Dissociation of Cytochrome *c* from Liposomes by Histone H1. Comparison with Basic Peptides[†,‡]

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ABSTRACT: The basic chromosomal protein histone H1 binds avidly to liposomes containing acidic phospholipids and with characteristics somewhat resembling the lipid association of cytochrome *c* (cyt *c*) [Kõiv et al. (1995) *Biochemistry 34*, 8018−8027]. Membrane association of histone H1 strongly attenuates the lipid lateral diffusion in large unilamellar vesicles containing phosphatidylglycerol (PG) as revealed by the decrease in the excimer to monomer ratio I_e/I_m of the pyrene fatty acid-containing phospholipid derivative 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-*sn*-glycero-3-phosphoglycerol (PPDPG) fluorescence. Similarly, an increase in fluorescence anisotropy of the membrane-incorporated probe, diphenylhexatriene (DPH), due to histone H1 indicates that the membrane becomes more rigid. Increasing the mole fraction of PG (X_{PG}) increases in a linear manner the concentration $[H1]_S$ required for the maximal decrease in I_e/I_m or increase in fluorescence anisotropy, thus allowing to estimate the binding site for H1 to be constituted by approximately 20 PG molecules. Domain formation is also supported by differential scanning calorimetry measurements. Subsequently, we studied the detachment of cyt *c* from PG-containing liposomes by H1 by measuring its efficiency in decreasing resonance energy transfer between PPDPG and the heme of cyt *c*. The A-site interaction of 1 μM cyt *c* with 25 μM PG/PC ($X_{PG} = 0.20$) liposomes is fully inhibited by low (0.1 μM) histone concentrations. Upon X_{PG} being increased, the concentration $[H1]_D$ required for complete detachment of cyt *c* increses. Irrespective of the [cyt *c*] present (varying between 0.1 and 10 μM), the C-site-mediated binding of cyt *c* to neat PG liposomes ($X_{PG} = 1.0$) is fully prevented at [H1] = 0.6 μM. These measurements indicate that the affinity of histone H1 to liposomes exceeds that of cyt *c*. The above effects of H1 were subsequently compared with those of different basic membrane-associating peptides. Notably, the effects of H1 were remarkably well-reproduced by polylysine (K_{19}). The high affinity of H1 to acidic phospholipids suggests that this feature might also contribute to its physiological function.

The requirement for acidic phospholipids for the membrane association and activity of peripheral proteins is common, and numerous examples, such as some coagulation factors, protein kinase C, and dnaA protein, can be cited [for a recent review, see Kinnunen et al. (1994)]. Although the physicochemical properties of the bilayer seem to be crucially involved, only a few peripheral proteins or their models appear so far to have been systematically investigated in this respect: myelin basic protein [e.g. Nabet et al. (1994)], protein kinase C [e.g. Newton (1993) and Pap et al. (1995)], poly-K [e.g. de Kruijff et al. (1985) and Laroche et al. (1990)], and cytochrome *c* (cyt *c*).[1] The binding of the latter to membranes containing acidic phospholipids has been extensively studied [e.g. Kimelberg and Lee (1969), Quinn and Dawson (1969), Steinemann and Läuger (1971), Vanderkooi et al. (1973), Teissie (1981), Waltham et al. (1986), Mustonen et al. (1987), Kozarac et al. (1988), and Demel et al. (1989)]. Yet, the exact mechanisms and the amino acid residues constituting the lipid binding site(s) of cyt *c* have remained elusive. We have forwarded evidence for two different mechanisms of interaction of this protein with acidic phospholipids and assigned these to putative distinct sites, nominated as A and C (Rytömaa et al., 1992; Rytömaa & Kinnunen, 1994). The A-site of cyt *c* should interact electrostatically with deprotonated acidic phospholipids while the C-site ligands presumably by hydrogen bonding to protonated acidic phospholipids (Rytömaa & Kinnunen, 1994, 1995). For an acidic phospholipid such as phosphatidylglycerol (PG), the A-site interaction of cyt *c* predominates at $X_{PG} = 0.20$ while the C-site interaction prevails when $X_{PG} = 1.0$. The A-site interaction of cyt *c* is dissociated by increasing ionic strength as well as by nucleotides. Instead,

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[1] Abbreviations: ACTH 1−24, adrenocorticotropin hormone amino acids 1−24; ACTH 1−13, adrenocorticotropin hormone amino acids 1−13; cyt *c*, cytochrome *c*; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DPH, diphenylhexatriene; DSC, differential scanning calorimetry; egg PC, egg phosphatidylcholine; PG, phosphatidylglycerol; H1, histone H1; K_3, Lys-Lys-Lys; K_{19}, poly(L-Lys); KRTLR, Lys-Arg-Thr-Leu-Arg; Myr-KRTLR, myristoylated KRTLR; PPDPG, 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-*sn*-glycero-3-phosphoglycerol; PKC, protein kinase C; RFI_m, relative monomer fluorescence intensity; $R(I_e/I_m)$, relative excimer to monomer ratio of PPDPG fluorescence; X_{PG}, mole fraction of PG; $[H1]_S$, concentration of H1 inducing maximal decrease in $R(I_e/I_m)$; $[H1]_D$, concentration of H1 required for the dissociation of cyt *c*.

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ATP does not affect the binding via the C-site, and also, increasing ionic strength reverses this interaction only when it causes the deprotonation of acidic phospholipids (Rytömaa & Kinnunen, 1994). Both the A- and C-site membrane associations of cyt *c* may further include hydrophobic interaction due to extended lipid anchorage in which one acyl chain of the acidic diacyl phospholipid is suggested to be accommodated within the hydrophobic cavity of cyt *c* (Dickerson et al., 1971), while the other chain remains intercalated within the bilayer (Kinnunen et al., 1994; Rytömaa & Kinnunen, 1995). The protonation behavior of acidic phospholipids could be relevant also to the activation of *Escherichia coli* dnaA protein. Due to the close vicinity of the two phosphate groups of cardiolipin, its protonation behavior is much more complex than that of other acidic phospholipids such as PG (Hübner et al., 1991). Interestingly, cardiolipin is a much more potent activator of dnaA than PG (Sekimizu & Kornberg, 1988; Sekimizu et al., 1988).

Linker histone H1 is a basic chromatin protein with a high affinity for DNA. H1 also binds to acidic phospholipids and is commonly used as an in vitro substrate for protein kinase C [e.g. Bazzi and Nelsestuen (1987a)]. These authors also demonstrated that the cationic amphiphile sphingosine reverses the association of H1 with acidic phospholipids (Bazzi & Nelsestuen, 1987b). Similar observation has been made for the membrane binding of cyt *c* (Mustonen et al., 1993). Histone H1 binds weakly to phosphatidylcholine, but the affinity for membranes increases considerably with increasing content of acidic phospholipids (Kõiv et al., 1995). The association of histone H1 with liposomes containing over 30 mol % phosphatidylserine becomes resistant for NaCl. In a manner similar to that of cyt *c* (Rytömaa & Kinnunen, 1995) and protein kinase C (Bazzi & Nelsestuen, 1991), the lipid associated H1 is not detached by an excess of subsequently added liposomes (A. Kõiv and P. K. J. Kinnunen, unpublished results).

