Partitioning of **a**-Factor Analogues into Membranes: Analysis of Binding and Importance for Biological Activity†

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ABSTRACT: Analogues of the **a**-factor mating pheromone of the yeast *Saccharomyces cerevisiae* were used to measure interactions of the pheromones with lipid vesicles and with isolated yeast membranes. The binding of the analogues of **a**-factor to vesicles and to membranes was best described as a partitioning of the pheromones into the lipid phase. The partitioning was enhanced by the negative surface potential of the membranes and was well described by the Gouy−Chapman theory of diffuse double layers. From the analysis of the binding of the pheromones to synthetic vesicles of known surface potential, effective charges and intrinsic partition coefficients were obtained for the pheromones. The information was used in subsequent experiments with yeast membranes to determine the intrinsic partition coefficients of the **a**-factor analogues and the charge density of the yeast membranes. Derivatives of **a**-factor with different alkyl chains in place of the normal C-terminal farnesyl displayed biological activity that paralleled the degree of partitioning of the pheromones into vesicles. Demethylation of the C-terminus decreased the partition coefficient by 6-fold and decreased the biological activity of the pheromone by greater than 2500-fold. The results show that **a**-factor can effectively partition into membrane bilayers and that the partitioning is probably involved in the subsequent recognition of the pheromone by the **a**-factor receptor.

The two haploid cell types of *Saccharomyces cerevisiae*, **a**-type and α-type, conjugate to form a diploid cell type in response to pheromones secreted by the haploid cells (Herskowitz & Marsh, 1987; Blumer & Thorner, 1991). The **a**-type cells secrete a pheromone known as **a**-factor, and the α-type cells secrete a pheromone known as α-factor. **a**-Factor is a dodecapeptide, which is modified posttranslationally at its C-terminal cysteine by farnesylation and methyl esterification, and α-factor is an unmodified tridecapeptide. The pheromones bind to receptors that are expressed specifically in haploid cells of the opposite mating type: **a**-cells express a receptor for α-factor, and α-cells express a receptor for **a**-factor. The receptors, which are encoded by the genes STE2 and STE3, respectively, are predicted to contain seven membrane-spanning segments and to belong to the family of receptors that are coupled to G proteins (Nakayama et al., 1985; Hagen et al., 1986).

The activity of **a**-factor peptide depends strongly on the *S*-farnesylation and *O*-methylation. Analogues of **a**-factor that lack both modifications are essentially inactive (Marcus et al., 1991). Recent studies suggest that the primary functional purpose of the modification is to increase the hydrophobicity of the peptide (Caldwell et al., 1994). It has been shown that replacement of the farnesyl group with less hydrophobic substituents results in a decrease in activity that is correlated with the size of the substituent (Marcus et al., 1991; Sherrill et al., 1995). It is not clear, however, whether these losses in activity are the result of changes in the direct interactions between the pheromone and the receptor itself or whether they result simply from a decrease in the nonspecific partitioning of the pheromone into the lipid bilayer of the plasma membrane.

The effect of *S*-farnesylation and *O*-methylation on the partitioning of model peptides into membranes has been examined by measuring the binding of fluorescently labeled tetrapeptides to synthetic vesicles (Silvius & l'Heureux, 1994). It was concluded that the modifications were sufficient to confer efficient membrane binding of hydrophilic di- and tetrapeptides to the model membranes. A similar conclusion was obtained from the study of the partitioning behavior of myristylated peptides corresponding to the amino terminus of Src (Buser et al., 1994). In addition to the effect of an isoprenyl or acyl group, it is also possible that hydrophobic residues within a modified peptide could also contribute to the partitioning of the peptide into membranes (Jacobs & White, 1989). Indeed, an analogue of **a**-factor with the farnesyl group replaced with a methyl group still interacts with membranes, although the affinity for membranes was not measured (Epand et al., 1993).

For this report, we measured the partitioning of **a**-factor analogues into synthetic phospholipid vesicles in order to determine the relationship between membrane binding and biological activity of the pheromones. The syntheses and biological activities of the **a**-factor analogues used in this

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[1] Abbreviations: DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetate; HPLC, high-pressure liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; PMSF, phenylmethanesulfonyl fluoride; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; Tris, tris(hydroxymethyl)aminomethane.

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study have been described recently (Sherrill et al., 1995). Some of these analogues contained the fluorescent, 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)[1] group. The fluorescence of the NBD probe was enhanced in the presence of vesicles, and the enhancement was used to calculate the binding affinities. In experiments with unlabeled derivatives of **a**-factor, the enhancement in the fluorescence of the tryptophan residue of the peptide was used to calculate the binding affinities. Analysis of binding experiments with membranes containing various negative surface potentials has allowed the contribution of electrostatics and hydrophobic effects to be differentiated.

MATERIALS AND METHODS

Synthesis and Labeling of the Pheromones. Synthesis of most of the pheromones used in this study has been described elsewhere (Sherrill et al., 1995). Demethylated decyl-NBD **a**-factor was produced by treatment of decyl-NBD **a**-factor with base. The peptide (1 mg) was dissolved in a solution of *N*,*N*-dimethylformamide/H_2O/0.5 M NaOH (20:1:1) (500 μL) and stirred at room temperature for 20 min. The reaction mixture was acidified with aqueous hydrochloric acid and dried under reduced pressure. The product was purified by HPLC and analyzed by mass spectrometry. The molecular mass [$(M+H^+)$] was 1730.8 (calculated, 1730.0).

