The Interaction of Bioactive Peptides with an Immobilized Phosphatidylcholine Monolayer

Henriette Mozsolits,* Tzong-Hsien Lee,* Hans-Jurgen Wirth,* Patrick Perlmutter,# and Marie-Isabel Aguilar*
*Department of Biochemistry & Molecular Biology and #Department of Chemistry, Monash University, Victoria 3168, Australia

ABSTRACT The interaction of three bioactive peptides, bombesin, β-endorphin, and glucagon with a phosphatidylcholine monolayer that was immobilized to porous silica particles and packed into a stainless steel column cartridge, has been studied using dynamic elution techniques. This immobilized lipid monolayer provides a biophysical model system with which to study the binding of peptides to a lipid membrane. In particular, the influence of temperature and methanol concentration on the affinity of each peptide for the immobilized lipid surface was assessed. For all test peptides, nonlinear retention plots were observed at all temperatures that contrasted sharply with the simple linear plots observed for the small unstructured control molecules N-acetyltryptophanamide and diphenylalanine. An analysis of the thermodynamics of the interaction of peptides with the immobilized monolayer was also carried out. The results revealed that while the peptides interacted with the monolayer predominantly through hydrophobic interactions, the relative contribution of ΔH^{O}_{assoc} and ΔS^{O}_{assoc} to the overall free energy of association was dependent on the temperature and methanol concentration. In particular, it was evident that under most conditions, the binding of the peptides to the immobilized lipid monolayer was enthalpy-driven, i.e., mediated by nonclassical hydrophobic interactions. Significant band-broadening and asymmetric and split peaks were also observed for bombesin, β-endorphin, and glucagon at different temperatures and methanol concentrations. These changes in affinity and peak shape are consistent with the formation of multiple conformational species during the interaction of these peptides with the lipid monolayer. In addition, the binding behavior of the three test peptides on an *n*-octylsilica surface that lacked the phospho headgroups of the phospholipid was significantly different from that observed with the immobilized phosphatidylcholine surface, indicating a specificity of interaction between the peptides and the lipid surface. Overall, these experimental results demonstrate that the biomimetic phosphatidylcholine monolayer provides a stable and sensitive system with which to explore the molecular mechanism of peptide conformational changes during membrane interactions.

INTRODUCTION

Peptide-lipid interactions play a critical role in the regulation of several biological phenomena, including the insertion and folding of membrane proteins, the translocation of polypeptides though membranes, and the cytolytic action of antimicrobial peptides. The unique feature of peptides, which distinguishes them from many other classes of nonpeptidic molecules that associate with membranes, is the induction of specific secondary structure(s) of the peptide upon binding to a lipid surface from predominantly extended structure(s) in an aqueous environment (Deber and Li, 1995). The interaction of peptide hormones with the cell membrane has also been suggested to be a key step in the molecular mechanism of hormone receptor subtype selection (Behling and Jelinski, 1990). In particular, the catalytic function of membranes in hormone action has been proposed to involve the induction of peptide secondary structure upon interaction with the phospholipid membrane surface, thereby facilitating the presentation of the hormone to the receptor in the correct conformation and enhancing the recognition of the hormone by the receptor (Sargent et al., 1988).

An important step in the characterization of peptide- and protein-lipid interactions is the analysis of the energetics of binding and insertion of the biomolecule into the membrane systems as a function of the associated conformational changes. The ability to measure the free energy of interaction of peptides and proteins with membrane surfaces under a range of experimental conditions, in which the structure of the peptide and/or lipid can be manipulated, would be a powerful adjunct to the existing surface-interactive and spectroscopic techniques that are used in the study of membrane-mediated events.

Small or large unilamellar vesicles have conventionally been used to study the interaction of peptides and proteins with biomembranes. More recently, however, a number of novel biophysical techniques have been developed to study the binding of biomolecules to model membrane surfaces. The types of model membrane systems prepared in these studies have involved lipid monolayers or bilayers either adsorbed or covalently attached to a support surface (Cornell et al., 1997; Gizeli et al., 1997; Linseisen et al., 1997; Mrksich and Whitesides, 1996). The techniques used to detect binding include acoustic techniques (Gizeli et al., 1997), surface plasmon resonance (Cooper et al., 1997; Duschl et al., 1994) and gated ion channel-based biosensors (Cornell et al., 1997; Heyse et al., 1998). In addition, a number of spectroscopic methods including solid state nu-

Received for publication 27 July 1998 and in final form 25 May 1999.

Address reprint requests to Dr. Marie-Isabel Aguilar, Dept. of Biochemistry and Molecular Biology, Monash University, Wellington Rd., Clayton, Victoria 3168, Australia. Tel.: 61-3-9905-3723; Fax: 61-3-9905-5882; E-mail: mibel.aguilar@med.monash.edu.au.

0006-3495/99/09/1428/17 $2.00

clear magnetic resonance spectroscopy (Picard et al., 1998) attenuated total internal reflection infrared spectroscopy (Axelsen et al., 1995), fluorescence microscopy (Duschl et al., 1994), circular dichroism (deJongh et al., 1994) and impedance spectroscopy (Steinem et al., 1996) have been utilized to monitor conformational changes of peptides and proteins upon binding to a model membrane surface. However, there are no readily available tools that allow the determination of the free energy of interaction of peptides with these model membrane systems under dynamic conditions as a function of changes in peptide and lipid structure.

In the present study, the interaction of two peptide hormones β-endorphin and glucagon and the bioactive peptide bombesin with an immobilized model membrane surface has been studied. These peptides have been shown (Cavatorta et al., 1989; Erne and Schwyzer, 1987; Zetta et al., 1984; Kimura et al., 1992; Pasta et al., 1988) to adopt a significant degree of α-helical structure upon binding to lipid micelles and/or vesicles, which is considered to be an important step in their mechanism of action. The immobilized model membrane was prepared through the covalent attachment of a modified phosphatidylcholine to an amorphous silica surface (Lee et al., 1999, submitted for publication). The interactive behavior of the peptides with the biomimetic membrane surface was then studied using dynamic elution techniques that allowed the measurement of the affinity and the free energy of interaction of the peptides for the lipid monolayer. The role of peptide structure and lipid mobility in the binding of the peptides to the lipid surface was further studied by the measurement of binding parameters over a range of temperatures. The experimental data demonstrate that the biomimetic phosphatidylcholine monolayer provides a stable and sensitive system with which to explore the thermodynamic properties of peptide-membrane interactions.

MATERIAL AND METHODS

Chemicals and reagents

Methanol (HPLC) grade was obtained from Mallinchrodt Baker Inc. (Paris, KY) and water was quartz-distilled and deionized in a Milli-Q system (Millipore, Bedford, MA). N-Acetyl-L-tryptophanamide, di-L-phenylalanine, and glucagon (porcine pancreas) were obtained from Sigma (St. Louis, MO). Bombesin and β-endorphin (human) were obtained from Auspep (Parkville, Vic, Australia). The sequences and molecular weights of all peptides used are listed in Table 1.

Apparatus

All binding measurements were performed on a Waters Assoc. (Milford, MA) liquid chromatograph 484 system consisting of two Model 6000A solvent delivery pumps, a U6K universal injector, a WISP Model 712B sample processor, and an M660 gradient programmer. The detector used was a Lambda-Max Model 484 LC spectrophotometer operating at 215 nm and the total system was controlled by Mellinium 2010 operating software. The operating temperature was controlled by immersing the column in a MultiTemp thermostatic circulator (LKB-Producter AB, Bromma, Sweden).

Binding studies

The immobilized phosphatidylcholine model membrane surface was prepared as previously reported (Lee et al., 1999, submitted for publication) by covalent attachment of 1,2-di-O-(12-aminododecanoyl)-*sn*-glycerol-3-O-phosphorylcholine to NCS-activated spherical silica particles, and the structure of the monolayer is shown in Fig. 1. The lipid density was determined from elemental analysis to be 1.08 μmol/m^2, which is similar to the estimated lipid density in biological membranes (Pidgeon and Venkataram, 1989). The *n*-octylsilica (C8)-modified silica particles were prepared as previously described (Lee et al., 1999, submitted for publication) by covalent attachment of octyldimethylchlorosilane to activated silica particles, and elemental analysis indicated a ligand density of 3.85 μmol/m^2. The phosphatidylcholine-modified silica and the C8-modified silica were each packed into a stainless steel cartridge with dimensions of 4 cm $\times$ 4.6 mm ID. The interaction of peptides with the immobilized model membrane and the C8 surface was monitored using isocratic elution conditions from 0 to 80% (v/v) methanol in milli-Q water as the mobile phase. A flow rate of 1 ml/min was used throughout and experiments were carried out at column temperatures of 5, 15, 25, 35, 45, 55, and 65°C. The solvents were filtered under vacuum and degassed by sparging with nitrogen. Peptide solutions were prepared by dissolving the solute at concentrations of 0.1 mg/ml in milli-Q water except glucagon, which was dissolved in 40% methanol. A 50-μl injection volume was used for all measurements. All data points were derived from duplicate measurements with retention times between replicates varying by $<$5%. The column dead volume (t_o) was taken as the retention volume of the initial breakthrough peak and was found to be 0.462 min for the phosphatidylcholine monolayer and 0.553 min for the C8 surface. The phase ratio for the phosphatidylcholine monolayer was determined as previously described (Ong and Pidgeon, 1995) to be 0.036 and 0.0256 for the C8 surface. All interactive parameters were calculated using Excel version 7.0.

RESULTS

Retention relationships

Theoretical considerations

In a recent study we reported the preparation and preliminary characterization of a phosphatidylcholine monolayer covalently attached to a solid phase silica-based material as

TABLE 1 Sequence and molecular weights of solutes

Solute	Sequence	Molecular Weight
N-Acetyltryptophanamide	N-Ac-W-NH_2	245
Diphenylalanine	FF	312
Bombesin	pyrQQRLGNQWAVGHLM	1640
β-Endorphin	YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE	3470
Glucagon	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT	3520

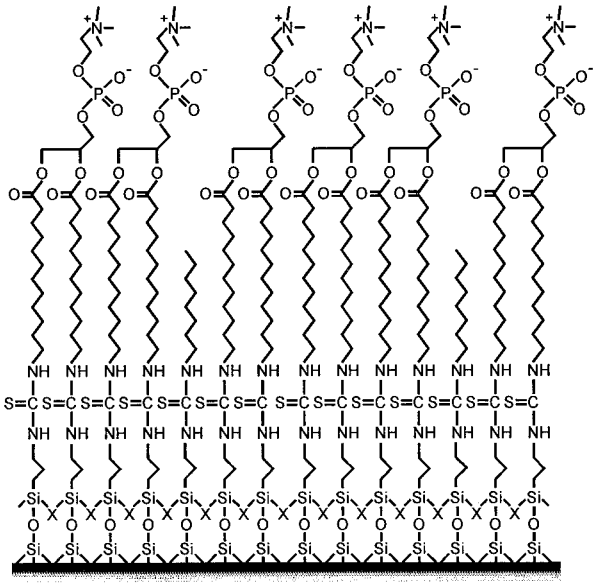

FIGURE 1 Chemical structure of the phosphatidylcholine monolayer chemically immobilized to the silica surface. X can either be an ether (—O—) linkage between adjacent silanes or exist as a free ethoxy ($—OC_2H_5$) group.

illustrated in Fig. 1 (Lee et al., 1999, submitted for publication). This model membrane system was used to study the interaction of peptides with the lipid surface in an analogous manner to the approach developed using reversed-phase high performance liquid chromatography (RP-HPLC). RP-HPLC has been increasingly used for the study of peptide interactions with hydrophobic, lipid-like surfaces and has been particularly successful in the analysis of the induction and stabilization of amphipathic α-helical and β-sheet peptides upon binding to *n*-alkylsilica materials (Lazoura et al., 1997; Steer et al., 1998; Blondelle et al., 1995b; Hodges et al., 1988, 1994; Krausse et al., 1995). In particular, significant changes in the interactive retention parameters that are related to the hydrophobic binding domain and the affinity of interaction can be correlated to changes in peptide secondary structure. In the present study, these elution techniques have been applied to the analysis of the interaction of the bioactive peptides bombesin, β-endorphin, and glucagon and a series of small control molecules with the immobilized phospholipid monolayer surface.