As the content of basic amino acid residues in proteins such as histones and myelin basic protein is unusually high, their binding to membranes containing acidic phospholipids has been modeled by for instance poly-Ks. Accordingly, studies have been carried out on the electrostatic association of different basic model peptides with acidic phospholipids [e.g. de Kruijff et al. (1985), Laroche et al. (1990), Kim et al. (1991), Mosior and McLaughlin (1991, 1992a,b), and Montich et al. (1993)]. While polylysines containing three or more residues interact with membranes (de Kruijff et al., 1985), their affinity increases with an increasing degree of polymerization (Kim et al., 1991). The separation of positively charged amino acids also affects the membrane binding affinity. When two alanine residues are inserted between lysines in pentalysine, the affinity for negatively charged membranes decreases by 1 order of magnitude (Mosior & McLaughlin, 1992b). The association of longer poly-K (1200−1700 amino acids) with dimyristoylphosphatidic acid (DMPA) membranes increases the ordering of the lipid acyl chains, and the gel to liquid crystal transition temperature of DMPA is elevated by ca. 14 °C (Laroche et al., 1990). The gel to liquid crystal transition of DMPA triggers a conformational change in K_{26} from an ordered β-sheet to random structure (Laroche et al., 1990), whereas the ordered β-sheet structure of longer poly-K is not strongly affected by the phase state of the membrane. Myristoylation of short basic peptides significantly increases their affinity to membranes (Buser et al., 1994), while the membrane affinity of myristoylated neutral peptides is much smaller than that of the corresponding basic peptides (Sigal et al., 1994). Likewise, both electrostatic attraction and hydrophobicity also contribute to the binding of the peptide hormone ACTH to membranes containing acidic lipids (Gysin & Schwyzer, 1984). Accordingly, ACTH 1−24 with a net positive charge of +6 binds to negatively charged membranes, and it has higher affinity than the amphipathic α-helical fragment 1−13 (Verhallen et al., 1984; de Kroon et al., 1991).

In order to develop further understanding on the characteristics of the A- and C-sites, we studied the detachment of cyt *c* from membranes containing acidic phospholipids by the strongly basic protein histone H1. More specifically, we compared the displacement of cyt *c* by H1 to that by different basic peptides, associating to membranes due to their charge or myristoylation or via an amphipathic helix. Cardiolipin either as such or complexed with cytochrome *c* oxidase forms the probable binding site for cyt *c* at the inner mitochondrial membrane (Vik et al., 1981; Speck et al., 1983). However, formation of the hexagonal H_{II} phase has been demonstrated as a consequence of cyt *c*−cardiolipin interaction (de Kruijff & Cullis, 1980). To avoid ambiguities in the interpretation of the results arising from the formation of the H_{II} phase, the present experiments were performed with PG-containing liposomes. In this context, it is also relevant to emphasize that we were not analyzing the lipid association of cyt *c* or H1 *per se* but are rather attempting to examine possible parallels as well as principal differences in the membrane association of these two peripheral proteins under conditions attempting to minimize interference due to factors compromising reliable interpretation of the data.

EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl-2-[10-(pyren-1-yl)decanoyl]-*sn*-glycero-3-phosphoglycerol (PPDPG) was purchased from K&V Bioware (Espoo, Finland) and diphenylhexatriene (DPH) from EGA Chemie (Steinheim, Germany). Horse heart cyt *c* (type VI, oxidized form), DMPC, DPPG, egg PC, and cholesterol were from Sigma. ACTH 1−24 and ACTH 1−13 were generously provided by Ciba-Geigy AG (Basel, Switzerland). K_3, Myr-KRTLR, and KRTLR were purchased from Bachem (Bubendorf, Switzerland), and K_{19} (on the average 19 amino acids) was from Sigma. The Na_2 salt of ATP was from Boehringer Mannheim (Germany). Egg PG was obtained from Sigma or Avanti Polar Lipids, Inc. No impurities were detected in the above lipids upon thin layer chromatography on silicic acid using chloroform/methanol/water/ammonia (65/20/2/2, v/v) as the solvent system and examination of the plates for pyrene fluorescence or after iodine staining. Histone H1 was purified from calf thymus (Johns, 1976). All other reagents were from Sigma.

Preparation of Liposomes. Lipids were dissolved in chloroform and mixed in this solvent to obtain the desired compositions. PPDPG ($X = 0.01$) was used as the fluorescent lipid probe. In fluorescence anisotropy measurements, DPH was included in a molar ratio of DPH/phospholipid of 1/500. The solvent was removed under a stream of nitrogen and the lipid residue subsequently maintained under reduced pressure for at least 2 h. The dry lipids were then hydrated in 20 mM Hepes and 0.1 mM ethylenediaminetetraacetic acid

(EDTA) (pH 7.0) at room temperature or at 50 °C (DPPG/DMPC liposomes) to yield a lipid concentration of 1 mM. To obtain unilamellar vesicles, the hydrated lipid dispersions were extruded with a LiposoFast small-volume homogenizer (Avestin, Ottawa, Canada). Samples were subjected to 19 passes through two polycarbonate filters (100 nm pore size, Nucleopore, Pleasanton, CA) installed in tandem (MacDonald et al., 1991). Minimal exposure of the lipids to light was ensured throughout the above procedure. Subsequently, the liposome solution was divided into proper aliquots and diluted with the buffer to a final lipid concentration of 25 μM.

Membrane Association of Histone H1 and Basic Peptides. Monomeric pyrene emits fluorescence with a maximum at 398 nm. However, when the pyrene concentration increases, an excited monomer may form a short-lived complex excimer (excited dimer) with an adjacent ground state pyrene. This excited dimer then relaxes back to two ground state pyrenes by emission with a maximum at about 480 nm. In the absence of quantum mechanical effects (Kinnunen et al., 1987) as well as nonradiative relaxation processes such as resonance energy transfer, I_e/I_m values for PPDPG reflect changes in the rate of lateral diffusion (Galla & Sackmann, 1974; Galla et al., 1979) as well as the distribution (i.e. local concentration) of the probe in the membrane (Galla & Hartmann, 1980; Somerharju et al., 1985; Hresko et al., 1986; Eklund et al., 1988). Comparison of data collected with inter- and intramolecular excimer-forming probes can be best described in terms of membrane free volume theory (Lehtonen & Kinnunen, 1994). A brief, recent review on the properties and uses of pyrene-labeled lipid probes has been written by us (Kinnunen et al., 1993). Notably, when absolute values for lipid lateral diffusion are not being sought, steady state I_e/I_m values give a reasonably good measure of qualitative changes in collision frequency of the probes, mirroring both lateral diffusion and local concentration of the pyrene-labeled lipids. The effect of histone H1 and basic peptides on the dynamics of phospholipid liposomes was studied by measurement of the pyrene excimer to monomer emission intensity ratio I_e/I_m (480 nm/398 nm) of the probe PPDPG. The measurements where conducted with our Perkin-Elmer LSB50 spectrofluorometer using excitation at 344 nm. Band widths of 2.5 and 4.0 nm were used for excitation and emission beams, respectively. The I_e/I_m values for 1 mol % of PPDPG included in liposomes composed of egg PG purchased from two suppliers Sigma and Avanti were 0.0525 and 0.0660, respectively. As both lipids revealed no impurities upon thin layer chromatography, the most feasible explanation for this difference is that it reflects variation in their acyl chain composition.

Fluorescence anisotropy measurements were conducted with our SLM 4800S spectrofluorometer. Polarized emission was measured in T-format and using Glan-Thompson calcite prisms. Excitation at 350 nm and emission at 450 nm were selected with monochromators, with respective band widths of 1 and 16 nm. Emission was also monitored with a long pass filter (430−455 nm). Values of steady state fluorescence anisotropy were calculated by the following equation (Lakowicz, 1983):

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}}$$

Differential Scanning Calorimetry. Binary large unilamellar liposomes composed of DMPC and DPPG in a 1/1 stoichiometry were used in DSC measurements. In order to have a large surface of liposomes accessible to binding of H1, we had to employ unilamellar instead of multilamellar liposomes. Importantly, the latter have been "traditionally" exploited in DSC, while there are no systematic studies characterizing large unilamellar liposomes by this method. Unilamellar DPPG/DMPC ($X_{PG} = 0.50$) liposomes (final lipid concentration of 1 mM) were maintained on ice overnight before the heat capacity scans were recorded using a high-sensitivity adiabatic differential scanning calorimeter (DASM-4, Biopribor, Pushchino, Russia) at a heating rate of 0.5 °C/min. The instrument was calibrated with a 1 mM sample of multilamellar liposomes of DPPC with an enthalpy of 35.6 kJ/mol for its main transition. Histone H1 was added to unilamellar liposomes maintained on ice and was thereafter allowed to equilibrate for 1 h prior to DSC.

