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Binding of amphiphilic peptides to phospholipid/cholesterol unilamellar vesicles: A model for protein–cholesterol interaction

(apolipoprotein A-I)

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ABSTRACT **In earlier studies, we prepared a docosapeptide, 1, designed with minimum homology as an amphiphilic α-helical model of apolipoprotein A-I (apo A-I) and described its lipid-binding characteristics, surface properties, and enzyme-activating ability. Although the affinity of 1 for egg lecithin unilamellar vesicles was comparable with that for the binding of apo A-I, the affinity of 1 for mixed lecithin/cholesterol (4:1 mol/mol) vesicles was less than that of apo A-I. It appeared possible that the 3-hydroxyl group of cholesterol may have a deleterious interaction with the hydrophobic portion of the amphiphilic helix of 1 that is inserted into the vesicles. Examination of the amphiphilic α-helical segments of apo A-I suggested that the preferential interaction of apo A-I with the mixed vesicles might be due to the presence of polar arginine residues in the otherwise hydrophobic regions of two of the helices. Therefore, we synthesized a model docosapeptide, 2, corresponding to the sequence of 1 but containing arginine rather than leucine at position 10 in the hydrophobic region of the α helix to assess the role of the alcohol function of cholesterol in protein–cholesterol interactions. The results of studies on the binding of 2 to unilamellar vesicles containing lecithin only, lecithin/cholesterol, lecithin/cholesterol hemisuccinate, or lecithin/cholesterol methyl ether were consistent with the postulate that the major role of cholesterol in the binding of proteins to phospholipid surfaces is the creation of free space between the phospholipid head groups that can accommodate the amphiphilic peptide chains at the interface.**

Recently, we described the lipid-binding characteristics, surface properties, and enzyme-activating ability of a docosapeptide, 1, designed with minimum homology as an amphiphilic α-helical model of apolipoprotein A-I (apo A-I) (1, 2). We found that the dissociation constant for the binding of 1 to egg lecithin unilamellar vesicles was approximately the same as that for the binding of apo A-I. In contrast, addition of cholesterol to the lecithin resulted in a lower affinity of 1 for the mixed vesicles compared with that of apo A-I. The mixed vesicles showed, however, the same increased capacity toward both ligands, suggesting that insertion of cholesterol in a phospholipid bilayer creates binding regions for amphiphilic proteins. The exact interaction of proteins with cholesterol thus still remained unspecified. In examining the regions of apo A-I that are thought to form amphiphilic α helices (3), we noted that, in two of these regions—those including Arg-116 and Arg-123—these amino acids were the only hydrophilic residues interrupting an otherwise hydrophobic portion of the helix. In considering our results with model peptide 1, it appeared possible that the 3-hydroxyl group of cholesterol, which is near the outer surface of the vesicle, may have a deleterious interaction with the hydrophobic portion of the amphiphilic helix, which is inserted into the vesicles in the spaces between the phospholipid polar head groups. If this were the case, the preferential interaction of apo A-I with the mixed lecithin/cholesterol vesicles might be due to the presence of the polar arginine residues in the otherwise hydrophobic region of the respective amphiphilic α helices (2, 4). Thus, by incorporating a polar residue into the hydrophobic region of an amphiphilic α helix, we could assess the role of the 3-hydroxyl function of cholesterol in peptide–cholesterol interaction. A very attractive feature of the use of 1 is that, because it is an idealized model for the predominant secondary structural characteristic of apo A-I—the amphiphilic α helix—and does not follow the amino acid sequence of the native apolipoprotein, specific amino acid replacements can be made on a rational basis and their effects on the chemical and physical properties of models can be tested quantitatively. In this article, we describe the synthesis of a model peptide 2 (Fig. 1) corresponding to the sequence of 1 but containing arginine rather than leucine at position 10 and a systematic study of the binding of 2 to single bilayer lecithin vesicles containing cholesterol or cholesterol derivatives.

