

Binding and Dissociation of Cytochrome *c* to and from Membranes Containing Acidic Phospholipids[†]

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ABSTRACT: Membrane association and detachment of cytochrome *c* (cyt *c*) in millisecond to second time domain were investigated by stopped-flow fluorescence spectroscopy monitoring the efficiency of energy transfer from a pyrene-fatty acid containing phospholipid derivative, 1-palmitoyl-2-[10-(pyren-1-yl)-decanoyl]-*sn*-glycero-3-phosphoglycerol (PPDPG, mole fraction $X = 0.01$) to the heme of the cyt *c*. Large unilamellar liposomes composed of egg phosphatidylcholine (eggPC) with varying content of the acidic phospholipid phosphatidylglycerol (eggPG) were employed. Unexpectedly, the rate of binding of cyt *c* to membranes was attenuated upon increasing the mole fraction of the acidic phospholipid (X_{PG}). For example, at 50 μ M phospholipid and 5 μ M cyt *c*, when X_{PG} was increased from 0.20 to 0.40 the half-time for the single-exponential decay in fluorescence increased from 4.7 to 8.6 ms. A similar observation was made for the membrane binding of another cationic protein, histone H1. We suggest that the formation of cooperative hydrogen-bonded networks by deprotonated and protonated PG in the vesicle surface retards the binding of cyt *c* to the liposome surface. However, once formed, the complex of cyt *c* with acidic phospholipids is stabilized by increasing X_{PG} . Accordingly, significantly prolonged half-times of dissociation of cyt *c* from liposomes by NaCl, ATP, and different cationic proteins are measured upon increasing X_{PG} . Differences between the latter cationic membrane binding ligands most likely reflect the varying relative contributions of hydrophobicity and Coulombic forces to their attachment to liposomes. Our data on the release and binding of cyt *c* to liposomes as a function of X_{PG} and in the presence of ATP also provide the first direct experimental evidence for multiple lipid binding sites in cyt *c*.

Although peripheral membrane proteins are abundant in cells and participate in diverse functions, ranging from blood coagulation to replication and signal transduction (1), the molecular level mechanisms and possible specific lipid–protein interactions have received only limited attention. Yet, the understanding of their mechanisms of association to membranes is of considerable interest as reversible attachment/detachment to and from membrane surfaces would offer excellent means for regulatory purposes (1). To this end, we have undertaken to compare the membrane association of different peripheral proteins, with particular interest in possible competition for lipid binding. At this stage we are focusing primarily on cytochrome *c* (cyt *c*)¹ as a model.

Cyt *c* is a small (13 kDa) and thoroughly studied cationic peripheral membrane protein which mediates single-electron transfer in the respiratory chain between cytochrome *c* reductase and cytochrome *c* oxidase of the inner mitochondrial membrane. Interestingly, cyt *c* has been shown recently to be also a key component in apoptosis, its release from mitochondria apparently representing the rate-limiting step in the commitment of a cell into the death program (2–4). Cyt *c* associates only weakly with zwitterionic phosphatidylcholine membranes (5, 6), whereas increased affinity is observed when acidic phospholipids are present (6–8). Membrane binding has been shown to induce conformational alterations in the protein and phospholipids, together with changes in the conformation and coordination of the heme group (9–16).

Cyt *c* is considered as a paradigm for the electrostatic binding of peripheral proteins to membranes, and this interaction is dependent on the ionic strength of the medium (1, 17–32). Cationic amphiphile sphingosine reverses the association of cyt *c* with phosphatidic acid-containing liposomes (6). Likewise, cyt *c* is dissociated from cardiolipin also by the cationic cytotoxic drug adriamycin, which competes for the negatively charged phosphates of the lipid at equimolar drug:lipid ratios (33). In addition to the electrostatic attraction, hydrophobic interaction has been demonstrated to contribute to the membrane association of cyt *c* (34).

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¹ Abbreviations: ACTH 1–24, adrenocorticotropin 1–24; cyt *c*, cytochrome *c*; eggPC, egg phosphatidylcholine; eggPG, egg phosphatidylglycerol; eggPA, egg phosphatidic acid; EDTA, ethylenediamine-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; H1, histone H1; F-H1, fluorescein-labeled histone H1; K₁₉, poly-L-Lys; Myr-KRTL, myristoylated Lys-Arg-Thr-Leu-Arg; PPDPG, 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-*sn*-glycero-3-phosphoglycerol; bisPDPC, 1,2-bis[(pyren-1-yl)decanoyl]-*sn*-glycero-3-phosphocholine; X_{PG} , mole fraction of PG; τ , half-time for fluorescence intensity changes; RFI, relative fluorescence intensity.

Two different lipid binding sites in cyt *c*, labeled as A- and C-site, and involving electrostatic and hydrogen bonding between the protein and acidic phospholipids, respectively, have been postulated (26, 29, 30). The former interaction dominates at low content of acidic phospholipid in the membrane and is reversed by ATP, this nucleotide competing with the deprotonated acidic phospholipid for the same binding site in cyt *c* (29). Compared to ADP and AMP, ATP is more effective and detaches cyt *c* nearly quantitatively from the liposomes up to $X_{PG} = 0.30$. This effect of ATP is highly pH dependent with decreasing efficiency under more acidic conditions. At pH 4.0 the nucleotides actually enhance the quenching of pyrene fluorescence by cyt *c* (26), thus indicating conformational changes in the protein (29, 35). The C-site mediated binding predominates at high content of the acidic phospholipid and is insensitive to ATP (26, 29).