Binding of cyt c to Liposomes. The lipid binding of cyt *c* was assessed as described previously (Mustonen et al., 1987; Rytömaa et al., 1992; Rytömaa & Kinnunen, 1994, 1995) by monitoring resonance energy transfer (Förster, 1948; Stryer, 1978) between the pyrene-containing lipid PPDPG and the heme of cyt *c*. The quenching of PPDPG fluorescence by the heme of cyt *c* was measured at 398 nm. Two milliliters of liposome solution was placed into a magnetically stirred four-window quartz cuvette in a holder thermostated with a circulating water bath at 25 °C or as indicated. Five or ten microliter aliquots of a 40 μM solution of cyt *c* were subsequently added, and the quenching of pyrene fluorescence by the heme of cyt *c* was observed. Thereafter, proper aliquots of the indicated basic peptides or histone H1 were included. Unless otherwise stated, changes in fluorescence were allowed to stabilize for approximately 40 s, whereafter the intensity of pyrene monomer fluorescence was recorded. Because of the low concentrations of both lipids and cyt *c* generally utilized, minimal interference by the inner filter effect is expected. Yet, in some experiments, higher concentrations of cyt *c* (up to 10 μM) were used and also a significant inner filter effect became evident. The rationale of these measurements was that in spite of high trivial absorption the concentration $[H1]_D$ required for the maximal detachment of cyt *c* from membranes can still be measured. As judged from its absorption spectra, the cyt *c* used was mainly in the oxidized form.

The merits as well as limitations of the use of pyrene-labeled lipids in energy transfer measurements have been discussed elsewhere (Kaihovaara et al., 1991; Rytömaa et al., 1992; Mostonen & Kinnunen, 1993; Kinnunen et al., 1993). In brief, the use of resonance energy transfer to assess the displacement of cyt *c* from PG-containing liposomes by H1 rests on the following mechanism. Due to spectral overlap, highly efficient resonance energy transfer from pyrene (donor) to heme (acceptor) of cyt *c* occurs upon the binding of the latter to liposomes labeled with the fluorescent lipid (Mustonen et al., 1987). To this end, detachment of cyt *c* from liposomes due to increasing ionic strength (i.e. increase in [NaCl]) or ATP can be followed by measurement of the reduction in quenching (Mustonen et al., 1987; Rytömaa et al., 1992; Rytömaa & Kunnunen, 1994, 1995). In the present study, we used H1 to detach cyt *c* from liposomes. Importantly, although we do not know either the orientations of the pyrene relaxation dipoles or that of heme

absorption, this point is essentially of no importance for the type of measurements performed. This stems from the fact that the increase in fluorescence due to detachment of cyt *c* from membranes by H1 is used to get the concentration of H1 fully dissociating cyt *c* (i.e. $[H1]_D$). Accordingly, the intermediate fluorescence intensities do not matter and the values of $[H1]_D$ can be obtained from the data completely irrespectively of the value of the coupling constant between the dipoles. In other words, for the way the data are collected, the values for $[H1]_D$ are absolutely independent of the actual value of the coupling constant. To this end, even in proximity measurements (using resonance energy transfer as a "spectroscopic ruler" (Stryer, 1978), the orientation of the dipoles is generally not known and is usually approximated using a value of $^2/_3$. Notably, even X-ray structure data (when available) would not be directly applicable to solution studies performed with liposomes, which, in addition are further impeded by the inherent complications of the two-dimensional systems (Drake et al., 1991). An important advantage of the use of resonance energy transfer between pyrene-labeled liposomes and the heme cyt *c* on our studies is that both cyt *c* and histone H1 can be used in their native state, without these proteins being labeled with fluorescent probes.

Our previous experiments using 20 mM Mes as the buffering medium revealed only insignificant differences in the membrane association of cyt *c* upon X_{PG} being increased from 0.3 to 1.0 (Rytömaa & Kinnunen, 1994). Interestingly, measurements in Hepes show clear differences in the kinetics of membrane association of cyt *c* when X_{PG} in liposomes is varied between 0.20 and 1.0 (Figure 4). More specifically, the efficiency of fluorescence quenching due to small amounts of cyt *c* (i.e. its apparent membrane affinity) is higher when $X_{PG} = 0.20$ than for neat PG liposomes. This difference between Mes and Hepes could result from binding of the buffering substances either to cyt *c* or to the liposomes. Importantly, this difference does not compromise the present data.

RESULTS

Binding of Histone H1 to Liposomes. In order to allow for an unambiguous interpretation of the subsequent fluorescence data on the detachment of cyt *c* from liposomes by histone H1, we first investigated the effects of H1 alone on the membrane lipid dynamics, as reflected in the I_e/I_m values for the membrane-incorporated probe PPDPG and the fluorescence anisotropy of membrane-incorporated DPH. As summarized in the introductory section, we have previously shown that the content of the acidic phospholipid critically influences the mode of membrane association of cyt *c* (Rytömaa & Kinnunen, 1994, 1995). Accordingly, the binding of histone H1 to liposomes was studied as a function of X_{PG}. Increasing X_{PG} from 0.20 to 1.0 progressively enhanced the increase in monomer emission intensity at 398 nm (RFI_m) observed after the addition of H1. The corresponding maximal decrease in I_e/I_m due to H1 was from 5 to 40%, while DPH emission anisotropy increased from 8 to 50% (Figure 1). In parallel with the increase 0.075 to 0.6 μM in I_e/I_m measurements and from 0.12 to 0.62 in fluorescence anisotropy measurements when X_{PG} was increased from 0.20 to 1.0. The approximately linear dependency of $[H1]_S$ on X_{PG} derived from both I_e/I_m and anisotropy measurements is depicted in Figure 2.

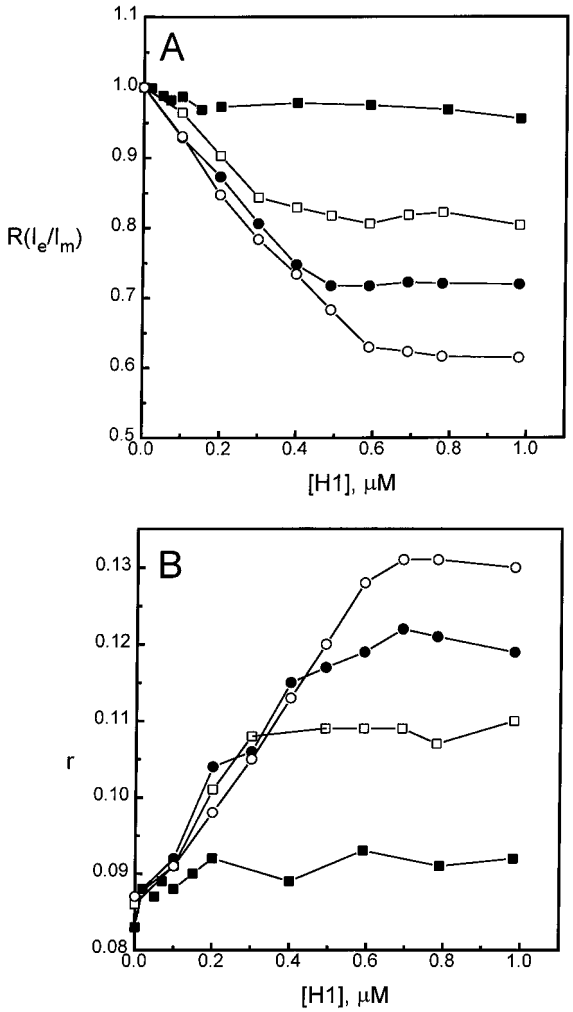

FIGURE 1: Effect of histone H1 on the relative excimer to monomer ratio $R(I_e/I_m)$ of pyrene fluorescence (A) and DPH anisotropy *r* (B) in eggPG/eggPC liposomes with $X_{PG} = 0.20$ (■), 0.50 (□), 0.75 (●), or 1.0 (○). The concentration of lipids in 20 mM Hepes and 0.1 mM EDTA (pH 7) was 25 μM.