Preparation of Vesicles. Chloroform solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) (Avanti Polar Lipids, Birmingham, AL) were mixed to give a POPC:POPG molar ratio of 4:1. The mixture was transferred into a round-bottom flask and dried by rotary evaporation. The lipids were hydrated with buffered solutions that contained 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), adjusted to pH 6.0 with potassium hydroxide, and various concentrations of potassium chloride. The concentration of lipid in the suspension was 10, 20, or 40 mM. After hydration, the lipid suspension was frozen and thawed 5 times and then extruded 10 times through a pair of Nucleopore filters (0.1 μm) (Hope et al., 1985). Vesicles lacking POPG were also prepared. The vesicles were always used immediately after extrusion.

Isolation of Yeast Plasma Membranes. Plasma membranes were isolated from the yeast strain RH272-6D (MATα, *lys2, ura3, leu2, his, pep4*-3, *bar1-1*) which had been transformed with a plasmid that contained the URA3 gene. The procedure for the isolation of the plasma membranes was based on a minor modification of a published procedure (Serrano, 1988). Cells grown to exponential phase in a synthetic medium (0.67% yeast nitrogen base, 0.002% histidine, 0.002% leucine, 0.002% lysine, 2% galactose) were harvested and resuspended in ice-cold TE buffer [10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetate (EDTA)] (one-fortieth the original volume) that contained phenylmethanesulfonyl fluoride (PMSF) (0.5 mM) and 2-mercaptoethanol (0.4 mM). The cells were placed in a Bead Beater apparatus (Biospec Products, Bartlesville, OK) together with an equal volume of glass beads (0.5 mm), and the suspension was mixed at high speed for 6 min. The apparatus was kept under ice during the mixing. The glass beads were removed by filtration through glass wool, and the cellular debris in the filtrate were removed by centrifugation at low speed (5000*g*, 4 °C) for 5 min. The supernatant was centrifuged at a higher speed (30000*g*, 4 °C) for 40 min to obtain a crude membrane fraction. The membrane pellet was resuspended in ice-cold TE buffer (one-third the original volume) that contained glycerol (20% v/v). The suspension was applied to the top of a discontinuous sucrose gradient [53% and 43% (w/w) in TE buffer] and centrifuged (100000*g*, 4 °C) overnight. The membranes that equilibrated at the interface between 43% and 53% sucrose were collected, diluted with TE buffer, and centrifuged (130000*g*, 4 °C) for 30 min. The resulting pellet was resuspended in TE buffer that contained glycerol (20% v/v). The protein concentration of each suspension was routinely estimated by diluting an aliquot into a solution of SDS (1%) and measuring the absorbance of the solution at 280 nm. To calculate the partition coefficient, it was necessary to estimate the concentration of phospholipid in the membrane suspension. This was accomplished by ashing aliquots from the membrane samples and analyzing for phosphate (Ames & Dubin, 1960). It was assumed that all the phosphate in the samples arose from phospholipid.

Binding Assays. The lipid vesicles were diluted into a solution of 10 mM MES−KOH, pH 6.0, with various concentrations of potassium chloride in a quartz cuvette at 20 °C. Separate batches of vesicles were prepared for each concentration of potassium chloride. The **a**-factor analogues were each dissolved in methanol. Small volumes of the concentrated stock solutions of the pheromone were added sequentially to each dilution of lipid vesicles. The final concentration of the NBD-labeled peptides added to the cuvette ranged from 0.01 to 0.2 μM for experiments using vesicles containing POPG and from 0.1 to 0.7 μM for experiments using vesicles containing only POPC. The final concentration of the unlabeled peptides ranged from 0.6 to 5 μM. The experiments were performed in a total volume of 2 mL with constant stirring of the solution using a Teflon-coated stir bar. Some of the experiments using the NBD-labeled pheromones were also performed in a total volume of 1 mL without constant stirring. In these cases, the sample was stirred after each addition of pheromone with the needle of the syringe used to deliver the pheromone. The results were independent of the method of stirring. The fluorescence was measured using an SLM/Aminco SPF-500C fluorometer. For the experiments in which the tryptophan fluorescence was measured, the samples were excited at a wavelength of 280 nm (7.5 nm bandwidth), and the emission was measured at 350 nm (2.5 nm bandwidth). For the experiments in which the NBD fluorescence was measured, the samples were excited at a wavelength of 466 or 487 nm (5 nm bandwidth for the experiments in 1 mL, 10 nm bandwidth for the experiments in 2 mL), and the emission was measured at 550 nm (5 nm bandwidth). For each measurement, the data were collected for a minimum of 30 s and averaged. The signal was stable over the time of measurement.

The binding experiments with isolated yeast membranes were performed in a total volume of 1 mL. Only the binding of decyl-NBD **a**-factor to these membranes was measured. The fluorometer settings were the same as the settings for the experiments with vesicles.

