Fusion of Phospholipid Vesicles Induced by α-Lactalbumin at Acidic pH†

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ABSTRACT: α-Lactalbumin (α-LA), lysozyme, and ribonuclease are found to induce fusion of phosphatidylserine/phosphatidylethanolamine vesicles at low pH. The fusogenic behavior and the binding to phospholipid vesicles of one of these proteins, α-LA, are studied at a wide range of conditions. The initial rate of fusion in the presence of α-LA increases with increasing acidity below pH 6, and the extent of α-LA binding to the vesicles is also found to increase with decreasing pH. Once bound to the vesicles in acidic media, the neutralization to pH 7 fails to dislodge the α-LA from the vesicles, and this irreversible binding also increases with decreasing pH. A segment of α-LA is found to be resistant to the proteolytic digestion when initially incubated with the vesicles at low pH. The amino acid composition of this fragment was determined, and from this the sequence of α-LA fragment, which appears to be inserted into the bilayer, is deduced. Hydrophobic labeling with dansyl chloride renders support that this segment indeed penetrates into the hydrophobic interior of bilayer. Since both the N-terminal and the C-terminal of this vesicle-bound protein are accessible to the externally added proteolytic enzymes, it is concluded that a loop of the polypeptide segment goes into the bilayer. These observations, taken together, suggest a possibility that the penetration by a loop of α-LA segment into the phospholipid bilayer is responsible for the fusion.

The membrane fusion is an essential process in a broad range of vital biological phenomena. Many of these functions such as exocytosis, fertilization, and the formation of muscle cells from fibroblasts require Ca^{2+} ions while some others, such as virus infection, do not involve this ion for the fusion (Papahadjopoulos et al., 1979). There have been extensive investigations on Ca^{2+}-induced fusion of biological membranes as well as phospholipid vesicles, but the molecular mechanism of this process is still poorly understood. The fusion process between the plasma membrane and animal virus envelope is somewhat better known as far as the triggering mechanism by envelope integral protein is concerned (White et al., 1983).

For the case of infection by enveloped animal viruses, it was observed that hydrophobic segments of envelope-integral proteins are exposed either from proteolysis as in the case of Sendai virus or by acidic pH as in the case of influenza and Semliki Forest viruses. It is believed that these exposed hydrophobic segments penetrate into the plasma membranes, which somehow leads to the fusion of the plasma membrane of host cell and the virus envelope membrane. From these and other observations, Lucy (1984) proposed that the fusion in the biological systems occurs as a result of the production of hydrophobic protein segments by endogeneous proteolysis. The possibility that this protein-induced membrane fusion may be a more general phenomenon than anticipated was indicated by the fusion of phospholipid vesicles by a number of water-soluble proteins such as cytochrome *c* (Gad et al., 1982), bovine serum albumin (Schenkman et al., 1981) and its fragments (Garcia et al., 1984), diphtheria toxin (Cabiaux et al., 1984; Blewitt et al., 1985), tetanus toxin (Cabiaux et al., 1985), clathrin (Steer et al., 1982; Blumenthal et al., 1983; Hong et al., 1985), and insulin (Farias et al., 1985) at low pH. These proteins expose hydrophobic amino acid side chains at low pH, and it has been suggested that the penetration of these into the vesicle bilayer is the cause of the membrane fusion (Hong et al., 1985). No actual demonstration of the penetration by a hydrophobic segment of a protein into bilayer under the condition of protein-induced vesicle fusion, however, has been made so far. These experimental results suggest that simple water-soluble proteins, if induce fusion under certain conditions, may serve as good model systems, which may eventually help clarify the fusion mechanism. Especially useful systems are the small proteins whose structural transitions at low pH are well-known.

There is a group of small water-soluble proteins, some known as peripheral proteins, that undergo partial conformational changes in an acidic media (Kuwajima et al., 1975; Dolgikh et al., 1981; Ohgushi & Wada, 1983; Dolgikh et al., 1984). Their CD[1] spectra in the near-UV range at low pH as examplified by the case of α-LA are those of random coil, indicating the exposure of aromatic amino acid side chains. On the other hand, the mean residue ellipticities in the far-UV range remain the same or are even increased slightly when the pH of the media is brought from high to low values. Hydrodynamic studies suggest that this intermediate state is still rather compact. One of these proteins, cytochrome *c*, was already found to fuse model membrane (Gad et al., 1982).

We have observed that some of these proteins fuse model membranes under acidic conditions. This paper describes the detailed studies of the first of these proteins, α-LA. We found that there is a correlation between the irreversible binding of this protein to the vesicles and the vesicle fusion induced by this protein. We also found that a portion of this protein penetrates into the bilayer under acidic condition. The vesicles used in the first part of the fusion studies were prepared with a 1:1 composition of phosphatidylserine (PS) and phosphatidylethanolamine (PE), because vesicles of this composition have been extensively studied with many fusogenic agents.

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[1] Abbreviations: α-LA, α-lactalbumin; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *N,N'-p*-xylylenebis(pyridinium bromide); PS, phosphatidylserine; PE, phosphatidylethanolamine; PMSF, phenylmethanesulfonyl fluoride; DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism; OD, optical density; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TLC, thin-layer chromatography; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

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Although α-LA-induced fusion of PS/PE vesicles takes place at lower pH than those for the membrane fusion brought about by the virus envelope proteins, the underlining mechanism of fusion caused by all these proteins may be basically the same.

Materials and Methods

Chemicals. PS, PE, α-LA (from bovine milk), cytochrome *c* (from horse heart), lysozyme (from chicken egg white), ribonuclease A (from bovine pancrease), trypsin (from bovine pancrease), chymotrypsin (from bovine pancrease), carboxypeptidase Y (from potato tuber), phenylmethanesulfonyl fluoride (PMSF), cyclodextrin, dansyl L-amino acids, and dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid) were purchased from Sigma. $TbCl_3 \cdot 6H_2O$ (99.99%) was obtained from Alfa, and dansyl chloride was from Pierce. All other chemicals were purchased in the highest purity available. α-LA, in a buffer solution (2 mM Tes, 2 mM histidine, 100 mM NaCl, pH 7.4) containing 1.0 mM EDTA was purified by passage through a column of Sephadex G-100. Of the two protein peaks separated, the slower component was identified as being the α-LA from the molecular weight determination by using SDS–PAGE. The other proteins purchased were used without further purification. All phospholipids migrated as single spots upon thin-layer chromatography on silica gel. Phospholipid concentration was determined according to the method of Vaskowsky et al. (1975). Proteins were determined with a Gilford 260 UV–visible spectrophotometer at 280 nm and also by the modified Lowry method (Markwell et al., 1978). Water was distilled twice, the second time in an all-glass apparatus.