Histones are basic proteins abundant in the cell nucleus and with a high affinity for DNA. They play a major role in chromatin condensation and regulation of gene expression (36–38). Recently, Hirai et al. (39) demonstrated association of histones with phosphatidylglycerol, -serine, -inositol, phosphatidic acid, and cardiolipin. The lipid binding properties of H1 could also have physiological relevance (40, 41). The linker histone H1 is commonly used as a substrate for protein kinase C (40). Binding of H1 to liposomes containing acidic phospholipids can be reversed by DNA (41). Attachment of H1 causes significant rigidification of the membrane, reflected in attenuated lipid lateral diffusion and parallel increase in diphenylhexatriene polarization (31). Formation of ternary complexes by H1, DNA, and liposomes containing sphingosine and acidic phospholipid has been demonstrated in our laboratory (41). The characteristics of the membrane binding of H1 resemble the lipid association of cyt *c* (41). In brief, the affinity of H1 to membranes is enhanced with increasing content of acidic phospholipids, and the interaction becomes resistant to NaCl. Histone H1 is detached from DNA by sphingosine-containing liposomes. In addition to the strong electrostatic attraction, the involvement of hydrophobic interaction is suggested by the weak attachment of histone H1 with neutral eggPC liposomes. The affinity of H1 to acidic phospholipids appears to exceed that of cyt *c*, and the latter is efficiently displaced by H1 from liposomes (31).

A cluster of basic amino residues in proteins such as histone H1 is thought to mediate their high affinity interaction with membranes containing acidic phospholipids. This electrostatic interaction has been modeled using synthetic basic peptides such as polylysine (42–49). In keeping with theoretical considerations, the membrane binding of such peptides varies as a function of the peptide–membrane distance, orientation, number of residues, ionic strength, and mole fraction of acidic lipids (42, 44, 49). Analogously to H1, poly-K also can detach cyt *c* from acidic phospholipids (31). McLaughlin and co-workers (50, 51) have demonstrated the affinity of basic peptides with covalently attached myristoyl moiety to acidic phospholipids to be very high when compared to the corresponding myristoylated neutral peptides. This is due to the additivity of the electrostatic attraction of basic amino residues and the hydrophobic interaction of the myristoyl chain with a lipid bilayer which together anchor the peptide firmly to membranes. Anal-

gously, the combination of hydrophobicity and electrostatic attraction causes the peptide hormone ACTH containing an amphipathic helical stretch and having a net charge of +6 to bind tightly to negatively charged membranes (52). Both myristoylated basic peptide KRTLRL and ACTH can displace cyt *c* from liposomes (31).

We report here on the binding of cyt *c* and H1 to liposomes containing varying mole fractions of the acidic phospholipid PG. Subsequently, we compared the detachment of cyt *c* from liposomes by increasing ionic strength and ATP as well as by histone H1, poly-L-lysine (K_{19}), myristoylated peptide (myr-KRTLRL), and ACTH 1–24. Our data reveal that the interaction of cyt *c* with liposomes is highly sensitive to their content of acidic phospholipid. This is further reflected in the competition of the above cationic ligands with cyt *c* for membrane binding.

EXPERIMENTAL PROCEDURES

Materials. Horse heart cytochrome *c* (type VI, oxidized form), eggPG (sodium salt), eggPC, fluorescein 5-isothiocyanate (FITC), and poly-L-lysine (K_{19} , average number of residues ≈ 19) were purchased from Sigma. 1-Palmitoyl-2-[10-(pyren-1-yl)decanoyl]-sn-glycero-3-phosphoglycerol (PPDPG) was from K&V Bioware (Espoo, Finland). Synthetic ACTH 1–24 was a gift from Ciba-Geigy AG (Basel, Switzerland). Myr-KRTLRL was from Bachem (Bubendorf, Switzerland), and the disodium salt of ATP was purchased from Boehringer Mannheim (Mannheim, Germany). No impurities were detected in the above lipids upon thin-layer chromatography on silicic acid coated plates using $CHCl_3/CH_3OH/H_2O/NH_3$ (65:20:2:2, by volume) as the solvent system and examining the spots either by fluorescence or after iodine staining. Histone H1 was purified from calf thymus (53) and was labeled with fluorescein 5-isothiocyanate (FITC) according to the method of Favazza et al. (54) so as to yield a fluorescein/histone H1 stoichiometry of approximately 1.1/1. Horse heart cyt *c* solution was stored at $-20^\circ C$ in 20 mM Hepes, 0.1 mM EDTA, pH 7. Water used was freshly deionized in a Milli RO/Milli Q (Millipore) filtering system.

Preparation of Liposomes. Lipids were dissolved in chloroform and stored at $-20^\circ C$. To prepare LUVs, appropriate amounts of lipids were first mixed in the solvent to obtain the desired compositions. Cyt *c*-induced nonbilayer structures have been demonstrated for cardiolipin but not for PG (17). Accordingly, to avoid ambiguities in the interpretation of the data, the latter lipid was used. In all experiments assessing membrane association of cyt *c*, PPDPG [1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-sn-glycero-3-phosphoglycerol] was included in liposomes as the fluorescence energy donor at a mole fraction $X = 0.01$. For monitoring the membrane association of fluorescein-labeled histone H1, another pyrene-labeled lipid bisPDPC [1,2-bis-[(pyren-1-yl)decanoyl]-sn-glycero-3-phosphocholine, at $X = 0.01$] was used. These contents of the fluorescent probes yield well-resolved emission signals, while minimal perturbation of the packing of the unsaturated matrix lipids can be expected. After mixing of the lipids the solvent was removed under a stream of nitrogen. The lipid residues were subsequently maintained under reduced pressure for 2 h and then hydrated at room temperature in 20 mM Hepes, 0.1 mM EDTA, pH 7 to yield a lipid concentration of 1–2 mM.

To obtain unilamellar vesicles the lipid dispersions were first vigorously mixed by vortexing and then extruded with a LiposoFast small-volume homogenizer (Avestin, Ottawa, Canada) by subjecting them to 19 passes through a stack of two polycarbonate filters (100 nm pore size, Nucleopore, Pleasanton, CA) installed in tandem. Minimum exposure of the lipids to light was ensured during the above procedure. Subsequently, the liposome solution was divided into proper aliquots and diluted with buffer to yield a lipid concentration of 50 μM . LUVs were freshly prepared and used within 1 day.