EXPERIMENTAL

Materials. Egg yolk lecithin was purchased from Avanti Chemical and cholesterol was from Supelco (Bellefonte, PA). The identity and purity of these compounds were ascertained by thin-layer chromatography (TLC) on silica gel G, using chloroform/methanol/water (70:30:5, vol/vol) for the former and hexane/diethyl ether/acetic acid (65:35:1, vol/vol) for the latter. Cholesterol hemisuccinate was purchased from Steraloids (Pawling, NY), and cholesterol methyl ether was synthesized by the method of Narayanan and Iyer (5). Both of these cholesterol derivatives were purified by crystallization from methanol, and they were identified and tested for purity by TLC on silica gel G using hexane/diethyl ether/acetic acid (70:30:1, vol/vol).

The solvents and reagents used for solid-phase peptide synthesis were purified by the methods described previously (6). Chloromethylated styrene–divinylbenzene copolymer (1% crosslinked) was obtained from Pierce. *tert*-butoxycarbonyl derivatives of L-alanine, tosyl-L-arginine, ε-2-chlorobenzyloxycarbonyl-L-lysine, L-glutamic acid γ-benzyl ester, L-leucine, and L-proline were purchased from Bachem Fine Chemicals (Torrance, CA).

The water used for experiments in surface chemistry was redistilled from deionized water in an all-glass apparatus.

Synthesis of Peptide. The peptide was synthesized with a Beckman model 990 automated synthesizer. Chloromethylated styrene–divinylbenzene copolymer was esterified by reaction with the cesium salt of *tert*-butoxycarbonyl alanine in dimethyl

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Abbreviations: apo A-I, apolipoprotein A-I; TLC, thin-layer chromatography; CD, circular dichroism; Mops, 3-(*N*-morpholino)propanesulfonic acid.

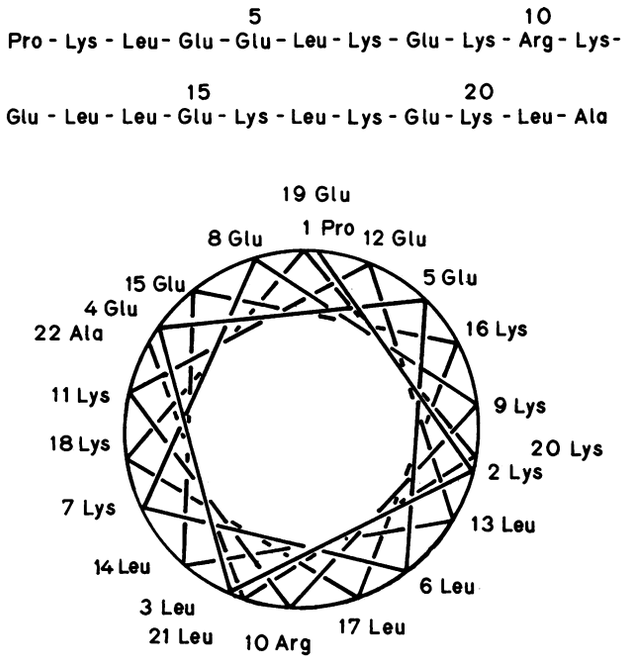

FIG. 1. Amino acid sequence of **2** and its axial projection when it forms an α helix.

formamide (7). The substitution level was 0.3 mmol/g of resin by titration with picric acid (8). The substituted resin, 1.7 g (0.5 mmol), was placed in the reaction vessel of the synthesizer, and the coupling steps were carried out using the symmetric anhydride method (9). After completion of the synthesis, the peptide was cleaved from the polymeric support by reaction with anhydrous HF in the presence of anisole at 0°C (10). The mixture of cleaved peptide and resin was washed with 20 ml of CH_2Cl_2, and then the peptide was extracted with two 50-ml portions of 10% aqueous acetic acid. The extract was lyophilized to obtain 1.24 g of crude peptide.