Vesicle Formation. Phospholipid vesicles composed of a 1:1 molar mixture of PS and PE were prepared by the ether injection technique (Deamer & Bangham, 1976) followed by 15 s of sonication. The multilamellar liposomes were removed by centrifugation at 6500 rpm for 30 min, and the supernatant was collected to obtain relatively homogeneous monolamellar vesicles. In order to perform the fusion and binding studies at various pH values, the phospholipid suspension in ether was injected into the buffer solution of a particular pH value. Subsequent centrifugation was also made at the same pH. The resulting vesicles were observed with the thin-section method and the negative staining with uranyl acetate on a Japan Electron JEM 100 CX-II electron microscope. Most of the vesicles (50-nm diameter) obtained were found to be monolamellar, and there was no discernible size difference between the vesicles obtained at pH 2 and those formed at pH 7. Since aggregation of the vesicles was observed 2 days after their preparation at pH 2, these were used within this time limit.

Fusion. For observation of the fusion process, the Tb–DPA method of Wilschut et al. (1980) was followed. This method monitors the extent of mixing of the vesicle contents upon fusion and has been widely used for model membrane fusion studies. Here, one population of vesicles was prepared in a solution containing 2.5 mM $TbCl_3$ and 50 mM sodium citrate and another population of the vesicles in a solution containing 50 mM DPA (sodium salt) and 20 mM NaCl. The vesicles were separated from nonencapsulated materials by gel filtration on a Sephadex G-75 column. The elution was made with a buffer solution containing 0.1 mM EDTA. After equal concentrations of these two types of vesicles and various concentrations of the protein were mixed, the fusion process was followed by using an Aminco-Bowman spectrofluorometer.

The leakage of the vesicles after the addition of fusogenic agents was monitored by using PS/PE vesicles containing 1.25 mM $TbCl_3$, 25 mM sodium citrate, and 25 mM DPA (Bentz et al., 1983). Again, the nonencapsulated materials were removed by gel filtration as described above. The decrease in the fluorescence intensity after the initiation of fusion results from the dissociation of the Tb–DPA complex when it leaks out into the bulk solution, which contains EDTA, and from the leak-in of outside solution into the fused vesicles.

The change in OD at 340 nm was also used for monitoring the fusion process. The formation of large vesicles as the result of fusion was also observed by electron microscope and by gel filtration. All the fusion experiments were performed at 18 °C.

α-LA Binding to Vesicles. The centrifugation method was used for the binding studies. The vesicles prepared at each pH by using the ether injection method were first centrifuged at 6800 rpm to remove heavy liposomes. The vesicles in the supernatant were then sedimented 8 times with progressively increasing angular velocity from 20 000 to 34 000 rpm, each operation lasting from 60–90 min. A Beckman SW 41 swinging-bucket rotor was used here. In this way, the size range of the vesicles was narrowed down. After the final precipitation, the supernatant solution was found to be optically clear as observed by the OD at 280 nm. Phosphate analysis confirmed that the phospholipid concentration of the supernatant was negligible. The last precipitated vesicles were resuspended in a buffer solution, and an aliquot of α-LA solution was added to bring the concentrations of both phospholipid and protein to predetermined values. After incubation at 18 °C for 30 min, the vesicles were precipitated by ultracentrifugation at 35 000 rpm, and the supernatant solution was analyzed for the protein concentration by measuring OD values at 280 nm. Preliminary tests showed that at least 15 min of incubation period is required prior to the ultracentrifugation to reach the binding equilibria.

In order to test the reversibility of the binding, the vesicles were incubated with α-LA in acidic media (pH 2, 3, and 4) at 18 °C for 30 min and centrifuged for 90 min at 35 000 rpm. The precipitates formed were then resuspended in a pH 7 buffer solution (ionic strength 0.1) and incubated for 48 h at 18 °C. The pH of this suspension remained constant at 7 throughout this period. A part of this suspension was centrifuged for 90 min at 35 000 rpm, and the protein concentration in the supernatant solution was determined (OD at 280 nm). The remaining suspension was passed through a Sepharose 2B column, and the eluent was analyzed both for the protein and for the phospholipid. The protein concentration was determined by using the modified Lowry method.

Proteolytic Digestion of Vesicle-Bound α-LA. The possibility of a segment of the α-LA being inserted into the phospholipid vesicle membrane was checked by the treatment of the vesicle–protein complex with trypsin, chymotrypsin, and carboxypeptidase Y. In these experiments, 2 mg of α-LA was incubated with 10 mL of PS/PE (1:1) vesicle suspension (1 mM P_i) for 60 min at 18 °C at several pH values. The vesicles with bound α-LA were sedimented by centrifuging for 90 min at 30 000 rpm in a Beckman SW 41 rotor. The pellets were resuspended in 5 mL of medium containing 150 mM KCl, 25 mM imidazole, and 100 μg of proteolytic enzyme, pH 7.5, and incubated for 1 h at 37 °C. The digestion was stopped by the addition of PMSF as a freshly dissolved enthanolic solution to 3 mM final concentration, followed by incubation of samples at 37 °C for 3 min. The vesicles were then pelleted by centrifugation for 90 min at 30 000 rpm in a Beckman SW 41 rotor. The pellets were resuspended in 2 mL of buffer (25 mM imidazole, 1 mM EDTA, pH 7.5).

In order to determine the molecular weight of the protected portion of the protein, the lipid was extracted in 3 volumes

of chloroform–methanol (2:1) and the protein was precipitated in 10% trichloroacetic acid at 0 °C, followed by centrifugation for 15 min in an Eppendorf microfuge. After two acetone washings, a part of the resulting pellet of the protein fragment was solubilized in a buffer of 40% glycerol, 2.5% SDS, 0.01 M H_3PO_4/Tris, 8 M urea, and 5% β-mercaptoethanol, pH 6.7. This dissolved protein was electrophoresed in 15% acrylamide gels in SDS. The remaining portion of the pellet was lyophilized. This extracted sample was analyzed for amino acid composition after being dissolved in 6 M HCl, sealed under nitrogen, and hydrolyzed for 24 h at 110 °C. A Durrum D-500 amino acid analyzer was used.