Stopped-Flow Fluorescence Measurements. Membrane association and detachment of cyt *c* were measured in the millisecond to second time range using stopped-flow spectrofluorometer equipped with a rapid-scanning emission monochromator (On-line Instrument Systems Inc., Bogart, GA) to monitor resonance energy transfer from a pyrene-fatty acid containing phospholipid derivative (fluorescence donor) to the heme (acceptor) of cyt *c*. Fluorescence excitation was provided by a water-cooled 450 W Xe arc lamp. The 20 mm path length and 1.5 mm diameter quartz glass fluorescence observation chamber was connected to a sample mixing jet having a dead time of ≤ 2 ms. Two syringes mounted in parallel were driven pneumatically at a gas pressure of 7 bar to inject the reactants into the rapid-mixing chamber. Temperature of the stopped-flow solution barrels and the flow cell was maintained by a circulating waterbath. When binding of cyt *c* to LUVs was measured, one of the syringes was filled with 0.5–5.0 μM cyt *c* solution and the other syringe with liposomes (15 or 50 μM total phospholipid) so as to yield the final concentrations of 0.25–2.5 μM protein and 7.5 or 25 μM lipid in the observation chamber. When release of cyt *c* from liposome surface was investigated, one syringe was filled with a solution containing cyt *c* and liposomes at concentration of 2 and 50 μM , respectively. The other syringe was filled with a solution of the cyt *c* detaching reactant so as to yield the indicated final concentrations in the mixing chamber. Excitation wavelength was 344 nm, while emission spectra from 364 to 516 nm were recorded. Although this emission wavelength range includes also excimer fluorescence at the low probe concentration used, its contribution is negligible to the integrated overall intensity which is dominated by pyrene monomer emission. Intensity of emission was measured by a photomultiplier tube through a rotating spoke wheel collecting 1000 spectra per second. Representative traces for the membrane binding and detachment of cyt *c* with the respective residuals are depicted in Figure 1. Also shown are steady-state emission spectra recorded for LUVs before and after the addition of cyt *c*. Photomultiplier output was digitized by a Pentium PC equipped with an A/D converter. All measurements were carried out at a constant temperature of 25 $^{\circ}\text{C}$ where all the lipids used are in a fluid, liquid-crystalline state. The same instrumental parameters were employed for the detachment of cyt *c* from liposomes by NaCl, ATP, histone H1, K₁₉, myristoylated cationic peptide KRTLRL, and ACTH 1–24. To allow for a meaningful comparison of the stopped-flow data, the concentrations of these membrane binding ligands were those yielding saturating response, i.e., maximum detachment of cyt *c* from liposomes in steady-state measurements (31). To assess the membrane association of histone H1, this protein was labeled

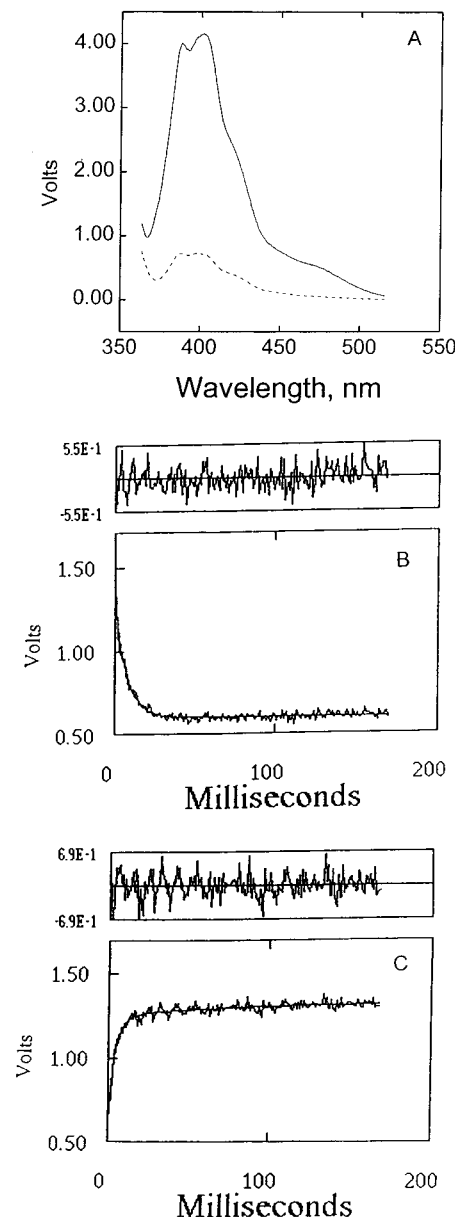


FIGURE 1: (A) Steady-state emission spectra of PPDPC-labeled eggPC/eggPG liposomes (25 μM total phospholipid, $X_{\text{PG}} = 0.30$) in the absence (—) and in the presence (---) of 1 μM cyt *c*. (B) Decrease in fluorescence intensity due to the binding of cyt *c* to eggPG/eggPC ($X_{\text{PG}} = 0.30$) unilamellar vesicles. Relative fluorescence intensity is given as the photomultiplier output voltage. The smooth line in the lower panel shows the fit of the single-exponential function $Y = Ae^{-kt} + C$. Residuals of the fit are illustrated in the upper panel. (C) Fluorescence increase following the detachment of cyt *c* from LUVs by 100 mM NaCl. The smooth line depicts the fit of data to the two-exponential equation $Y = A_1e^{-k_1t} + A_2e^{-k_2t} + C$. Upper panel shows the residuals. In both experiments the final concentrations of lipids and cyt *c* in the stopped-flow cell were 25 and 1 μM , respectively, in 20 mM Hepes, 0.1 mM EDTA, pH 7.0. Temperature was 25 $^{\circ}\text{C}$.

with fluorescein isothiocyanate while bisPDPC ($X = 0.01$) was included in liposomes as a fluorescence donor (41). This energy transfer couple utilizes the overlap between pyrene excimer emission and fluorescein absorption spectra. Analogously to the studies on cyt *c*, the quenching of pyrene emission due to Förster resonance energy transfer between the bisPDPC and the fluorescein moiety made it possible to monitor the binding of H1 to vesicles. Because of energy

losses due to quantum yield (being less than unity) as well as the involvement of multiple donors for a single acceptor, the magnitude of quenching is significantly larger than the emission from fluorescein. Furthermore, as the latter parameter provides no additional information, only the decrease in the intensity of pyrene emission was measured.