The interaction of peptides with the lipid monolayer can be described in terms of a number of discrete steps including electrostatic and/or hydrophobic binding to the lipid surface followed by reorientation and partitioning of the peptide into the hydrophobic phase. The overall standard free energy of interaction of a peptide with the immobilized lipid monolayer, ΔG^{O}_{imm}, can be described (Ben-Tal et al., 1996) by the following relationship

$$\Delta G^{O}_{assoc} = \Delta G^{O}_{solv} + \Delta G^{O}_{imm} + \Delta G^{O}_{lip} + \Delta G^{O}_{conf} \qquad (1)$$

where ΔG^{O}_{solv} is the standard free energy change associated with changes in the solvation of the peptide upon binding made up of electrostatic and hydrophobic contributions, ΔG^{O}_{imm} is the standard free energy change associated with immobilization of the peptide in the lipid layer, ΔG^{O}_{lip} is the standard free energy associated with the lipid perturbation effects, and ΔG^{O}_{conf} is associated with peptide conformational changes upon interaction with the lipid surface. By analogy with the theoretical relationships derived for interactive chromatography, the binding of a peptide to the immobilized lipid layer using dynamic elution can be expressed by the isocratic capacity factor, k', according to the following expression

$$k' = (t_r - t_o)/t_o \qquad (2)$$

where t_r is the retention time of the solute and t_o is the dead time of the column. In RP-HPLC, under conditions where hydrophobic interactions are the dominant interactive force, a linear relationship is generally observed (Horváth et al., 1976) between log k' and φ, the mole fraction of organic solvent required to elute the solute from the hydrophobic surface, according to

$$\log k' = \log k_o - S\varphi \qquad (3)$$

where log k_O is the capacity factor in the absence of organic solvent and S is the slope of plots of log k' versus φ. Peptides and proteins interact with hydrophobic surfaces in an orientation-specific manner via a hydrophobic contact area. If this hydrophobic contact area changes as a result of conformational or orientational changes, then the binding properties of the peptide will also be altered. As a consequence, several studies have demonstrated that the log k' value is a physical parameter that is highly sensitive to the conformational status of the peptide or protein upon interaction with hydrophobic surfaces (Blondelle et al., 1995b; Hodges et al., 1994; Krausse et al., 1995; Lazoura et al., 1997; Lee et al., 1997; Steer et al., 1998). As such, the log k' value represents an important probe of changes in peptide orientation during their interaction with the immobilized lipid monolayer as a function of peptide or lipid conformation. Furthermore, it has been well-established that the k' value can be related (Horváth et al., 1976) to the peptide-lipid association constant, K_a, through the following expression

$$K_a = (C_s/C_m) = k'\Phi \qquad (4)$$

where C_s and C_m are the concentration of the solute in the stationary phase and the mobile phase, respectively, and Φ is the phase ratio equal to V_s/V_m, where V_s and V_m are the volume of the stationary phase and mobile phase, respectively. More significantly, the standard unitary free energy of interaction with the immobilized lipid surface, ΔG^{O}_{assoc}, can then be obtained from the following

$$\Delta G^{O}_{assoc} = -RT \ln K_a \qquad (5)$$

Hence, peptide retention can be directly related to the overall standard free energy of interaction ΔG^{O}_{assoc} according to

$$\ln k' = \ln \Phi - \Delta G^{O}_{assoc}/RT \qquad (6)$$

The experimentally determined k' values therefore represent a measure of the total change in free energy associated with the binding of a peptide to the lipid surface. In the absence of organic solvent, the ΔG^{O}_{assoc} value will incorporate the contributions of all factors described in Eq. 1. However, the ln k' values obtained in the presence of organic solvent will not reflect the contribution of electrostatic terms to ΔG^{O}_{assoc}.

The relationship between ΔG^{O}_{assoc} and ΔH^{O}_{assoc}, the enthalpy of solute-lipid association and ΔS^{O}_{assoc}, the entropy of solute-lipid interaction can be expressed by

$$\Delta G^{O}_{assoc} = \Delta H^{O}_{assoc} - T\Delta S^{O}_{assoc} \quad (7)$$

Calculation of ΔH^{O}_{assoc} and ΔS^{O}_{assoc} allows the relative contribution of each parameter to the overall ΔG^{O}_{assoc} to be assessed from comparison of the magnitude of the ΔH^{O}_{assoc} value with the T ΔS^{O}_{assoc} term. In particular, it is generally accepted that classical hydrophobic interactions are entropy-driven at low temperatures and become increasingly enthalpy-driven at high temperatures (Tanford, 1980). However, nonclassical hydrophobic effects have been observed for the interaction of small molecules and peptides with lipid bilayers in which the hydrophobic interactions are enthalpy-driven at room temperature (Beschiaschvili and Seelig, 1992; Seelig and Ganz, 1991). The ability to derive ΔH^{O}_{assoc} and ΔS^{O}_{assoc} values for the interaction of peptides and proteins with lipids would therefore provide important information on the driving forces underlying peptide and protein-membrane interactions. Using the dynamic elution techniques described in this study, the dependence of ln k' values on temperature can be used to derive ΔH^{O}_{assoc} and ΔS^{O}_{assoc} by combination of Eqs. 6 and 7 to give

$$\ln k' = -\frac{\Delta H^{o}_{assoc}}{RT} + \frac{\Delta S^{o}_{assoc}}{R} + \ln \Phi \quad (8)$$

When enthalpy and entropy changes are independent of temperature, plots of ln k' versus $1/T$, i.e., van't Hoff plots, are linear and values of ΔH^{O}_{assoc} and ΔS^{O}_{assoc} can be determined according to Eq. 8. In the case of nonlinear van't Hoff plots that indicate a strong dependence of ΔH^{O}_{assoc} and ΔS^{O}_{assoc} on temperature, a second-order quadratic expression can be used (Haidacher et al., 1996; Cole et al., 1992) to evaluate thermodynamic data as follows

$$\ln k' = a + \frac{b}{T} + \frac{c}{T^2} + \ln \Phi \quad (9)$$

where ΔH^{O}_{assoc} is given by

$$\Delta H^{O}_{assoc} = -R\frac{d \ln k'}{d(1/T)} = -R\left(b + \frac{2c}{T}\right) \quad (10)$$

and ΔS^{O}_{assoc} is equal to

$$\Delta S^{o}_{assoc} = R\left(a - \frac{c}{T^2}\right) - \ln \Phi \quad (11)$$

Thus, elution techniques can be used to derive important thermodynamic data associated with peptide-lipid interactions as a function of both peptide and lipid structure.

In order to investigate the thermodynamic behavior of peptide interactions with membranes, the binding behavior of bombesin, β-endorphin, and glucagon with the phosphatidylcholine-immobilized model membrane surface was investigated with isocratic elution over a range of methanol concentrations. The influence of peptide conformation and ligand mobility on peptide binding behavior was further analyzed by varying the operating temperature to gain further insight into the factors that contribute to the free energy of interaction.

Experimental dependence of retention on solvent composition and temperature

The values of log k' for all molecules listed in Table 1 were derived from the retention times obtained with methanol concentrations between 0 and 80% at a flow rate of 1 ml/min and at temperatures between 5 and 65°C. The values of log k' were calculated according to Eq. 2. N-acetyltryptophanamide and diphenylalanine were used as low molecular weight standards that do not undergo any significant conformational changes either in solution or upon adsorption to a hydrophobic surface. Any variation observed in the log k' will therefore not be related to structural changes of the solute molecules. N-acetyltryptophanamide has also been previously used as a neutral model compound in studies on peptide-lipid interactions, and its location and orientation at the lipid interfacial region has been established by fluorescence techniques (Davis et al., 1996; Mishra and Palgunachari, 1996). Fig. 2, *a* and *b* show the plots of log k' versus percent methanol ($\varphi \times 100$) for the control molecules N-acetyltryptophanamide and diphenylalanine, respectively. It can be seen that there is a linear decrease in solute retention with increasing methanol concentration over the temperature range employed with the correlation coefficients (r^2) for the linear regression analysis between 0.9 and 1.00. This linear dependency of log k' on percent methanol indicates that hydrophobic interactions were the predominant interaction controlling the binding of these molecules to the immobilized lipid monolayer.

The influence of lipid mobility on the binding behavior of N-acetyltryptophanamide and diphenylalanine was further analyzed from the dependence of log k' on temperature for each methanol concentration shown in Fig. 3. For N-acetyltryptophanamide (Fig. 3 *a*) there was a linear decrease in log k' with temperature at each methanol concentration. However, for diphenylalanine (Fig. 3 *b*), between 5 and 25°C, there was no change in the log k' value between 0 and 40% methanol. However, from 25 to 65°C, there was a decrease in log k' that became steeper with increasing methanol concentrations. The use of methanol to desorb the solutes from the immobilized lipid surface influences the interactive process in two ways. First, increasing methanol concentrations decreases the solute retention as a result of

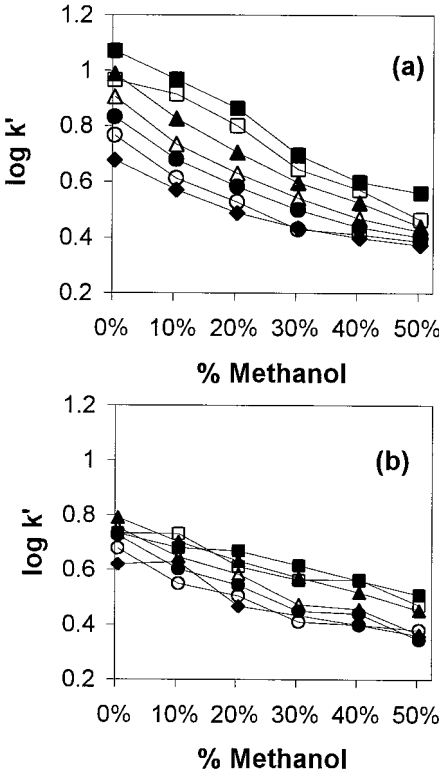

FIGURE 2 Plot of log k' versus percent methanol for (*a*) N-acetyltryptophanamide and (*b*) diphenylalanine after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C; (■) 5°C, (□) 15°C, (▲) 25°C, (△) 35°C, (●) 45°C, (○) 55°C, (◆) 65°C. See Materials and Methods for other conditions.

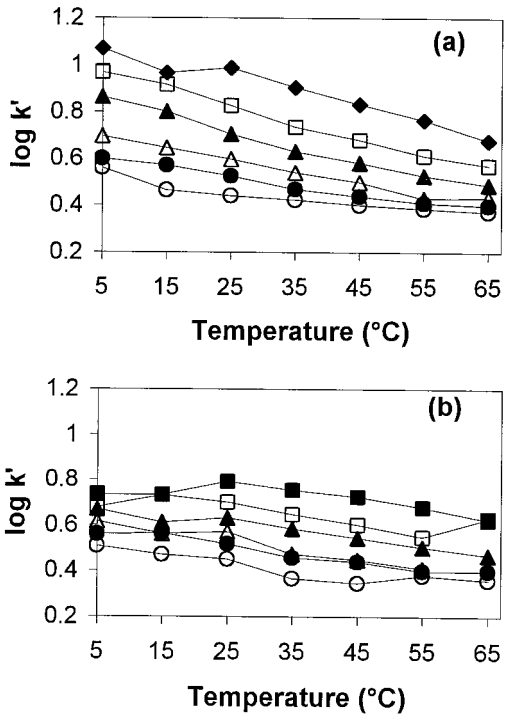

FIGURE 3 Plot of log k' versus temperature for (*a*) N-acetyltryptophanamide and (*b*) diphenylalanine after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C at the following methanol concentrations: (■) 0%, (□) 10%, (▲) 20%, (△) 30%, (●) 40%, (○) 50%. See Materials and Methods for other conditions.

decreases in the surface tension of the mobile phase (Horváth et al., 1976), thereby decreasing the hydrophobic interaction between the solute and the lipid surface. Second, increasing methanol concentrations may also increase the fluidity of the immobilized lipid ligands via specific solvation effects, thereby increasing the ability of the solute to penetrate into the lipid monolayer. The change in retention at 25°C for diphenylalanine may also reflect a phase transition in the immobilized phosphatidylcholine ligands. Preliminary studies on the measurement of the phase transition of this monolayer system by changes in fluorescence anisotropy associated with the insertion of diphenylhexatriene have revealed a transition between 25 and 35°C. The results in Fig. 3 therefore demonstrate that the binding of N-acetyltryptophanamide is unaffected by a change in lipid fluidity, which is consistent with the previous findings (Davis et al., 1996; Mishra and Palgunachari, 1996) that N-acetyltryptophanamide does not penetrate significantly into the acyl chains. In contrast, the nature of the interaction of diphenylalanine with the immobilized monolayer alters with changes in lipid conformation, indicating that this molecule can partially penetrate into the acyl chains of the immobilized lipids.

