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The Role of Protein Charge in Protein–Lipid Interactions. pH-Dependent Changes of the Electrophoretic Mobility of Liposomes through Adsorption of Water-Soluble, Globular Proteins

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ABSTRACT: The role of electrostatics in the adsorption process of proteins to preformed negatively-charged (phosphatidylcholine/phosphatidylglycerol) and neutral (phosphatidylcholine) liposomes was studied. The interaction was monitored at low ionic strength for a set of model proteins as a function of pH. The adsorption behavior of trypsin inhibitor (pI = 4.6), myoglobin (pI = 7.4), ribonuclease (pI = 9.6), and lysozyme (pI = 10.7) with preformed liposomes was investigated, along with changes in the electrophoretic mobility of liposomes through the adsorption of charged proteins. Mean protein charge was determined by acid/base titration. Significant adsorption of the proteins to negatively-charged liposomes was only found at pH values where the number of positive charge moieties exceeds the number of negative charge moieties on the protein by at least three charge units. Negligible adsorption to liposomes composed of zwitterionic lipids was observed in the pH range tested (4–9). The absolute value of the electrophoretic mobilities of negatively-charged, empty liposomes decreased after adsorption of positively-charged proteins. With increasing protein to phospholipid ratio, the drop in the electrophoretic mobility leveled off and reached a plateau; protein adsorption profiles showed a similar shape. Analysis of the data demonstrated that neutralization of the liposome charge due to the adsorption of the positively-charged proteins is the controlling factor in their adsorption. The plateau level reached depended on the type of protein and the pH of the incubation medium. This pH dependency could be ascribed to the mean positive charge of the protein. The effective charge of myoglobin, ribonuclease, and lysozyme (defined as the number of phosphatidylglycerol groups neutralized by one adsorbed protein molecule) was calculated from the charge differences between empty liposomes and protein-coated liposomes using the Gouy–Chapman theory. For lysozyme and myoglobin, an excellent correlation was found between the effective charge and the mean protein charge.

The interaction between proteins and liposomes can be mediated by electrostatic and/or hydrophobic forces or by covalent bonds. Intrinsic membrane proteins can be embedded within the liposomal bilayer through hydrophobic interactions between the hydrophobic parts of the protein and the hydrocarbon chains of the phospholipids (PL).[1] The association of water-soluble proteins with liposomes is thought to be primarily dependent on the overall electrostatic, Coulombic attraction between the membrane- and protein-associated charges (Kimelberg & Papahadjopoulos, 1971; Jain & Zakim, 1987). However, others have reported that the association was mainly dependent on hydrophobic interactions (Law et al., 1986; Cevc et al., 1990; Maitani et al., 1990). In this case, the charge characteristics of the protein played only a minor role. The relative significance of these interactions is difficult to assess; it may vary from case to case. The physicochemical properties of the proteins, such as size, shape, charge, the presence of hydrophobic patches on the surface of the protein, and the conformation of the protein (e.g., the formation of amphipathic α-helices), as well as the liposome characteristics and the incubation conditions can affect the binding characteristics (Cruz et al., 1989; Parente et al., 1990).

Several studies have shown that the association of positively-charged proteins with negatively-charged membranes is enhanced compared to the association with neutral membranes (Kimelberg, 1976; Yoshimura & Sone, 1987; Seelig & Macdonald, 1989). Low molecular weight synthetic polypeptides have been employed to unravel the role of the sign and the number of charges, the charge distribution, and the overall hydrophobicity of the polypeptide in the polypeptide–membrane interaction (Surewicz & Epand, 1984; Jacobs & White, 1989; De Kroon et al., 1990; Mosior & McLaughlin, 1992a,b). De Kroon et al. (1990) showed that the affinity of positively-charged mono- and divalent peptides for cardiolipin vesicles is determined by both hydrophobic and electrostatic interactions while the main determinant for zwitterionic membranes is the overall hydrophobicity of the peptide. The distribution of the charged moieties in divalent, positively-charged peptides had little influence on the affinity of the peptide for cardiolipin. Recently, the membrane association of a larger polypeptide, melittin (26 amino acids), has been evaluated in a quantitative way (Kuchinka & Seelig, 1989; Stankowski & Schwarz, 1990; Beschiaschivili & Seelig, 1990). Although the binding of melittin to liposomes is due to hydrophobic effects, electrostatic

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[1] Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; PL, phospholipid(s).

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Table I: Some Physical Parameters of the Model Proteins[a]

protein	molecular weight[b]	pI	dimensions (nm)	no. of S–S bonds	% nonpolar residues[b]	no. of α-helices	no. of β-strands
myoglobin	17600	6.9, 7.4	4.4 × 4.4 × 2.5	0	50	8	0
ribonuclease	13700	9.6	3.8 × 2.8 × 2.2	4	51	3	7
lysozyme	14300	10.7	4.5 × 3.0 × 3.0	4	40	4	5
trypsin inhibitor	20100	4.6		2	49	0	12

[a] Source: Protein Data Bank and Dautrevaux et al. (1969), Koide and Ikenaka (1973), Sweet et al. (1974), Reghetti and Caravaggio (1976), McLachlan (1979), and Wei et al. (1990). [b] Calculated from the amino acid composition.

interactions increase the concentration of peptide adjacent to the membrane and thus enhance the binding. These electrostatic effects were analyzed with the Gouy–Chapman theory. The net molecular charge calculated for melittin was substantially lower than that predicted on the basis of its molecular structure. For high molecular weight proteins that contain several positively- and negatively-charged groups, either homogeneously distributed or regionally enriched, the situation is even more complex. Little detailed information is available at the present time about the role of the protein charge in the association with liposomes.

Therefore, in the present study, the role of protein charge on association with preformed, negatively-charged liposomes composed of phosphatidylcholine/phosphatidylglycerol (PC/PG) was investigated for a set of model proteins. The ionic strength was kept low to promote electrostatic interactions. Protein adsorption, electrophoretic mobility, and liposome size were measured under identical conditions as a function of the pH. Mean protein charge was assessed by acid/base titration. Myoglobin, ribonuclease, lysozyme, and trypsin inhibitor were used as model proteins because they are single-domain globular proteins with known three-dimensional structures. Some of their properties relevant for this study are summarized in Table I. They are similar in size and dimension, but they differ in other physicochemical properties. This will help to reduce the effect of molecular size on the association with liposomes (Williams & Weiner, 1989). Furthermore, the surface hydrophobicity of these proteins is considered to be relatively low in the native state. For instance, the retention time of these proteins on hydrophobic interaction chromatography columns is short compared to the retention time of bovine serum albumin (Kato et al., 1983; Heinitz et al., 1988), a protein that often has been used in protein–lipid interaction studies (Cruz et al., 1989; Cevc et al., 1990). Lysozyme, ribonuclease, and myoglobin show no major conformational changes between pH 4 and pH 10 (Tanford, 1962). Therefore, by varying the pH within this range, the effect of charge on protein–liposome interactions can be studied for a protein without major changes in their conformation. Charge differences between empty liposomes and liposomes with myoglobin, ribonuclease, or lysozyme adsorbed on their surface were calculated from the electrophoretic mobility data using the Gouy–Chapman theory. A satisfactory correlation was found between the calculated effective charge, defined as the number of PG molecules neutralized by one adsorbed protein molecule, and the mean protein charge, determined on the basis of acid/base titration, of lysozyme and myoglobin.