Hydrophobic Labeling. The segment of α-LA that penetrates into the hydrophobic interior of phospholipid bilayer was labeled with dansyl chloride by using the method described by Sikorski and Daczynska (1982). Dansyl chloride (5 mg) was dissolved in acetone in a round-bottomed flask, and the solvent was evaporated to obtain a thin layer of solute on the inside wall of the flask. PS/PE (1:1) vesicle suspension (1 mL) was introduced into this flask, and it was gently shaken for 3 h at 4 °C. The suspension was passed through a Sephadex G-10 column (1 × 40 cm) to remove the unbound dansyl chloride. Any residual free dansyl chloride was eliminated by washing the eluted vesicles with a buffer solution containing 0.5% cyclodextrin. The vesicles were then pelleted by ultracentrifugation and resuspended in a buffer solution (glycine hydrochloride, pH 3.0). After 2 mL of 0.1% α-LA solution (pH 3) was added, the suspension was incubated for 30 min at 18 °C. The vesicles were again sedimented for 90 min at 25000 rpm, and the pellets were suspended in a buffer solution (pH 7.4). The suspension was incubated with 100 μg of trypsin for 1 h at 37 °C. The proteolysis was terminated with the addition of PMSF to make its final concentration of 3 mM, and 9 volumes of acetone was added to the suspension. This was left to stand for 2 h at −20 °C. The precipitated protein was collected by centrifugation and washed with chloroform followed by SDS–PAGE.

For a second set of experiments, the vesicle-bound α-LA was first digested with trypsin and then labeled with dansyl chloride. The labeling procedure and the method of removal of the unreacted dansyl chloride were essentially the same as before.

The α-LA segment protected from the tryptic digestion and extracted was also labeled with dansyl chloride with somewhat different approach. α-LA solution (1 mg/mL, Tes buffer, pH 7.4) was mixed with 50 μL of dansyl chloride solution in acetone (20 mg/mL) in a cap tube and vortexed. The tube was then sealed by melting the Parafilm lining the cap and incubated for 15 min in a water bath, temperature being maintained at 50 °C. After 50 μL of β-mercaptoethanol was added into this tube, the solution was incubated again for 15 min at 50 °C. The solution was passed through a Pasteur pipet filled with Bio-Gel P6 to remove the unbound dansyl chloride. This sample was then subject to SDS–PAGE.

In order to identify the amino acid residues that form covalent bonds with the dansyl group, the labeled protein fragment was subject to a 6 N HCl treatment. After the removal of HCl by evaporation in a rotary evaporator, the labeled amino acids were separated by two-dimensional TLC and compared with labeled standard amino acids.

Results

Fusogenic Properties of Proteins. Figure 1 gives typical time courses of the fluorescence intensity change when proteins were added to the vesicle suspensions containing equal populations of Tb-containing vesicles and DPA-containing vesicles

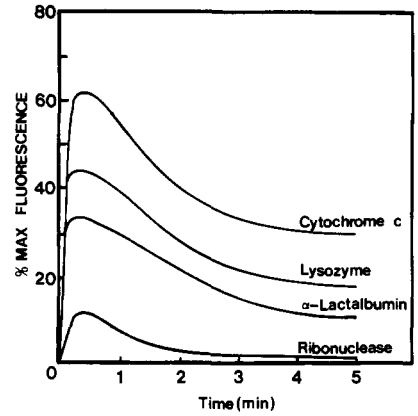

FIGURE 1: Fusion of PS/PE vesicles induced by several proteins at pH 3 (50 mM glycine, 11 mM HCl, 48 mM NaCl). In all cases the protein concentrations were 80 μg/mL, 18 °C. At time zero, 0.1 mL of α-LA solution was added into the spectrofluorometer cuvette, which contains 0.9 mL of vesicle suspension (0.05 mM P_i).

at pH 3. The percent maximum fluorescence values in this figure represent the fluorescence intensity during the fusion, divided by the fluorescence intensity when the vesicle-entrapped components were completely mixed by detergent treatment (Wilschut et al., 1980). These curves indicate that the proteins induce a fast fusion of PS/PE vesicles, followed by the dissociation of Tb–DPA complex due to the vesicle leakage. The fusion curves are similar to those of Ca^{2+}-induced fusion of PS vesicles (Wilschut et al., 1980). After the fusion was initiated, the percent maximum fluorescence values increased linearly for about 15 s and then decreased slowly.

An additional proof of the fusion came from the electron microscopic studies, which showed the enlargement of the vesicles. The diameter of monolamellar vesicles increased more than 10-fold after the addition of α-LA at low pH, and sometimes multiple lamellar liposomes could also be seen.

Although it is certain that these proteins induce fusion of PS/PE vesicles, it is not possible to correlate the values of percent maximum with the "extent" of fusion at very low pH because of two inherent problems in acidic media. The first is the decrease in the fluorescence intensity due to the dissociation of Tb–DPA complex following the protonation of DPA (Barela & Sherry, 1976). In order to improve the situation, Ellens et al. (1985) developed a new system of the monitoring fusion process in which one population of vesicles contains a fluorophore, ANTS, and another population of vesicles contains its quencher, DPX. When the contents of these two populations of vesicles are mixed upon fusion, the fluorescence is quenched. This method extends the range of quantitative fusion monitoring from pH 5 of the Tb–DPA system down to pH 4. Another potential problem at low pH is the possibility of fusion of vesicles induced by the protons themselves. It was found that large unilamellar PS vesicles fuse at pH 2 without any added fusogens (Ellens et al., 1985). In the present case of PS/PE vesicles, no change in percent maximum fluorescence was observed at pH 2 for 15 min without added protein. Also, there was no size difference between the vesicles at pH 7 and those produced at pH 2 as observed by an electron microscope. Therefore, we may conclude that the increase in percent maximum fluorescence even at this pH is solely due to the added protein. Thus, the increase in the initial rate of fusion with decreasing pH down from pH 6 is a real phenomenon at least qualitatively.