RESULTS

Binding of the Pheromones to Lipid Vesicles. The structures of the pheromones examined in this study are presented in Figure 1. Stepwise addition of the derivatives of **a**-factor to lipid vesicles resulted in an enhanced fluores-

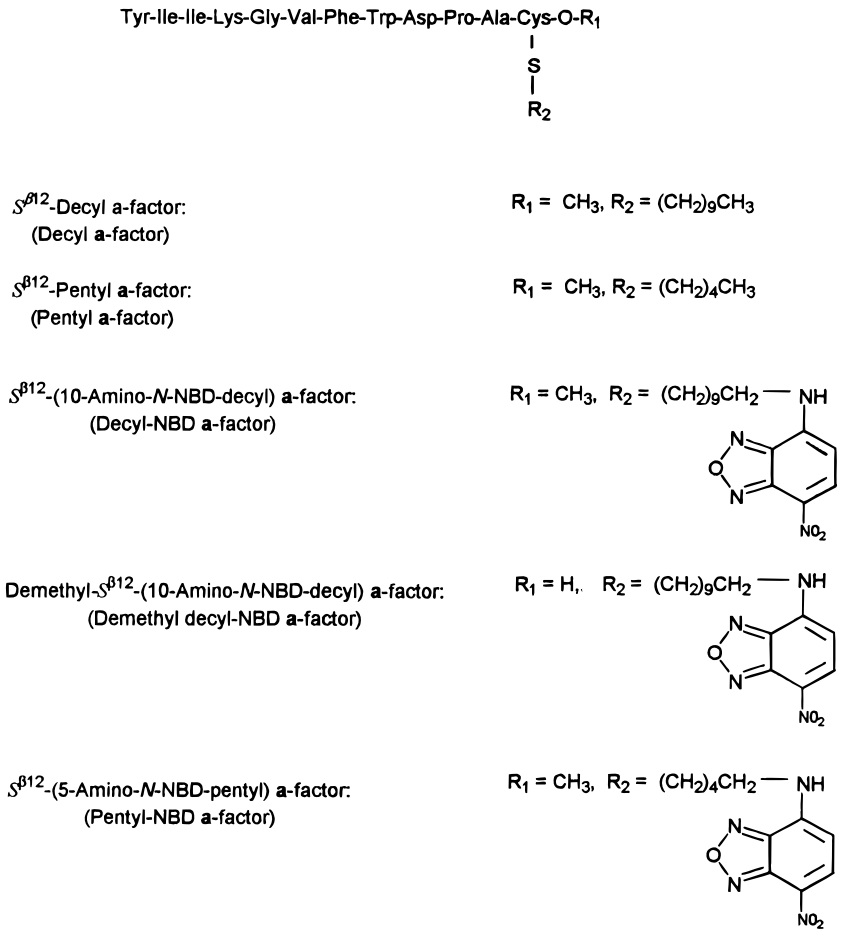

FIGURE 1: Structure of the **a**-factor analogues. Common names used in the text are indicated within the parentheses.

cence signal, compared to that observed in the absence of vesicles, as the pheromones bound to the membranes. In the absence of vesicles, the fluorescence per mole of pheromones remained constant as the concentration of pheromone was increased. In the presence of vesicles, however, the molar fluorescence decreased in a linear manner as the concentration of pheromone was increased (Figure 2). This effect was observed with both the NBD fluorescence of decyl-NBD **a**-factor (Figure 2A) and the tryptophan fluorescence of decyl **a**-factor (Figure 2B). The effect was greatest at the smallest ratio of lipid to peptide and decreased as the ratio increased.

The lack of a proportional increase in the fluorescence as a function of the concentration of pheromone made the analysis of the binding somewhat complicated. If the pheromones were simply partitioning into the lipid bilayer, their fluorescence would be expected to increase proportionately to their concentration. It is possible, therefore, that the lack of a proportionate increase in fluorescence could reflect a decreased binding of the pheromones to the vesicles, due either to a saturation of binding sites or to a change in the charge density of the vesicles as the cationic peptide binds to the vesicle surface. The observation that the effect occurs even at high ratios of lipid to peptide, however, argues against these possibilities. Alternatively, the lack of a proportionate increase in fluorescence could reflect attractive or repulsive interactions between peptides in the aqueous or membrane phases (Schwarz et al., 1986). Such interactions, however, would not be expected to result in linear changes in fluorescence with respect to peptide concentration. Finally, the effect could simply reflect a fluorescence artifact that arises from the concentration-dependent self-quenching of the fluorophores on the surface of the vesicles (Nichols & Pagano, 1981). We feel that the latter explanation is the most reasonable.

Regardless of the reason for the decreased molar fluorescence, it was possible to correct for this effect by linear extrapolation of the molar fluorescence values to a concentration of pheromone of zero. At this extreme, neither saturation of binding sites nor self-quenching of fluorescence is important. The enhancement of fluorescence in the presence of vesicles was calculated as the ratio of the extrapolated molar fluorescence of the pheromone in the presence of lipid, F, to the molar fluorescence in the absence of lipid, F_o. Binding curves for the pheromones were generated by plotting the enhancement as a function of the concentration of lipid. The data were fit to the equation:

$$FF_o^{-1} = [(F_{max} - F_o)F_o^{-1}][M](M_{50} + [M])^{-1} + 1 \quad (1)$$

where [M] is the concentration of accessible lipid in the outer leaflet of the vesicle bilayer, F_{max} is a constant corresponding to the fluorescence of the bound pheromone, and M_{50} is a constant corresponding to the concentration of lipid at which the enhancement is half-maximal. We assumed that 50% of the total lipid was present in the outer leaflet of the vesicles used in our assays.