Analysis of the Stopped-Flow Data. Each experiment was repeated five to ten times, and the values were averaged for data analysis. Data were fitted using either single- or two-exponential equations:

$$Y = Ae^{-kt} + C \quad (1)$$

$$Y = A_1e^{-k_1t} + A_2e^{-k_2t} + C \quad (2)$$

which were solved with nonlinear least-squares fitting procedures by both the Levenberg–Marquardt algorithm and the Successive Integration method using the dedicated software provided by Olis. The best-fit parameters A_1 and A_2 represent the amplitudes of the processes; k_1 and k_2 are the respective rate constants, and C is a background constant to account for the baseline at infinite time. The fluorescence intensity traces were practically identical for each repeated measurement (i.e., the fitted curve for the first shot was identical with that up to the fifth or tenth repeat). Unfortunately, the commercial software used does not allow to obtain accurate values for the relative amplitudes for the two exponential processes measured. Although this information would have given further insight into the mechanisms involved, we decided at this stage to pursue changes in the rates of fluorescence intensity only. Importantly, while the extent of fluorescence quenching correlates to the number of cyt *c* molecules attached to liposomes, the calculation of the actual number of cyt *c* molecules bound to vesicles is not possible. More specifically, this is due to the fact that at low surface coverage one cyt *c* causes the quenching of a large number of probes, whereas when approaching higher occupancies the signal is significantly attenuated (55). Notably, in the former region the correlation between the number of cyt *c* molecules on the liposome surface and the decrease in fluorescence emission is linear, whereas under the latter condition this is no longer true. Although this drawback does not jeopardize the present data, it must be understood that the rate constants of the fluorescence decays would in essence be apparent only and merely represent changes in fluorescence intensity in arbitrary units per unit time. Accordingly, the data are presented as half-times for changes in fluorescence emission, ranging from 3.3 ms to 6.66 s with average errors of ± 0.1 and ± 10 ms, respectively. It should be stressed that in the case of two-exponential processes the half-times of the slower component bear little information on the actual rate constant. Likewise, because of the above limitations (i.e., lack of information on the amplitudes as well as on the actual rate constants of binding and detachment of cyt *c*), true kinetics of the different processes could not be obtained, and we had to restrict our study to collecting qualitative data on the changes in lipid–protein interaction as a function of X_{PG} .

RESULTS

Membrane Association of Cytochrome *c*. To study the attachment of cyt *c* to eggPG/eggPC liposomes by the

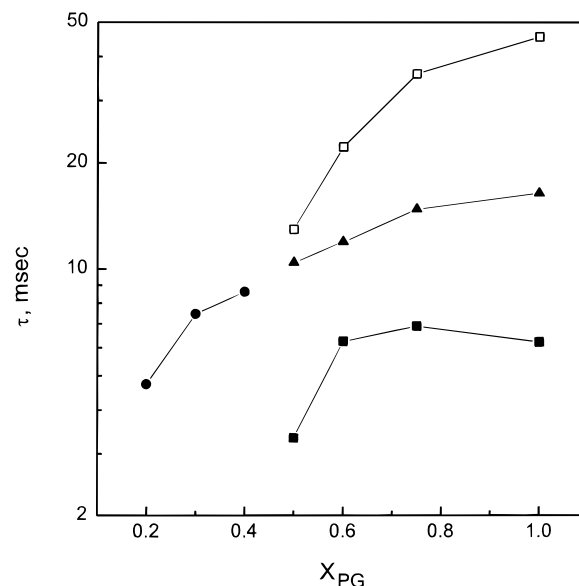


FIGURE 2: Dependency of the half-times (τ) of the membrane binding the cyt *c* on the content of the acidic phospholipid in the liposomes. The fluorescence decays recorded with X_{PG} varying between 0.20 and 0.40 were single-exponential (●), whereas with X_{PG} in the range of 0.50–1.0 a two-exponential process was evident. The half-times for the fast (■) and slow (□) processes are depicted. Data illustrating the C-site mediated binding of cyt *c* (1 μ M) to liposomes were measured in the presence of 5mM ATP (▲).

different postulated lipid binding sites (A-site and C-site) of cyt *c*, the mole fraction (X) of PG in LUVs was varied between 0.2 and 1.0. The rate of quenching of pyrene fluorescence due to resonance energy transfer between PPDGP and the heme moiety of cyt *c* was then used to quantitate the half-times for the membrane association of cyt *c*. The small amplitude of the fluorescence decrease prohibited reliable assessment of the binding at $X_{PG} = 0.1$. On the other hand, the range of X_{PG} from 0.2 to 1.0 allows for the measurement of A- and C-site interaction, respectively. Thus, the region $X_{PG} < 0.2$ would not have added significantly to the present study. In keeping with our previous steady-state fluorescence data, the association of cyt *c* with eggPG/eggPC liposomes (25 μ M) was fully saturated in the presence of 1 μ M protein with little difference in fluorescence intensity at $X_{PG} = 0.2$ or 1.0 (29). Under these conditions the A-site mediated attachment of cyt *c* to acidic phospholipid at $X_{PG} = 0.20$ is evident as a single-exponential decay of pyrene fluorescence with a half-time of 4.7 ms. Increasing X_{PG} to 0.40 progressively attenuated the binding, and a half-time of 8.6 ms was measured. Notably, at $X_{PG} = 0.50$ fluorescence decay was best fitted with a two-exponential process with half-times of 3.3 and 13.0 ms. Increasing X_{PG} further decreased the rates of the two processes until at $X_{PG} = 1.0$ values of 6.2 and 45.5 ms were evident (Figure 2).

