed from the extracellular side. Only seven residues (4−10) out of 13 in α-factor are shown here for clarity. The residues at the N- and C-terminus of α-factor are buried inside the transmembrane helices of Ste2p in this model. The Lys^7 residue of α-factor mainly interacts with the first extracellular loop. Two residues of the α-factor receptor shown to interact with Gln^{10} of α-factor have been included (shown in box).

length of the side chain was systematically decreased by shortening the side chain by one methylene group, all analogues had approximately the same activity but exhibited from 3-fold to 60-fold lower receptor affinity (*30*). Most significantly, as the number of methylenes decreased from 4 to 1 the binding affinity decreased by factors of 3, 60, and 26, respectively (*30*). As all analogues have an ammonium group at the terminus of the position 7 side chain, the discontinuous nature of the decrease would be consistent with a binding region with a discontinuous polarity and thus consistent as well with the results reported herein for the NBD fluorescent reporter group.

Although mutagenesis experiments have suggested that residues in the first, fifth, and sixth transmembrane domains influence agonist specificity and binding (*9*, *13*−*15*), there is no supporting biophysical evidence to characterize any binding-site interactions in the mutant receptors. The results of this study present direct evidence concerning the environment of the side chain of the Lys residue in position 7 of α-factor when it is bound to the receptor. Since the ϵ-ammonium group on Lys^7 in the wild-type pheromone contributes to the free energy of binding, it is likely that this group interacts with a polar patch in the binding pocket. Our observation that in [K^7(NBD),Nle^{12}]-α-factor the NBD group is in a nonpolar environment whereas the same group in [Orn^7(NBD),Nle^{12}]-α-factor is in a more polar environment would be consistent with the receptor binding pocket for the Lys^7 side chain having both polar and nonpolar character. Alternatively, the differences between these pheromones might reflect changes in the overall conformation of the bound ligand or binding to different subsites on the receptor. Given the present knowledge concerning the bound conformation of α-factor and the receptor binding site, we cannot distinguish between these alternatives. In the case of [K^7(*ah*NBD),Nle^{12}]-α-factor the long spacer arm is very flexible and likely sticks out from the binding pocket of the pheromone, placing the NBD group in a polar environment.

On the basis of the binding of the fluorescent α-factors to Ste2p and other published and preliminary data, we are currently testing a hypothetical α-factor binding model depicted in Figure 6. In this model both the N- and C-terminal ends of the pheromone interact with the outer regions of the transmembrane domains of Ste2p, while residues in the center of α-factor mainly interact with the extracellular domains of the receptor. This simplified model was developed on the basis of the following observations: (1) a bend structure around the Pro^8-Gly^9 residues of α-factor is important for biological activity (*29*−*32*); (2) mutation (F55V) in TM1 converts a nonbinding synergist [D-Ala^{10},-Nle^{12}]-α-factor to an agonist with good receptor affinity (*13*), suggesting that residues at the C terminus of α-factor interact with TM1 of Ste2p; and (3) interactions occur between the side chains of Gln^{10} of α-factor and of the S47 and T48 residues at the junction between the N-terminal region and TM1 domain of the receptor (B. K. Lee, F. Naider, and J. M. Becker, manuscript in preparation). In addition, in our two-dimensional model, the residues near the middle of α-factor (residues 5−10) are spatially restricted into the three distinct receptor regions (residues 47−49, extracellular loops 1 and 3) that determine the ligand specificity between *S. cerevisiae* and *Saccharomyces kluyveri* α-factors (*10*, *33*). These peptides are invariant except for five residues found in the central regions of the pheromones.

Although verification of this model awaits further mutagenesis studies, it provides plausible explanations for the results of the fluorescence results reported in this paper. If Lys^7 of α-factor interacts with residues in the first extracellular loop, the changes in the environment of the NBD group in [K^7(NBD),Nle^{12}]-α-factor as compared to [Orn^7(NBD),-Nle^{12}]-α-factor could be very reasonable as the amino acid sequence of this loop contains both polar and nonpolar groupings of residues. Furthermore, the α-factor is an amphipathic molecule with the amino terminus (WHWL) hydrophobic and the center (LKPGQ) hydrophilic. We believe it is not unreasonable that the Trp side chains face down into the hydrophobic TM domains and the central residues face out into the extracellular loops. Obviously this simplified working model will require careful experimental examination.

In conclusion, we have synthesized three fluorescent α-factors to probe the pheromone/receptor interaction. The studies have provided the first biophysical evidence of the environment in the vicinity of the side chain of Lys^7 of α-factor as it binds to its receptor. Additional probes containing fluorescent and photo-cross-linking groups are being evaluated to further define the nature of the binding pocket of Ste2p.

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