Figure 2 shows the initial rate of percent maximum fluorescence increase during the fusion as a function of α-LA concentration as well as pH. The initial rates were determined

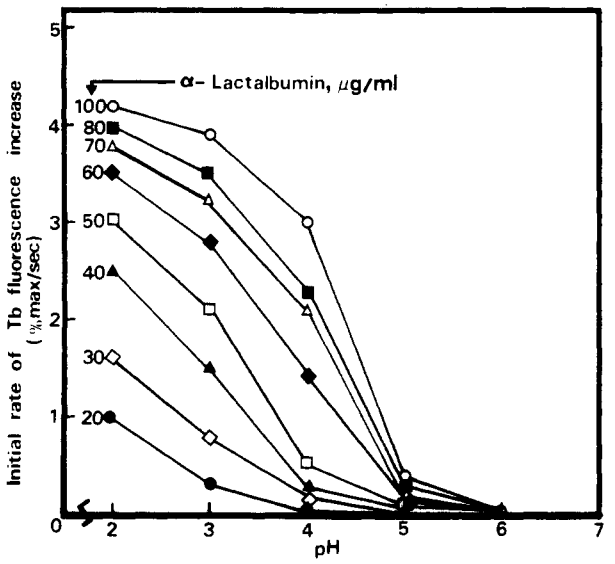

FIGURE 2: Dependences of initial rate of Tb fluorescence increase on pH upon fusion of PS/PE vesicles induced by various concentration of α-LA. The buffer solutions for pH of 2, 3, 4, and 5 have the same compositions as given in Figure 1. The pH 6 buffer contained 2 mM Mes and 80 mM NaCl while the pH 7 buffer contained 2 mM Tes, 2 mM L-histidine, and 100 mM NaCl. The fusion was initiated by adding various amounts of α-LA solution to make up the final concentrations as indicated in the figure, 18 °C.

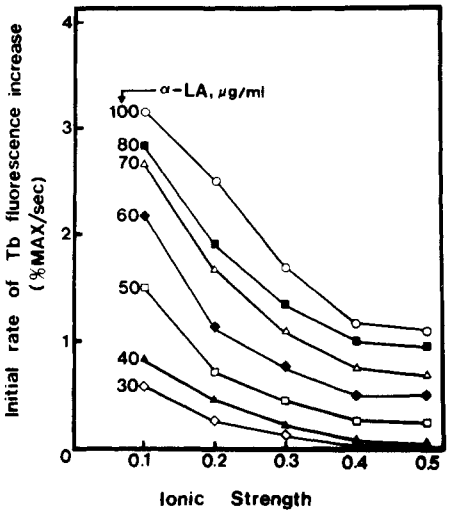

FIGURE 3: Effect of ionic strength on α-LA-induced PS/PE vesicle fusion at pH 3.0 (50 mM glycine, 11 mM HCl, 48 mM NaCl). The experimental condition was the same as in Figure 2 except for the fact that the ionic strength was increased by adding NaCl, 18 °C.

from the initial slopes of the curves represented in Figure 1. The increase in the initial rate with increasing α-LA concentration indicates that the α-LA really is the cause of the fusion. This figure demonstrates the increased fusion rate with decreasing pH. However, it should be emphasized that this gives only a qualitative trend in view of the problems with the Tb–DPA method in acidic media as discussed above. It is of interest to note that the curves here are similar to the profiles for the clathrin-induced fusion of vesicles (Hong et al., 1985).

In order to see the relative effect of electrostatic interaction on the fusion, the experiments were performed with increasing ionic strength (0.1–0.5) at pH 4, and the results are shown in Figure 3. The initial rate of fusion decreases with ionic strength, but it approaches a finite value for each α-LA concentration. This indicates that the charge–charge interaction is only partially responsible for the fusion. The fusion of the PS/PE vesicles induced by polylysine, on the other hand,

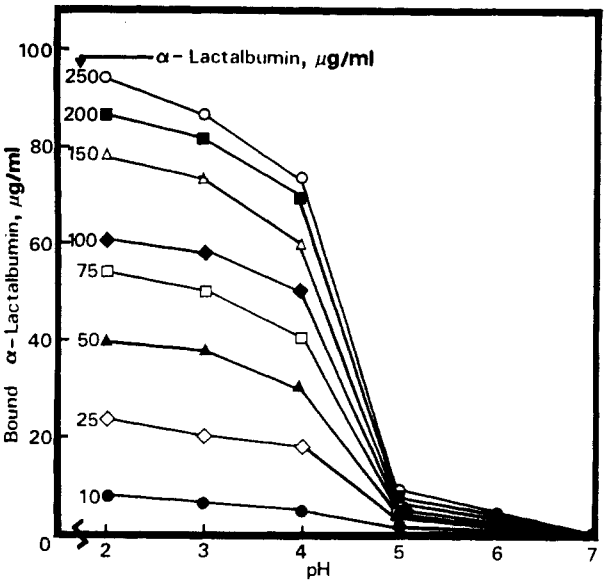

FIGURE 4: pH-dependent α-LA binding to PS/PE vesicles at several total protein concentrations. The buffer compositions for each pH is the same as in Figure 2. The concentration of the vesicle was fixed at 1 mM P_i/mL. See the text for details. The numbers in the figure represent the total α-LA concentration, 18 °C.

approached zero at high ionic strength (J. Kim and H. Kim, unpublished results). Therefore, it appears that nonpolar interaction is also involved in the α-LA-induced fusion of PS/PE vesicles at acidic pH.

α-LA Binding to PS/PE Vesicles. Figure 4 gives the pH dependency of the α-LA binding to PS/PE vesicles at several total protein concentrations. The binding isotherms obtained by replotting Figure 4 in terms of concentration for each pH are found to be hyperbolic, which indicates that there is no cooperativity between binding sites. It is very interesting to note that the binding profile in Figure 4 is similar to the fusion profile shown in Figure 2, suggesting a correlationship between these two events. Here again the binding increases with increasing acidity below pH 6, and there is practically no binding between pH 6 and pH 7. It should be kept in mind, however, that no quantitative comparison between the fusion and binding can be made here. First of all, the fusion data given in Figure 2 are only qualitative at low pH levels while binding can be regarded as quantitative. Second, the fusion process depicted in Figure 2 represents a kinetic process while Figure 4 gives the equilibrium binding property of the vesicles after the fusion process is over.