Purification of the Peptide. The lyophilized product (1.24 g) was suspended in 10 ml of 0.2 M acetic acid, and the mixture was filtered and then loaded onto a Sephadex G-25 Superfine column (2.2 × 55 cm) equilibrated with 0.2 M acetic acid. Elution was performed using the same solvent at a rate of 15 ml/hr, and 4-ml fractions were collected, monitoring the absorbance at 250 nm. The first small peak (K_{av} = 0.35) and the ascending part of the second peak (K_{av} = 0.67) were shown to have essentially the same composition by TLC on cellulose, using 1-butanol/acetic acid/water/pyridine (15:3:12:10, vol/vol). On the basis of dry weight, 47% of the starting material was recovered in these combined fractions after lyophilization. The lyophilized material was dissolved in 25 ml of 0.02 M phosphate buffer (pH 7.0) and applied to a CM Sephadex C-25 column (2 × 40 cm) equilibrated with the same buffer. After elution with 150 ml of the initial buffer, a linear gradient of NaCl (0–0.3 M/400 ml) in the original buffer was applied to the column. Fractions of 8 ml were collected, and the UV absorbance was monitored at 230 nm. The main fractions were pooled, lyophilized, and desalted on a Sephadex G-25 column. The final dry weight after lyophilization was 291 mg, a yield of 23% based on the lyophilized crude peptide isolated after HF cleavage.

TLC on cellulose using 1-butanol/acetic acid/water/pyridine (15:3:12:10, vol/vol), showed a single spot (R_F = 0.61) positive to both ninhydrin and Sakaguchi reagents. High-performance liquid chromatography on a C_{18} column using as the eluting solvent a 10–90% linear gradient of acetonitrile in 50 mM tetraethylammonium phosphate buffer (pH 3.15) showed a single symmetric peak for the final product at ≈28 vol % of acetonitrile.

The amino acid composition was measured after hydrolysis of the peptide with 5.5 M HCl for 24 hr at 110°C (Beckman Spinco model 121 amino acid analyzer). The peptide was also subjected to automated Edman degradation (Beckman model 890-C protein peptide sequencer) (11).

Molecular Properties in Solution. Circular dichroism (CD) spectra (Cary 60 spectropolarimeter) of the peptide over the range 1.2–16.5 μM were measured in 0.1 M phosphate buffer, pH 7.0/0.16 M KCl. The effect of the presence of 50% trifluoroethanol in the same buffered solution (vol/vol) was also measured (12). The mean residue ellipticity at 222 nm ($[\Theta]_{222\,nm}$) was used to calculate the α helicity according to the equation, % α helix = $([\Theta]_{222\,nm} + 3000)/(36{,}000 + 3000)$ (13). The apparent molecular weight in aqueous solution was obtained by gel permeation chromatography using a Sephadex G-50 column (1 × 100 cm) calibrated with globular proteins.

Monolayer Studies of the Peptide at the Air-Water Interface. The surface pressure (π) of monolayers of the peptides at the air-water interface was monitored, using a Lauda film balance, as a function of the area of the monolayer (a). The peptide solution (70 μM) was spread on the surface of the buffer [0.02 M 3-(*N*-morpholino)propanesulfonic acid (Mops)/0.16 M KCl, pH 7.4], and it was compressed and expanded between 700 and 200 cm^2 at a rate of 2.2 cm^2/sec.