Overall, these results represent the behavior that would be anticipated for molecules that do not undergo any significant conformational interconversion during interaction with the immobilized phospholipid molecules. In addition, the relatively small size of these control molecules ensures that the degree of penetration of the molecules into the phospholipid monolayer will only be dependent on the lipid mobility and will not be influenced by any changes in solute conformation over the range of experimental conditions used.

The interactive behavior of bombesin with the immobilized phospholipid monolayer was investigated under the same conditions as described for the control molecules. The dependence of log k' versus percent methanol for the interaction of bombesin with the immobilized phosphatidylcholine between 5 and 65°C is shown in Fig. 4 *a*. While there was a decrease in retention with increasing temperature at each methanol concentration, there were significant fluctuations in the retention plots, particularly at 30% methanol, which were not apparent for the control molecules. These changes reflect variations in the affinity of bombesin for the phosphatidylcholine ligands under these conditions. This nonlinear relationship between log k' and percent methanol indicates that there is a significant change in the nature of the interaction of the peptides over the range of methanol concentration and temperature used in these studies. As the control molecules did not exhibit these large changes, the molecular basis for the results seen with bombesin is likely to be related to changes in conformation. In addition, these structural changes may also be associated with changes in

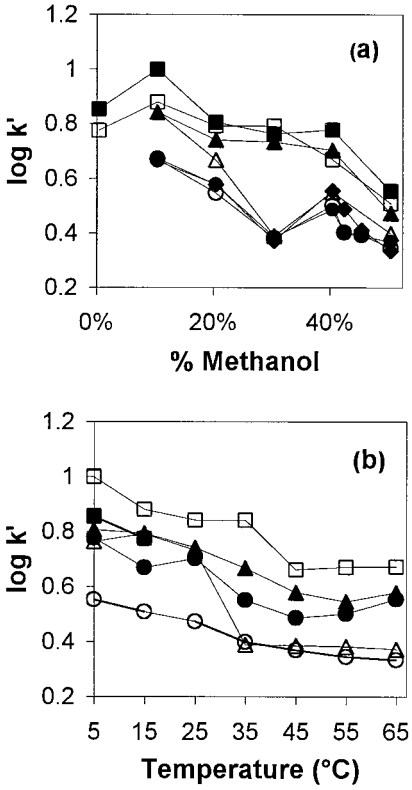

FIGURE 4 Plot of (*a*) log k' versus percent methanol and (*b*) log k' versus temperature for bombesin after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C. For (*a*): (■) 5°C, (□) 15°C, (▲) 25°C, (△) 35°C, (●) 45°C, (○) 55°C, (◆) 65°C. For (*b*): (□) 10%, (▲) 20%, (△) 30%, (●) 40%, (○) 50%. See Materials and Methods for other conditions.

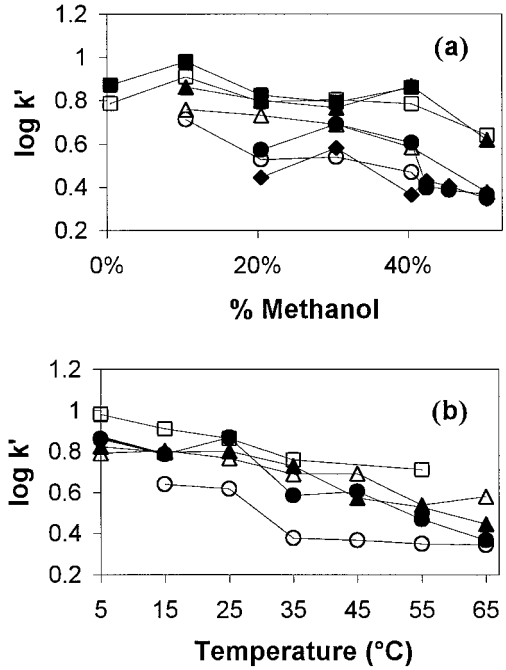

FIGURE 5 Plot of (*a*) log k' versus percent methanol and (*b*) log k' versus temperature for β-endorphin after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C. For (*a*) (■) 5°C, (□) 15°C, (▲) 25°C, (△) 35°C, (●) 45°C, (○) 55°C, (◆) 65°C. For (*b*): (□) 10%, (▲) 20%, (△) 30%, (●) 40%, (○) 50%. See Materials and Methods for other conditions.

the degree of insertion of bombesin into the lipid monolayer, although the extent of insertion will be restricted by the covalent attachment of the phosphatidylcholine to the silica support.

The dependence of log k' on temperature for bombesin is shown in Fig. 4 *b*, and it can be seen that there was a transition in the binding of this peptide that started at 35°C at 10% methanol and at 25°C at the higher methanol concentrations. The much greater fluctuations seen in this plot compared to the linear nature of the corresponding data for the control molecules in Fig. 3 suggest that peptide conformation and lipid mobility strongly influence the binding of bombesin to the immobilized lipid monolayer.

The dependence of log k' versus percent methanol for the interaction of β-endorphin with the immobilized phosphatidylcholine surface is shown in Fig. 5 *a*. There was a decrease in retention with increasing temperature at each methanol concentration, and at each temperature a decrease in retention was generally observed, similar to the results obtained with bombesin. In addition, there was a sharp decrease in retention between 40 and 50% methanol. However, at higher temperatures, β-endorphin could not be eluted at the lower methanol concentrations, which suggests a significant change in the nature of the interaction. This change could result from a combination of structural changes, which both the peptide and the lipid layer can undergo at higher temperatures. The dependence of log k' on temperature is shown in Fig. 5 *b*, and it is evident that log k' values decreased with increasing temperature. A clear transition between 25 and 35°C was only apparent at 40% and 50% methanol, and comparison with the results for bombesin demonstrate how the interactive behaviors of different peptides are clearly evident using these elution techniques.

The dependence of log k' versus percent methanol for the interaction of glucagon with the immobilized phosphatidylcholine surface is shown in Fig. 6 *a*. The binding of glucagon to the model membrane surface was much stronger than that of bombesin and β-endorphin, and as a consequence much higher levels of methanol between 50 and 80% (compared to 0–50% for bombesin and β-endorphin) were required to elute glucagon from the surface. While reasonably linear plots were observed for glucagon, this peptide could not be eluted under a range of conditions including 50% and 60% methanol at 5°C, all methanol concentrations at 15°C, and 50% methanol at 25°C. This discontinuity in the effect of temperature on the ability to elute glucagon from the immobilized lipid monolayer suggests that changes in both peptide conformation and the lipid mobility have exerted a profound effect on the affinity of glucagon for the immobilized lipid monolayer surface.

In order to establish the specificity of interaction of these peptides with the immobilized lipid monolayer relative to

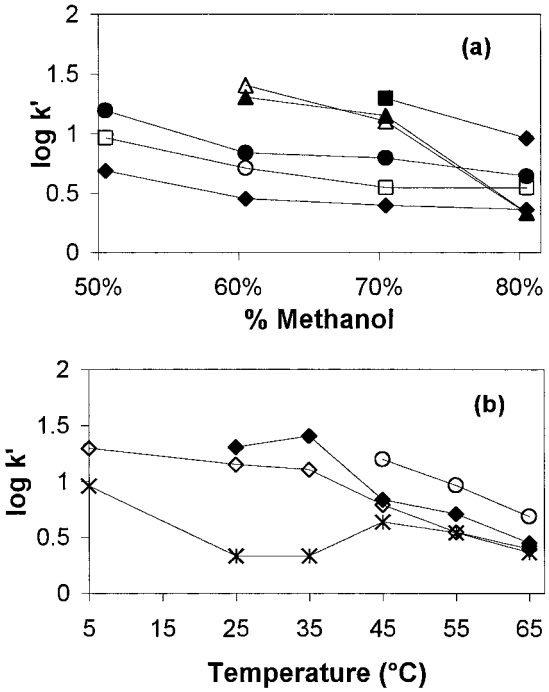

FIGURE 6 Plot of (*a*) log k' versus φ and (*b*) log k' versus temperature for glucagon after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C. For (*a*): (■) 5°C, (▲) 25°C, (△) 35°C, (●) 45°C, (○) 55°C, (◆) 65°C. For (*b*): (○) 50%, (◆) 60%, (◇) 70%, (∗) 80% methanol. See Materials and Methods for other conditions.

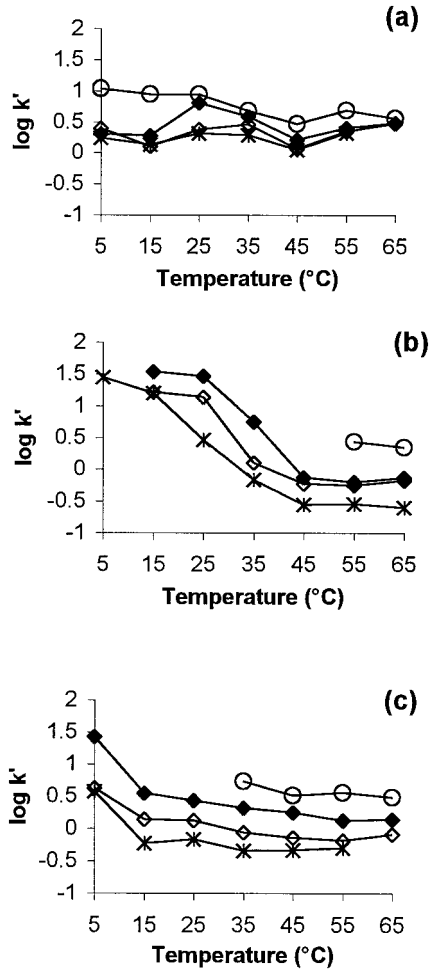

FIGURE 7 Plots of log k' versus temperature for (*a*) bombesin, (*b*) β-endorphin, and (*c*) glucagon after elution from the *n*-octylsilica (C8) support at temperatures between 5 and 65°C; (○) 50%, (◆) 60%, (◇) 70%, (∗) 80% methanol. See Materials and Methods for other conditions.

conventional *n*-alkyl ligands, the binding behavior of the three test peptides was determined using an *n*-octylsilica (C8) surface, which contains the same number of atoms as the immobilized phosphatidylcholine phase and was coupled to the same silica as used for the immobilized phosphatidylcholine and was packed into a column of identical column dimensions. Binding data were obtained under identical conditions of flow rate, solvent composition, and temperature. The dependence of log k' versus temperature for the interaction of bombesin, β-endorphin, and glucagon with the C8 surface is shown in Fig. 7. Comparison of these results with those presented in Figs. 4–6 clearly indicates that bombesin and β-endorphin interacted with the C8 surface much more strongly than with the immobilized lipid monolayer, since much higher methanol concentrations were required to elute these peptides from the C8 material. In contrast, similar methanol concentrations were required to elute glucagon from both surfaces. However, glucagon also generally exhibited higher log k' values, and hence a higher affinity for the phospholipid monolayer. In addition, all three peptides exhibited much more defined transitions in the log k' values on the C8 surface, which may reflect differences in the degree of structural stabilization of the peptides in contact with each surface and differences in the nature of the structural transition of the *n*-alkyl ligands compared to the phospholipid ligands. These results also contrasted with the linear dependence of log k' values on temperature observed for the control molecules on the C8 surface (results not shown). Overall, these differences in the relative affinity of the test peptides for each surface clearly demonstrate specificity of interaction of the peptides with the immobilized phosphatidylcholine monolayer.