By determining the amount of α-LA released after neutralization of the vesicles, which had been incubated with the protein at acidic pH, it was found that the binding reaction is only partially reversible. When 1 mM P_i of vesicles was first incubated with 250 μg/mL of α-LA at pH 4 and then diluted with a pH 7 buffer solution, about 50% of bound protein was released. The release was slow and continued until about 15 h. From this point on there was no further release until after 48 h. When the same composition of vesicle–protein mixed solution that was incubated at pH 2 and pH 3 was brought to pH 7, about 7% and 23% of the bound α-LA were released, respectively. It is clear that the extent of the nonpolar interaction between the protein and PS/PE vesicles increases with decreasing pH. This irreversible binding was also demonstrated by the gel filtration of the α-LA–vesicle suspension, incubated first at low pH and brought to pH 7, through Sepharose 2B column. A major protein peak eluted with the phospholipid, and then a minor protein fraction followed. When the vesicle fraction with the attached protein was re-

Table I: Amino Acid Composition of α-LA Fragments Protected from Proteolytic Digestion of α-LA–Vesicle Mixture[a]

	trypsin		chymotrypsin		carboxypeptidase Y	
amino acid	residue/mol of α-LA segment	nearest integer	residue/mol of α-LA segment	nearest integer	residue/mol of α-LA segment	nearest integer
Ala	0.99	1	1.91	2	3.02	3
Arg	0.02	0	0.04	0	1.12	1
Asn	nd		nd		nd	
Asp	2.10	2	3.09	3	7.83	8
Cys	nd		nd		nd	
Gln	nd		nd		nd	
Glu	0.04	0	1.10	1	6.92	7
Gly	1.11	1	1.02	1	5.82	6
His	0.95	1	1.04	1	3.04	3
Ile	2.97	3	3.11	1	7.84	8
Leu	4.09	4	4.92	5	10.33	10
Lys	3.96	4	4.94	5	10.23	10
Met	1.02	1	0.93	1	1.03	1
Phe	1.06	1	0.02	0	4.03	4
Pro	nd		nd		nd	
Ser	0.03	0	0.98	1	7.01	7
Thr	1.07	1	0.92	1	7.20	7
Trp	nd		nd		nd	
Tyr	1.16	1	1.06	1	3.82	4
Val	2.03	2	1.97	2	6.03	6

[a] The vesicles and α-LA were allowed to interact at pH 3 and then brought to pH 7.3 prior to the proteolytic digestion.

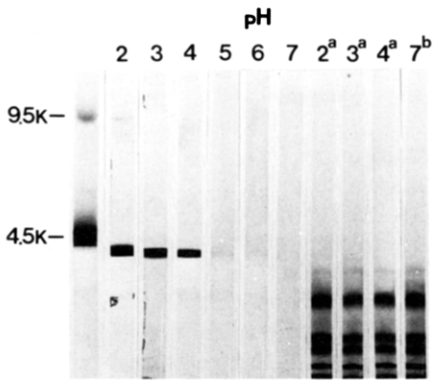

FIGURE 5: SDS–PAGE of protected segment of α-LA after trypsin digestion of α-LA–vesicle suspension that had been incubated at the pH indicated and then brought to pH 7.5 before the trypsin treatment at 37 °C. The procedure of the extraction of this segment is given in the text. The marker proteins used are as follows: 9.5K unit; the larger fragment after the cyanogen bromide treatment of α-LA and 4.5K unit; the smaller fragment of the cyanogen bromide treatment of α-LA. (a) Control patterns obtained by incubating α-LA at pH 2, 3, and 4 in the absence of PS/PE vesicles and then by digesting with trypsin at 37 °C after bringing the pH to 7.3. (b) α-LA was digested at 37 °C and pH 7 in the absence of vesicles without prior exposure to acidic media.

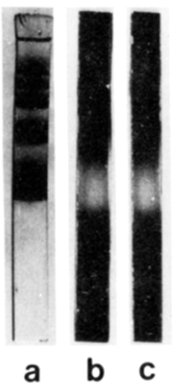

FIGURE 6: SDS–PAGE patterns of α-LA fragment labeled with dansyl chloride: (a) Coomassie brilliant blue stained trypsin digests of α-LA–vesicle mixture; (b) same digest showing the dansyl-labeled polypeptide; (c) dansyl-labeled segment of α-LA extracted from the vesicles after trypsin digestion of α-La–vesicle mixture followed by the removal of the soluble polypeptide fragments.

cycled through the column, no minor peak appeared, indicating that no further release of the protein from the vesicles occurred.

Segment of α-LA Penetrating into the Membrane. Figure 5 shows the results of SDS–PAGE of the extracted segment of α-LA after the trypsin treatment of this protein, which had been incubated with PS/PE vesicles at several pH values. A protein band with estimated molecular weight of approximately 4000 was observed for each pH value. No band with this apparent molecular weight is seen in the control patterns, which were obtained by incubating the α-LA at pH 2, 3, and 4 in the absence of PS/PE vesicles and then digesting with trypsin at 37 °C after bringing the pH to 7.3. These control patterns are identical with the pattern obtained by digesting α-LA at pH 7 without prior exposure to acidic media. It is clear that the same segment is protected from trypsin digestion when the protein is bound to the vesicles at several pH values and that the degree of protection is stronger at low pH. It is most likely that a portion of this M_r 4000 unit is buried into the hydrophobic core of the bilayer.

Essentially the same result was obtained when chymotrypsin was used except the molecular weight of the protected segment of α-LA was about 5000. Table I gives the amino acid compositions of these segments obtained with α-LA being incubated with vesicles at pH 3. Triplicate repetitions of the experiment gave the same results. Table I also gives the amino acid composition of the protected part of the α-LA after the carboxypeptidase Y treatment. The compositions of the segments that were protected from tryptic digest after being incubated with vesicles at pH 2 and 4 were found to be the same as that given in Table I. This indicates that an identical segment is protected from tryptic digestion at all the pH values studied.