Preparation of Unilamellar Vesicles. Unilamellar vesicles of lecithin, lecithin/cholesterol, lecithin/cholesterol hemisuccinate, and lecithin/cholesterol methyl ether were prepared and purified according to described methods (2, 4, 14). One milliliter of a solution of lecithin, lecithin and cholesterol, or lecithin and cholesterol derivative (4:1, mol/mol) in pure ethanol (10 mg of lecithin/ml) was injected rapidly into 50 ml of 0.16 M KCl. After concentration of the solution to a volume of 5 ml, using an Amicon XM100 membrane, the vesicles were purified using a Sepharose CL-4B column (2.5 × 60 cm), and the eluant was monitored by a differential refractometer (Waters Associates). Fractions (5 ml) were collected, and the phosphorus (15) and cholesterol (16) concentrations were measured. The peak fractions obtained for the unilamellar vesicles were concentrated to 5 ml using an Amicon XM100 membrane. The stability of the vesicles was checked by chromatography on a Sephadex CL-4B column (1.5 × 30 cm) monitored through the refractive index of the eluate (model R401 refractometer, Waters Associates).

Binding of the Peptides to Unilamellar Vesicles. The binding of the peptide to the unilamellar vesicles was quantitated by rapid ultrafiltration sampling of the free peptide, combined with the determination of peptide concentration using *o*-phthalaldehyde (1, 2, 4). Incubation of the peptide with the vesicles was carried out with 0.34–0.36 mM lecithin and 4–25 μM peptide in 0.02 M Mops/0.16 M KCl (pH 7.4) and 22°C.

RESULTS

The amino acid composition obtained after acid hydrolysis of the peptide agreed well with the one expected for **2** (Table 1). Automated Edman degradation of the peptide also gave the predicted sequence (Table 2).

The mean residue ellipticity at 222 nm (-4873 ± 70 deg cm^2 $dmol^{-1}$, corresponding to an α helicity of ≈20%) remained constant throughout the entire concentration range used for the CD measurements in neutral aqueous solution. In the presence of 50% trifluoroethanol, the estimated helicity increased to 60% ($[\Theta]_{222\,nm} = -20{,}256$ deg $cm^{-2}dmol^{-1}$).

Gel permeation chromatography on Sephadex G-50 showed an apparent M_r of 3000, even at 2.5 mM peptide. This value is

Table 1. Amino acid analysis of the synthetic peptide

Amino acid	Expected value	Observed value
Glutamic	6	6.26
Proline	1	1.08
Alanine	1	1.10
Leucine	6	5.74
Lysine	7	6.88
Arginine	1	0.86

in good agreement with the calculated M_r for the monomeric form of the peptide (2, 17). Thus, in contrast to the case of a synthetic amphiphilic docosapeptide, 1, where both the concentration dependence of the CD spectrum at 222 nm and the gel permeation chromatography results were consistent with the formation of a tetramer at high peptide concentration (1, 2), we were unable to detect any molecular self-association in aqueous solution with the arginine-containing peptide, even in the millimolar range.

The arginine-containing peptide was found to form a stable monolayer at the air-water interface. The π-a curve showed a discontinuity at around $\pi = 6$ dyn/cm, indicating the collapse of the monolayer. Over the range $0.1 < \pi < 5$ dyn/cm, this curve was analyzed using the empirical equation $\pi[a - a_o(1 - \kappa\pi)] = nRT$, where $a_o = 21.3$ Å^2 per residue and $\kappa = 2.7 \times 10^{-2}$ cm/dyn are constants (4) and the M_r calculated was 2300.

Analysis of unilamellar vesicles of lecithin, lecithin/cholesterol, lecithin/cholesterol hemisuccinate, and lecithin/cholesterol methyl ether was performed with a Sepharose CL-4B column. When a constant amount of lecithin was used, the elution profiles (monitored by refractive index) of concentrated vesicle solutions prepared by the injection of ethanolic solutions of the lipids were quite similar to each other. Under the conditions described in *Experimental*, only 3–5% fused vesicles were found. The K_{av} values for the main peaks for the mixed vesicles were 0.40 ± 0.01, not significantly different from the values for pure lecithin vesicles. In the cases of the mixed vesicles, the peaks corresponding to cholesterol and to phosphorus-containing material were coincident, and the molar ratios of lecithin and cholesterol were similar to that in the original ethanolic solution (4:1) in every fraction checked. On storage at 4°C for 1 week, none of the vesicle solutions showed evidence for more than 1% fusion.