The parameter M_{50} reflects the concentration of lipid at which half the pheromone is bound and half the pheromone is free. It can thus be represented as a dissociation constant:

$$M_{50} = [P_F][M]([P_B])^{-1} \quad (2)$$

In this equation, $[P_F]$ is the concentration of free pheromone,

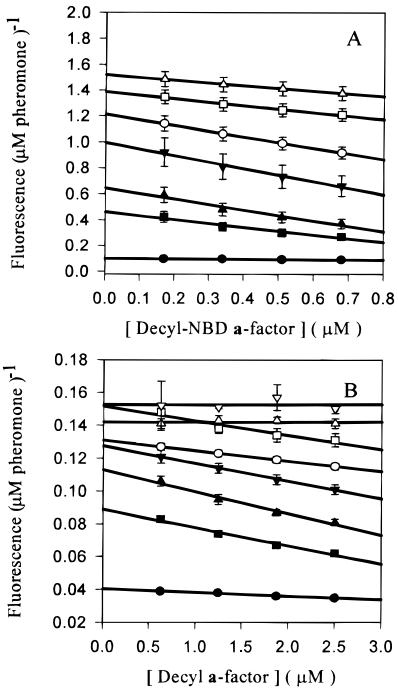

FIGURE 2: Correction for the apparent quenching of fluorescence. The molar fluorescence of each peptide is plotted as a function of the molar concentration of the peptide. For experiments with decyl-NBD **a**-factor and POPC vesicles (A), the fluorescence of NBD was measured, and the concentrations of lipid were: (●) 0 μM, (■) 10 μM, (▲) 20 μM, (▼) 50 μM, (○) 100 μM, (□) 250 μM, and (△) 500 μM. For experiments with decyl **a**-factor and POPC vesicles (B), the fluorescence of tryptophan was measured, and the concentrations of lipid were (●) 0 μM, (■) 20 μM, (▲) 50 μM, (▼) 100 μM, (○) 200 μM, (□) 0.5 mM, (△) 1 mM, and (▽) 2 mM. Values of the molar fluorescence at each concentration of lipid were obtained by extrapolation to zero concentration of peptide. Concentrations of lipid are given in terms of the accessible lipid and are half the total concentration of lipid. All data points are averages of at least three measurements.

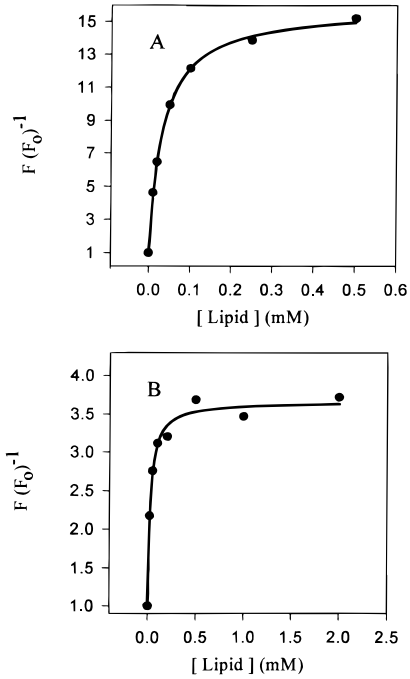

FIGURE 3: Binding curves. The enhancement of fluorescence for decyl-NBD **a**-factor (A) and decyl **a**-factor (B) is plotted as a function of the concentration of POPC vesicles. The enhancement corresponds to the ratio of the extrapolated fluorescence in the presence of lipid, F, to the extrapolated fluorescence in the absence of lipid, F_o. The lines were generated according to eq 1 in the text using values of F_{max} and M_{50} obtained from curve fitting.

and $[P_B]$ is the concentration of bound pheromone. The value of M_{50} obtained from a binding curve therefore reflects the affinity of the pheromone for the lipid bilayer under the conditions of the experiment. A low value of M_{50} corresponds to a high affinity, and a high value of M_{50} corresponds to a low affinity.

The affinity of an amphiphilic peptide for a membrane can also be presented as a partition coefficient. This value, which corresponds to the ratio between the mole fraction of peptide in the membrane phase and the mole fraction of peptide in the aqueous phase, can easily be calculated from the value of M_{50}, by substitution from eq 2:

$$K_p = [P_B][M]^{-1}([P_F][H_2O]^{-1})^{-1} = [H_2O]M_{50}^{-1} \quad (3)$$

The binding of decyl-NBD **a**-factor and decyl **a**-factor to vesicles composed entirely of POPC is well described by eq 1 (Figure 3). As described above, the values of F used to calculate the fluorescence enhancements in these experiments were obtained by extrapolation to zero concentration of pheromone, and the values of M_{50} obtained from the curve fitting thus reflect the binding at infinitely low concentrations of pheromone. Similar values of M_{50} were obtained, however, if the enhancement data at each concentration of pheromone were first fit to eq 1 to obtain concentration-dependent values of M_{50}, and if these values were then linearly extrapolated to zero concentration of pheromone. That the final values of M_{50} were independent of the method of analysis argues that they reflect the true binding parameters.

Table 1: Binding Affinities of the Pheromones for Uncharged Vesicles

pheromone[a]	M_{50} (mM)[b]	K_p ($\times 10^{-5}$)[c]
decyl **a**-factor	0.025 ± 0.005	22 ± 4
decyl-NBD **a**-factor	0.030 ± 0.005	18 ± 4
demethyl-decyl NBD **a**-factor	0.18 ± 0.01	3 ± 0.2
pentyl **a**-factor	2.5 ± 0.5	0.22 ± 0.04
pentyl-NBD **a**-factor	3.3 ± 0.3	0.17 ± 0.02

[a] The final concentration of the NBD-labeled pheromones added to the vesicle suspensions ranged from 0.1 to 0.7 μM, and the final concentration of the unlabeled pheromones ranged from 0.6 to 5 μM. [b] Values of M_{50} were determined by fitting data from binding experiments to eq 1 in the text. The binding experiments were performed with vesicles composed solely of POPC. The buffer in these experiments was 10 mM MES−KOH, pH 6.0 with 200 mM KCl, but the values of M_{50} did not depend on the ionic strength of the solution. [c] Values of K_p were calculated from the values of M_{50} using eq 3 from the text.