We also carried out preliminary studies varying the reactant concentrations. Increasing the cyt *c* concentration accelerated the binding almost linearly. For example, at 25 μ M phospholipid and at [cyt *c*] of 0.25 and 2.5 μ M, half-times of 44.2 and 3.7 ms, respectively, were observed at $X_{PG} = 0.30$. Changing the lipid concentration had a less pronounced effect and was more clearly observed at low cyt *c* concentrations. Thus at [cyt *c*] = 0.25 μ M and at $X_{PG} =$

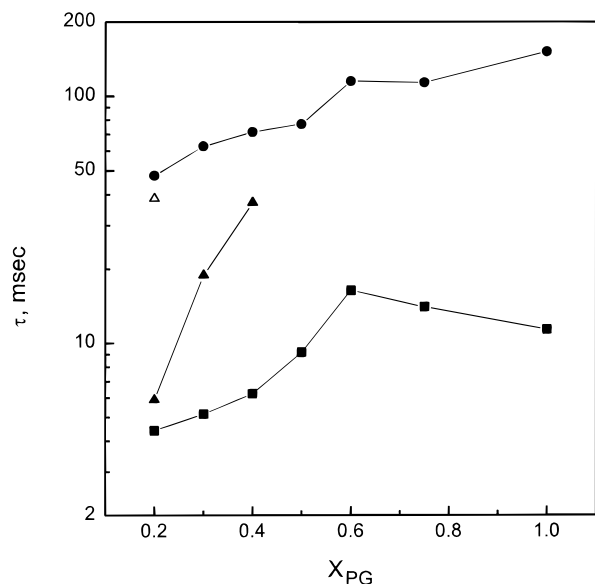


FIGURE 3: Half-times (τ) for the fast and slow components of dissociation of cyt *c* by 100 mM NaCl (■ and ●) and 5 mM ATP (▲ and △) as a function of X_{PG} .

0.30, half-times of 44.2 and 53.6 ms were measured at 25 and 7.5 μ M total phospholipid, respectively.

The A-site mediated association of cyt *c* to liposomes is prevented by ATP, and we have postulated that this inhibition results from a competition between ATP and deprotonated acidic phospholipid for the same cationic binding site in cyt *c* (29). However, at $X_{PG} \geq 0.5$ the attachment of cyt *c* to vesicles is mostly due to the C-site of the protein and is no longer sensitive to ATP (29). To study the membrane association of cyt *c* by the C-site, the measurements were conducted in the presence of 5 mM ATP while X_{PG} was varied between 0.5 and 1.0. These data were best fitted with a single-exponential decay, and at $X_{PG} = 0.5$ a half-time of 10.4 ms was evident (Figure 2). Increasing X_{PG} attenuated the rate of binding, and at $X_{PG} = 1.0$ a half-time of 16.4 ms was measured. This blocking of the A-site of cyt *c* by ATP provides further support to the notion that the fast component measured in the absence of ATP arises from an electrostatic interaction between cyt *c* and deprotonated PG. More detailed elucidation of the above process should also facilitate the development of a proper overall kinetic model.

Detachment of Cyt *c* from Liposomes by ATP and NaCl. We then proceeded to study the release of cyt *c* by 5 mM ATP from eggPG/eggPC LUVs. At $X_{PG} = 0.20$ the increase in pyrene fluorescence was two-exponential with half-times of 5.9 and 38.5 ms (Figure 3). Increasing X_{PG} to 0.30 and further to 0.40 caused the increase in RFI to become single-exponential and also progressively reduced the rate of detachment of cyt *c* by ATP, the half-times increasing from 18.9 to 37.0 ms, respectively. At $X_{PG} = 0.50$ ATP was without effect. Instead of releasing cyt *c*, further quenching of fluorescence with complex kinetics was evident when ATP was added at X_{PG} in the range of 0.50–1.0 (data not shown), in keeping with ATP-induced conformational changes in membrane-bound cyt *c* (26).

The electrostatic association of cyt *c* with acidic phospholipids is sensitive to ionic strength, and increasing [NaCl] both dissociates cyt *c* from membranes and decreases the pK_a for the acidic phospholipid (5, 26, 56). When the content

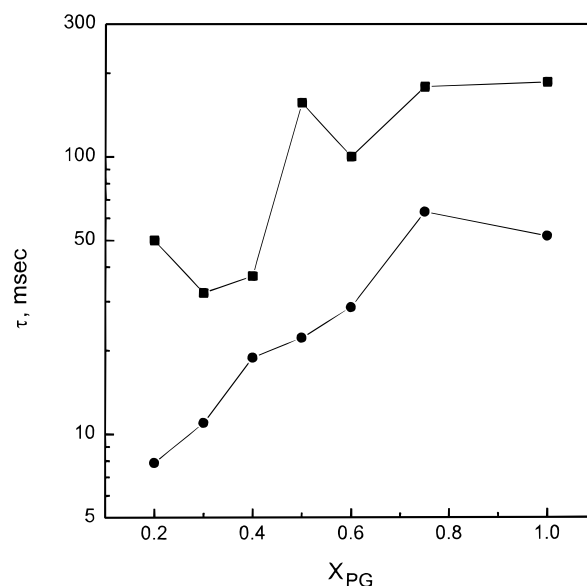


FIGURE 4: Half-times for the binding of F-H1 to liposomes measured as a function of X_{PG} in eggPG/eggPC vesicles. The two data sets represent the values for the fast (●) and slow (■) components of the two-exponential processes.

of the acidic phospholipid PG in vesicles increases, progressively higher salt concentrations are required to detach cyt *c* from their surface (29). We measured the dissociation of cyt *c* from eggPG/eggPC liposomes by 100 mM NaCl. At $X_{PG} = 0.20$ the increase in fluorescence was two-exponential with half-times of 4.4 and 47.6 ms (Figure 3). Upon increasing X_{PG} to 0.50, the rates for both the fast and slow process were attenuated; whereafter, upon increasing X_{PG} further from 0.60 to 1.0, the slow component remained unaltered while the rate of the fast process actually slightly increased.