Binding studies were performed within 3 days of preparation of the vesicles. Even after incubation of the vesicles with the peptide at its maximum concentration used in the binding studies for 3 hr at 22°C at pH 7.4, the elution profiles for each type of vesicle, monitored by the refractive index and by cholesterol and phosphorus concentration, did not change. The profiles for the binding of the peptide to each type of vesicle are shown in Fig. 2. The unilamellar vesicles used in these experiments contained lecithin only, lecithin/cholesterol, lecithin/cholesterol hemisuccinate, or lecithin/cholesterol methyl ether. The molar ratio of lecithin to cholesterol or cholesterol derivatives in the mixed vesicles was 4:1. All of the binding curves measured show a saturation, and the data obtained were analyzed as a single Langmuir isothermal absorption (17). $P_f = (N \cdot PC \cdot P_f/P_b) - K_d$, where P_f and P_b correspond to the free and bound peptide concentrations, respectively, PC is the concentration of lecithin, N is the upper limit of P_b/PC, and K_d is the dissociation constant. The plots of P_f vs. $PC \cdot P_f/P_b$ for the data of Fig. 2 are shown in Fig. 3. In each case, a straight line was obtained, indicating that the binding of the peptide obeys the law expressed in the equation. The parameters, N and K_d, obtained from Fig. 3 for each binding curve are listed in Table 3. Addition of 20 mol % of cholesterol to the lecithin vesicles resulted in an increase of 46% in the maximum number of peptide molecules bound to the vesicles and a decrease in K_d by a factor of one-half.

Table 2. Edman degradation results for peptide **2**

Cycle	Amino acid	Yield, nmol*	TLC	Spot test
1	Proline	300		
2	Lysine	—	Lysine	
3	Leucine	310		
4	Glutamic	100	Glutamic acid	
5	Glutamic	150	Glutamic acid	
6	Leucine	250		
7	Lysine	—	Lysine	
8	Glutamic	125	Glutamic acid	
9	Lysine	—	Lysine	
10	Arginine	—		Arginine
11	Lysine	—	Lysine	
12	Glutamic	75	Glutamic	
13	Leucine	190		
14	Leucine	250		
15	Glutamic	100	Glutamic acid	
16	Lysine	—	Lysine	
17	Leucine	190	Lysine	
18	Lysine	—	Lysine	
19	Glutamic	30	Glutamic acid	
20	Lysine	—	Lysine	
21	Leucine	110		
22	Alanine	25	Alanine	

* A 350-nmol sample of peptide was subjected to degradation. The phenylthiohydantoins were identified and quantitated by gas chromatography.