Determination of thermodynamic parameters

The log k' values for the control molecules and the three test peptides were used to calculate the ΔG^{O}_{assoc} values according to Eq. 6. The results for N-acetyltryptophanamide and diphenylalanine are plotted against temperature in Fig. 8, while the corresponding data for bombesin, β-endorphin, and glucagon are presented in Fig. 9. For the control molecules, the ΔG^{O}_{assoc} values were either constant or decreased slightly with increasing temperature. These results indicate that the ΔG^{O}_{assoc} values for these molecules are unaffected by the change in lipid mobility and structure, particularly at the phase transition between 25 and 35°C. In contrast, the ΔG^{O}_{assoc} values for bombesin, β-endorphin, and glucagon

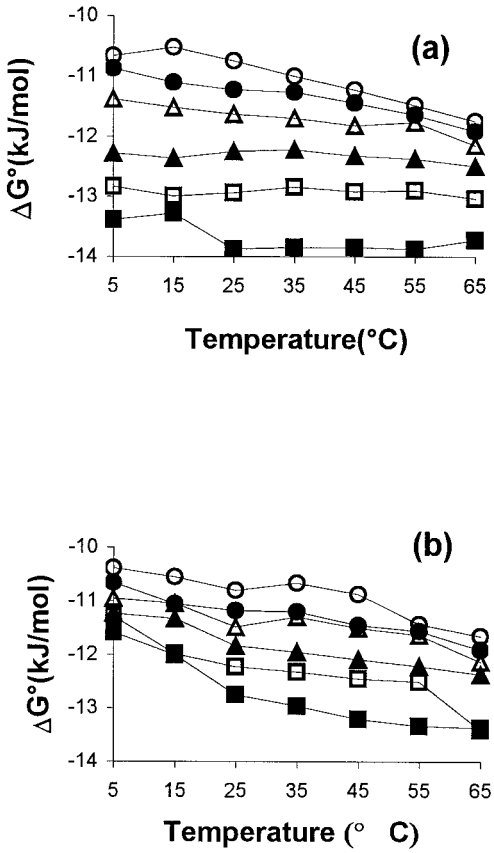

FIGURE 8 Plots of ΔG^{O}_{assoc} versus temperature for (*a*) N-acetyltryptophanamide and (*b*) diphenylalanine after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C: (■) 0%, (□) 10%, (▲) 20%, (△) 30%, (●) 40%, (○) 50% methanol. See Materials and Methods for other conditions.

exhibited significant fluctuations under the various operating conditions. The ΔG^{O}_{assoc} values are derived from affinity data that depend both on peptide conformation and the degree of insertion into the lipid monolayer. The nonlinear nature of the dependence of ΔG^{O}_{assoc} on temperature therefore reflects the changes in the relative contribution of the different factors involved in the binding of the peptides to the lipid monolayer, particularly changes in peptide and lipid conformation. The relative magnitude of the ΔG^{O}_{assoc} values observed in this study are similar to values previously determined by others. In addition, the relative trends in the temperature dependency of ΔG^{O}_{assoc} seen in Figs. 8 and 9 are in agreement with previous studies on peptide (Jacobs and White, 1989) and polypeptide partitioning into lipid vesicles (Russell et al., 1996), but contrast with other studies on the binding of small peptides to lipid vesicles (Wimley and White, 1993; Wimley et al., 1998). These differences arise from the nature of the peptide and the model membrane system in which significant differences exist in the extent of peptide structural changes and lipid insertion effects that dramatically alter the energetics of peptide-lipid interactions.

An analysis of the thermodynamics of the interaction of peptides with the immobilized monolayer in terms of the

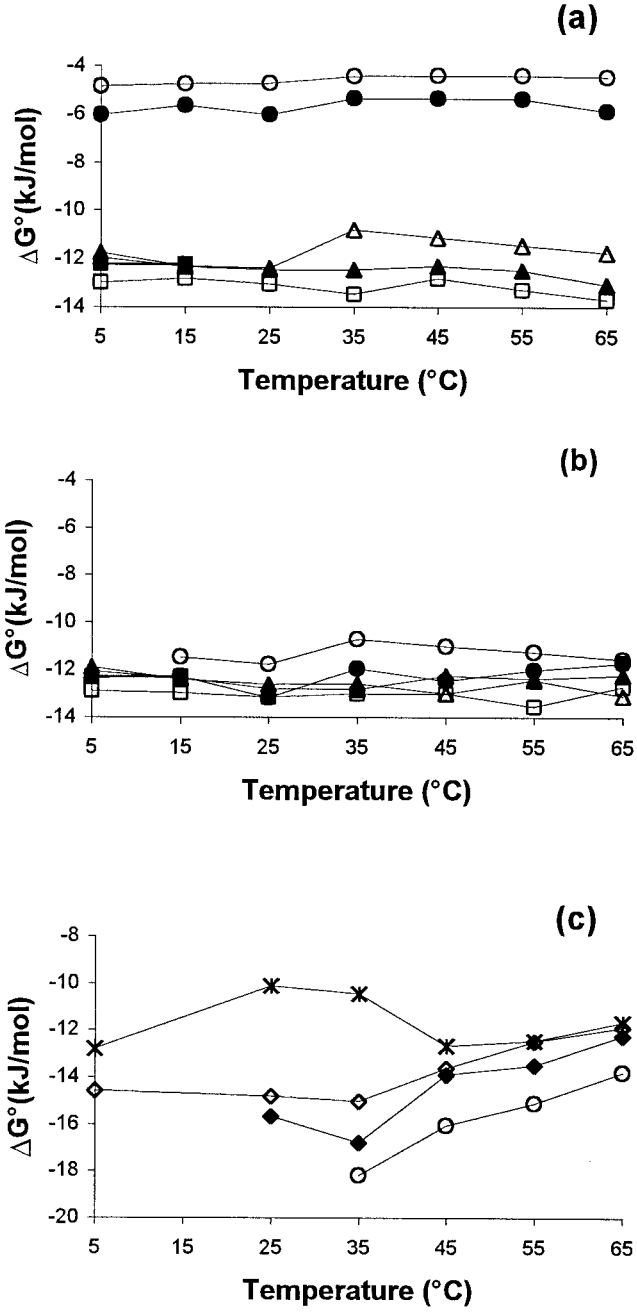

FIGURE 9 Plots of ΔG^{O}_{assoc} versus temperature for (*a*) bombesin, (*b*) β-endorphin, and (*c*) glucagon after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C: (■) 0%, (□) 10%, (▲) 20%, (△) 30%, (●) 40%, (○) 50%, (◆) 60%, (◇) 70%, (*) 80% methanol. See Materials and Methods for other conditions.

relative contribution of ΔH^{O}_{assoc} and ΔS^{O}_{assoc} to the overall free energy of association was also carried out (Haidacher et al., 1996; Ong and Pidgeon, 1995). Plots of ln k' versus $1/T$ (van't Hoff plots) were fitted to Eq. 9 for each solute and the data for N-acetyltryptophanamide is shown in Fig. 10 *a*. Excellent fits were obtained with r^2 values >0.96. ΔH^{O}_{assoc} and ΔS^{O}_{assoc} values were then determined according to Eqs. 10 and 11, respectively, and are plotted against temperature for N-acetyltryptophanamide in Fig. 10, *b* and *c*. Compari-

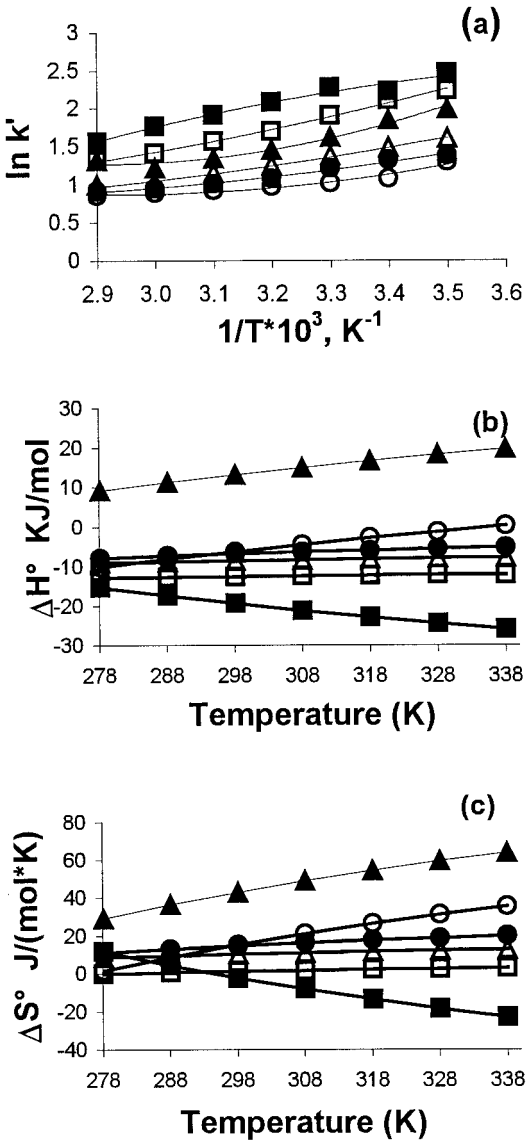

FIGURE 10 Plots of (*a*) ln k' versus 1/temperature, (*b*) ΔH^{O}_{assoc} versus temperature, and (*c*) ΔS^{O}_{assoc} on temperature for N-acetyltryptophanamide after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C (278–338 K) at the following methanol concentrations: (■) 0%, (□) 10%, (▲) 20%, (△) 30%, (●) 40%, (○) 50%. See Materials and Methods for other conditions.

son of the magnitude of the ΔH^{O}_{assoc} and ΔS^{O}_{assoc} values indicates that under most conditions, the interaction of N-acetyltryptophanamide with the immobilized lipid monolayer was enthalpy-driven rather than entropy-driven, which is in contrast to the thermodynamic properties of a classical hydrophobic interaction (Tanford, 1980). As N-acetyltryptophanamide has been shown to be located at the interfacial region between the polar headgroup and the acyl chains (Davis et al., 1996; Mishra and Palgunachari, 1996), the results of the present study indicate that enthalpic factors associated with the solvation (in the presence and absence of methanol) and van der Waals interactions of this molecule dominate the binding rather than entropic factors. In addition, it can be seen that at 10%, 30%, and 40% methanol there was little change in either ΔH^{O}_{assoc} or ΔS^{O}_{assoc} with temperature, demonstrating that the relative contribution of each parameter to ΔG^{O}_{assoc} remained relatively constant between 5 and 65°C. However, at 20% and 50% methanol, both ΔH^{O}_{assoc} and ΔS^{O}_{assoc} increased with temperature, while at 0% methanol both parameters decreased over the experimental temperature range. Similar results were obtained for diphenylalanine. Increases in ΔH^{O}_{assoc} and ΔS^{O}_{assoc} with temperature indicate an increasingly entropy-driven interaction at higher temperatures, while decreases in ΔH^{O}_{assoc} and ΔS^{O}_{assoc} over the temperature range reflect an increasingly enthalpy-driven binding of the solute to the lipid monolayer. These results therefore demonstrate that the nature of the interaction of the two control molecules changes with different methanol concentrations. These effects relate to the degree of insertion of these molecules into the lipid monolayer which change with increased lipid fluidity and the changes in solvation at each methanol concentration.

The van't Hoff plots and the dependence of ΔH^{O}_{assoc} and ΔS^{O}_{assoc} on temperature for bombesin, β-endorphin, and glucagon are shown in Figs. 11, 12, and 13. While the plots of ln k' versus $1/T$ were clearly nonlinear, r^2 values >0.9 were generally obtained with the use of the quadratic function of Eq. 9. The dependence of ΔH^{O}_{assoc} and ΔS^{O}_{assoc} on temperature for bombesin (Fig. 11 *b*) indicates that at all methanol concentrations, except for 0% where there was no change in the enthalpy and entropy, there was an increase in both parameters with increased temperature. Thus, at lower temperatures the interaction of bombesin with the lipid monolayer is enthalpy-driven with large negative ΔH^{O}_{assoc} values, while at higher temperatures this interaction becomes more entropy-driven with ΔS^{O}_{assoc} becoming increasingly positive. However, the results of the present study indicate that for bombesin, in the presence of methanol, enthalpic factors associated with peptide and lipid structure and solvation in methanol dominate the interaction with the immobilized lipid monolayer.

The corresponding van't Hoff plots and the plots of ΔH^{O}_{assoc} and ΔS^{O}_{assoc} on temperature for β-endorphin and glucagon are shown in Figs. 12 and 13, respectively, and indicate that these peptides exhibited different behavior to bombesin. For β-endorphin, both ΔH^{O}_{assoc} and ΔS^{O}_{assoc} values remained reasonably constant with temperature at 0%, 10%, and 30% methanol. However, both parameters decreased with temperature at 20% and 40% methanol and increased with temperature at 50%. Thus the relative contribution of enthalpic and entropic factors to the free energy of interaction for β-endorphin was dependent on the concentration of methanol. For glucagon (Fig. 13), the ΔH^{O}_{assoc} and ΔS^{O}_{assoc} values increased with temperature at 50% and 80% methanol, while they decreased with temperature at 60% and 70% methanol. The different trends observed for each of the test peptides thus highlight the different nature of the interaction of these peptides with the immobilized phosphatidylcholine monolayer.