Binding experiments with the other pheromones allowed values of M_{50} to be obtained for each pheromone (Table 1). As can be seen from the similar values of M_{50} for the labeled and unlabeled peptides, the presence of the NBD modification on the decyl and pentyl **a**-factor did not significantly affect the partitioning of the pheromones into these membranes. The values of M_{50} for the *O*-methyl, *S*-decyl derivatives were 100-fold smaller than the values for the corresponding pentyl derivatives. This result indicates that five methylene groups increase the affinity of the pheromone for the membrane by 100-fold. Removal of the *O*-methyl group resulted in a 6-fold decrease in the affinity of the decyl-NBD **a**-factor for the vesicles.

Table 2: Binding Affinities (M_{50} Values, μM) of the Pheromones for Negatively-Charged Vesicles at Various Concentrations of Salt[a]

	[K^+] (mM)[c]				
pheromone[b]	4.5	29.5	54.5	104.5	204.5
decyl-NBD **a**-factor	0.5 ± 0.1	1 ± 0.5	2 ± 0.5	2.5 ± 0.5	5 ± 1
demethyl decyl-NBD **a**-factor	4 ± 0.5	25 ± 5	50 ± 5	75 ± 5	100 ± 10
pentyl **a**-factor	N/D[d]	N/D	150 ± 50	N/D	N/D
pentyl-NBD **a**-factor	15 ± 2	75 ± 5	170 ± 25	410 ± 40	550 ± 50

[a] Values of M_{50} were determined by fitting data from binding experiments with POPC/POPG (4:1) vesicles to eq 1 of the text. The error corresponds to the asymptotic standard error of the M_{50} parameter and was calculated by the curve-fitting program. [b] The final concentration of the NBD-labeled pheromones added to the vesicle suspensions ranged from 0.01 to 0.2 μM, and the final concentration of pentyl **a**-factor ranged from 0.6 to 5 μM. [c] The concentration of potassium ions includes both the concentration of potassium chloride added to the solutions and the concentration of potassium hydroxide used to adjust the pH. [d] Not determined.

POPC vesicles are electrically neutral, but the binding of the charged pheromones to the surface of these vesicles could result in changes in the surface potential of the membrane, and these changes could affect the binding affinity of the membrane for the pheromones (McLaughlin, 1977). In order to test whether or not the binding of positively-charged pheromones to the POPC vesicles was altering the surface charge of these vesicles and causing electrostatic effects, the binding experiments were repeated using solutions containing various concentrations of potassium chloride. In no case was the value of M_{50} significantly affected by the concentration of potassium chloride in the buffer (data not shown). These experiments indicate that the binding of the pheromones to the vesicles does not significantly alter the surface charge of the vesicles and also that changes in the concentration of salt do not alter the hydrophobic interactions between the pheromones and vesicles. The results support the conclusion that the concentration dependence of the molar fluorescence of the bound pheromones results from quenching of the fluorescence rather than from a decreased affinity of the membranes for the pheromones due to the buildup of a positive surface potential on the bilayers.

In order to examine the effects of physiological surface charge on the binding of the pheromones, binding experiments were repeated with vesicles composed of 80% POPC and 20% POPG. In these experiments, the values of M_{50} for the peptides were from 2-fold to 220-fold lower than those obtained from experiments with POPC vesicles (Table 2). The values of M_{50} also depended on the concentration of potassium chloride in the buffer; as the concentration of potassium chloride in the buffer was increased, the values of M_{50} determined from these experiments increased. Clearly, in addition to the hydrophobic effects, there is a significant electrostatic component to the binding of the pheromones to charged vesicles.

The electrostatic contribution to the binding, which arises from the presence of negatively-charged POPG in the vesicles, can be described quantitatively. The magnitude of the electrostatic attraction depends on the charge of the pheromone, z_p, and the surface potential of the vesicles, ψ_o. In order to differentiate the contribution of the electrostatic attraction to the overall binding affinity of the pheromones, it is necessary to determine the effect of the surface potential on the concentrations of pheromone directly adjacent to the lipid bilayer. In general, the concentration of a charged molecule adjacent to a charged surface is related to the bulk concentration of the molecule in the aqueous phase by a Boltzmann function (McLaughlin, 1977). In the case of the pheromones, this function is of the form:

$$[P_M] = [P_F] \exp[-z_p F \psi_o (RT)^{-1}] \quad (4)$$

where $[P_M]$ represents the concentration of pheromone adjacent to the membrane and $[P_F]$ represents the bulk concentration of the pheromone in the aqueous solution. The value of the true partition coefficient under these circumstances depends on the concentration of pheromone adjacent to the surface:

$$K_p = [P_B][M]^{-1}([P_M][H_2O]^{-1})^{-1} \quad (5)$$

This partition coefficient is independent of the effects of electrostatics. Combination of eqs 2, 4, and 5 gives the relationship between this partition coefficient and the values of M_{50} determined from the vesicles containing POPG:

$$K_p = [H_2O](M_{50})^{-1} \exp[z_p F \psi_o (RT)^{-1}] \quad (6)$$

The surface potential depends on the charge density of the surface and the ionic strength of the solution and can be described by a simple form of the Gouy−Chapman equation (McLaughlin, 1977):

$$\psi_o = 2RTz_e^{-1}F^{-1} \sinh^{-1}[\sigma(8C_e\epsilon_r\epsilon_o RT)^{-1/2}] \quad (7)$$

In this equation, z_e represents the valence of the electrolyte in solution, σ represents the charge density of the surface, C_e represents the millimolar concentration of the electrolyte in solution, ϵ_r represents the dielectric constant of the aqueous solution, ϵ_o represents the permittivity of free space, R is the universal gas constant, F is the Faraday constant, and T is the absolute temperature.