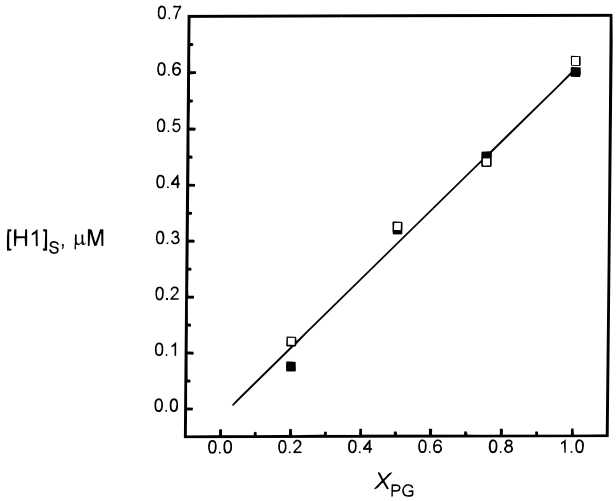

FIGURE 2: Concentration of $[H1]_S$ producing saturation response in I_e/I_m (■) and *r* (□) as a function of X_{PG}. The values were taken from Figure 1.

Histone H1-induced phase separation of negatively charged phospholipids was also revealed by DSC. Differential heat capacity scans for DPPG/DMPC ($X_{PG} = 0.50$) large unilamellar vesicles were measured both in the absence and in the presence of 20 μM H1. While the liposomes as such

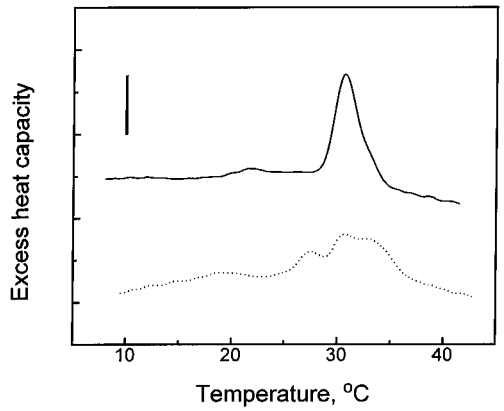

FIGURE 3: DSC scans for DPPG/DMPC (X_{PG} = 0.50) large unilamellar liposomes recorded in the absence (—) and presence (···) of 20 μM H1. The total concentration of lipids in 20 mM Hepes and 0.1 mM EDTA (pH 7) was 1 mM. The calibration bar corresponds to 5 kJ/°C mol^{-1}.

revealed one major peak (together with a minor shoulder on the high-temperature side) in the presence of H1, most of the enthalpy is contained in at least two overlapping endotherms (Figure 3), thus demonstrating H1-induced segregation of PG. To this end, the above lipid composition was chosen as the formation of a domain enriched in the acidic phospholipid DPPG due to H1 was anticipated to result in the splitting of the original heat absorption peak (recorded in the absence of H1) to lower and higher melting components. The former should represent H1 "free" membrane, enriched in DMPC, while the latter should be due to domain enriched in DPPG and scavenged by H1. As is evident in the tracings depicted, reliable estimation of the base lines in the presence of H1 is not possible. Accordingly, the total enthalpies measured for the combined peaks must be understood to be tentative only. Yet, for the liposomes as such and in the presence of H1, the estimated excess heat capacities were 27.5 and 28.5 kJ/mol, respectively, thus indicating that no major perturbation (except for the domain formation discussed above) of the bilayer by H1 is taking place. It is important to emphasize that these results are not directly comparable to the above fluorescence data since for DSC lipids containing saturated acyl chains are required whereas the I_e/I_m measurements were conducted with fluid membranes (i.e. lipids derived from egg yolk) abundant in unsaturated acyl chains.

Dissociation of Cytochrome c from PG-Containing Liposomes by Histone H1. The detachment of cyt *c* from liposomes by histone H1 was studied by measurement of the decrease in the extent of resonance energy transfer between PPDPG and the heme of cyt *c*. The effects of histone H1 on the membrane association of cyt *c* by its A- and C-site were studied separately using liposomes with X_{PG} = 0.20 and 1.0, respectively. Under the conditions used in the present study (Hepes buffer), the apparent affinity of the A-site of cyt *c* to liposomes appears to exceed that of the C-site (Figure 4). This could be due to a higher affinity of cyt *c* to membranes at X_{PG} = 0.20. Alternatively, the difference between kinetics at X_{PG} = 0.20 and 1.0 may also be apparent only and could also result from different efficiencies of resonance energy transfer (i.e. dipole−dipole coupling) further caused by different orientations of the heme of the membrane-bound cyt *c* with respect to the relaxation dipole of pyrene. At 25 μM total phospholipid and X_{PG} = 0.20, the A-site interaction of 1 μM cyt *c* was fully inhibited at $[H1]_D$ = 0.1 μM. Also the C-site binding at X_{PG} = 1.0 was fully dissociated by histone H1, although the concentration $[H1]_D$ required was higher, 0.6 μM (Figure 4). However, unlike the histone concentrations $[H1]_S$ causing a maximal decrease in I_e/I_m in the absence of cyt *c* (Figure 2), the dependency of $[H1]_D$ on X_{PG} clearly deviates from linear (Figure 5) and for X_{PG} < 1.0 clearly exceeds $[H1]_S$, as illustrated in Figure 6 by the difference $[H1]_D - [H1]_S$ *vs* X_{PG}. However, for neat PG liposomes, $[H1]_S$ equals $[H1]_D$. We then maintained X_{PG} at 1.0 and varied [cyt *c*] present between 0.1 and 10 μM, whereafter histone H1 was added (Figure 7). Interestingly, although the magnitude of fluorescence recovery due to H1 decreases due to trivial absorption at high cyt *c* concentrations, the concentration of histone $[H1]_D$ required to fully dissociate cyt *c* is independent of cyt *c* concentration (Figure 7). The lack of dependency of $[H1]_D$ on [cyt *c*] is further depicted in Figure 8. The above data suggest that the affinity of H1 to PG-containing membranes is high and exceeds that of cyt *c*. The concentration of histone $[H1]_D$ completely detaching cyt *c* presumably produces complete coverage of the liposome surface and/or scavenges all available PG.