The hydrophobic labeling of vesicle-bound α-LA with dansyl chloride gave further support to the possibility of penetration of a segment of the protein into the hydrophobic interior of PS/PE vesicles. Figure 6a shows the Coomassie blue stained SDS–PAGE patterns of vesicle-bound α-LA, which was hydrophobically labeled and subsequently treated with trypsin. Figure 6b is the fluorescence emission by the same electrophoresis pattern. Of the several fragments from tryptic treatment, only one major band is labeled by dansyl chloride. Figure 6c shows the fragment that was extracted from the

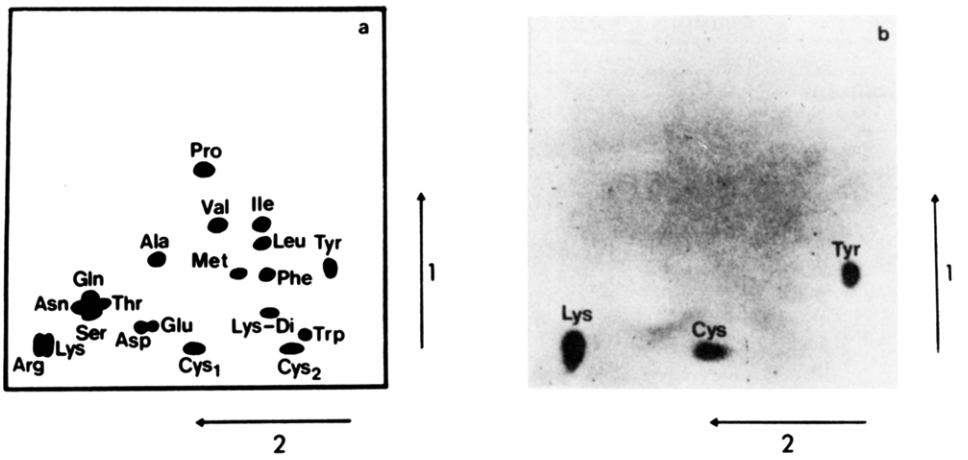

FIGURE 7: TLC patterns of dansylated amino acids obtained by 6 N HCl treatment of labeled segment of α-LA. (a) Dansylated standard amino acids. (b) Dansylated amino acids obtained from the hydrophobically labeled α-LA. (1) Developing solvent, H_2O–formic acid (50:1.5 v/v). (2) Developing solvent, benzene–acetic acid (9:1 v/v). (Cys_1) Dansyl-L-cysteic acid. (Cys_2) *N*,*N*′-Didansyl-L-cysteine. (Lys-Di) Didansyl-L-lysine.

vesicles after the trypsin treatment and then labeled. It is clear that this fragment has an identical molecular weight with the dansyl group labeled main fragment shown in Figure 6b. Since the labeling procedure used in obtaining Figure 6b is such that only the segment present within the bilayer is labeled, one may conclude that a segment of α-LA is really buried inside the bilayer. The same results were obtained when the vesicle-bound α-LA was labeled with dansyl chloride and then incubated with trypsin.

Figure 7b shows the two-dimensional TLC of the dansyl-labeled amino acids obtained by the 6 N HCl treatment of the fragment protected from trypsin digestion. This may be compared with the TLC pattern of standard dansyl-labeled amino acids shown in Figure 7a. The amino acid residues in the polypeptide that can be dansylated are lysine, tryosine, cysteine, and histidine. The spot for the dansylated histidine is missing from Figure 7a because the standard kit obtained from Sigma (DNA-L-23) did not include this particular standard. However, by comparing the R_f values of this compound in the literature (Niederwieser, 1972) with those of Tyr, Lys, and Cys, one can deduce the position of His relative to others. This predicts the position of His in Figure 7b to be above and slightly left of the position held by Tyr, clearly separated from it. It can be seen that among these four amino acid residues in the trypsin-resistant fragment given in Table I, histidine is the only residue that was not labeled, indicating that this residue is situated outside of the bilayer.

Discussion

The main points of interest from this investigation are that the pH-dependent fusion of phospholipid vesicles induced by α-LA parallels with the pH-dependent binding of this protein to the vesicles and that the irreversible binding, with one segment of this protein going into the bilayer, is involved in these events. Although the techniques of monitoring the fusion process are such that a quantitative correlationship between this and the binding cannot be made, there is no doubt that the fusion by α-LA is more extensive at low pH. Thus, it is possible that the penetration of α-LA segment into PS/PE vesicles may be responsible for the fusion.

It appears that the binding of α-LA to the vesicles is closely related to the structural state of this protein. The mean residue ellipticity of this protein at 272 nm, $[\theta]_{272}$, in neutral media has a finite negative value, showing that one or more of the aromatic amino acid side chains is located in an asymmetric environment (Dolgikh et al., 1981). As the pH is lowered, however, the $[\theta]_{272}$ value is increased approaching zero, which makes the $[\theta]_{272}$ vs. pH profile resemble those of Figure 2 and Figure 4. The collapse of the negative ellipticity may mean the exposure of the aromatic group to the aqueous medium, or they simply exist inside the protein but have symmetrical environment. From the following two observations, it is more likely that the aromatic groups and possibly other hydrophobic groups as well are exposed to the surface at low pH. When α-LA is added to the 1,8-anilinonaphthalene at acidic pH, there is an increase in the extrinsic fluorescence intensity (Kronman et al., 1966), and this protein has a pronounced tendency to form oligomers at low pH (Kronman & Andreotti, 1964). From the observation that the acrylation of lysyl side chains brings about changes in CD spectra identical with the change induced by acidification, Kronman et al. (1974) concluded that the latter change is caused by the protonation of carboxylate side chains, which results in the breakdown of ion pair bonds between lysyl α-amino groups and carboxylate groups. This may explain the steep changes that take place below the isoelectric point of this protein in $[\theta]_{272}$, binding of α-LA to PS/PE vesicles and the fusion of these vesicles induced by this protein.

When α-LA in an acidic medium, with its exposed hydrophobic group and positive net charge, is in contact with negatively charged phospholipid vesicles, there may be an electrostatic interaction between the positively charged lysyl amino side groups and negatively charged head groups of PS and also an interaction between the hydrophobic surface of this protein and the acyl chains of the phospholipids. The involvement of the hydrophobic interaction was suggested by Hanssens et al. (1980) who observed a large enthalpy change and intrinsic fluorescence change when phosphatidylcholine (PC) and α-LA were brought together under acidic conditions. The patterns of increase in the enthalpy of binding and the increase in the extent of binding with lowering pH are similar. Our results also generally support the notion that hydrophobic interaction is involved in binding of α-LA to PS/PE vesicles. First, the binding and fusion in acidic pH do occur under high ionic strength condition where the electrostatic effect is largely suppressed. Second, once the protein binds to the vesicles in acidic pH, a large portion of it cannot be dislodged from the vesicles under the condition where no binding occurs. This kind of very strong binding usually involves hydrophobic interaction.