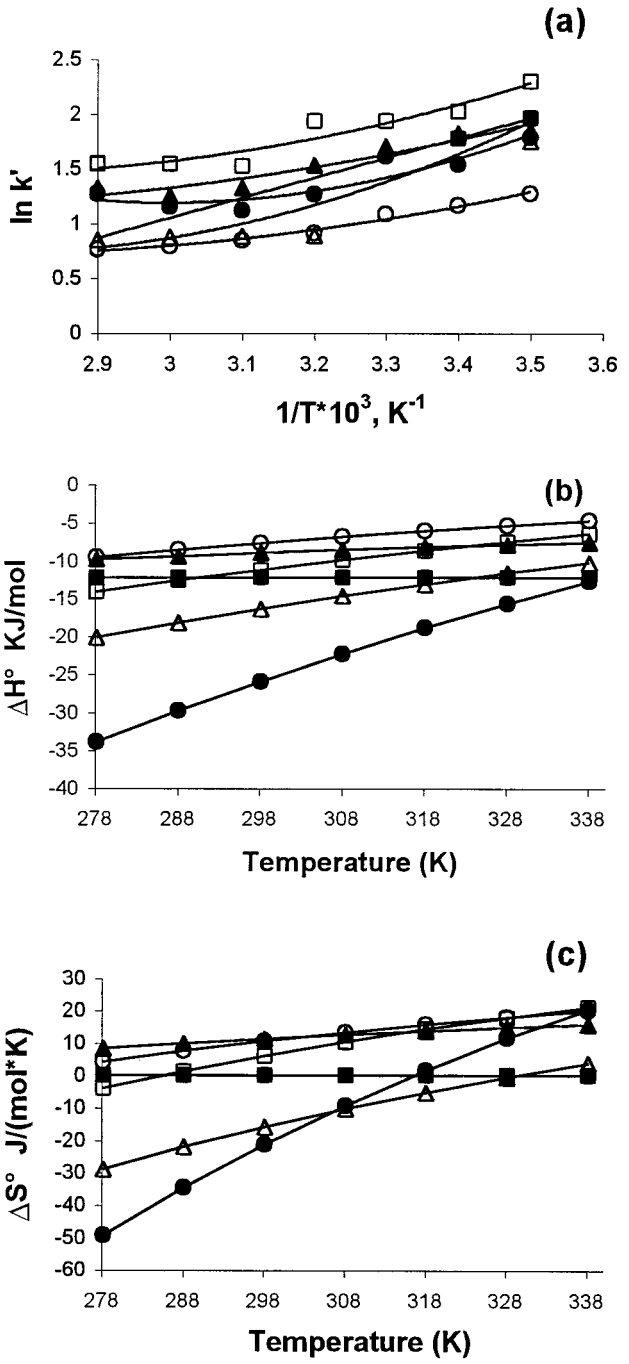

FIGURE 11 Plots of (*a*) ln k' versus 1/temperature, (*b*) ΔH^{O}_{assoc} versus temperature, and (*c*) ΔS^{O}_{assoc} on temperature for bombesin after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C (278–338 K) at the following methanol concentrations: (■) 0%, (□) 10%, (▲) 20%, (△) 30%, (●) 40%, (○) 50%. See Materials and Methods for other conditions.

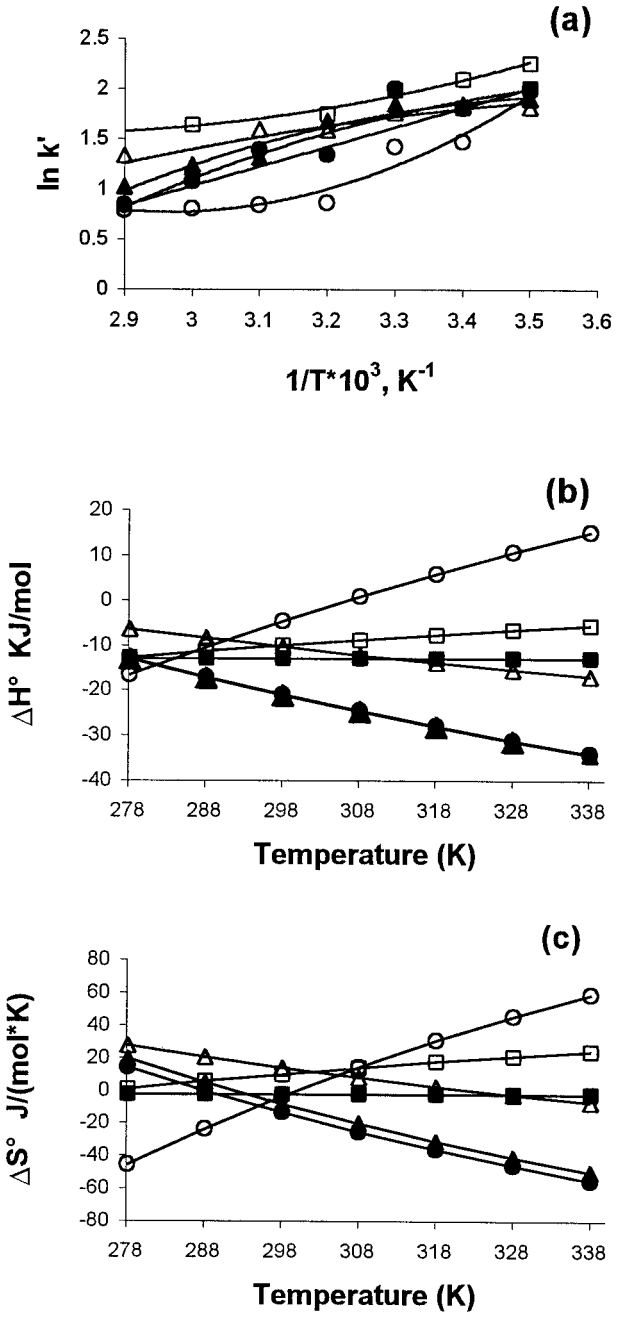

FIGURE 12 Plots of (*a*) ln k' versus 1/temperature, (*b*) ΔH^{O}_{assoc} versus temperature, and (*c*) ΔS^{O}_{assoc} on temperature for β-endorphin after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C (278–338 K) at the following methanol concentrations: (■) 0%, (□) 10%, (▲) 20%, (△) 30%, (●) 40%, (○) 50%. See Materials and Methods for other conditions.

Dependence of bandwidth on experimental parameters

The dynamic behavior of the interaction of peptides with the immobilized phospholipid surface can be further characterized through analysis of the elution peak widths (Lee et al., 1997; Purcell et al., 1993; Steer et al., 1998). For low molecular weight conformationally rigid molecules that interact through a single binding site and with a unique orientation in the lipid monolayer, the experimentally observed elution peak will appear as a sharp Gaussian- (or near-Gaussian)-shaped peak. However, there are two major factors that can lead to atypical band-broadening in the elution profile, namely changes in peptide conformation and changes in the degree of penetration of the peptide into the

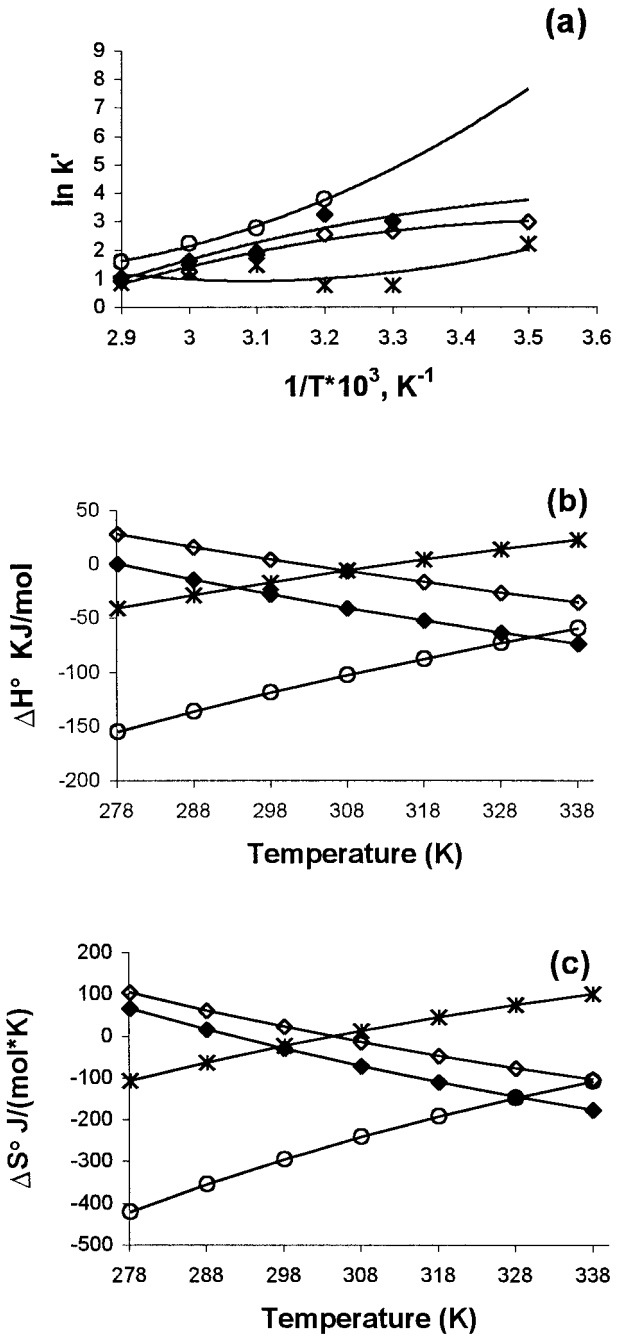

FIGURE 13 Plots of (*a*) ln k' versus 1/temperature, (*b*) ΔH^{O}_{assoc} versus temperature, and (*c*) ΔS^{O}_{assoc} on temperature for glucagon after elution from the immobilized phosphatidylcholine monolayer at temperatures between 5 and 65°C (278–338 K) at the following methanol concentrations: (○) 50%, (◆) 60%, (◇) 70%, (∗) 80% methanol. See Materials and Methods for other conditions.

lipid monolayer. Peptide conformation can be influenced by experimental parameters such as temperature and organic solvent concentration. Additionally, peptide secondary structure can be induced upon binding of the peptide to the immobilized lipid monolayer. Furthermore, the degree of lipid mobility will also be influenced by temperature and the presence of organic solvent. The degree of penetration of the peptide into the lipid monolayer will thus be affected by changes in the peptide conformation and changes to the mobility of the lipid. When peptide solutes undergo conformational transitions during the interaction process, the changes in peptide structure will result in the formation of different contact regions on the peptide surface, which in turn may result in a different affinity of the peptide for the lipid than the initial/original conformational species. The unfolding of a peptide in solution is associated with an equilibrium between folded and extended conformations with the possible formation of a range of structurally less stable intermediates. However, these transiently formed intermediates may be "trapped" by the immobilized lipid surface and may exhibit small but significant differences in lipid affinity. The extent of band-broadening during the interaction of the peptide with the immobilized lipid surface depends on the rate of interconversion between the folded and unfolded states and the surface residence time for each peptide conformer. If the rate of interconversion is faster or slower that the elution time scale, no band-broadening will be observed because the fully folded or fully unfolded form will be the predominant species present, and a single narrow peak will be observed. However, if the rate of interconversion is similar to the elution time scale, structural intermediates may be trapped at the phospholipid surface. These intermediates may have slightly different affinities for the surface as a result of changes in the lipid binding domain or changes in the degree of penetration of the peptide as a result of the structural changes. These differences in relative affinities will then lead to a broadened peak or, alternatively, if there is sufficient column resolution, partially or fully resolved peaks.

Fig. 14 shows the elution profiles for N-acetyltryptophanamide, bombesin, and β-endorphin obtained at 35°C between 0 and 50% methanol. A single narrow peak was observed for the control molecule N-acetyltryptophanamide, which confirms that there are no significant contributions to the bandwidth from secondary equilibria associated with conformational changes or differences in penetration during the interaction of this control molecule with the immobilized lipid monolayer. In contrast, the elution profiles for bombesin, β-endorphin, and glucagon exhibited anomalous band-broadening, and in some cases asymmetric peak-splitting, results that are indicative of the contribution of additional secondary equilibria to the mass transfer processes.