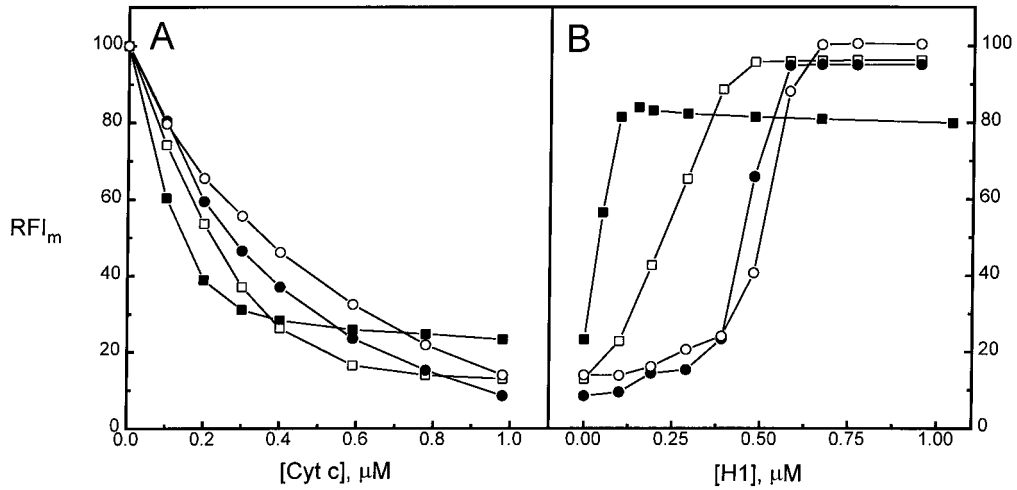

FIGURE 4: Effect of histone H1 on the association of cyt *c*. Quenching of pyrene monomer fluorescence (RFI_m) is illustrated as a function of [cyt *c*] (A) and subsequently added [H1] (B). X_{PG} in liposomes was 0.20 (■), 0.50 (□), 0.75 (●), or 1.0 (○). Otherwise, the conditions were as indicated in the legend for Figure 1.

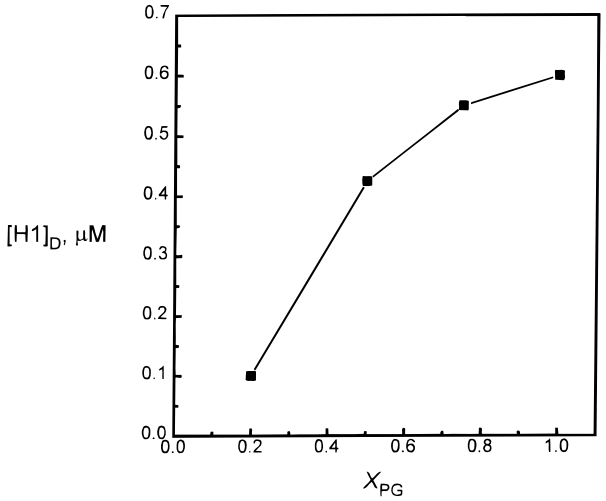

FIGURE 5: Histone concentration $[H1]_D$ required for the dissociation of cyt *c* from liposomes as a function of X_{PG}. The values were taken from Figure 4.

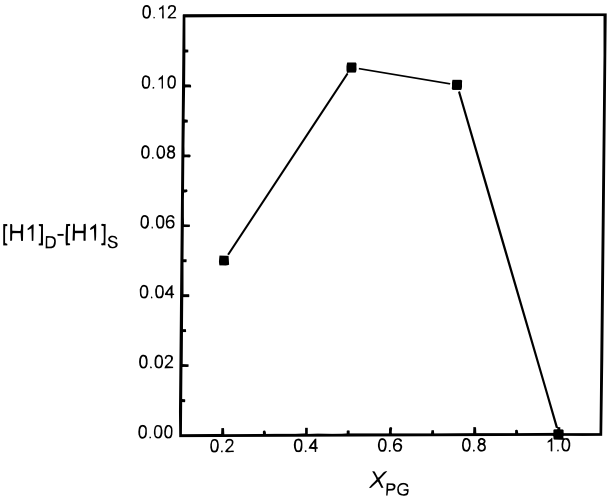

FIGURE 6: Difference between $[H1]_D$ and $[H1]_S$ as a function of X_{PG}. The values were taken from Figures 1 and 4.

Dissociation of Cytochrome c from PG-Containing Liposomes by K_3 and K_{19}. Histone H1 is strongly basic with approximately 29% of its amino acids being Lys. Therefore, it was of interest to compare the detachment of cyt *c* by H1 to that due to a basic model peptide, K_{19}. At $X_{PG} = 0.20$, the peptide itself in the absence of cyt *c* had only minor effects on the I_e/I_m of pyrene fluorescence of liposomes (Figure 9). However, when X_{PG} was increased, K_{19} decreased I_e/I_m in a manner somewhat similar to that of histone H1 (Figure 9). Unfortunately, the I_e/I_m *vs* X_{PG} data does not allow us to obtain $[K_{19}]_S$, thus impeding comparison with H1. K_{19} also dissociated cyt *c* bound to membranes via either its A-site or C-site, and a lower peptide concentration (≈0.3 μM) was required at $X_{PG} = 0.20$ than at $X_{PG} = 1.0$ (≈1.5 μM) (Figure 10). Similar to that of H1, the concentration of cyt *c* present did not strongly affect the $[K_{19}]_D$ required to fully dissociate cyt *c* from liposomes at $X_{PG} = 1.0$ (Figure 10).

The affinity of poly-Ks to acidic phospholipids increases considerably for longer peptides (Kim et al., 1991). This is readily understood in terms of the larger free energy gain upon charge neutralization of the higher polymers achieved upon their membrane association. Accordingly, when the same amount of charge but in the form of K_3 instead of K_{19} was added to liposomes in the absence of cyt *c*, practically no effect on I_e/I_m was observed. Likewise, K_3 did not dissociate cyt *c* liposomes at either $X_{PG} = 0.20$ or 1.0 (data not shown).

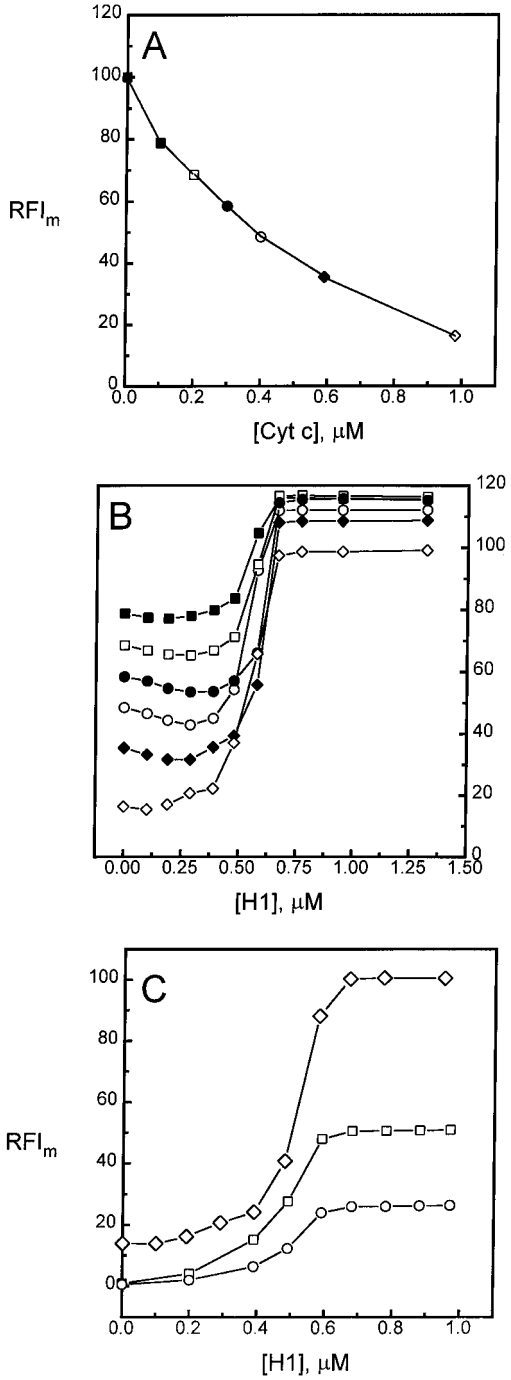

FIGURE 7: Effect of varying [cyt *c*] on the histone H1-induced dissociation of cyt *c* from neat PG liposomes (A) Final cyt *c* concentrations were 0.1 (■), 0.2 (□), 0.3 (●), 0.4 (○), 0.6 (◆), or 1.0 μM (◇). (B) Dissociation of cyt *c* by histone H1. The cyt *c* concentrations are as indicated in panel A. (C) The conditions were as in panel B, but the cyt *c* concentrations were 1.0 (◇), 5.0 (□), and 9.90 μM (○). Otherwise, the conditions were as described in the legend for Figure 1.