A suggestion of the penetration of an α-LA segment into the PS/PE vesicle bilayer comes from the proteolytic digestion

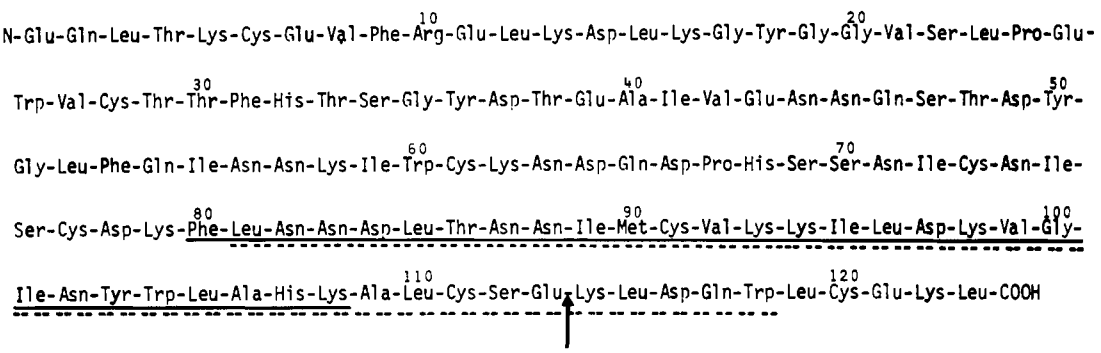

FIGURE 8: Complete amino acid sequence of bovine α-LA (Brew et al., 1970) showing segments inaccessible to various proteolytic enzymes when this protein is present with PS/PE vesicles: (−−) segment protected from the chymotrypsin digestion; (—) segment protected from the trypsin digestion. The arrow indicates the last bond broken by carboxypeptidase Y.

of vesicle-bound protein. The protected segments from the digestion were analyzed for the amino acid composition, and the results were given in Table I. By taking advantage of the fact that the single methionine residue in the intact α-LA is also present in these segments, we were able to deduce the segments of the whole amino acid sequence (Brew et al., 1970), and the results are shown in Figure 8. Only the segments shown here can have the amino acid compositions given in Table I. The calculated molecular weights of the protected fragments from the tryptic and chymotryptic digestions are 3488 and 4605, respectively. These values are close to the approximate molecular weights obtained from SDS–PAGE of these fragments. Figure 8 also indicates the position of the sequence up to which the cleavage of the C-terminal sequence takes place by the carboxypeptidase Y treatment. The segment of the α-LA protected from all these enzymes is from 81 (Leu) to 108 (Lys), and a portion of this may be buried inside the bilayer.

More definite support of the insertion of a protein segment into the bilayer is provided by the hydrophobic labeling with dansyl chloride. Figure 6 is the result of labeling by the dansyl chloride that was already present in the hydrophobic interior of the bilayer, and this is a strong indication that the segment that is protected from the proteolysis is indeed buried within the bilayer. The TLC experiment following the digestion by 6 N HCl of the hydrophobically labeled segment showed that His-107 is located outside of the bilayer. Therefore, a segment within the boundary of Leu-81 and Ala-106 appears to be buried within the bilayer. Since both the N-terminal and the C-terminal ends are susceptible to the digestion, it is clear that these terminal segments are situated outside of the vesicles and that the segment goes into the membrane forming a loop. And increasing number of this loop of the fixed segment of the α-LA goes into the hydrophobic interior of the membrane as pH is decreased.

It is generally assumed that the portion of an integral protein that goes into the membrane is highly hydrophobic, and in fact, various methods have been proposed for predicting these segments of the proteins (Kyte & Doolittle, 1982; Argos et al., 1982; Eisenberg et al., 1984; Kuhn & Leigh, 1985). For the case of the segment of α-LA that penetrates into the bilayer, however, the profiles obtained by using these methods are not particularly hydrophobic. The two-dimensional helical wheel diagram (Schiffer & Edmundson, 1967) of the segment of sequence from amino acid 85 to amino acid 106 showed that the hydrophobic residues are situated mainly on one side of the helix surface. This suggests the possibility that this segment folds to form a helical loop and the hydrophobic sides of this loop face the acyl chains of the phospholipid in the bilayer. In order to pursue this point further, we are currently investigating the topology of this segment in the bilayer by the method of hydrophobic labeling with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) and amino acid sequencing following the protocol of Brunner et al. (1985).

Since an α-helix that fully spans the phospholipid bilayer requires about 25 amino acid residues (Engelman et al., 1980), the loop segment of α-LA goes into the bilayer only part of the way if it forms α-helix structure. This may mean that the protein loop disturbs only the outer half of a bilayer.

The qualitative observation that both fusion rate and the extent of binding as well as the probability of one particular segment of α-LA penetrating into vesicles increases with decreasing pH suggests a possibility that the penetration of the bilayer by a loop of the α-LA segment is responsible for the fusion of PS/PE vesicle. Unfortunately, this is only a tentative conclusion because the binding and the related studies provide information on the state of α-LA after the fusion process is completed while the Tb–DPA monitoring indicates the ongoing fusion process. A better picture may be obtained by performing the kinetic study of the binding reaction and comparing it with the fusion kinetics, but the ultracentrifugation method employed here cannot provide this kind of information.

The studies on the fusion induced by some other proteins also point to the penetration by the segment of proteins into the hydrophobic interior of the vesicle following the binding of the protein through its exposed hydrophobic surface. For some proteins, such as lysin (Hong & Vacquier, 1986), there already is an exposed hydrophobic surface, but for others the hydrophobic surface is exposed as a result of either proteolysis, as in the case of Sendai virus protein (White et al., 1983), or low pH, as in the cases of bovine serum albumin (Schenkman et al., 1981) and its fragment (Garcia, et al., 1984), insulin (Farias et al., 1985), diphtheria toxin (Cabiaux et al., 1984) and tetanus toxins (Cabiaux et al., 1985), clathrin (Hong et al., 1985), and Semliki Forest and influenza virus (White et al., 1983). Although there has been no demonstration of the penetration of a protein segment into the bilayer for these proteins, it is interesting that many of these proteins can form potentially amphiphilic α-helix segments. Thus, for the case of bovine serum albumin fragment P-9 (amino acids 307–385), there are three possible amphiphilic α-helix segments (Garcia et al., 1984). For the case of diphtheria toxin, fragment B has an amphiphilic segment (Cabiaux et al., 1984; Lambotte et al., 1980), the structure of which is very similar to that of melittin (Terwillinger & Heisenberg, 1982). Melittin also induces fusion of model membranes (Morgan et al., 1983; Eytan & Almany, 1983). Therefore, the penetration of amphiphilic α-helices of the protein segments may be the cause of the fusion induced by proteins. Although the results of the present study suggest that the fusion of PS/PE vesicles may

be caused by the penetration of a loop within the bilayer of a segment of α-LA about half-way into the bilayer at low pH, these observations are not sufficient to give a picture of the molecular mechanism of fusion. A detailed structure of the penetrating loop within the bilayer and the influence of this segment on the properties of the phospholipid phase must be clarified first, and these are currently being investigated in our laboratory.