MATERIALS AND METHODS

Materials. Myoglobin (horse heart, 95–100% purity), lysozyme (hen egg white, 3 × crystallized), and trypsin inhibitor (soybean, type I-s, chromatographically purified) were obtained from Sigma Chemical Co. (St. Louis, MO). Ribonuclease (bovine pancreas, analytical grade) was obtained from Boehringer Mannheim (FRG). Egg L-α-phosphatidylcholine type V-E (PC) was purchased from Sigma Chemical Co. Egg phosphatidylglycerol (PG) was a gift from Nattermann GmbH (Cologne, FRG). Amberlite IR-120, IRA-400, and IRA-402 were obtained from BDH Chemicals Ltd. (Poole, U.K.). IR-120 was converted to the hydrogen form and IRA-400 and IRA-402 to the hydroxide form. All other chemicals were of analytical grade.

Protein Acid/Base Titration. The proteins were titrated as described earlier (Janssen & Van Wilgenburg, 1978; Bos et al., 1989). Proteins were desalted by gel filtration on PD-10 columns (Pharmacia, Uppsala, Sweden) and subsequently by passing the protein solution over a mixed-bed cat/anion-exchange column (Amberlite IR-120 and IRA-400 for myoglobin and trypsin inhibitor; Amberlite IR-120 and IRA-402 for ribonuclease and lysozyme). Titrations were performed at 25 °C in a total volume of 3 mL, containing 0.01 M KCl and a protein concentration of about 2 mg/mL. For trypsin inhibitor, 6 mL of a 1 mg/mL protein solution was used, as at higher concentrations the protein precipitated. Lysozyme precipitated during deionization as well, but after addition of 0.01 M KCl, the precipitate dissolved. The starting pHs (±standard deviations) of the deionized protein solutions were 4.92 ± 0.03 (n = 5), 7.13 ± 0.04 (n = 8), 9.71 ± 0.05 (n = 7), and 10.30 ± 0.06 (n = 5) for trypsin inhibitor, myoglobin, ribonuclease, and lysozyme, respectively. From the starting pH, the proteins were titrated to pH 10 by addition of 10-μL aliquots of an 0.025 M KOH solution. Subsequently, proteins were titrated to pH 3–4 with 0.025 M HCl. From the acid/base titration experiments, the mean protein charge that is due to the binding of protons (Z_H) can be calculated.

Preparation of Liposomes. Negatively-charged liposomes were prepared from PC and PG at a molar ratio of 9:1. Lipids were dissolved in a chloroform/methanol (9:1) mixture and evaporated to dryness in a rotary evaporator. The lipid film was evacuated for at least 1 h. Glass beads and hydration medium were added to the lipid film to reach a final PL concentration of 40 μmol/mL. The hydration media consisted of 0.01 M sodium acetate (in the pH range 3–6) or of 0.01 M Tris (in the pH range 7–9) or of 0.01 M glycine (pH 10) adjusted to the desired pH by adding HCl or NaOH. The ionic strength was kept constant at 0.010 ± 0.001 M by addition of NaCl. The film was hydrated by hand-shaking at room temperature. Liposomes were sized by repeated extrusion sequentially through polycarbonate membrane filters (Nucleopore; Costar, Cambridge, MA) with pore diameters of 0.4 and 0.2 μm under nitrogen pressure up to 0.8 MPa. Jousma et al. (1987) showed that under these conditions mainly unilamellar vesicles are obtained. Lipid phosphate of the final preparation was determined by the colorimetric method of Fiske and Subbarow (1925). Liposome dispersions were stored at 4 °C under nitrogen and used within 1 week. Liposomes prepared at pH 3 or at pH 10 were used within 1 day to prevent the presence of hydrolysis products in the liposome dispersions during the experiments. Neutral liposomes were prepared from PC in a similar manner.

Assessment of the Adsorption of Proteins to Liposomes. Liposomes were incubated with protein at room temperature (22 ± 2 °C) at a final PL concentration of 5.0 μmol/mL. The electrophoretic mobility of liposomes in the presence of protein (measured at pH 4, 7.3, and 9) did not change between 2 and 6 h of incubation with protein. Therefore, an incubation time of 2 h was chosen for all experiments. To prevent desorption of proteins from liposomes, electrophoretic mobility measurements and the removal of nonadsorbed protein were performed without dilution.

The removal of free, nonadsorbed protein was performed by centrifugation of the liposome dispersion at 200000*g* for 1 h. The protein concentration in the supernatant was determined by absorbance measurements at 280 nm and/or by the method of Lowry et al. (1951). The amount of protein adsorbed to the liposomes can be calculated from the difference between the protein concentration of the supernatant of the liposome dispersion with protein and that of a protein solution without PL treated identically as the protein-containing liposome dispersion.

Electrophoretic Mobility and Other Analytical Assays. Mobility of the particles was measured in a PC-4 cell with a Malvern zeta-sizer 2C unit (Malvern Ltd., Malvern, U.K.) at a temperature of 25 °C. The mean particle size was determined by dynamic light scattering with a Malvern 4700 system using a 25-mW He–Ne laser and the Automeasure version 3.2 software (Malvern Ltd.). The viscosity and refractive index values of pure water were used for these measurements. The distribution of particle sizes was quantitated using the polydispersity index. This index ranges from 0.0 for entirely monodisperse up to 1.0 for a completely polydisperse dispersion. Atomic coordinates for myoglobin, ribonuclease, and lysozyme were obtained from the Protein Data Bank (Brookhaven National Laboratory).

RESULTS

Determination of Mean Protein Charge in the Absence of Lipids. Acid/base titrations of myoglobin, ribonuclease, lysozyme, and trypsin inhibitor were performed in order to determine the mean protein charge that resulted from the binding of protons (Z_H) (Figure 1). The titration curves were similar to those published earlier in the literature (Tanford & Wagner, 1954; Tanford & Hauenstein, 1956; Janssen et al., 1972). A biphasic titration curve was found for myoglobin with a transition point at pH 4.0. A concomitant reduction of the Soret absorbance of myoglobin was observed (data not shown), which implies that the protein unfolds below pH 4.0. Trypsin inhibitor was soluble in 10 mM KCl solution between pH 4.6 and 10, but precipitated at pH values lower than 4.6. Water, hydrogen, and hydroxyl ions appeared able to react with the acidic and basic groups. However, the mean protein charge of precipitated trypsin inhibitor may deviate from that of soluble trypsin inhibitor. In adsorption and electrophoretic mobility experiments, which were performed at a concentration of 1.0 mg/mL buffer (see below), trypsin inhibitor was soluble at all pH values tested. At pH 4, the Z_H values of myoglobin, ribonuclease, and lysozyme were approximately equal and amounted to +12 to +14. At each pH value above pH 4, lysozyme showed the highest Z_H value of the four proteins, followed by ribonuclease, myoglobin, and finally trypsin inhibitor.