Dissociation of Cytochrome c from PG-Containing Liposomes by Myr-KRTLR and KRTLR. The experiments comparing K_3 and K_{19} revealed that enhanced membrane association due to electrostatics suffices for the detachment of cyt *c* by the higher polymer. Accordingly, it was of interest to study if enhanced membrane binding of the short

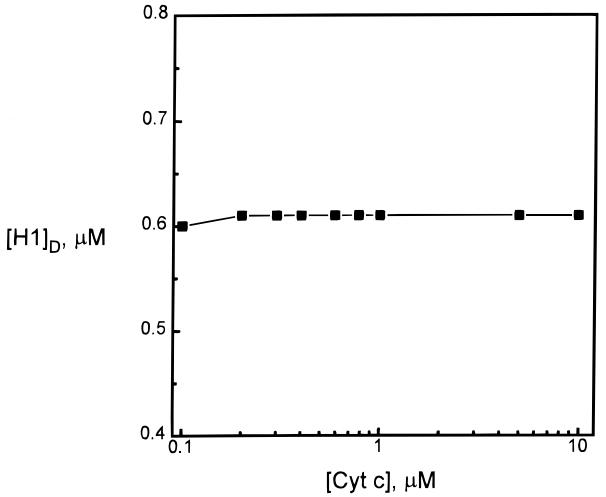

FIGURE 8: Apparent histone H1 concentration $[H1]_D$ required to fully dissociate cyt *c* as a function of [cyt *c*]. The values were taken from Figure 6.

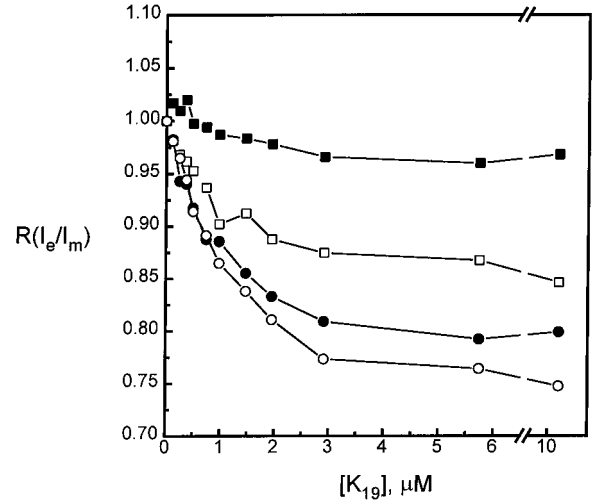

FIGURE 9: Effect of K_{19} on the relative excimer to monomer ratio $R(I_e/I_m)$ of pyrene fluorescence from PPDPG/egg PG/egg PC liposomes at $X_{PG} = 0.20$ (■), 0.50 (□), 0.75 (●), or 1.0 (○). The total lipid concentration was 25 μM in 20 mM Hepes and 0.1 mM EDTA (pH 7).

cationic peptide KRTLR due to myristoylation would render it capable of dissociating cyt *c* from liposomes. This turned out to be the case, and Myr-KRTLR detached cyt *c* bound to membranes via its A-site, although compared with that of K_{19} a higher concentration (2 μM) was required (Figure 11A,B). In keeping with the data on K_3, the nonmyristoylated peptide had no effect.

Interestingly, both Myr-KRTLR and KRTLR were unable to dissociate cyt *c* bound via its C-site from the membrane surface (Figure 11A,B). Judged by the decrease in I_e/I_m at $X_{PG} = 1.0$ and in the absence of cyt *c*, the above peptides do appear to partition into the membrane, whereas at $X_{PG} = 0.20$, only Myr-KRTLR is able to diminish I_e/I_m (Figure 12A,B).

Dissociation of Cytochrome c from PG-Containing Liposomes by ACTH 1−24 and ACTH 1−13. To complement the above model peptide studies, we then utilized two synthetic fragments of the cationic polypeptide hormone ACTH, i.e. ACTH 1−24 and 1−13. Both of these peptides should associate with liposomes due to the amphipathic helix formed by residues 1−12 (Verhallen et al., 1984). The membrane association of ACTH 1−24 and ACTH 1−13 decreased I_e/I_m (Figure 12C,D). Notably, ACTH 1−24 which contains the positively charged cluster (residues 15−18) dissociated both A- and C-site membrane association of cyt *c*, while ACTH 1−13 had essentially no effect (Figure 11C,D). The concentrations of ACTH 1−24 required for the detachment of cyt *c* bound via its C-site are much higher than those needed to dissociate A-site-bound cyt *c*.

DISCUSSION

Membrane binding of cyt *c* and the chromosomal strongly basic protein histone H1 seem to share similar features, and also the latter protein interacts strongly with acidic phospholipids (Kõiv et al., 1995). Yet, there are also distinct differences in their membrane association. Thus, H1 bound to membranes containing 30 mol % of either phosphatidylserine or PG is not dissociated by NaCl (Kõiv et al., 1995), whereas cyt *c* is detached even from neat PG membranes. The affinity of histone H1 to negatively charged membranes thus appears to be higher, and also, the binding mechanisms are likely to be different.

The membrane association of the highly basic histone H1 ($M_w \approx 22\,000$, with 29% of amino acids being Lys) induces rigidification of the membrane as shown by the decrease in I_e/I_m of PPDPG or the increase in fluorescence anisotropy of DPH in liposomes when X_{PG} varies between 0.50 and 1.0. However, very little change in I_e/I_m due to H1 binding to $X_{PG} = 0.20$ liposomes was observed, thus suggesting that in addition to rigidification also segregation of PG takes place due to the membrane association of H1 as demonstrated by DSC. While decreased lateral diffusion decreases I_e/I_m, the patching of PG increases this parameter, thus resulting in an insignificant net change. Since $[H1]_S$ is approximately linearly dependent on X_{PG}, the stoichiometry of H1 and PG in the complex formed should remain constant. Accordingly, 0.6 μM histone H1 is required to induce maximal change in I_e/I_m in neat PG liposomes at [PG] = 25 μM, thus yielding a PG/H1 stoichiometry of 40/1. Since approximately half of the PG resides in the inner leaflet of the liposomal bilayer, the binding site for H1 can be estimated to be constituted by ≈20 PG molecules.

The concentration of histone H1 required to dissociate cyt *c*, i.e. $[H1]_D$, is nonlinearly dependent on X_{PG}, and with X_{PG} varied in the range of 0.20−0.75 in liposomes, $[H1]_D$ exceeds $[H1]_S$ (Figure 6). A possible explanation could be cyt *c*-induced translocation of PG from the inner to the outer leaflet of liposomes. The facilitated translocation of PG from the inner to the outer leaflet by cyt *c* could be anticipated due to the neutralization of negative charges of PG at the outer leaflet upon complexation with the protein. The resulting net charge difference may thus provide the driving force for the augmented flip-flop of PG. At $X_{PG} = 1.0$, there should be no difference between $[H1]_D$ and $[H1]_S$, as observed. In the case of histone H1, similar translocation of PG due to charge gradient across the bilayer would be prevented by the rigidification of the membrane by H1, as shown by both I_e/I_m and fluorescence anisotropy measurements.