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Registry No. RNase, 9001-99-4; cytochrome *c*, 9007-43-6; lysozyme, 9001-63-2.

REFERENCES

Argos, P., Rao, J. K. M., & Hargrave, P. A. (1982) *Eur. J. Biochem. 128*, 565–575.

Barela, T. D., & Sherry, A. D. (1976) *Anal. Biochem. 71*, 351–357.

Bentz, J., Düzgunes, N., & Nir, S. (1983) *Biochemistry 22*, 3320–3330.

Blewitt, M. G., Chung, L. A., & London, E. (1985) *Biochemistry 24*, 5458–5464.

Blumenthal, R., Henkart, M., & Steer, C. J. (1983) *J. Biol. Chem. 258*, 3409–3415.

Brew, K., Castellino, F. J., Vanaman, T. C., & Hill, R. L. (1970) *J. Biol. Chem. 245*, 4570–4582.

Brunner, J., Franzusoff, A. J., Luscher, B., Zugliani, C., & Semenza, G. (1985) *Biochemistry 24*, 5422–5430.

Cabiaux, V., Vandenbranden, M., Falmagne, P., & Ruysschaert, J.-M. (1984) *Biochim. Biophys. Acta 775*, 31–36.

Cabiaux, V., Lorge, P., Vandenbranden, M., Falmagne, P., & Ruysschaert, J. M. (1985) *Biochem. Biophys. Res. Commun. 128*, 840–849.

Deamer, D., & Bangham, A. D. (1976) *Biochim. Biophys. Acta 443*, 629–634.

Dolgikh, D. A., Gilmanshin, R. I., Brazhnikow, E. V., Bychkova, V. E., Semisotnow, G. V., Venyaminov, S. Yu., & Ptitsyn, O. B. (1981) *FEBS Lett. 136*, 311–315.

Dolgikh, D. A., Kolomiets, A. P., Bolotina, I. A., & Ptitsyn, O. B. (1984) *FEBS Lett. 165*, 88–92.

Eisenberg, D., Schwarz, E., Komaromy, M., & Wall, R. (1984) *J. Mol. Biol. 179*, 125–142.

Ellens, H., Bentz, J., & Szoka, F. C. (1985) *Biochemistry 24*, 3099–3106.

Engelman, D. M., Henderson, R., McLachlan, A. D., & Wallace, B. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2023–2027.

Eytan, G. D., & Almary, T. (1983) *FEBS Lett. 156*, 29–32.

Farias, R. N., Vinals, A. L., & Morero, R. D. (1985) *Biochem. Biophys. Res. Commun. 128*, 68–74.

Gad, A. E., Silver, G. L., & Eytan, G. A. (1982) *Biochim. Biophys. Acta 659*, 124–132.

Garcia, L. A. M., Araujo, P. S., & Chaimovich, L. (1984) *Biochim. Biophys. Acta 772*, 231–234.

Hanssens, I., Houthuys, C., Heereman, W., & Van Cauwelaert, F. H. (1980) *Biochim. Biophys. Acta 602*, 539–557.

Hong, K., & Vacquier, V. D. (1986) *Biochemistry 25*, 543–549.

Hong, K., Yoshimura, R., & Papahadjopoulos (1985) *FEBS Lett. 191*, 17–23.

Kronman, M. J., & Andreotti, R. E. (1964) *Biochemistry 3*, 1145–1151.

Kronman, M. J., Blum, R., & Holms, L. G. (1966) *Biochemistry 5*, 1970–1978.

Kronman, M. J., Holmes, L. G., & Robbins, F. M. (1974) *J. Biol. Chem. 246*, 1909–1921.

Kuhn, L. A., & Leigh, J. S., Jr. (1985) *Biochim. Biophys. Acta 828*, 351–361.

Kuwajima, K., Nitta, K., & Sugai, S. (1975) *J. Biochem. (Tokyo) 78*, 205–211.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Lambotte, P., Falmagne, P., Capiau, C., Zanen, J., Ruysschaert, J. M., & Dirkx, J. (1980) *J. Cell Biol. 87*, 837–840.

Lucy, J. A. (1984) *FEBS Lett. 166*, 223–231.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.

Morgan, G. D., Williamson, H., Fuller, S., & Hudson, B. (1983) *Biochim. Biophys. Acta 732*, 668–674.

Niederwieser, A. (1972) *Methods Enzymol. 25*, 60–99.

Ohgushi, M., & Wada, A. (1983) *FEBS Lett. 164*, 21–24.

Papahadjopoulos, D., Poste, G., & Vail, W. J. (1979) *Methods Membr. Biol. 10*, 1–121.

Schenkman, S., Arauje, P. S., Kijkman, R., Quina, F. H., & Chaimovich, H. (1981) *Biochim. Biophys. Acta 649*, 633–641.

Schiffer, M., & Edmundson, A. B. (1967) *Biophys. J. 7*, 121–135.

Sikorski, A., & Daczynska, R. B. (1982) *Biochim. Biophys. Acta 690*, 302–305.

Steer, C. J., Kausner, R. D., & Blumenthal, R. (1982) *J. Biol. Chem. 257*, 8533–8540.

Terwillinger, R. C., & Heisenberg, D. (1982) *J. Biol. Chem. 257*, 6010–6022.

Vaskowsky, R. E., Kostetsky, E. Y., & Vasendin, I. M. (1975) *J. Chromatogr. 114*, 129–141.

White, J., Kieliean, M., & Helenius, A. (1983) *Q. Rev. Biophys. 16*, 151–195.

Wilschut, J., Düzgunes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.