The experimental bandwidth for all molecules was measured at 4σ (13.4% peak height) and is plotted against temperature and percent methanol in Fig. 15 for N-acetyltryptophanamide, bombesin, β-endorphin, and glucagon. The results for N-acetyltryptophanamide demonstrate that a fairly constant bandwidth value was observed over the range of temperatures and organic solvent concentrations used, with a small decrease observed with increasing percent methanol, a result that is anticipated from the known relationship between retention and bandwidth (Hearn and Aguilar, 1986; Stadalius et al., 1985). Similar results were

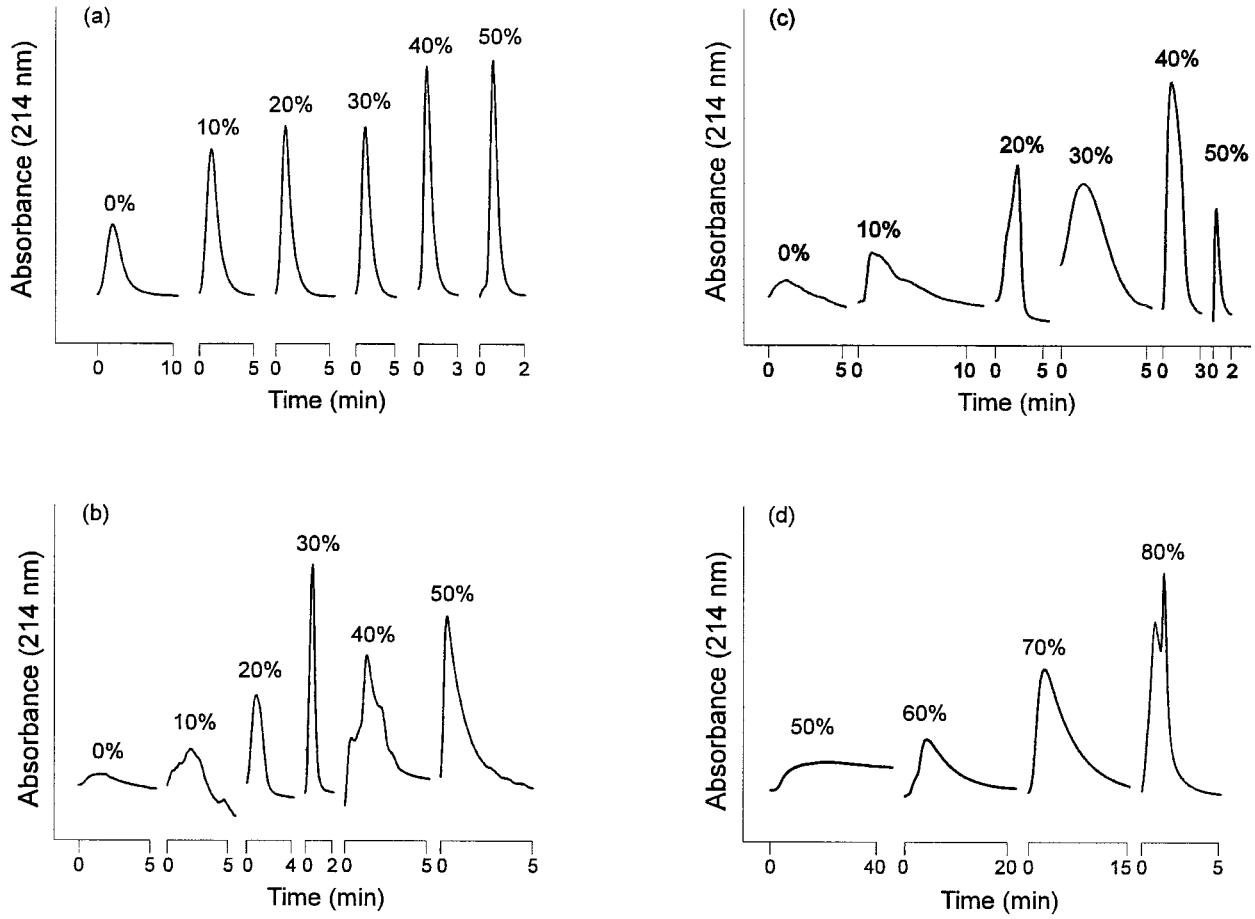

FIGURE 14 Elution profiles for (*a*) N-acetyltryptophanamide, (*b*) bombesin, (*c*) β-endorphin, and (*d*) glucagon obtained at 35°C with the immobilized phosphatidylcholine monolayer at different methanol concentrations. See Materials and Methods for other conditions.

also obtained for diphenylalanine. In contrast to the control molecules, the bandwidth plots for bombesin and β-endorphin shown in Fig. 15, *b* and *c*, respectively, exhibited large fluctuations over the range of experimental conditions used. The experimental bandwidth plots for glucagon are shown in Fig. 15 *d* and it is apparent that much larger bandwidth plots were observed for this peptide than for bombesin or β-endorphin. In addition, there were several conditions where glucagon could not be eluted. In contrast, the elution profiles obtained for bombesin, β-endorphin, and glucagon with the C8 surface did not exhibit any peak splitting or strongly asymmetric peak shapes. The dependence of 4σ on percent methanol and temperature for these peptides eluted from the C8 surface is shown in Fig. 16, and it can be seen that significant band-broadening was only observed at lower temperatures (≤25°C). Relatively flat bandwidth plots were obtained for both control molecules on the C8 surface (results not shown). Significant band-broadening has been previously observed during the interaction of these peptides with other *n*-alkyl ligands (Purcell et al., 1993), which was shown to be due to conformational changes of the peptide upon binding to the *n*-alkyl ligands. However, in comparison with peptide interactions with the lipid monolayer, other interactive equilibria associated with peptide interactions with the phospholipid headgroup and partial insertion of the peptide into the lipid monolayer are eliminated with the C8 surface. These additional processes will therefore not contribute to the binding behavior with the C8 surface, thereby giving rise to less complex elution profiles.

Overall, the increases in bandwidth observed with the immobilized phosphatidylcholine monolayer correlated with the observed fluctuations in retention behavior for these molecules, indicating that the conformational changes that give rise to the changes in retention times observed in the previous section, occur on a time scale equivalent to the separation time. Moreover, the results demonstrate that subtle differences in both structure and orientation of the peptide at the lipid surface can be monitored by these elution techniques.

DISCUSSION

The bioactivity of the three test peptides used in this study has been shown to be mediated through interactions with biomembranes. Bombesin was originally isolated from the skin of European frogs and has a wide range of biological activity and pharmacological effects in mammalian tissues,

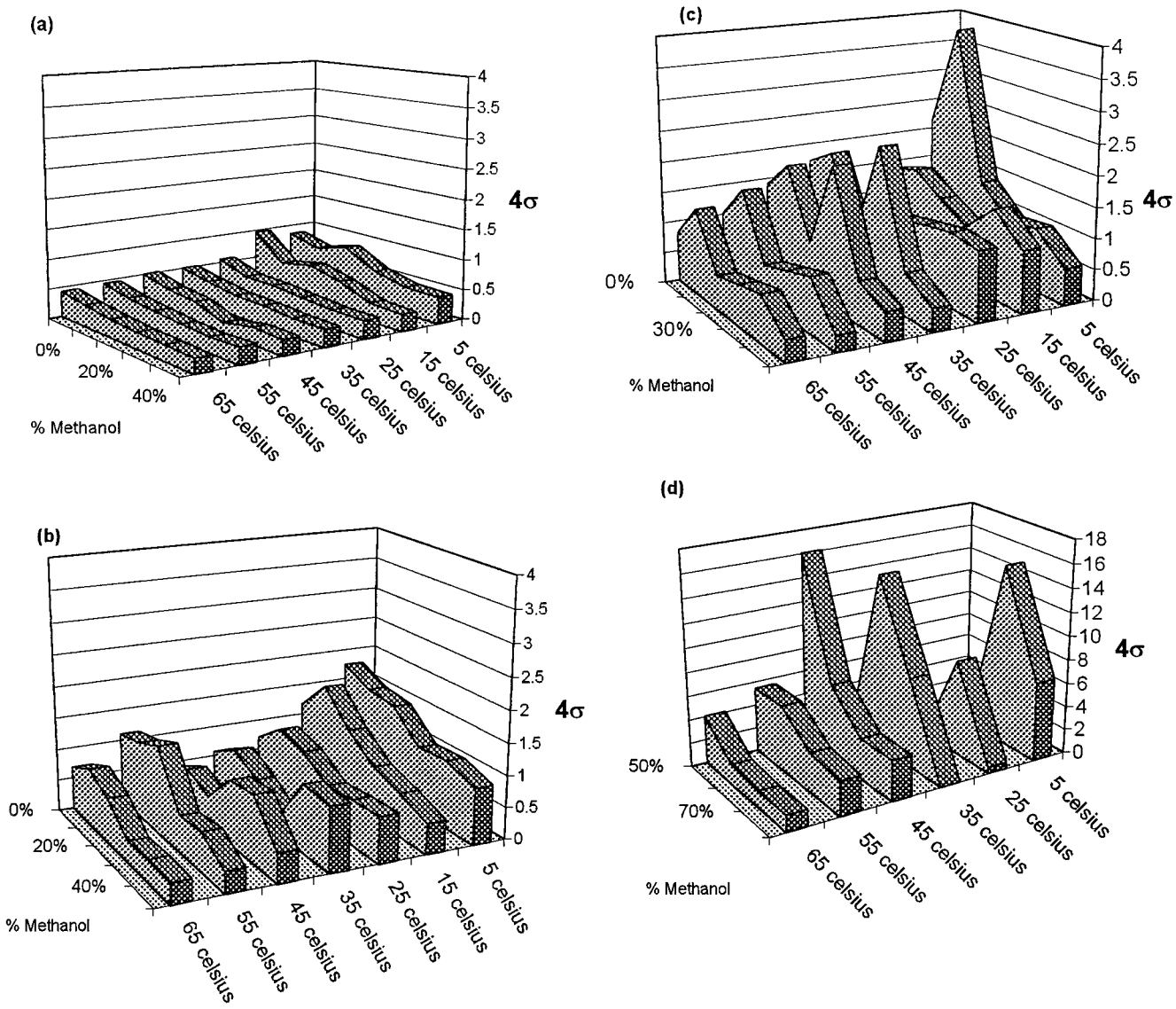

FIGURE 15 Dependence of bandwidth (4σ) on temperature and percent methanol for (*a*) N-acetyltryptophanamide, (*b*) bombesin, (*c*) β-endorphin, and (*d*) glucagon after elution from the phosphatidylcholine monolayer at temperatures between 5 and 65°C. See Materials and Methods for other conditions.

and it has been suggested that the biological activity of bombesin depends on the relatively hydrophobic C-terminal portion of the peptide that has been shown to adopt an α-helical conformation in the presence of lipids (Cavatorta et al., 1989; Erne and Schwyzer, 1987). Human β-endorphin is involved in the regulation of a number of pharmacological activities mediated through binding to specific receptors in the brain. Studies have indicated that the central and C-terminal residues adopt an α-helical conformation and that the lipid membrane plays a key role in the induction of this structure, thereby facilitating the binding of β-endorphin to its membrane-bound receptor (Zetta and Kaptein, 1984; Pasta et al., 1990; Zetta et al., 1990). Glucagon acts to increase blood glucose levels in response to low blood sugar levels, an effect that is thought to be mediated through the adoption of an α-helical conformation at the C-terminus (Kimura et al., 1992; Pasta et al., 1988, Wu et al., 1982). Thus, all three peptides are known to adopt a significant degree of secondary structure upon binding to a membrane surface, which is considered to be a central step in the biological function of these peptides (Schwyzer, 1991). The ability to determine thermodynamic parameters associated with peptide-lipid interactions as a function of peptide and lipid structure would therefore provide significant insight into the role of membrane interactions in the function of bioactive peptides.