The concentration $[H1]_D = 0.1$ μM (corresponding to a H1/PG stoichiometry of 1/50) required to fully dissociate the A-site interaction of cyt *c* at $X_{PG} = 0.20$ is much lower than the concentration of cyt *c* present, 1.0 μM. This is likely

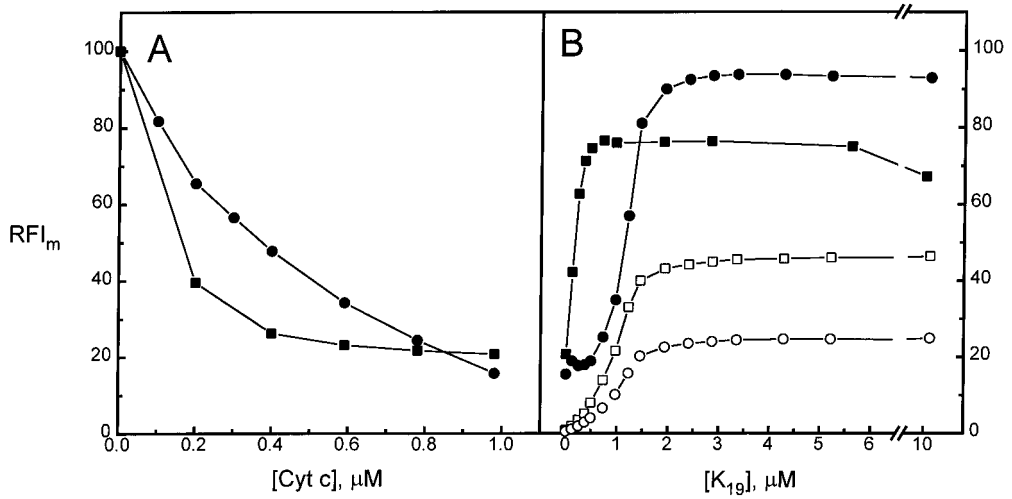

FIGURE 10: Effect of X_{PG} and [cyt *c*] on the K_{19}-induced dissociation of cyt *c* from liposomes: (A) binding of cyt *c* to liposomes at X_{PG} = 0.20 (■) or 1.0 (●) and (B) dissociation of cyt *c* from liposomes by K_{19} at X_{PG} = 0.20 (■) or 1.0 (●, □, and ○). The final [cyt *c*] was 1.0 (■ and ●), 5.0 (□), or 9.9 μM (○). Otherwise, the conditions were as described in the legend for Figure 1.

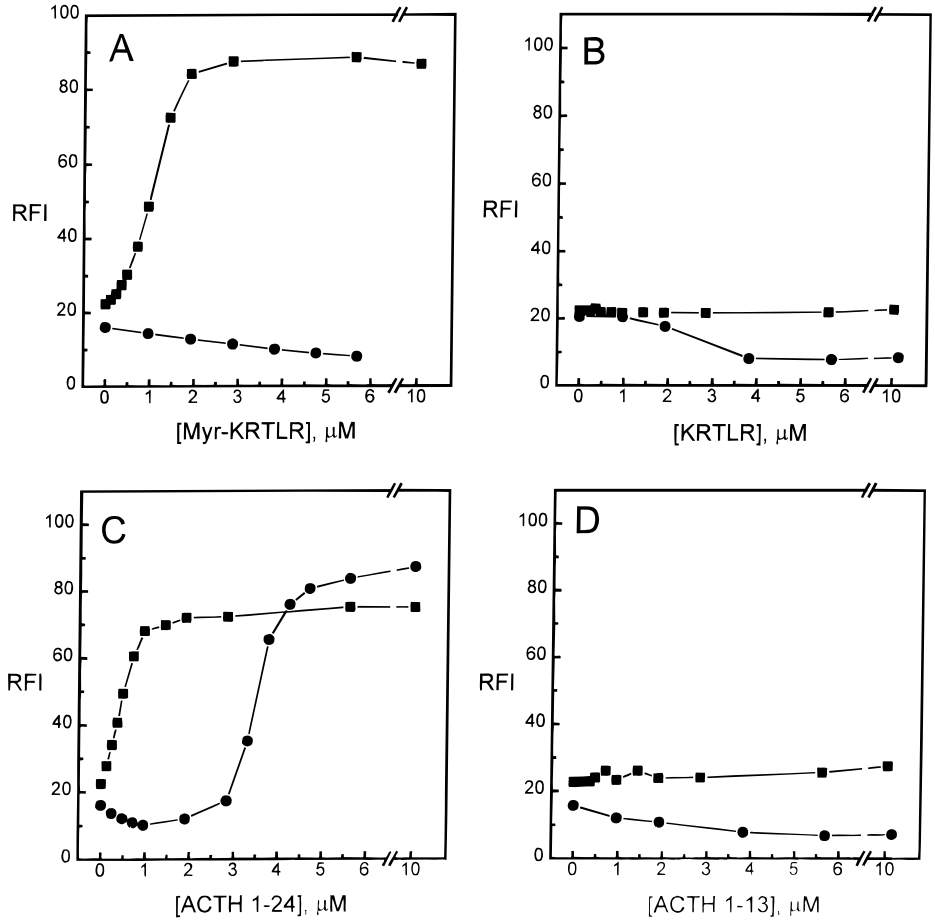

FIGURE 11: Effect of Myr-KRTLR (A), KRTLR (B), ACTH 1−24 (C), and ACTH 1−13 (D) on the association of cyt *c* with PPDPG/egg PG/egg PC liposomes at X_{PG} = 0.20 (■) and X_{PG} = 1.0 (○). The peptides were included subsequent to the membrane association of 1.0 μM cyt *c*. Otherwise, the conditions were as described in the legend for Figure 1.

to reflect different stoichiometries for the association of these proteins and acidic phospholipids. We have previously estimated the binding site for cyt *c* to be constituted by approximately 9.5 phospholipids (Mustonen et al., 1987). As was discussed above, our DSC as well as fluorescence measurements reveal the membrane association of H1 to be accompanied by the formation of domains enriched in PG, each H1 binding site being constituted by approximately 20 acidic phospholipids. This should result in a lower negative charge density elsewhere in the liposome surface so as to reduce the attachment of cyt *c* to membranes. Interestingly, the extent of dissociation of C-site interaction of cyt *c* by histone H1 does not seem to depend on the amount of cyt *c* present but rather on [H1] and the amount of PG present. Notably, even though there is little or no change in RFI_m at low [H1], this does not imply lack of dissociation of cyt *c* by H1. Accordingly, even small amounts of membrane-associated histone H1 should increase the degree of depro-