The charge density of a membrane surface can be calculated from the lipid composition of the vesicles, and a value for ψ_o in eq 6 can be obtained directly from eq 7 using this value together with the other constants. Values for z_p in eq 6, however, are somewhat more difficult to assign. First, it is assumed in the Gouy−Chapman and Boltzmann equations that ions behave as point charges. This assumption is not necessarily valid for peptides, however, because they are often relatively large compared to the Debye length, and because they can contain multiple ionizable groups (McLaughlin, 1977; Langner et al., 1990; Stankowski, 1991). Second, in our experiments, the surface potential was modulated by varying the concentration of salt in the aqueous solution. Because the concentration of salt also affects the Debye length, and because the Debye length in turn affects how an ion of finite size interacts with the surface potential (McLaughlin, 1977; Mosior & McLaughlin, 1992), the effective charges of the peptides may not be constant under our experimental conditions. Finally, the exact pK_a values of the ionizable groups on the peptides are not known and would be difficult to determine under the experimental conditions of the binding experiments. For these reasons, values for z_p in eq 7 were not assigned *a priori*, but were obtained from the curve-fitting procedure.

The data from Table 2 for the binding of the pheromones to vesicles containing 20% POPG were fit to the combined eqs 6 and 7 (Figure 4). The charge density used in eq 7, $\sigma = -0.0457$ C m^{-2}, was calculated from the mole fraction

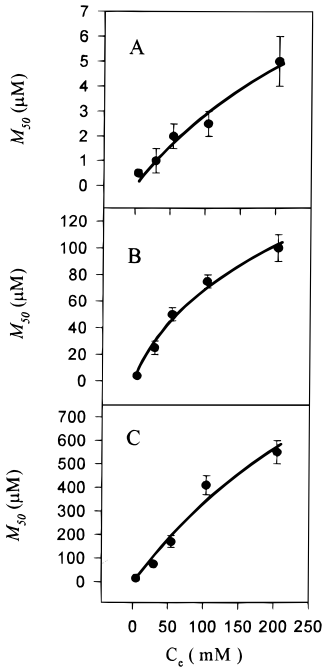

FIGURE 4: Effects of electrostatics on binding to POPC/POPG (4:1) vesicles. The values of M_{50} from experiments with decyl-NBD **a**-factor (A), demethyl decyl-NBD **a**-factor (B), and pentyl-NBD **a**-factor (C) were plotted as a function of the concentration of salt in the buffer. The data were fit to eqs 6 and 7 as described in the text to obtain values of K_p, and z_p. These values were used to generate the lines shown.

Table 3: Binding Parameters for the Pheromones Binding to Negatively-Charged Vesicles[a]

pheromone	K_p ($\times 10^{-5}$)	z_p
decyl-NBD **a**-factor	23 ± 6	1.1 ± 0.2
demethyl decyl-NBD **a**-factor	1.5 ± 0.3	0.8 ± 0.1
pentyl-NBD **a**-factor	0.18 ± 0.06	1.1 ± 0.2

[a] Values of K_p and z_p were obtained by fitting the values of M_{50} from Table 2 to a combination of eqs 6 and 7 of the text.

of negative lipids in the vesicles by assuming a surface area of 0.70 nm^2 per lipid (Evans et al., 1987). The change in charge density due to adsorption of ions from the electrolyte onto the surface of the membrane (Eisenberg et al., 1979) was considered insignificant and was ignored. Values of K_p and z_p obtained from curve fitting are presented in Table 3. These values were used in eq 6 to generate the lines shown in Figure 4.

It is clear from the results of the curve fitting that the binding data are well described by the combination of the Gouy−Chapman and Boltzmann equations. In addition, the values of z_p obtained from the fitting procedure are reasonably close to those expected by assigning formal charges to the ionizable groups on the peptide at pH 6.0. In the case of demethyl decyl-NBD **a**-factor, a net charge of 0 would be expected if formal charges are assigned to the ionizable groups at pH 6.0. That an effective charge of +0.8 was estimated for this peptide suggests that the C-terminus of the peptide may be protonated under the conditions of the binding experiments. It should also be noted that the pH adjacent to a negatively-charged membrane is significantly lower than that of the bulk solution, and the lower pH at the surface could be at least partly responsible for the more positive estimated effective charge.

Binding of the Pheromones to Yeast Membranes. Binding of decyl-NBD **a**-factor to isolated yeast membranes displayed similar behavior as the binding to lipid vesicles containing 20% POPG. The values of M_{50} increased as a function of salt concentration and are shown in Figure 5. The presence or absence of low levels of the pheromone receptors did not affect the nonspecific binding of the pheromone to the membranes as measured by enhancement of fluorescence. Under the conditions of our assays, the concentration of **a**-factor receptor was estimated by Western blot analysis to be less than 1 nM (data not shown). At these low concentration, the receptor could not bind a significant fraction of the total amount of pheromone present in a binding assay.