Binding of Histone H1 to Liposomes and Displacement of Cyt *c*. Histone H1 labeled with fluorescein isothiocyanate can be used as a resonance energy transfer acceptor for the intramolecular excimer forming pyrene-labeled lipid, bisP-DPC (31, 41). We used this method to monitor the binding of H1 to eggPG/eggPC liposomes. Decrease in fluorescence upon membrane association of H1 was two-exponential over the range of X_{PG} studied from 0.2 to 1.0, and at $X_{PG} = 0.20$ half-times for fluorescence quenching were 7.9 and 50 ms (Figure 4). In resemblance to cyt *c* the half-times for the fast and slow components were progressively increased upon increasing X_{PG} , and at $X_{PG} = 1.0$ values of 51.8 ms and 0.185 s were measured for the fast and slow component, respectively. To this end, similar two-exponential binding was observed also using another acidic phospholipid, phosphatidic acid, which has two ionizable hydroxyls in the phosphate group. At $X_{PA} = 1.0$ the values for τ were 30.4 ms and 0.163 s.

Association of H1 with liposomes containing PG attenuates lipid lateral diffusion and rigidifies the membrane as revealed by the decrease in I_e/I_m values for the intermolecular excimer forming pyrene-labeled lipid probe PPDPG as well by increase in fluorescence emission anisotropy of diphenyl-hexatriene (31). The H1 binding site was calculated to be constituted by approximately 20 phospholipids. We measured the rate of the formation of this domain by monitoring I_e/I_m vs time. As the signal is maximal at $X_{PG} = 1.0$, the

measurement was carried with neat PG LUVs only. Our data reveal the formation of the H1-induced lipid domain in the time range 0–200 ms to be a single-exponential process with a half-time of 58.5 ms. This is slightly slower than the fast component of the membrane binding of fluorescein-labeled H1 for which $\tau = 51.8$ ms was evident. The difference of approximately 6.6 ms is likely to represent the time required for the simultaneous scavenging of the acidic phospholipids into the membrane domain underneath H1 and forming the protein binding site in the membrane.

The affinity of H1 to acidic phospholipids seems to exceed that of cyt *c*, in keeping with the complete release of cyt *c* from membranes by H1 (31). The dissociation of cyt *c* by H1 from liposomes was two-exponential over the range of X_{PG} studied, from 0.20 to 1.0. At $X_{PG} = 0.20$, where cyt *c* is associated to liposomes via its A-site, the release of cyt *c* from LUVs had half-times of 17.1 ms and 0.145 s (Figure 5). Upon increasing X_{PG} , the rate of dissociation decreased progressively. At $X_{PG} = 1.0$, values of 0.204 and 1.61 s were measured.

Dissociation of Cyt *c* from Liposomes by Other Cationic Membrane Binding Ligands. We recently compared the abilities of K_3 and K_{19} to dissociate cyt *c* from eggPG/eggPC liposomes. In brief, while K_{19} reversed the membrane binding of cyt *c*, the shorter peptide K_3 failed to do so (31). The dissociation of cyt *c* by K_{19} from liposomes was two-exponential, and at $X_{PG} = 0.20$ the fast and slow processes had half-times of 3.8 and 42.4 ms (Figure 5). Increasing X_{PG} attenuates in a progressive manner both processes, and at $X_{PG} = 1.0$ values of 10 ms and 2.5 s are observed.

Both myristoyl and palmitoyl chains covalently linked to proteins and peptides greatly enhance their membrane association (57, 58). McLaughlin and co-workers demonstrated myristoylated basic peptide to bind to phospholipid vesicles with an apparent rate on the order of magnitude of 10^{-7} , whereas the values for myristoyl moiety and peptide were 10^{-4} and 10^{-3} , respectively (50, 51). These data suggest that the hydrophobic and electrostatic binding energies were additive and that their combination strongly enhances membrane association. In this mechanism the acyl chain intercalates hydrophobically into the interior of the bilayer, while basic amino residues interact electrostatically with negative charges of the acidic phospholipid on the membrane surface. This mechanism is in agreement with our studies demonstrating that the myristoylated but not the nonmyristoylated KRTLRL releases cyt *c* from liposomes (31). The increase in fluorescence intensity was two-exponential, and at $X_{PG} = 0.20$, 3 μ M myr-KRTLRL detached cyt *c* from eggPG/eggPC liposomes with values of τ of 22.0 ms and 0.203 s (Figure 5). Increasing affinity of cyt *c* to the vesicles was observed upon increasing X_{PG} , and at $X_{PG} = 0.30$ and 0.40 strong suppression of the release of cyt *c* by myr-KRTLRL was evident. At $X_{PG} \geq 0.50$, myr-KRTLRL no longer dissociated cyt *c* from membranes.

The amphipathic helix containing peptide ACTH 1–24 has a net charge of +6 and thus associates with membranes by both electrostatic and hydrophobic interactions (52). Our earlier steady-state measurements showed ACTH 1–24 to detach cyt *c* from eggPG/eggPC liposomes (31). Studies on dissociation of cyt *c* from liposomes by ACTH 1–24 revealed this process to be two-exponential, and at $X_{PG} = 0.20$ the measured half-times were 5.7 and 63.4 ms (Figure