Adsorption of Proteins to Liposomes. The adsorption of myoglobin, ribonuclease, lysozyme, and trypsin inhibitor to preformed empty liposomes composed of PC and PC/PG (9:1) was measured at pH 4, 7.3, and 9. The mean vesicle size of PC and PC/PG liposomes was 0.24 and 0.18 μm (polydispersity index of 0.3 and 0.1–0.2), respectively. The protein to PL ratio was fixed at 200 μg of protein/μmol of PL (corresponding to 10–15 nmol of protein/μmol of PL), and the ionic strength was kept at 0.01 M. The results depicted in Figure 2 show that the basic proteins (ribonuclease and lysozyme) adsorbed substantially to PC/PG liposomes at all three pH values tested. However, the pH dependence of the adsorption with liposomes differed. At pH 4, the adsorption capacity of the liposomes for lysozyme and ribonuclease was about 60 μg of protein/μmol of PL. At pH 9, the adsorption of lysozyme to liposomes almost doubled, whereas the adsorption of ribonuclease was similar at pH 4 and 9. Significant adsorption of myoglobin and trypsin inhibitor to negatively charged liposomes occurred only at pH 4; at this pH value, these proteins are positively charged. At pH 7.3 and 9, the mean protein charge is zero or negative. None of the four proteins was adsorbed in detectable amounts (adsorption data were in the experimental "noise" level: <5 μg/

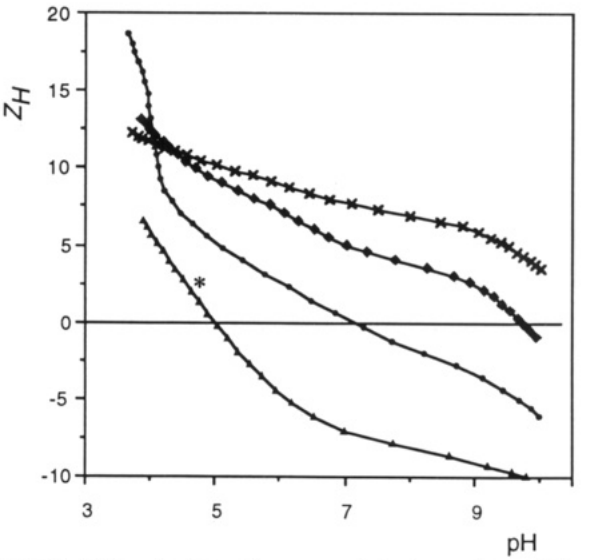

FIGURE 1: Determination of mean protein charge by acid/base titration. Proteins were titrated in 10 mM KCl from the isoionic point to pH 10 with KOH and subsequently with HCl to pH 3.5. Representative HCl titration curves are shown. No differences with the KOH titration curves were observed. Titrations were performed 5–8 times. Myoglobin (●); ribonuclease (♦); lysozyme (×); trypsin inhibitor (▲). (Asterisk) Trypsin inhibitor precipitated below pH 4.6. Titrating continued after the precipitation process started.

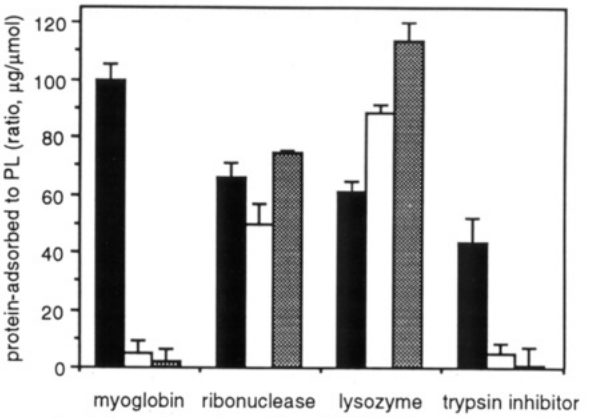

FIGURE 2: Adsorption of protein to PC/PG liposomes at different pH values. Empty liposomes (5.0 μmol/mL) were incubated with the indicated protein (1.0 mg/mL) at pH 4 (black bars), pH 7.3 (white bars), and pH 9 (grey bars) at room temperature for 2 h. The amount of liposome-adsorbed protein was determined after centrifugation as described under Materials and Methods. Error bars indicate standard deviations (n = 4).

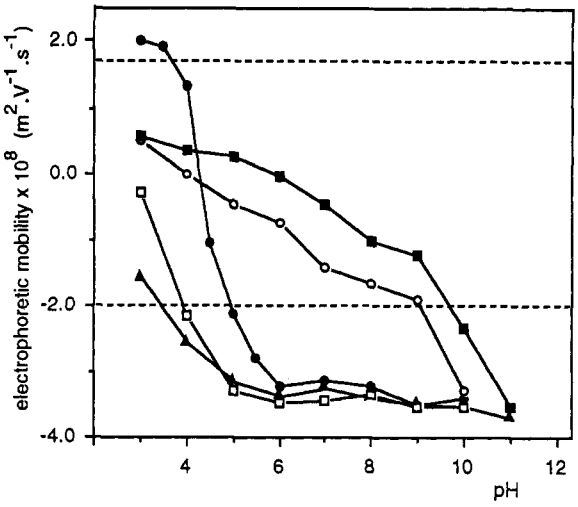

FIGURE 3: Electrophoretic mobilities of PC/PG liposomes with or without protein as a function of pH. Empty liposomes (5.0 μmol/mL) were incubated with protein (1 mg/mL) at room temperature. After 2 h, the electrophoretic mobilities of the undiluted dispersions were measured. Empty liposomes (▲); liposomes incubated with myoglobin (●), ribonuclease (○), lysozyme (■), or trypsin inhibitor (□). The liposomes incubated at pH 11 were prepared by adjusting the pH of the liposomes prepared at pH 10. Standard deviations were typically 0.2×10^{-8} $m^2 \cdot V^{-1} \cdot s^{-1}$. Protein-containing dispersions with mobilities in the range between the dashed lines were aggregated.

μmol of PL) to liposomes composed of zwitterionic lipids (data not shown). These results point to the critical importance of electrostatic attraction between the membrane- and protein-associated charges for adsorption to occur.

Effect of Protein Adsorption on Electrophoretic Mobility. The alteration of the electrophoretic mobility of PC/PG liposomes due to the adsorption of proteins is shown in Figure 3 as a function of the pH. The incubation conditions were the same as in the adsorption experiments. In the absence of protein, a constant negative electrophoretic mobility of the liposomes was found in the range from pH 5 to pH 11. The negative signs indicate migration toward the anode. The electrophoretic mobility of empty PC/PG liposomes became less negative at lower pH values. This deviation is likely to be caused by the protonation of PG. Incubation of liposomes with myoglobin, ribonuclease, lysozyme, or trypsin inhibitor resulted in alteration of the electrophoretic mobility of liposomes only at pH values where the mean charge of the protein is positive. A considerable reduction of the electrophoretic mobility of empty liposomes was observed when the pH of the incubation medium was 1–2 units below the p*I* of the protein. At pH values above the p*I* of the protein, no alteration of the electrophoretic mobility of the liposomes was observed. For myoglobin, a sharp reduction in the negative electrophoretic mobility of liposomes was found when the pH was lowered from 5 to 4. At pH 4, the liposomes showed a considerable positive mobility. Incubation of neutral PC liposomes with protein did not affect the electrophoretic mobilities of these liposomes significantly (data not shown). These results agree with the outcome of the adsorption experiments with PC liposomes mentioned above.