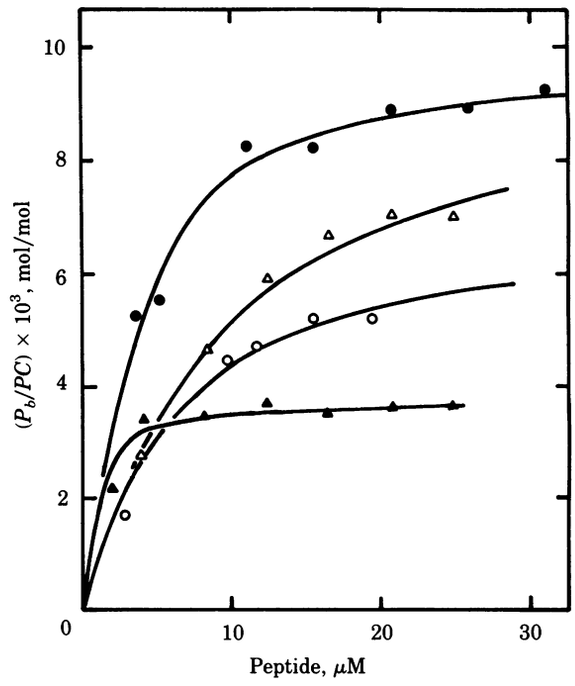

FIG. 2. Binding of **2** to various vesicles. ○, Binding to lecithin (*PC*) vesicles (*PC* = 0.361 mM in 0.02 M Mops/0.16 M KCl, pH 7.4); ●, binding to lecithin/cholesterol vesicles (molar ratio 4:1, *PC* = 0.34 mM); △, binding to lecithin/cholesterol methyl ether vesicles (molar ratio 4:1, *PC* = 0.313 mM; and ▲, binding to lecithin/cholesterol hemisuccinate vesicles (molar ratio 4:1, *PC* = 0.374 μM). Theoretical curves were calculated according to the Langmuir equation, and the parameters are listed in Table 3.

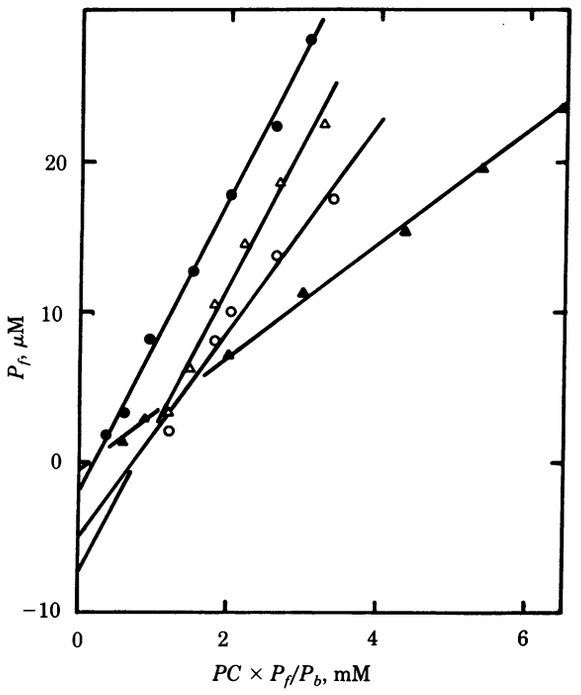

FIG. 3. Linearized plots of the data in Fig. 2 used to obtain N and K_d values. Symbols are the same as those in Fig. 2. The straight lines represent least-squares fits. The K_d values are obtained from the intercepts on the ordinate and the N values are from the slopes.

In other words, the peptide had a higher affinity for the cholesterol-containing vesicles than for those which did not contain cholesterol. This is exactly opposite to the case of the amphiphilic synthetic peptide that contained leucine in place of arginine (2). The effect of added cholesterol methyl ether was an increase in the limiting number of peptide molecules bound similar to that seen in the instance of cholesterol (42%), but the K_d increased for the case of the methyl ether by 70% compared with plain lecithin vesicles. The presence of cholesterol hemisuccinate in the vesicles caused a decrease in the capacity of the vesicles for the peptide (44%); but there was a remarkable increase in the binding affinity of the peptide for the vesicles (the value of K_d was $\frac{1}{10}$ that observed for pure lecithin vesicles).