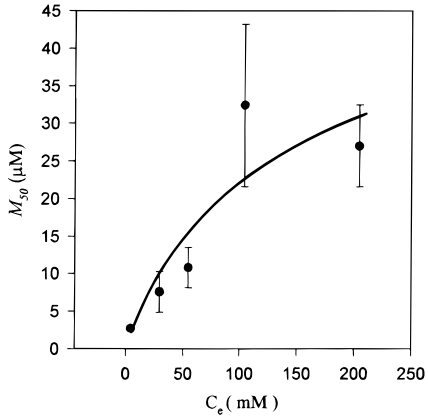

FIGURE 5: Binding of decyl-NBD **a**-factor to isolated yeast membranes. The values of M_{50} from experiments with yeast membranes and decyl-NBD **a**-factor were plotted as a function of the concentration of salt in the buffer. Using the value of z_p obtained from the data in Figure 4A, the values of M_{50} were fit to eqs 6 and 7 to determine the charge density of the yeast membranes and the value of K_p. The line was generated using $K_p = (8 \pm 6) \times 10^5$ and $\sigma = -0.02 \pm 0.01$ C m^{-2} in eqs 6 and 7.

The data obtained from the experiments with yeast membranes were analyzed in a manner similar to that described for the lipid vesicles. The charge density of the yeast membranes was unknown, however, and was left as a parameter during the curve-fitting procedure. A value for the effective charge of decyl-NBD **a**-factor was assumed to be the same as that obtained from the experiments with lipid vesicles. This assumption seems reasonable since the experiments were performed under identical conditions.

Values of K_p and the charge density of the yeast membranes obtained from the curve-fitting procedure were $(8 \pm 6) \times 10^5$ and -0.02 ± 0.01 C m^{-2}, respectively. The charge density of the yeast membranes obtained from the curve-fitting procedure is in good agreement with values obtained by others using an unrelated membrane probe (Cerbon & Calderon, 1994). The partition coefficient of the yeast membranes is within experimental error of the partition coefficient determined from experiments with synthetic vesicles (Tables 1 and 3). This comparison is not exact, however, as the yeast membrane preparations consist of an equal mixture of wrong-side-out or unsealed vesicles and right-side-out sealed vesicles (Monk et al., 1989). The presence of the wrong-side out and unsealed vesicles allows the decyl-NBD **a**-factor to interact not only with the extracellular but also with the cytosolic leaflet of the plasma membrane. Since the lipid composition of the two leaflets differs, the partitioning of the peptide into the two leaflets is probably also different. In addition, the presence of unsealed vesicles in the binding assays results in an underestimation of the concentration of accessible lipid in

the assays. For these reasons, the partition coefficient for decyl-NBD **a**-factor determined with yeast membranes and the charge density of the yeast membranes are probably not as accurate as the analogous values obtained with the synthetic vesicles.

Biological Activity of a Pheromone Lacking the C-Terminal Methyl Ester. The biological activity of the demethyl decyl-NBD **a**-factor was determined as the concentration of peptide required for half-maximal arrest of the growth of α-cells as previously described (Sherrill et al., 1995). No arrest of growth was observed for a concentration of peptide as high as 5 μM. For comparison, the activity of the decyl-NBD **a**-factor is 2.0 ± 0.5 nM (Sherrill et al., 1995).

DISCUSSION

We have used the fluorescence of tryptophan or NBD to measure the partitioning of *S*-alkyl derivatives of **a**-factor into lipid vesicles and isolated yeast membranes. The partitioning of the peptides into the apolar environment of the lipid bilayer results in an increase in the quantum yield of tryptophan and NBD. Ideally, the binding affinity of a peptide for a membrane can be calculated directly from the fluorescence signal of a fixed concentration of the fluorescent peptide at various concentrations of membrane. By curve fitting, the fluorescence of the bound form of the peptide is obtained, together with the concentration of membrane required for half-maximal enhancement of the fluorescence signal. In these calculations, the quantum yield of the bound peptide is implicitly assumed to be independent of the surface concentration of the peptide. This procedure has been used to determine the partition coefficient of many different peptides (Surewicz & Epand, 1984; Pouny et al., 1992; Rapaport et al., 1992).

The assumption that the quantum yield of the fluorophore in the bound form of the peptide is constant may not be valid for tryptophan and NBD, however. The molar fluorescence of the **a**-factor analogues in the presence of vesicles indicated that the quantum yield of NBD and tryptophan in the lipid bilayer decreased at increasing surface concentrations of peptide (Figure 2). This effect probably results from self-quenching of the fluorophores in the relatively small volumes of the vesicle surface. In order to correct for the effect, it was necessary to measure the fluorescence at different concentrations of peptide and to extrapolate the values of molar fluorescence to infinitely low concentrations of peptide. Although this correction has a relatively small effect on the binding constants obtained from these calculations, it is important to note that accurate constants cannot be obtained from fluorescence-based binding experiments performed at a single concentration of peptide. After correcting for the concentration dependence of the molar fluorescence, partition coefficients can be determined in the usual manner.

The partition coefficients of the decyl derivatives of **a**-factor are 100-fold greater than those of the pentyl derivatives. This result indicates that each methylene group adds approximately 0.5 kcal mol^{-1} to the free energy of partitioning. Although this value is somewhat smaller than the value of 0.8 kcal mol^{-1} per methylene group seen by others for the partitioning of myristylated peptides and *S*-alkylated, *O*-methylated peptides into vesicle membranes (Peitzsch & McLaughlin, 1993; Silvius & l'Heureux, 1994), it is not clear whether or not the difference is significant. It is also possible that the nature of the peptide can affect the interactions of alkylated peptides with membranes, or that the relatively short alkyl chains used in our peptides do not show the same dependence on added methylene groups as do longer alkyl chains.