The effect of different protein to PL ratios on the electrophoretic mobility of PC/PG liposomes is shown in Figure 4 for three pH values of the aqueous medium. A sharp decrease in the negative electrophoretic mobility was observed when liposomes were incubated with positively-charged proteins at protein to PL ratios up to 50–100 μg of protein/μmol of PL. For ribonuclease and lysozyme, the initial slope of the curve was dependent on the pH of the incubation medium. The electrophoretic mobility plateaued with increasing protein to PL ratios. Concomitantly, lysozyme adsorption was saturable at high protein to PL ratios (Figure 5). At protein to PL ratios of 25 and 50 μg of lysozyme/μmol of PL, more than 85% of the initial amount of lysozyme was adsorbed to the liposomes at the three pH values tested. At higher protein to PL ratios, the maximal adsorption was dependent on the pH; more lysozyme was adsorbed at pH 9 than at pH 4. The adsorption of lysozyme to PC/PG liposomes is correlated with the induced alterations of the electrophoretic mobilities of the liposomes in Figure 6. A nearly linear reduction of the electrophoretic mobility was observed with the amount of lysozyme adsorbed. At pH 4, one molecule of adsorbed lysozyme is more effective in reducing the electrophoretic mobility of liposomes than at pH 9.

In some cases, the reduction of the electrophoretic mobility in the presence of protein was accompanied by aggregation and visible precipitation of the liposomes. This was especially true when the absolute value for the electrophoretic mobility was less than approximately 2×10^{-8} $m^2 \cdot s^{-1} \cdot V^{-1}$ (corresponding with a ζ-potential of about 30 mV). Aggregation was not observed for liposomes with an electrophoretic mobility in this range (e.g., PC/PG liposomes with lower charge densities) in the absence of protein. Distortion of the electroosmotic profile and inhomogeneity in the particle size distribution of precipitated liposome dispersions may cause deviations in the mobility measurements. However, no discontinuities in the curves (Figures 3 and 4) or broadening of the electrophoretic mobility peaks were observed, and the measurements were reproducible. This indicates that further evaluation of the mobility data is warranted.

Desorption of Protein from Liposomes. The reversibility of the adsorption process of the electrostatically driven interaction was tested (Jain & Zakim, 1987). This was done as follows. Liposomes (5.0 μmol/mL) were incubated with protein (1.0 mg/mL) for 2 h. Subsequently, the dispersions were diluted 10 times with the incubation medium or with a solution containing the free protein at equilibrium concentration (this protein solution was obtained as the supernatant after centrifugation of identically treated protein–liposome dispersions). The electrophoretic mobility of these liposomes in the range from pH 3 to 7 is shown in Figure 7. The electrophoretic mobility of liposomes diluted with a solution of the protein (equilibrium concentration) agreed well with the electrophoretic mobility obtained with the undiluted liposomes (compare Figure 3 with Figure 7). For myoglobin, the curves for both dilution media were equal. However, for lysozyme and ribonuclease, the electrophoretic mobility of liposomes diluted with the incubation medium was different from that of liposomes diluted with the free protein solution. For these two proteins, the electrophoretic mobility as measured after dilution with incubation medium moved toward the mobility of empty liposomes, indicating that protein desorption occurred at least to a certain degree.

Liposomes were incubated with proteins at pH 4, and then the pH was adjusted to pH 8.5 (trypsin inhibitor, myoglobin) by the addition of Tris or to pH 11 (ribonuclease, lysozyme) by the addition of glycine/NaOH (Table II). The electrophoretic mobility of empty liposomes and liposomes incubated with protein differed at pH 4. After the pH was raised to a value above the p*I* of the protein, the electrophoretic mobility of the liposomes was similar to the electrophoretic mobility of empty liposomes that were treated identically as the liposomes incubated with protein. Liposomes incubated with

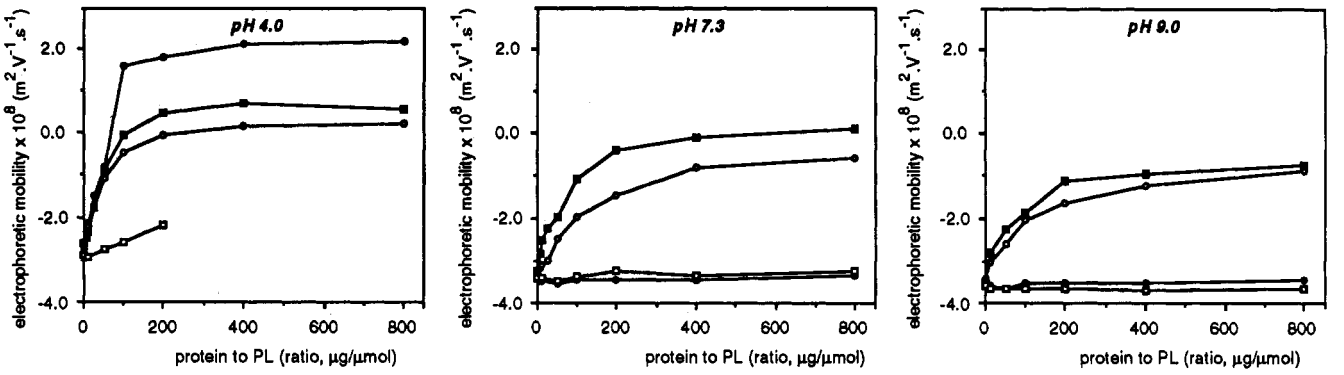

FIGURE 4: Electrophoretic mobilities of PC/PG liposomes at different pH values as a function of the initial protein to PL ratio. Empty liposomes (5.0 μmol/mL) were incubated with protein at room temperature for 2 h, and the electrophoretic mobilities were measured. Liposomes incubated with myoglobin (●), ribonuclease (○), lysozyme (■), or trypsin inhibitor (□). Standard deviations were typically 0.2 × 10^{-8} $m^2 \cdot V^{-1} \cdot s^{-1}$. At pH 4, trypsin inhibitor was not soluble above a concentration of 1 mg/mL.

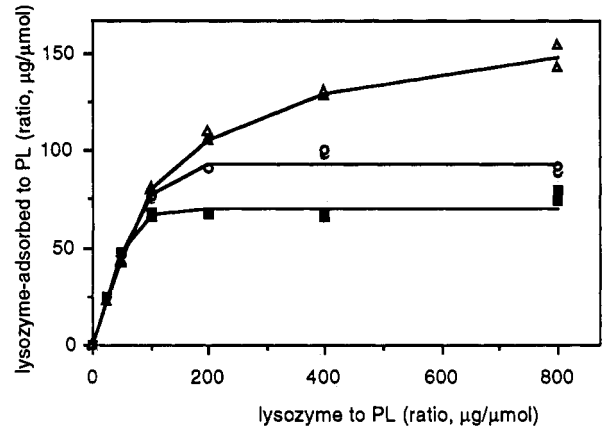

FIGURE 5: Adsorption of lysozyme to negatively-charged liposomes at different pH values as a function of the initial lysozyme to PL ratio. Empty liposomes (5.0 μmol/mL) were incubated with 0.125–4.0 mg/mL protein at pH 4 (■), pH 7.3 (○), and pH 9 (△) during 2 h at room temperature. The amount of vesicle-adsorbed protein was determined after centrifugation as described under Materials and Methods.