The interaction of peptides with membrane surfaces involves a number of intermediate steps, which include 1) initial binding/attraction of the peptide to the membrane surface, either through electrostatic or hydrophobic interactions, depending on the sequence of the peptide and the structure of the phospholipid; 2) induction or further stabilization of secondary structure upon interaction of the peptide with the phospholipid surface; 3) reorientation of the

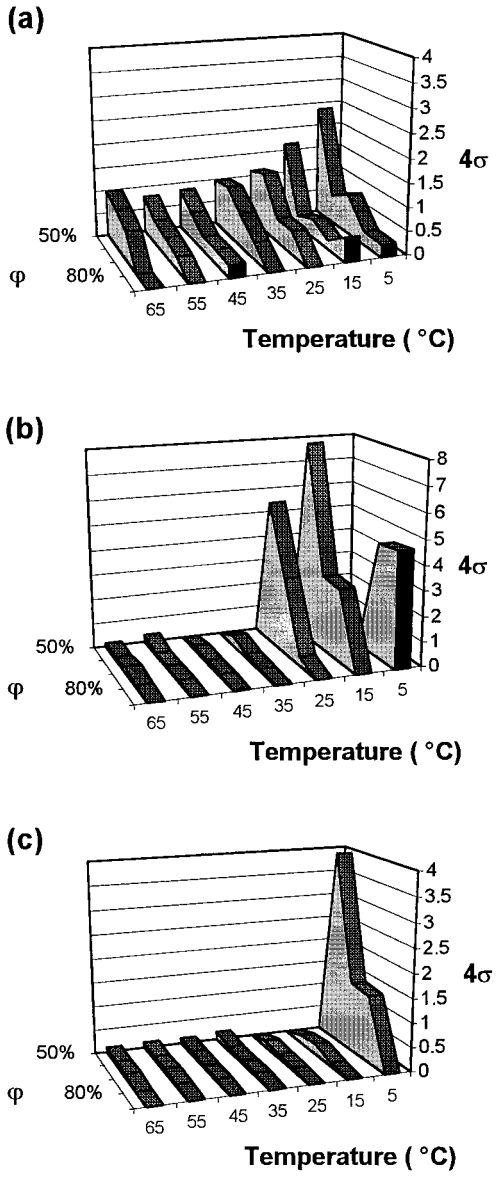

FIGURE 16 Dependence of bandwidth (4σ) on temperature and percent methanol for (*a*) bombesin, (*b*) β-endorphin, and (*c*) glucagon after elution from the *n*-octylsilica (C8) support at temperatures between 5 and 65°C. See Materials and Methods for other conditions.

peptide at the phospholipid surface, resulting in the partial insertion of the peptide in the membrane; 4) complete partitioning of the peptide in the membrane interface to either lie parallel to the surface or fully insert into the membrane.

The experimental design of the immobilized monolayer described in this study limits the degree of peptide insertion and therefore represents a system that models the initial binding and possible partial insertion of a peptide to a membrane surface, i.e., steps 1, 2, and 3.

The results presented in this study indicate that bombesin, β-endorphin, and glucagon all exhibited complex binding behavior upon adsorption to and elution from the immobilized phosphatidylcholine monolayer. Methanol was used to desorb the peptides from the phospholipid monolayer and a decrease in log k' values was observed with increasing methanol concentrations, which is evidence that these peptides interacted predominantly through hydrophobic interactions. Two additional experimental factors will also alter both the peptide structure and the lipid mobility, and consequently the binding behavior of each peptide, namely the methanol concentration and the temperature, as shown in Fig. 17. At low temperatures (5–25°C), under fully aqueous conditions the peptides will exist as an extended coil and will adopt helical structure upon adsorption to the immobilized lipids, as illustrated in Fig. 17 *a*. The degree of secondary structure will vary depending on the methanol concentration (Fig. 17 *b*), which has been previously documented for β-endorphin (Yang et al., 1977) and other peptides, including melittin (Lakowicz et al., 1990). While organic solvents do not represent a true physiological solution, the use of increasing amounts of methanol thus provides a solution environment in which the conformation of the peptide can be manipulated before binding to the immobilized lipid. At intermediate temperatures that correspond to a transition in either the peptide secondary structure and/or the lipid mobility, as shown in Fig. 17 *c*, complex binding behavior was observed for each of the test peptides. At high temperatures, the peptide will most likely exist as a fully extended coil and will be able to penetrate the lipid chains more extensively than at lower temperatures as a result of the increased lipid mobility. In each case, the binding behavior in terms of the peptide affinity and elution profiles is a reflection of the conformational heterogeneity of both the peptide solutes and the immobilized phospholipid.

The nature of the interaction of the control molecules and the test peptides with the immobilized lipid monolayer was also further assessed through the determination of the enthalpy and entropy terms associated with the binding of the solutes to the lipid monolayer. Nonlinear van't Hoff plots were observed for each peptide, which indicates a strong dependence of the ΔH^{O}_{assoc} and ΔS^{O}_{assoc} values on temperature. Furthermore, trends in the dependence of these two parameters on temperature varied with each peptide and methanol concentration. Since ΔH^{O}_{assoc} values reflect van der Waals interactions between the solutes, the solvent and the immobilized lipid molecules and the ΔS^{O}_{assoc} values relate to the changes in mobility of the solute, lipid, and water molecules, these results indicate that the relative contribution of enthalpy and entropy to the overall free energy of interaction depends on the conformational status of both the peptide and the lipid. In particular, the results indicate that the interaction of bombesin, β-endorphin, and glucagon with the immobilized lipid monolayer is mediated by nonclassical hydrophobic interactions, as previously observed for small solutes and peptides (Beschiaschvili and Seelig, 1992; Seelig and Ganz, 1991). However, these results contrast with previous studies on the interaction of polypeptides with lipid vesicles (Russell et al., 1996) and small nonpeptidic drug molecules with immobilized lipid monolayers (Ong and Pidgeon, 1995). These differences

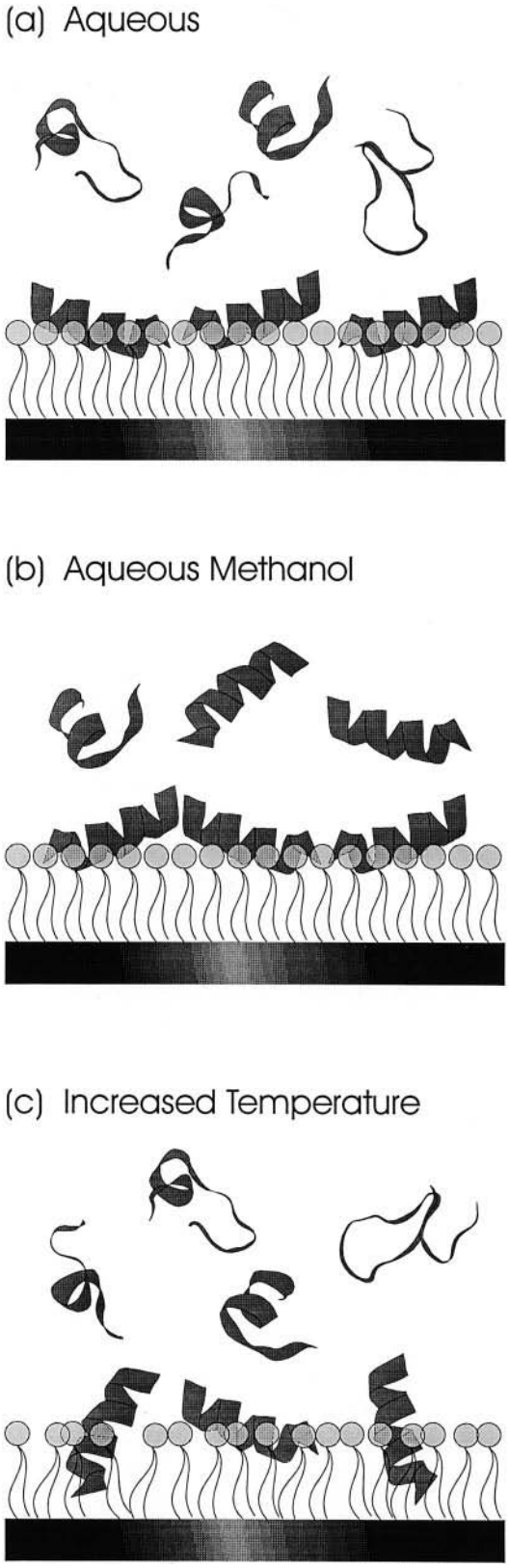

FIGURE 17 Schematic illustration of the interaction of peptides with the immobilized phosphatidylcholine monolayer under the following conditions: (*a*) fully aqueous solution and low temperatures (5–25°C); (*b*) aqueous methanol solution at low temperatures (5–25°C); and (*c*) aqueous methanol solution at intermediate temperatures where the peptide and/or phospholipid undergo conformational interconversions.

illustrate how the interaction of peptides with the lipid monolayers is significantly more complex than that of small organic molecules. Thus it is apparent that the interaction of peptides with lipid systems may not necessarily be mediated by classical hydrophobic interactions that are entropy-driven, but rather enthalpic factors may play a more dominant role depending on the structure of the peptide and the lipid. The presence of methanol may influence the relative contribution of enthalpy and entropy to the free energy of binding through differences in solvation structure relative to water. However, methanol can still participate in a significant degree of H-bonding, and enthalpic dominance was also observed in the absence of methanol. In the case of the immobilized lipid monolayer, the dominance of enthalpy in the free energy of binding may reflect a strong interaction of the peptide with the more solvated interfacial region of the lipid monolayer, due to the inability of the peptides to insert into the hydrophobic core of the lipid chains. This conclusion is consistent with previous studies (Schwyzer et al., 1992) which showed that β-endorphin and glucagon orient parallel to lipid multilayers. In this regard, the immobilized lipid monolayer provides an ideal system with which to study the interaction of peptides that bind to the interfacial region of lipid membranes. Furthermore, the preparation of lipid monolayers with different lipid densities and immobilized lipid bilayers will help to dissect the relative contribution of peptide binding and insertion to the overall energetics of binding.

The interactive behavior of bombesin, β-endorphin, and glucagon on the immobilized phosphatidylcholine monolayer also contrasted to that observed with a C8 support and those previously observed with other RP-HPLC support materials (Purcell et al., 1992, 1993). The differences in relative affinities of the peptides for the immobilized phospholipid compared to the RP-HPLC materials demonstrate the contribution of the phospholipid head to the interaction of peptides with membranes. In addition, while significant band-broadening was observed with the C8 support at lower temperatures, less complex elution profiles were observed for the three peptides with the *n*-octyl ligands, further demonstrating the specificity of the phospholipid surface, and that the interaction of these peptides with the immobilized phosphatidylcholine monolayer is more complex than with the purely aliphatic *n*-alkyl ligands. However, the concept of specificity of binding as generally defined for other macromolecular interactions is very difficult to apply to peptide-lipid interactions due to the complex interplay of electrostatic and hydrophobic interactions and the changes in both peptide secondary structure and degree of insertion upon binding of the peptide to the phospholipid surface. Most peptides bind to a hydrophobic surface (hence the widespread use of RP-HPLC for peptide analysis and purification) and so it is difficult to define what is specific binding and what is nonspecific; rather, it is related to the comparative strength of binding and the nature of the orientational effects.

In summary, since the test peptides exhibited complex elution profiles while the control molecules exhibited single peaks, the results suggest that the immobilized lipid monolayer can be used to monitor the influence of peptide conformation and lipid mobility on the interaction of peptides with membrane surfaces. While the relative contribution of solvent-induced structural changes in the peptide could not be readily differentiated, in the present study, from the solvent-induced dynamic changes in the immobilized lipid molecules, the preparation of immobilized monolayers with different ligand densities together with the analysis of the lipid transition behavior as a function of methanol concentration will allow the role of lipid rigidity in these interactions to be further explored. In addition, the preparation of lipid monolayers composed of different phospholipids varying in both the nature of the acyl chain and the lipid headgroup will provide an experimental system that will allow the influence of different lipids on the interaction of peptides with membranes to be analyzed. Furthermore, while the studies described here were carried out in aquo-organic mixtures in order to allow direct comparison with conventional RP-HPLC-based systems, the role of pH and ionic strength on peptide-lipid interactions can be readily assessed through the use of different buffer compositions. The elution techniques described in this study can also be applied to the analysis of peptide binding to stabilized bilayer systems prepared using polymerizable phospholipids (Ringsdorf et al., 1988). Since the determination of lipid affinity is critical to the understanding of the function of membrane-active peptides, the column-based design of the analytical system presented here provides a potentially powerful method for the rapid on-line analysis and screening of the lipid-binding properties of peptide analogs that can be used in the development of structure-function relationships (Ong and Pidgeon, 1995) and the design of novel membrane-active peptides. In particular, these types of studies could provide thermodynamic information that is not readily accessible by other techniques to assist in the delineation of the role of the lipid membrane in hormone receptor subtype selectivity and, ultimately, peptide function (Behling and Jelinski, 1990). Finally, the application of spectroscopic techniques such as oriented CD (Vogel, 1987) and in situ CD (Blondelle et al., 1995a) to the analysis of the conformation of adsorbed peptides will further extend the biophysical potential of the immobilized lipid monolayer systems. The overall conclusion that can be drawn from the present study is that the immobilized phosphatidylcholine monolayer combined with the dynamic elution techniques provides a stable membrane mimetic material with which to study the molecular basis of the surface interaction of bioactive peptides with biological membranes.

The financial support of the Australian Research Council is gratefully acknowledged.