DISCUSSION

The chemical and physical characteristics of **2** correspond closely to those expected for the replacement of a leucine residue by an arginine residue in the hydrophobic portion of the amphiphilic α helix formed by **1**. In particular, unlike the CD spectra of **1**, which show a dependence on the concentration of the peptide consistent with the postulation of a monomer-tetramer equilibrium for **1**, the spectra measured for **2** showed no concentration dependence over a wide range. The ellipticity at 222 nm for solutions of **2** was comparable with that found for the monomeric form of **1**, and the α helicity calculated was only 20%. Force-area curve measurements at the air-water interface for monolayers of **2**, as well as gel filtration experiments, provided no evidence for the presence of a species of this peptide other than the monomer. Presumably, unlike peptide **1**, where the hydrophobic region of the peptide molecules can interact strongly, giving rise to tetramerization, the presence of the positively charged arginine residues in the corresponding region of **2** prevents such an interaction. On the other hand, as can be seen from Table 3, **2** does interact somewhat more strongly with lecithin/cholesterol (4:1 mol/mol) vesicles ($K_d = 2.0 \times 10^{-6}$ M) than does **1** ($K_d = 2.8 \times 10^{-6}$ M), while the reverse is true for the binding to phospholipid vesicles ($K_d = 5.0 \times 10^{-6}$ M and 1.9×10^{-6} M, respectively). Thus, the arginine residue at position 10 does have a modest positive effect on the binding of the model peptide to the cholesterol-containing vesicles, as has been predicted. The slight contribution of this polar interaction is further supported by the fact that, when the polar alcohol group of cholesterol is etherified, the affinity of **2** for the mixed vesicles decreases from $K_d = 2.0 \times 10^{-6}$ M to $K_d = 7.4 \times 10^{-6}$ M. The presence of cholesterol in the vesicles increases their capacity to both **1** and **2**. The percentage increase is the same for **1** as for **2**. Furthermore, replacement of cholesterol by its methyl ether does not alter the binding capacity of the vesicles. These observations are all consistent with a mode of binding in which the surface bound peptides have the same conformation with and without cholesterol. The decreased capacity of the vesicles toward **2** as compared with that toward **1** also suggests that **2** is bound to the surface in a somewhat less compact conformation than that of the ideal helical peptide **1**.

Table 3. Parameters for the binding of **2** to vesicles calculated from the data of Fig. 3

Vesicles	$K_d \times 10^6$, M	$N \times 10^3$, mol of peptide/mol of lecithin
Lecithin	5.0 ± 1.1	6.9 ± 0.5
Lecithin/cholesterol (4:1)	2.0 ± 0.4	9.8 ± 0.2
Lecithin/cholesterol methyl ether (4:1)	7.4 ± 1.3	9.6 ± 0.6
Lecithin/cholesterol hemisuccinate (4:1)	0.4 ± 0.2	3.7 ± 0.1

Values are mean ± SEM.

Finally, replacement of cholesterol by its hemisuccinate results in a considerable decrease in the capacity of the vesicle to bind **2**—from 9.8×10^{-3} mol of peptide/mol of lecithin to 3.7×10^{-3} mol of peptide/mol of lecithin. The hemisuccinate of cholesterol has a highly hydrophilic head group and a length comparable with that of the phospholipids. These features are conducive to a mixed monolayer in which no free space is created between the phospholipid head groups by the introduction of the cholesterol derivative. The increased affinity ($K_d = 0.4 \times 10^{-6}$ M) presumably reflects electrostatic attraction of the positively charged peptide by the carboxylate groups.

The following conclusions emerged from these considerations: (*i*) Only an energetically modest interaction occurs between the polar group of cholesterol and the appropriately positioned hydrophilic groups of surface-bound peptides and proteins. This interaction is not the major force for binding of proteins to the phospholipid/cholesterol surface, as also noted by Lala *et al.* (18), who observed that the biological role of cholesterol can be fulfilled by its methyl ether. (*ii*) The hydrophobic portion of cholesterol is not a major contributor to the binding of proteins either; K_d values for phospholipid vesicles are of the same order of magnitude as those for mixed cholesterol/phospholipid vesicles.

It is then reasonable to postulate that the major role of cholesterol in the binding of proteins to phospholipid surfaces is the creation of free space between the phospholipid head groups that can accommodate the amphiphilic peptide chains at the interface. Some specific interaction does occur between the cholesterol head group and certain side chains of the peptide, resulting in the formation of weak complexes at the molecular level, but these interactions do not lead to any *specific* recognition of the mixed surface of the proteins.

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