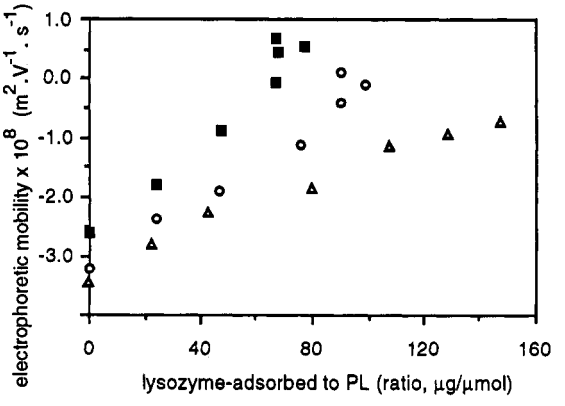

FIGURE 6: Alteration of the electrophoretic mobility of liposomes through adsorption of lysozyme. The electrophoretic mobilities of liposomes incubated with lysozyme as depicted in Figure 4 are plotted against the ratio of adsorbed lysozyme/μmol of PL as depicted in Figure 5. pH 4 (■); pH 7.3 (○); pH 9 (△).

myoglobin, ribonuclease, and lysozyme were visibly aggregated at pH 4, but after the pH was raised, dissociation of the aggregates occurred. For ribonuclease and lysozyme, no changes in mean particle sizes and polydispersity indexes were measured between these dissociates and empty liposomes. However, for myoglobin, the mean particle size of these dissociates (0.26 μm) increased compared to that of empty liposomes (0.19 μm).

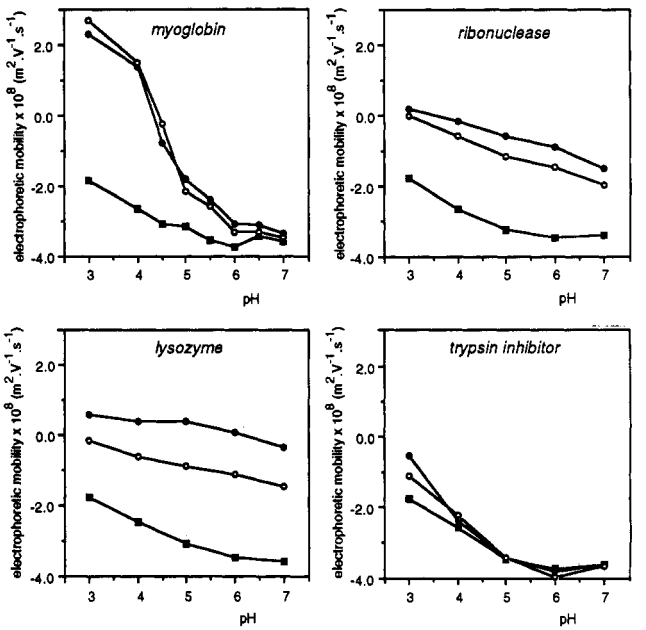

FIGURE 7: Desorption of proteins from liposomes. Empty liposomes (5.0 μmol/mL) were incubated with 1.0 mg/mL protein at room temperature during 2 h. The electrophoretic mobilities were measured of empty liposome dispersions diluted 10 times with buffer (■) and of protein–liposome dispersions diluted 10 times with buffer (○) or diluted 10 times with the supernatant obtained by centrifugation of an identically treated protein–liposome dispersion (●).

Table II: Effect of Increasing the pH of Protein–Liposome Dispersions on Their Electrophoretic Mobility

protein	electrophoretic mobility × 10^8 ($m^2 \cdot V^{-1} \cdot s^{-1}$) at pH 4[a]→[b]pH > p*I*		pH > p*I* (without protein)[c]
myoglobin	1.5	−3.0	−3.0
ribonuclease	0.0	−3.5	−3.5
lysozyme	0.5	−3.7	−3.5
trypsin inhibitor	−1.9	−2.9	−3.0

[a] Empty PC/PG liposomes (5.0 μmol/mL) were incubated with 1.0 mg/mL protein during 2 h at room temperature. [b] (→) The pH of liposomes incubated with protein at pH 4 was adjusted to pH 8.5 (myoglobin, trypsin inhibitor) by the addition of Tris or to pH 11 (ribonuclease, lysozyme) by the addition of glycine/NaOH. [c] The pH of empty liposomes was adjusted from pH 4 to pH 8.5 or 11, and the electrophoretic mobilities were measured. The electrophoretic mobility of empty liposomes at pH 4 was −2.8 × 10^{-8} $m^2 \cdot V^{-1} \cdot s^{-1}$.

DISCUSSION

Attempts were made to establish the role of electrostatics in the association of four relatively hydrophilic model proteins

with liposomes. The critical importance of the electrostatic interaction as the driving force for the interaction was clearly demonstrated by manipulating the charge of the proteins (by changing the medium pH) as well as the charge of the liposomes (with or without PG). For adsorption to occur, the protein and the liposomes should be oppositely charged. If only one species was charged, no interaction occurred. For example, none of the proteins showed adsorption to liposomes composed of zwitterionic lipids only.

Desorption was observed for both ribonuclease and lysozyme upon dilution of the bathing medium. This desorption process was not studied at greater dilutions. Although our data showed that the initial adsorption process was completely driven by electrostatic interactions, it is unclear if other, nonspecified (hydrophobic?) forces also participated in the interaction. It has been shown that lysozyme, but not ribonuclease, increased the $^{22}Na^+$ permeability of negatively-charged vesicles, indicating that lysozyme perturbed the liposomal bilayers (Kimelberg & Papahadjopoulos, 1971). Kim and Kim (1989) reported that a 6000 molecular weight segment of lysozyme was protected from tryptic digestion when incubated with phosphatidylethanolamine/phosphatidylserine vesicles, indicating vesicle penetration of lysozyme.

Our study showed that the behavior of myoglobin was different from lysozyme and ribonuclease. Specifically, adsorption to PC/PG liposomes at pH 4 continued after vesicle neutralization, no desorption occurred upon dilution of the liposome dispersion with the bathing medium, and irreversible alterations of the vesicle size were observed. The tertiary structure of myoglobin is formed by packing of eight amphipathic α-helices, which constitute more than 75% of its amino acid sequence. The secondary and tertiary structures of myoglobin are, however, easily disturbed under acidic conditions (Puett, 1973). The Soret absorbance (409 nm) of myoglobin in 10 mM sodium acetate solution started to decrease when the pH was lowered to pH 3.9, which is just below the experimental pH. Thus, after initial electrostatic interaction of native myoglobin with PC/PG liposomes, myoglobin may partially unfold to semirandom coils at the membrane/water interface, which would permit the nonpolar residues to contact the apolar region of the bilayer. Alternatively, the amphipathic α-helices of myoglobin may interact with the membrane instead of unfolding into semirandom coils. The presence or capability of formation of amphipathic α-helices has often been linked to the penetration of proteins into phospholipid bilayers (Parente et al., 1990; Thiaudière et al., 1991). Hanssens et al. (1985) suggested that for globular proteins partial unfolding of the tertiary structure is a prerequisite for such interactions.