REFERENCES

Axelsen, P. H., W. D. Braddock, H. L. Brockman, C. M. Jones, R. A. Dluhy, B. K. Kaufman, and F. J. Puga II. 1995. Use of internal relectance infrared spectroscopy for the *in situ* study of supported lipid monolayers. *Appl. Spectrosc.* 49:526–531.

Behling, R. W., and L. W. Jelinski. 1990. Importance of the membrane in ligand-receptor interactions. *Biochem. Pharmacol.* 40:49–54.

Ben-Tal, N., A. Ben-Shaul, A. Nicholls, and B. Honig. 1996. Free energy determinants of α-helix insertion into lipid bilayers. *Biophys. J.* 70: 1803–1812.

Beschiaschvili, G., and J. Seelig. 1992. Peptide binding to lipid bilayers. Nonclassical hydrophobic effect and membrane-induced pK shifts. *Biochemistry.* 31:10044–10053.

Blondelle, S. E., J. M. Ostresh, R. A. Houghten, and E. Pérez-Payá. 1995a. Induced conformational states of amphipathic peptides in aqueous/lipid environments. *Biophys. J.* 68:351–359.

Blondelle, S. E., E. Pérez-Payá, G. Allicotti, B. Forood, and R. A. Houghten. 1995b. Peptide binding domains determined through chemical modification of the side chain functional groups. *Biophys. J.* 69: 604–611.

Cavatorta, P., A. Spisni, A. G. Szabo, G. Farruggia, L. Franzoni, and L. Masotti. 1989. Conformation of bombesin in buffer and in the presence of lysolecithin micelles: NMR, CD and fluoroescence studies. *Biopolymers.* 29:441–463.

Cole, L. A., J. G. Dorsey, and K. A. Dill. 1992. Temperature dependence of retention in reversed-phase liquid chromatography. 2. Mobile-phase considerations. *Anal. Chem.* 64:1324–1327.

Cooper, M. A., D. H. Williams, and Y. R. Cho. 1997. Surface plasmon resonance analysis of glycopeptide antibiotic activity at a model membrane surface. *Chem. Commun.* 1997:1625–1626.

Cornell, B. A., V. L. B. Braach-Maksvytis, L. G. King, P. D. J. Osman, B. Raguse, L. Wieczorek, and R. J. Pace. 1997. A biosensor that uses ion-channel switches. *Nature.* 387:580–583.

Davis, D. M., D. McLoskey, D. J. S. Birch, P. G. Gellert, R. S. Kittlety, and R. M. Swart. 1996. The fluorescence and circular dichroism or protein in reverse micelles: application to the photophysics of human serum albumin and N-acetyltryptophanamide. *Biophys. Chem.* 60:63–77.

Deber, C. M., and S.-C. Li. 1995. Peptides in membranes: helicity and hydrophobicity. *Biopolymers (Peptide Science).* 37:295–318.

de Jongh, H. H. J., E. Goormaghtigh, and J. A. Killian. 1994. Analysis of circular dichroism spectra of oriented protein-lipid complexes: toward a general application. *Biochemistry.* 33:14521–14528.

Duschl, C., M. Liley, G. Corradin, and H. Vogel. 1994. Biologically addressable monolayer structures formed by templates of sulfur-bearing molecules. *Biophys. J.* 67:1229–1237.

Erne, D., and R. Schwyzer. 1987. Membrane structure of bombesin studied by infrared spectroscopy. Prediction of membrane interactions of gastrin-releasing peptide, neuromedin B and neuromedin C. *Biochemistry.* 26:6316–6319.

Gizeli, E., M. Liley, C. R. Lowe, and H. Vogel. 1997. Antibody binding to a functionalized supported lipid layer: a direct acoustic immunosensor. *Anal. Chem.* 69:4808–4813.

Haidacher, D., A. Vailaya, and Cs. Horváth. 1996. Temperature effects in hydrophobic interaction chromatography. *Proc. Natl. Acad. Sci. USA.* 93:2290–2295.

Hearn, M. T. W., and M. I. Aguilar. 1986. High performance liquid chromatography of amino acids, peptides and proteins. LXVII. Evaluation of bandwidth relationships of peptides related to b-endorphin, separated by gradient elution reversed phase high performance liquid chromatography. *J. Chromatogr.* 352:35–66.

Heyse, S., O. P. Ernst, Z. Dienes, K. P. Hofmann, and H. Vogel. 1998. Incorporation of rhodopsin in laterally structured supported membranes: observation of transducin activation with spatially and time-resolved surface plasmon resonance. *Biochemistry.* 37:507–522.

Hodges, R. S., P. D. Semchuck, A. K. Taneja, C. M. Kay, J. M. R. Parker, and C. T. Mant. 1988. Protein design using model synthetic peptides. *Peptide Res.* 1:19–30.

Hodges, R. S., B.-Y. Zhu, N. E. Zhou, and C. T. Mant. 1994. Reversed-phase liquid chromatography as a useful probe of hydrophobic interac-

tions involved in protein folding and protein stability. *J. Chromatogr. A.* 676:3–15.

Horváth, Cs., W. Melander, and I. Molnár. 1976. Solvophobic interactions in liquid chromatography with non-polar stationary phase. *J. Chromatogr.* 125:129–156.

Jacobs, R. E., and S. H. White. 1989. The nature of the hydrophobic binding of small peptides at the bilayer interface: implications for the insertion of transbilayer helices. *Biochemistry.* 28:3421–3437.

Kimura, S., D. Erne, and R. Schwyzer. 1992. Interaction of glucagon with artificial lipid membranes. *Int. J. Pept. Protein Res.* 39:431–442.

Krausse, E., M. Beyermann, M. Dathe, S. Rothemund, and M. Bienert. 1995. Location of an amphipathic α-helix in peptides using reversed-phase HPLC retention behavior of D-amino acid analogs. *Anal. Chem.* 67:252–258.

Lakowicz, J. R., I. Gryczynski, W. Wiczk, G. Laczko, F. C. Prendergast, and M. L. Johnson. 1990. Conformational distributions of melittin in water/methanol mixtures from frequency-domain measurements of non-radiative energy transfer. *Biophys. Chem.* 36:99–115.

Lazoura, E., I. Maidonnis, E. Bayer, M. T. W. Hearn, and M.-I. Aguilar. 1997. Conformational analysis of neuropeptide Y-[18-36] analogs in hydrophobic environments. *Biophys. J.* 72:238–246.

Lee, T.-H., P. E. Thompson, M. T. W. Hearn, and M.-I. Aguilar. 1997. Conformational stability of a type II′ β-turn motif in human growth hormone [6-13] peptide analogs at hydrophobic surfaces. *J. Peptide Res.* 49:394–403.

Linseisen, F. M., M. Hetzer, T. Brumm, and T. M. Bayerl. 1997. Differences in the physical properties of lipid monolayers and bilayers on a solid support. *Biophys. J.* 72:1659–1667.

Mishra, V. K., and M. N. Palgunachari. 1996. Interaction of model class A2, class A1 and class Y amphipathic helical peptides with membranes. *Biochemistry.* 35:11210–11220.

Mrksich, M., and G. M. Whitesides. 1996. Using self-assembled monolayers to understand the interactions of man-made surfaces with proteins and cells. *Annu. Rev. Biophys. Biomol. Struct.* 25:55–78.

Ong, S., and C. Pidgeon. 1995. Thermodynamics of solute partitioning into immobilized artificial membranes. *Anal. Chem.* 67:2119–2128.

Pasta, P., G. Carrea, R. Longhi, and L. Zetta. 1990. Circular dichroism and proteolysis of human β-endorphin in surfactant and lipid solutions. *Biochim. Biophys. Acta.* 1039:1–4.

Pasta, P., G. Vecchio, and G. Carrea. 1988. Conformation and proteolysis of glucagon and insulin in surfactant and lipid solutions. *Biochim. Biophys. Acta.* 953:314–320.

Picard, F., M.-J. Paquet, E. J. DuFourc, and M. Auger. 1998. Measurement of the lateral diffusion of dipalmitoylphosphatidylcholine adsorbed on silica beads in the absence and presence of melittin: a ^{31}P two-dimensional exchange solid state NMR study. *Biophys. J.* 74:857–868.

Pidgeon, C., and U. V. Venkataram. 1989. Immobilized artificial membrane chromatography: supports composed of membrane lipids. *Anal. Biochem.* 176:36–47.

Purcell, A. W., M.-I. Aguilar, and M. T. W. Hearn. 1992. The thermodynamic behavior of peptides in reversed phase HPLC. *J. Chromatogr.* 593:103–117.

Purcell, A. W., M.-I. Aguilar, and M. T. W. Hearn. 1993. The dynamics of peptides in RP-HPLC. *Anal. Chem.* 65:3038–3047.

Ringsdorf, H., B. Schlarb, and J. Venzmer. 1988. Molecular architecture and function of polymeric oriented systems: models for the study of organisation, surface recognition and dynamics of biomembranes. *Angew. Chem. Int. Ed. Engl.* 27:113–158.

Russell, C. J., T. E. Thorgeirrsson, and Y.-K. Shin. 1996. Temperature dependence of polypeptide partitioning between water and phospholipid bilayers. *Biochemistry.* 35:9526–9532.

Sargent, D. F., J. W. Bean, and R. Schwyzer. 1988. Conformation and orientation of regulatory peptides on lipid membranes. Key to the molecular mechanism of receptor selection. *Biophys. Chem.* 31: 183–193.

Schwyzer, R. 1991. New principle in QSAR: membrane requirements. *J. Receptor Research.* 11:45–57.

Schwyzer, R., S. Kimura, and D. Erne. 1992. Conformation, orientation and accumulation of peptides on the surface of lipid membranes. *In* Peptides: Chemistry and Biology. *Proceedings of the 12th American Peptide Symposium.* J. A. Smith and J. E. Rivier, editors. Escom (Leiden) 168–170.

Seelig, J., and P. Ganz. 1991. Nonclassical hydrophobic effect in membrane binding equilibria. *Biochemistry.* 30:9354–9359.

Stadalius, M. A., H. S. Gold, and L. R. Snyder. 1985. Optimization model for the gradient elution separation of peptide mixtures by reversed phase high performance liquid chromatography. Verification of band width relationships for acetonitrile-water mobile phases. *J. Chromatogr.* 327: 27–45.

Steer, D. L., P. E. Thompson, S. E. Blondelle, R. A. Houghten, and M.-I. Aguilar. 1998. Comparison of the binding of α-helical and β-sheet peptides to a hydrophobic surface. *J. Peptide Res.* 51:401–412.

Steinem, C., A. Janshoff, W. P. Ulrich, M. Sieber, and H.-J. Galla. 1996. Impedance analysis of supported lipid bilayer membranes: a scrutiny of different preparation techniques. *Biochim. Biophys. Acta.* 1279: 169–180.

Tanford, C. 1980. The Hydrophobic Effect: Formation of Micelles and Biological Membranes. Wiley, New York.

Vogel, H. 1987. Comparison of the conformation and orientation of alamethicin and melittin in lipid membranes. *Biochemistry.* 26:4562–4572.

Wimley, W. C., K. Hristova, A. S. Ladokhin, L. Silvestro, P. H. Axelson, and S. H. White. 1998. Folding of β-sheet membrane proteins: a hydrophobic hexapeptide model. *J. Mol. Biol.* 277:1091–1110.

Wimley, W. C., and S. H. White. 1993. Quantitation of electrostatic and hydrophobic membrane interactions by equilibrium dialysis and reverse-phase HPLC. *Anal. Biochem.* 213:213–217.

Wu, C.-S., A. Hachimori, and J. T. Yang. 1982. Lipid-induced ordered conformation of some peptide hormones and bioactive oligopeptides: predominance of helix over β-form. *Biochemistry.* 21:4556–4562.

Yang, J. T., T. A. Bewley, G. C. Chen, and C. H. Li. 1977. Conformation of β-endorphin and β-lipotropin: formation of helical structure in methanol and sodium dodecyl sulfate solutions. *Proc. Natl. Acad. Sci. USA.* 74:3235–3238.

Zetta, L., R. Consonni, A. deMarco, R. Longhi, E. Manera, and G. Vecchio. 1990. Opioid peptides in micellar systems: conformational analysis by CD and one-dimensional and two-dimensional ^{1}H-NMR spectroscopy. *Biopolymers.* 30:899–909.

Zetta, L., and R. Kaptein. 1984. Interaction of β-endorphin with sodium dodecyl sulfate in aqueous solution ^{1}H-NMR investigation. *Eur. J. Biochem.* 145:181–186.