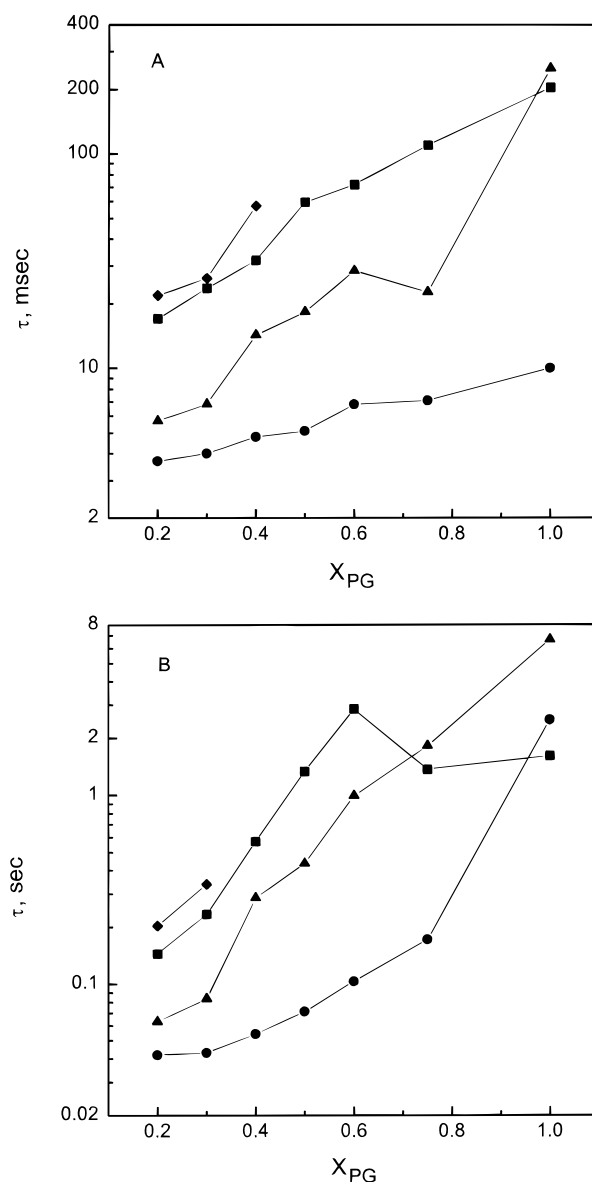


FIGURE 5: Comparison of the half-times (τ) for the dissociation of cyt *c* from eggPG/eggPC vesicles upon increasing X_{PG} by 0.6 μ M histone H1 (◆), 3 μ M poly-L-lysine (●), 5 μ M ACTH 1–24 (▲), and 3 μ M myr-KRTLRL (◈). For all these ligands the increase in fluorescence was two-exponential, and the corresponding half-times are compiled separately for the fast (A) and slow (B) components.

5). Upon increasing X_{PG} both components became slower, and at $X_{PG} = 1.0$ ACTH 1–24 displaces cyt *c* only sluggishly, with $\tau = 0.25$ and 6.66 s.

DISCUSSION

While binding of a peripheral protein to the surface of a lipid bilayer is a complex process, electrostatic attraction between a cationic protein and acidic phospholipids seems to be commonly involved (see ref 1 for a brief, recent review). We report here on the association of cyt *c* and histone H1 with liposomes containing varying amounts of the acidic phospholipid PG, measured using stopped-flow resonance energy transfer. Our previous steady-state measurements indicated that upon increasing X_{PG} from 0.1 to 0.4 the number of binding sites for cyt *c* in the membrane surface increased (29). However, the present data reveal that upon increasing X_{PG} in this range the binding of cyt *c*

becomes slower, i.e., the affinity of the vesicle surface for cyt *c* is diminished. This is unexpected as increasing negative surface charge density would be anticipated to enhance Coulombic attraction and thus to accelerate the membrane association of cyt *c*. As the converse is true, it follows that a process must be involved which retards the lipid binding of cyt *c*. Similar dependency on X_{PG} was observed also for the fluorescein-labeled histone H1, thus making it unlikely that a unique property of cyt *c* would be in question.

Despite extensive studies the properties of acidic phospholipid-containing bilayers have remained incompletely understood. Degree of protonation of PG increases upon increasing X_{PG} (56). Yet, this does not explain the deceleration of cyt *c* binding, as when X_{PG} is increased the surface net negative charge also increases. Therefore, an energy barrier dependent on X_{PG} must be involved. Such a barrier could be provided by highly cooperative hydrogen-bonded networks formed by deprotonated and protonated PG (59–63) which would further stabilize the lateral distribution of deprotonated PG molecules in the membrane. The distribution of charges appears to be critically dependent on X_{PG} , and different types of lipid head-group arrays appear to be formed below and above $X_{PG} = 0.50$. Further studies using different physicochemical techniques are required to characterize this process and its influence on membrane properties. Due to Coulombic repulsion, the distances separating deprotonated PGs bearing negative charge should be maximal. In other words, clusters of deprotonated acidic phospholipids are not present in the bilayer before the binding of the cationic protein. This also means that the binding of cyt *c* to the vesicle is not a simple bimolecular reaction. To this end, in combination with the actual number of membrane bound cyt *c* molecules being inaccessible to this approach, the complex nature of the binding process itself prohibits straightforward development of a meaningful kinetic model at this stage.

Once formed the complex of cyt *c* and lipid appears to be quite stable. This is clearly evident from the decreasing rate of release of A-site bound protein by, for example, ATP upon increasing X_{PG} . The enhanced stability of the membrane bound cyt *c* is likely to result from an attenuated off-rate for the equilibrium of attachment/detachment of cyt *c* upon increasing content of the acidic phospholipid, the latter stabilizing the lipid domain providing the binding site for cyt *c*. Additional stabilization could be due to the proposed extended lipid anchorage of cyt *c* to the lipid vesicle surface (1, 30, 64). The mechanism(s) of detachment of cyt *c* by NaCl also involves salt-induced changes in the degree of protonation of the acidic phospholipid (26, 29, 30, 56). Similarly to the release of cyt *c* by ATP, NaCl also should release only the A-site bound cyt *c*. However, because increasing [NaCl] augments deprotonation of PG, conversion from C-site to A-site association with subsequent detachment of cyt *c* from the membrane should commence. As to the increase in τ of the fast component as a function of X_{PG} , both increasing affinity of cyt *c* to the membrane as well as an increasing affinity of the membrane for protons could be involved.