From Figure 3, it can be derived that myoglobin showed adsorption to PC/PG liposomes at pH values at and below pH 5.5. At pH 5.5, the mean protonic charge of myoglobin was about +4 as determined by acid/base titration. Myoglobin contains about 23 carboxyl groups that are titrated in the acidic pH region (Friend & Gurd, 1979). Adsorption of trypsin inhibitor to PC/PG liposomes was only observed at pH 4; the electrophoretic mobility curves indicate that trypsin inhibitor does not adsorb to the liposomes at higher pH values. The mean charge of trypsin inhibitor at pH 4 was slightly positive; the exact value could not be determined as the protein precipitated below pH 4.6 during the titration. Trypsin inhibitor contains 31 carboxyl groups, 2 histidines, 10 lysines, and 9 arginines. Both ribonuclease and lysozyme showed adsorption to PC/PG liposomes at pH values at and below pH 9. At pH 9, the mean charge determined for ribonuclease was about +3 [all 11 carboxyl groups are titratable beneath pH 9 (Tanford & Hauenstein, 1956), so about 14 positive charges should be exposed], and the mean charge determined for lysozyme was about +6 [8 out of the 11 carboxyl groups are titratable below pH 9 (Tanford, 1962), so about 14 positive charges should be exposed]. These two proteins may even show adsorption at higher pH values. The electrophoretic mobility of PC/PG liposomes incubated with lysozyme at pH 10 differed from the electrophoretic mobility of empty liposomes, indicating lysozyme adsorption at pH 10. Kim and Kim (1989) reported adsorption of lysozyme to liposomes composed of phosphatidylethanolamine/phosphatidylserine at pH 10 (mean charge of lysozyme is +4). They found no lysozyme adsorption at pH 11 (mean charge of lysozyme is about 0). Thus, despite the electrostatic repulsion through the presence of 8–23 negatively charged moieties on the surface of these proteins, these results indicate that adsorption to negatively-charged liposomes can already take place when the number of positive charges exceeds the number of negative charges by approximately 3 charge units.

Mosior and McLaughlin (1992a) used basically the same two techniques (direct binding and electrophoretic mobility) to study the binding of basic peptides to the same type of liposomes (PC/PG). They also found that basic peptides did not bind to PC but did bind to PC/PG membranes. Their results with simple peptides implied that "a cluster of arginine and lysine residues with interspersed electrically neutral amino acids can bind a significant fraction of a cytoplasmic protein to the plasma membrane if the cluster contains more than five basic residues". The results of these studies with peptides appear to complement the present studies with intact proteins.

The reduction of the electrophoretic mobility per microgram of adsorbed lysozyme is higher at pH 4 than at pH 7.3 or 9 (Figure 6). Apparently, lysozyme at low pH is more effective in neutralizing the negative charge of the liposomes than at higher pH values. Such a behavior can be ascribed to the progressive gain in positive charges when lowering the pH. The effect of reduction of the liposomal charge is that the adsorption of lysozyme becomes increasingly more difficult. The electrophoretic mobility of liposomes incubated with lysozyme at saturation levels was slightly positive (pH 4), zero (pH 7.3), or slightly negative (pH 9). Under these conditions, the amount of lysozyme adsorbed per micromole of PL was less at pH 4 than at pH 9, which, again, can be explained by the higher positive charge of lysozyme at pH 4. At pH 9, about 30–40% of the surface of the liposomes was occupied with lysozyme under saturation conditions. This percentage is based on calculations assuming a molecular surface area of 0.70 nm^2 for both PC and PG (Crommelin, 1984) and 13.5 nm^2 for lysozyme (Wei et al., 1990) and adsorption only to the outside of the unilamellar liposomes. This implies that the maximal adsorption of lysozyme to liposomes is not a result of steric hindrance, but of liposome electroneutrality.

The question arises whether the neutralization of the charge of the liposomes is quantitatively related to the protein charge. Therefore, from the electrophoretic mobility data, the number of PG groups which are effectively neutralized by one protein molecule was calculated.

The ζ-potential, ζ, was obtained from the observed electrophoretic mobility, u, by using the Henry equation:

$$\zeta = (3u\eta/2\epsilon_r\epsilon_0)f(r_v/\lambda) \quad (1)$$

where η is the viscosity of the aqueous phase, ϵ_0 is the permittivity of free space, ϵ_r is the relative permittivity, λ is

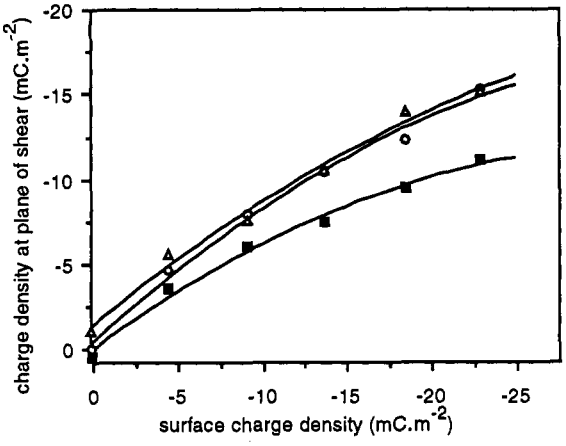

FIGURE 8: Charge density at the plane of shear (σ_E) of empty liposomes as a function of the surface charge density (σ_0). The charge densities at the plane of shear were calculated from the electrophoretic mobilities of liposomes composed of various PC/PG ratios using eq 1 and 2. pH 4 (■); pH 7.3 (○); pH 9 (△). The lines drawn were obtained by fitting the points with second-order polynomials: $\sigma_E = 0.0 + 0.73\sigma_0 + 0.011\sigma_0^2$ (pH 4); $\sigma_E = -0.3 + 0.91\sigma_0 + 0.012\sigma_0^2$ (pH 7.3); $\sigma_E = -1.3 + 0.82\sigma_0 + 0.009\sigma_0^2$ (pH 9). The correlation coefficients were 0.992 (pH 4), 0.994 (pH 7.3), and 0.991 (pH 9).

the Debye screening length, and r_v is the radius of the particles. The radius of the particles was obtained from the dynamic light-scattering measurements; the term $f(r_v/\lambda)$ varies from 1 for small r_v/λ (Hückel equation) to $^2/_3$ for large r_v/λ (Smoluchowski equation).

From the ζ-potential, the charge density at the hydrodynamic plane of shear, σ_E, can be calculated with the Gouy–Chapman equation [e.g., see Shaw (1969) and Cevc (1990)]:

$$\sigma_E = (8N\epsilon_r\epsilon_0 kTC)^{1/2} \sinh(0.5ze\zeta/kT) \qquad (2)$$

where e is the charge of a proton, z is the valency, k is the Boltzmann constant, T is the absolute temperature, N is the Avogadro constant, and C is the electrolyte concentration.