Although the partitioning of the decyl **a**-factor derivatives into membranes is 100-fold greater than the pentyl derivatives, the biological activity of the decyl derivatives is only approximately 10−30-fold greater than that of the pentyl derivatives (Sherrill et al., 1995). The lack of an exactly proportional relationship between membrane partitioning and biological activity may in part be caused by the different experimental conditions used for the binding and activity assays. As seen in Figure 2, the surface concentration of a bound pheromone, which corresponds to the ratio between the molar concentration of bound pheromone and the molar concentration of accessible lipid, varies in strict proportion to the partition coefficient only at very low concentrations of membrane. In other words, only at low concentrations of membrane would a change in the affinity of the pheromone for the membrane cause an equivalent change in the surface concentration of the bound pheromone. At high concentrations of membrane, the surface concentration of bound pheromone would be relatively insensitive to changes in the affinity of the pheromone for the membrane. Since the occupancy of the pheromone receptor is presumably dependent on the surface concentration of the pheromone, the biological activity of a pheromone may consequently depend not only on the aqueous concentration of the pheromone but also on the concentration of the membrane. Although the biological activity assays were performed at low cell densities, the density of cells may have been high enough that the full difference in membrane affinities between the pentyl and decyl **a**-factor derivatives may not have been reflected in the amount of peptide partitioned into the plasma membrane. In any case, the difference in biological activities of the pentyl and decyl derivatives is presumably at least partly explained by the difference in affinities of these derivatives for the membrane.

Although the decrease in biological activity of the pentyl derivatives relative to the decyl derivatives is consistent with a decrease in the partition coefficient, the change in biological activity due to the attachment of the NBD fluorophore is not consistent with a change in the partition coefficient. The biological activities of the NBD-labeled pheromones are 6−10-fold lower than the biological activities of the unlabeled pheromones (Sherrill et al., 1995) despite having identical partitioning behavior into membranes (Table 1). Attachment of NBD to the end of a flexible alkyl chain can affect the penetration of the alkyl chain into the lipid bilayer (Chattopadhyay & London, 1987). As was observed for NBD attached to the end of a fatty acyl chain of phosphatidylcholine (Chattopadhyay & London, 1988), the NBD of decyl-NBD **a**-factor is accessible to collisional quenching with cobalt ions (data not shown). This result suggests that the NBD is not deeply embedded in the bilayer. The effect of NBD on the penetration of the alkyl group may alter the interactions of the pheromone with the receptor without significantly affecting the membrane partitioning and may partly explain the decrease in the biological activity of the NBD derivatives of the pheromones.

Demethylation of the C-terminus of decyl-NBD **a**-factor also had a larger effect on the biological activity than on the partitioning behavior. Demethylation decreased the biological activity by more than 2500-fold but only decreased

the partitioning of the pheromone into lipid vesicles by about 6-fold. The effect of the demethylation on membrane partitioning is consistent with the observation of Silvius and l'Heureux (1994) that the partitioning of demethylated *S*-farnesylated peptides into neutral vesicles is decreased 10−20-fold relative to that of the methylated peptides. The lack of biological activity of demethyl decyl-NBD **a**-factor cannot, therefore, be explained simply by a reduced partitioning of the pheromone into membranes. Instead, the methyl group may be necessary for specific interactions between the pheromone and the receptor, either by direct contact with the receptors or by helping to position the pheromone in the membrane in a manner that is recognized by the receptor. The dependence of the partitioning of the demethyl decyl-NBD **a**-factor on electrostatics suggests that the C-terminus is protonated and that the reduced activity does not result from the presence of a negative charge on the C-terminus of the peptide.

The values of M_{50} for the partitioning of the pheromone derivatives into negatively-charged vesicles depend on the ionic strength of the solution (Table 2). That this effect is well described by the combined Gouy−Chapman and Boltzmann equations argues that the main consequence of the negative lipids is the generation of a negative surface potential that causes an increase in the concentration of the positively-charged pheromones close to the surface of the negatively-charged membranes. At the lowest ionic strength, however, the value of M_{50} measured for decyl-NBD **a**-factor was only an order of magnitude higher than the concentration of pheromone used in the experiments (Table 2). Because the use of low molar ratios of lipid to bound peptide may result in errors in the measurements that are not adequately corrected by the linear extrapolations of the molar fluorescence, these values may reflect an upper limit on the true binding constants. Nonetheless, the similarity in the values of K_p extrapolated from the binding data using the Gouy−Chapman theory, and the experimental values of K_p obtained for the binding of the pheromones to uncharged vesicles, suggests that there are not specific interactions between the anionic phospholipids and the pheromones as has been seen for other peptides (Kim et al., 1991; Buser et al., 1994).

Now that the nonspecific interactions between the **a**-factor analogues and membranes have been defined, it should be easier to analyze and understand the specific interactions between the pheromones and the **a**-factor receptor. Others have used fluorescent ligands to monitor such interactions (Tota & Strader, 1990; Hwang et al., 1992; Turcatti et al., 1995), and it should be possible to apply similar approaches to study the yeast receptors. A requirement for such assays is that the various forms of the ligand display distinct spectroscopic signals. As observed in this report and by others, the NBD probe is very sensitive to the polarity of its environment. The polarity of the receptor binding site may be sufficiently different from that of the membrane that the binding of the pheromone to its receptor may be measured. Use of these biophysical approaches to examine the interactions between ligands and their receptors should provide a more detailed view of this important problem than is currently possible.

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