For fluorescence-labeled H1 over the X_{PG} range of 0.2–1.0 and for cyt *c* when X_{PG} exceeds 0.5, the membrane binding of these proteins (i.e., decay of fluorescence)

becomes two-exponential. These data could be interpreted as the formation of two different types of PG domains in the membrane, one consisting of deprotonated PG and the other of hydrogen-bonded PG, the latter being responsible for the slow membrane association of the above proteins. The slower process with a half-time in the range of 10–40 ms could also be due to changes in the conformation of the proteins after their binding to the bilayer surface. The third possibility is that the initial fast binding process is followed by slow alterations in the membrane lateral order in a manner causing more of the pyrene-labeled probe to diffuse within the quenching radii of the proteins. We consider the last alternative to be more likely. Similar reasoning would also explain the slow changes in fluorescence following the initial rapid release of cyt *c* by NaCl, histone H1, and the other cationic ligands. As noted previously, these ligands as such cause alterations in fluorescence signals from the pyrene-labeled phospholipids (31). Yet, the sign of these changes parallel those produced when cyt *c* is detached from the liposomes by them. Accordingly, the increase in fluorescence intensity most likely involves contributions from two processes, i.e., release of cyt *c* as well as changes in fluorescence due to the membrane binding of the cationic ligands. Yet, as these processes must take place simultaneously, the increase in fluorescence does measure the detachment of cyt *c*. Similarly, in addition to the release of cyt *c* the addition of NaCl also reduces the protonation of PG. Accordingly, alterations in lipid distributions are not unexpected. Notably, Träuble (56) proposed in his insightful work a mechanism for electrostatic coupling between the two leaflets of bilayers containing acidic phospholipids. Changes in the net charge as well as in the charge distribution on the outer surface of the vesicles are likely to occur in the kind of experiments described here. Accordingly, transmembrane as well as lateral electric field gradients are anticipated. To conclude, changes in the organization of the membrane lipids induced by the attachment/detachment of the different surface-associating protein ligands are likely to be complex.

Notably, at $X_{PG} \geq 0.5$, the mode of lipid–cyt *c* interaction also becomes different. This is clearly demonstrated by the loss of the ability of ATP to detach cyt *c* when $X_{PG} \geq 0.5$. The C-site mediated binding of cyt *c* measured in the presence of ATP and varying X_{PG} between 0.5 and 1.0 is single-exponential, the fast component being absent. Interestingly, the rate of this process decreases upon increasing X_{PG} . Analogously to the discussion above, the reason for this could be intermolecular hydrogen bonding between PG head-groups competing for the interaction with cyt *c*. ATP appears to induce a change in the conformation of cyt *c* bound to membrane via its C-site (26). Accordingly, the difference in the half-times measured with X_{PG} in the range of 0.5–1.0 and in the absence and presence of ATP is not unexpected. Conversely, these data strongly support the concept of the fast process measured in the absence of ATP to represent electrostatically driven binding of cyt *c* to liposomes.

The detachment of cyt *c* from LUVs by H1 and the cationic membrane-binding peptides myr-KRTLRL, ACTH 1–24, and K₁₉ are similar in that for all of them the increase in fluorescence due to the release of cyt *c* from liposomes was two-exponential. Likewise, the fast and the slow

processes were both attenuated upon increasing X_{PG} . Although the concentrations of the ligands were such that they all produced saturating responses in steady-state measurements, there were also marked differences in the dissociation of cyt *c* by them. Thus, myr-KRTL only displaced cyt *c* when $X_{PG} \leq 0.4$. Interestingly, H1, K_{19} , and ACTH 1–24 all revealed complex dependencies of the values for τ on X_{PG} . On a molar basis, H1 is more effective than K_{19} in releasing cyt *c*. Yet, the half-time for the release of cyt *c* by 0.6 μM H1 at $X_{PG} = 0.20$ is approximately 17 ms, i.e., approximately 5-fold compared to 3.7 ms observed for 3 μM K_{19} . Accordingly, it seems likely that similarly to the binding of cyt *c* to membranes containing acidic phospholipids, also for H1 the association of H1 to membranes involves hydrogen bonding between protonated PG and H1. The differences in the release of cyt *c* by these ligands are likely to reflect differences in the relative contributions of hydrophobicity, Coulombic attraction, and hydrogen bonding in causing their attachment to the vesicles. Half-times for the membrane association of fluorescein-labeled H1 vary from 7.8 to 51.8 ms, at $X_{PG} = 0.20$ and 1.0, respectively. Accordingly, attachment of H1 to LUVs in the presence of cyt *c* is significantly retarded, with the corresponding half-times of 17 ms and 0.204 s. This is readily comprehensible as charges in the membrane surface should be neutralized by the bound cyt *c*, thus reducing the electrostatic attraction of H1 to the vesicle surface.

Current conceptualization of biomembranes emphasizes their functional dynamics (65). In brief, biomembranes are best understood as adaptive, liquid-crystalline supramolecular structures with intrinsic coupling between their ordering and functions (64–66). One of the keenly investigated issues concerns the formation and functional significance of domains enriched in acidic phospholipids (63, 66–68). Domains enriched in acidic phospholipids have been shown to promote the membrane association of proteins such as cyt *c* (22, 26, 29, 30), dnaA protein (69), histone H1 (41), and v-Src (50, 51). Also the converse is true, and the formation of lipid domains induced by the attachment of a protein to bilayer surface has been shown. For histone H1, for instance, this domain has been estimated to be comprised of approximately 20 phospholipid molecules (31). Our present data extend our previous studies and demonstrate that changes in the local concentration of acidic phospholipids can have pronounced influence on the mode of Coulombic attachment of peripheral membrane proteins. Although we used PG as the acidic phospholipid, there is no a priori reason for the properties determined by electrostatics to be different for phosphatidylserine, for instance. To this end, secondary peripheral lipid–protein interactions are likely to be abundant also for the extramembrane domains of integral membrane proteins (64). Accordingly, relatively simple yet effective mechanisms would be available for the regulation of a range of cellular processes by alterations in the local concentration of acidic phospholipids.

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