To determine the relationship between σ_E and the surface charge density, σ_0, liposomes composed of varying concentrations of PG were prepared, and their electrophoretic mobility was measured. The surface charge densities of the liposomes were calculated assuming a molecular surface area of 0.70 nm^2 for both PC and PG and complete dissociation of PG (Crommelin, 1984). In Figure 8, it is shown that σ_E indeed increased with the surface charge density. At higher surface charge densities, the difference between σ_0 and σ_E became pronounced. σ_0 (or ψ_0) always has to exceed σ_E (or ζ-potential) because the hydrodynamic plane of shear does not coincide with the surface, but is positioned at a finite distance from it. The largest absolute difference between σ_0 and σ_E can be expected at high surface potential, because the potential decreases exponentially with distance from a charged surface. In addition to the plane of shear effect, relaxation effects can cause deviations from simple theories for membranes with high surface charge densities and high potentials. These relaxation effects were neglected, as estimates of maximum errors caused by ignoring relaxation effects under those conditions did not exceed 10% of the calculated value (Hiemenz, 1986). At pH 4, the σ_E of the vesicles deviated from the σ_E of the corresponding vesicles prepared at pH 7.3 and 9. For the phosphate group of PG, an intrinsic pK value of about 1.5 has been reported (Tocanne & Teissié, 1990). This indicates that PG is fully ionized when the interfacial pH is above pH 4, being 2.5 pH units above the pK value of PG. The Bolzmann equation reveals that the interfacial pH of empty PC/PG (9:1) liposomes under the chosen ionic strength conditions is 1.2 pH units lower than the bulk pH, indicating that at a bulk pH of 4 a part of the PG molecules is not dissociated as the interfacial pH value is 2.8. However, the situation changes in the presence of protein. A less negative surface potential through adsorption of positively charged proteins decreases the difference between the bulk and the interfacial pH, and hence the percentage of nonionized PG molecules is decreased. Therefore, we assume that PG will be fully ionized upon protein association at pH 4.

Table III: Effective Charge and Mean Charge of Proteins

	pH 4		pH 7.3		pH 9	
protein	Z_p[a]	Z_H[b]	Z_p	Z_H	Z_p	Z_H
myoglobin	14	14 ± 1[c]	–[d]	0 ± 0	–	–4 ± 0
ribonuclease	11	12 ± 1	10	5 ± 1	7	3 ± 0
lysozyme	12 ± 1[e]	12 ± 1	8 ± 1	8 ± 0	6 ± 1	5 ± 2

[a] Z_p represents the effective protein charges that were calculated with eq 1–3. The electrophoretic mobility measurements in combination with the protein adsorption data obtained at a protein to phospholipid ratio of 200 μg/μmol were used; the Z_p values for lysozyme were calculated from the data collected at different lysozyme to phospholipid ratios (in the range from 50 to 800 μg of lysozyme/μmol of PL). [b] Z_H represents the mean protein charge as determined by acid/base titration. [c] ± standard deviation (five to eight titrations per protein). [d] (–) means the Z_p value could not be calculated as neither protein adsorption nor significant alteration of the electrophoretic mobility of liposomes could be detected. [e] ± standard deviation (four to five determinations at different protein to phospholipid ratios; see footnote *a*).

From the σ_E calculated from the experimental data (electrophoretic mobility and ζ-potential), σ_0 was estimated by using the second-order relationship between these two parameters as described in the legend of Figure 8. The relation between σ_E and σ_0 of liposomes adsorbed with protein may deviate from these equations. However, for the calculations described below, only liposomes adsorbed with proteins with an σ_E value between –6 and +6 mC/m^2 (corresponding with electrophoretic mobilities of $\pm 1.8 \times 10^{-8}$ m^2·s^{-1}·V^{-1}) were used. The difference between σ_E and σ_0 of empty liposomes is small for these low surface charge densities.

The difference in surface charge density between empty liposomes and liposomes incubated with protein, $\Delta\sigma_0$, is directly related to the adsorption of the protein:

$$\Delta\sigma_0 = \sigma_{0,\text{lip+protein}} - \sigma_{0,\text{empty lip}} = 2eXZ_p/A_L \qquad (3)$$

where X is the extent of protein adsorption (moles per mole), A_L is the average surface area of a PL molecule, and Z_p is the effective valency of the protein, which in fact represents the number of PG molecules neutralized by one protein molecule. A factor of 2 is included because the protein is considered to have access only to the outside of the unilamellar vesicles. The possible penetration of proteins into the lipid membrane, thereby increasing the surface area, was not taken into account since the value of X is very small. Table III gives the value of Z_p as calculated from the experimental results and the mean protein charge as determined by acid/base titration. At pH 4, an excellent correlation was found between the effective charge and the mean protein charge of lysozyme, myoglobin, and ribonuclease. However, at pH 7.3 and 9, the effective charge of ribonuclease was considerably higher than that predicted on the basis of the mean protein charge; for lysozyme, effective charges and mean protein charges correlated very well at all three pH values. The differences between the surface charge densities of empty liposomes and liposomes incubated with trypsin inhibitor and with myoglobin at the higher pH values were too small to allow realistic determination of the Z_p values.

Major assumptions in the calculations are that the liposomes are treated as membranes with a smooth surface and a uniform distribution of the structural surface charges. In spite of the low ionic strength of the incubation buffers, the protein dimensions were on the order of the Debye screening length (3 nm). This may interfere with the description of the electrostatic pattern as described in eq 2 and 3. The distribution of charged groups perpendicular to the membrane surface as well as the electrolyte penetration in the interfacial region was not taken into account. Localization of the charges at some distance from the interface will contribute more to the ζ-potential than charges at the interface. Nevertheless, the results show a good correlation between the effective charge and the mean protein charge for two out of three proteins. Examination of the three-dimensional structures of myoglobin, lysozyme, and ribonuclease reveals that the charges are most segregated on ribonuclease. Other possible explanations for the noncompliance of ribonuclease include (1) pK and surface pH shifts upon membrane association, (2) discrete charge effects, (3) lipid lateral phase separation, (4) and binding of ions other than hydrogen or hydroxyl ions to the proteins and phospholipids.

Electrostatic repulsion between charged vesicles contributes to the physical stability of liposomes against aggregation or fusion. As the negative charge of the PC/PG liposomes was reduced through the adsorption of positively-charged proteins, the electrostatic repulsion between the liposomes was reduced. However, if there is no electrostatic repulsion between two liposomes, this will not necessarily result in aggregate formation, as many uncharged liposomes are fully stabilized by hydration energies. After adsorption of proteins to negatively-charged liposomes, aggregation was observed for protein–liposome dispersions as soon as the ζ-potential of the liposomes adsorbed with protein reached a value lower than about 30 mV. This was seen for all four proteins tested and was independent of the pH. Adsorption of myoglobin to PC/PG liposomes at pH 3 and 3.5 resulted in positively-charged liposomes with a ζ-potential of about plus 30 mV, and no aggregation was observed. These results suggest that a strong electrostatic repulsion between liposomes adsorbed with protein is required to stabilize the protein-containing liposomes against aggregation.

For lysozyme and ribonuclease, the aggregation process was fully reversible; if the pH of aggregated liposome dispersions was raised to a value above the pI of the protein—the ζ-potential of the liposomes is consequently reduced to values below −30 mV—dissociation of the aggregates was observed. The mean particle size (measured by dynamic light scattering) of these dissociates was similar to that of the original empty liposomes without protein. This indicates that the aggregation process induced by the adsorption of lysozyme or ribonuclease was not accompanied by disruption or fusion of the vesicles. The aggregates are presumably composed of intact vesicles interconnected by proteins. For myoglobin, the particle size of the dissociates was increased compared to the particle size of empty liposomes, indicating that myoglobin induced alterations of the vesicle structure at low pH.

In conclusion, the major role of electrostatics in the adsorption process of four relatively hydrophilic globular proteins and the physical stability of liposomes adsorbed with proteins have been demonstrated. Adsorption to negatively-charged liposomes occurred only when the mean charge of the proteins was positive. Upon protein adsorption, the charge on the liposomes was reduced. Consequently, the driving force for further protein adsorption decreased. For lysozyme and myoglobin, the extent of charge neutralization as determined by electrophoretic mobility measurements could be quantitatively ascribed to the mean charge of the proteins. For ribonuclease at pH 4, also an excellent relationship was found, but at pH 7.3 and 9, deviations were observed. At the present time, one can only speculate about the reason for these deviations.

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REFERENCES

Beschiaschvili, G., & Seelig, J. (1990) *Biochemistry 29*, 52–58.
Bos, O. J. M., Labro, J. F. A., Fischer, M. J. E., Wilting, J., & Janssen, L. H. M. (1989) *J. Biol. Chem. 264*, 953–959.
Cevc, G. (1990) *Biochim. Biophys. Acta 1031-3*, 311–382.
Cevc, G., Strohmaier, L., Berkholz, J., & Blume, G. (1990) *Stud. Biophys. 138*, 57–70.
Crommelin, D. J. A. (1984) *J. Pharm. Sci. 73*, 1559–1563.
Cruz, M. E., Corvo, M. L., Forge, J. S., & Lopes, F. (1989) in *Liposomes in the Therapy of Infectious Diseases and Cancer* (Lopez-Berestein, G., & Fidler, I. J., Eds.) pp 417–426, Alan R. Liss, New York.
Dautrevaux, M., Boulanger, Y., Han, K., & Biserte, G. (1969) *Eur. J. Biochem. 11*, 267–277.
De Kroon, A. I. P. M., Soekarjo, M. W., De Gier, J., & De Kruijff, B. (1990) *Biochemistry 29*, 8229–8240.
Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.
Friend, S. H., & Gurd, F. R. N. (1979) *Biochemistry 18*, 4612–4619.
Hanssens, I., Van Ceunebroeck, J.-C., Pottel, H., Preaux, G., & Van Cauwelaert, F. (1985) *Biochim. Biophys. Acta 817*, 154–164.
Heinitz, M. L., Kennedy, L., Kopaciewicz, W., & Regnier, F. E. (1988) *J. Chromatogr. 443*, 173–182.
Hiemenz, P. C. (1986) *Principles of colloid and surface chemistry*, 2nd ed., p 756, Marcel Dekker, Basle and New York.
Jacobs, R. E., & White, S. H. (1989) *Biochemistry 28*, 3421–3437.
Jain, M. K., & Zakim, D. (1987) *Biochim. Biophys. Acta 906*, 33–68.
Janssen, L. H. M., & Van Wilgenburg, M. T. (1978) *Mol. Pharmacol. 14*, 884–889.
Janssen, L. H. M., De Bruin, S. H., & Van Os, G. A. J. (1972) *Int. J. Pept. Protein Res. 4*, 339–342.
Jousma, H., Talsma, H., Spies, F., Joosten, J. H. G., Junginger, H. E., & Crommelin, D. J. A. (1987) *Int. J. Pharm. 35*, 263–274.
Kato, Y., Kitamura, T., & Hashimoto, T. (1983) *J. Chromatogr. 266*, 49–54.
Kim, J., & Kim, H. (1989) *Arch. Biochem. Biophys. 274*, 100–108.
Kimelberg, H. K. (1976) *Mol. Cell. Biochem. 10*, 171–190.
Kimelberg, H. K., & Papahadjopoulos, D. (1971) *J. Biol. Chem. 246*, 1142–1148.
Koide, T., & Ikenaka, T. (1973) *Eur. J. Biochem. 32*, 417–431.
Kuchinka, E., & Seelig, J. (1989) *Biochemistry 28*, 4216–4221.
Law, S. L., Lo, W. Y., Pai, S. H., Teh, G. W., & Kou, F. Y. (1986) *Int. J. Pharm. 32*, 237–241.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Maitani, Y., Nakagaki, M., & Nagai, T. (1990) *Int. J. Pharm. 64*, 89–98.
McLachlan, A. D. (1979) *J. Mol. Biol. 133*, 557–563.
Mosior, M., & McLaughlin, S. (1992a) *Biochemistry 31*, 1767–1773.
Mosior, M., & McLaughlin, S. (1992b) *Biochim. Biophys. Acta 1105*, 185–187.
Parente, R. A., Nadasdi, L., Subbarao, N. K., & Szoka, F. C. (1990) *Biochemistry 29*, 8713–8719.
Puett, D. (1973) *J. Biol. Chem. 248*, 4623–4634.
Righetti, P. G., & Caravaggio, T. (1976) *J. Chromatogr. 127*, 1–28.
Seelig, A., & Macdonald, P. M. (1989) *Biochemistry 28*, 2490–2496.
Shaw, D. J. (1969) *Electrophoresis*, Academic Press, London.
Stankowski, S., & Schwarz, G. (1990) *Biochim. Biophys. Acta 1025*, 164–172.
Surewicz, W. K., & Epand, R. M. (1984) *Biochemistry 23*, 6072–6077.
Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., & Blow, D. M. (1974) *Biochemistry 13*, 4214–4228.
Tanford, C. (1962) *Adv. Protein Chem. 17*, 69–165.
Tanford, C., & Wagner, M. L. (1954) *J. Am. Chem. Soc. 76*, 3331–3336.
Tanford, C., & Hauenstein, J. D. (1956) *J. Am. Chem. Soc. 78*, 5287–5291.
Thiaudière, E., Siffert, O., Talbot, J.-C., Bolard, J., Alouf, J. E., & Dufourcq, J. (1991) *Eur. J. Biochem. 195*, 203–213.
Tocanne, J.-F., & Teissié, J. (1990) *Biochim. Biophys. Acta 1031*, 111–142.
Wei, A.-P., Herron, J. N., & Andrade, J. D. (1990) in *Developments in Biotherapy* (Crommelin, D. J. A., & Schellekens, H., Eds.) Vol. 1, pp 305–313, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Williams, N. A., & Weiner, N. D. (1989) *Int. J. Pharm. 50*, 261–266.
Yoshimura, T., & Sone, S. (1987) *J. Biol. Chem. 262*, 4597–4601.