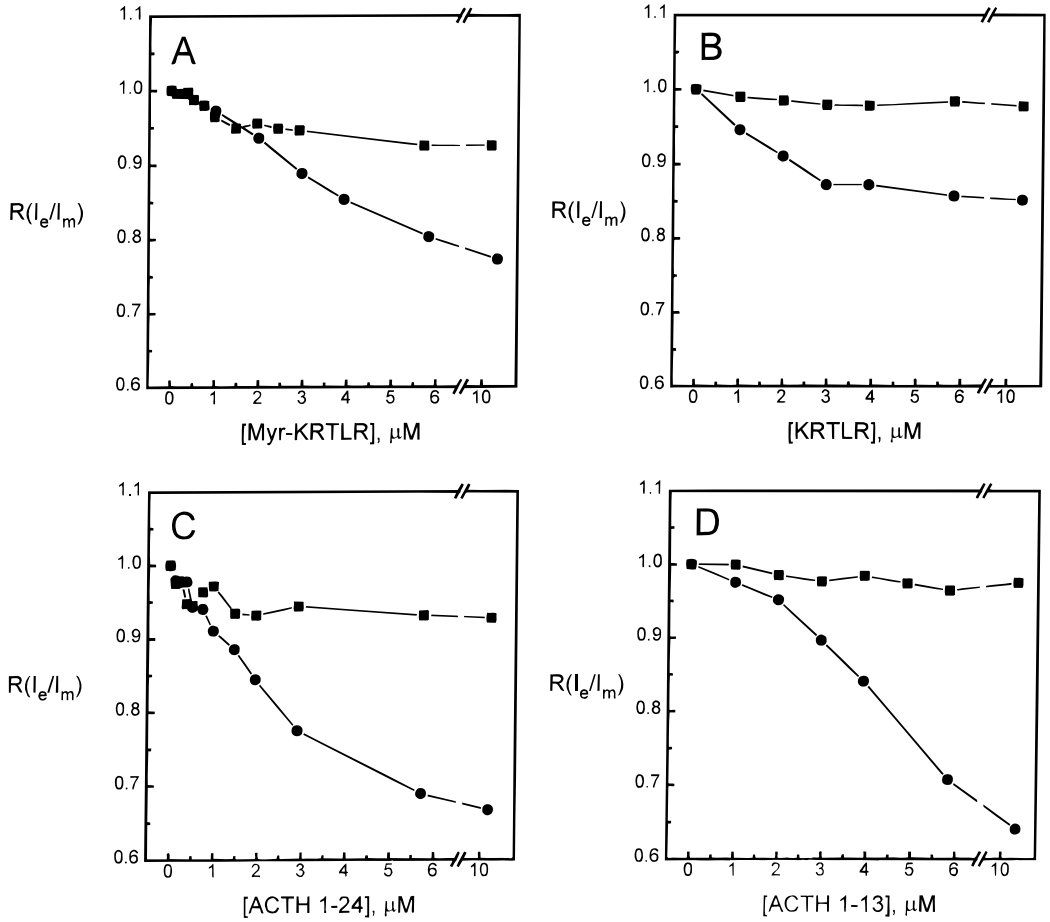

FIGURE 12: Effects of Myr-KRTLR (A), KRTLR (B), ACTH 1−24 (C), and ACTH 1−13 (D) on the relative excimer to monomer ratio $R(I_e/I_m)$ of pyrene fluorescence in liposomes at $X_{PG} = 0.20$ (■) and $X_{PG} = 1.0$ (●). Otherwise, the conditions were as described in the legend for Figure 1.

tonation of acidic phospholipids (due to reduced negative surface charge density), thus changing the cyt *c* lipid interaction to A-type binding, the latter then causing more efficient quenching of pyrene fluorescence than the C-site interaction (see Figure 4). As part of the membrane-associated cyt *c* becoming also detached by histone H1, we may expect no observable net change in RFI_m.

Due to the high content of Lys in the strongly basic H1, we then modeled the detachment of cyt *c* by this histone using poly-Ks. The present results indicate that the dissociation of cyt *c* from membranes by basic peptides seems to be governed by their high affinity to acidic phospholipids, resulting in competition with cyt *c* for electrostatic association with deprotonated acidic phospholipids. Accordingly, the increase in the degree of deprotonation of acidic phospholipids upon charge neutralization should also contribute (Träuble, 1977). For poly-Ks, the number of charges, i.e. the degree of polymerization, is important. Thus, even though K_3 has been reported to bind to membranes containing acidic phospholipids (de Kruijff et al., 1985), this peptide does not dissociate membrane-bound cyt *c*. However, increasing the number of lysine residues per peptide also increases their affinity to membranes (Kim et al., 1991), and K_{19} reverses both A- and C-type membrane interaction of cyt *c*. Similar to the case for H1, it appears to be the amount of PG available as well as the $[K_{19}]$ present which determines the detachment of cyt *c* as the same $[K_{19}]_D$ is required to dissociate both 1 and 10 μM cyt *c* (Figure 10). The 1/16 stoichiometry of K_{19}/PG observed at $[K_{19}]_D$ remains constant at both $X_{PG} = 0.20$ and 1.0. It follows that the binding site for K_{19} can be estimated to be constituted by ≈8 PG molecules at the outer monolayer of liposomes. Poly-K-induced segregation of acidic phospholipids has been reported (Galla & Sackmann, 1975; Hartmann & Galla, 1977, 1978). Upon the amount of membrane-associated K_{19} being increased, this results simultaneously in a diminishing surface density of PG^- (because of charge neutralization) which then allows for a progressive increase in the degree of deprotonation of PG in the liposomes (Träuble, 1977). As a result, the membrane association of cyt *c* changes from C-site to A-site.

Covalently attached myristoyl and palmitoyl chains have been suggested to be important in the enhancement of the membrane association of proteins (Schmidt, 1989; James & Olson, 1990). For example, endogenous PKC substrate $pp60^{v\text{-}src}$ is myristoylated at glycine 2 (Kaplan et al., 1988), while the positively charged lysine residues also play a role in the membrane association of $pp60^{v\text{-}src}$ (Silverman & Resh, 1992; Buser et al., 1994; Sigal et al., 1994). Myr-KRTLR is a synthetic peptide that inhibits PKC by interacting with phosphatidylserine and competing with the protein substrate of PKC (O'Brian et al., 1990). Myristoylation of this peptide is also crucial for its inhibitory effect on PKC. Our data show this peptide to reverse the A-site membrane association of cyt *c*. It seems reasonable to assume that the basic residues of the myristoylated peptide compete with cyt *c* for binding to PG. However, the hydrophobicity of the peptide appears to be important, and if the membrane anchorage of

the peptide is not promoted by the myristoyl chain, detachment of cyt *c* from liposomes is no longer observed. The partitioning of Myr-KRTLR to neat PG could be reduced and inhibited by cyt *c*. Alternatively, even though Myr-KRTLR would bind to neat PG liposomes, it may not be able to dissociate cyt *c*. The former explanation is more likely to be valid.

In addition to covalently linked fatty acids, amphipathic helices (Kaiser & Kezdy, 1984; Segrest et al., 1990) are common structural motifs causing hydrophobic association of proteins with membrane surfaces, as exemplified by ACTH. For this peptide hormone, the formation of an amphipathic helix by residues 1−12 or 1−16 has been suggested (Verhallen et al., 1984), while residues 11−24 should interact electrostatically with membranes containing acidic phospholipids (Gysin & Schwyzer, 1984). Although ACTH 1−13 has a net positive charge of +2, it is unable to interfere with the binding of cyt *c* to PG, in contrast to ACTH 1−24. Accordingly, the dissociation of cyt *c* bound via A-site by ACTH 1−24 should not be due to the hydrophobicity of the peptide associating it to liposomes but more likely due to a competition with cyt *c* for electrostatic interaction with deprotonated acidic phospholipids. Since ACTH 1−24 is also able to dissociate cyt *c* bound via its C-site, the peptide-induced increase in the deprotonation of PG probably takes place.

The membrane association of peripheral membrane proteins can result from electrostatic forces, hydrogen bonding, or hydrophobicity. In addition to the importance of understanding the regulation of the functions of peripheral membrane proteins by lipids, it is important to know how the membrane association of different proteins is regulated and which proteins compete for the same binding site on membrane surfaces. We are at present investigating the possibility that there could be distinct classes of peripheral proteins whose membrane binding is determined by specific physicochemical features of the bilayer. To this end, although competition for membrane binding by H1 and cyt *c* under physiological conditions is rather unlikely, the present results demonstrate that the affinity of H1 to acidic phospholipids far exceeds that of cyt *c*. In addition, some of the features of lipid binding of H1 are surprisingly well-retained by K_{19}. Both H1 and the membrane-associating peptides K_{19}, Myr-KRTLR, and ACTH 1−24 seem to have two effects which influence the binding of cyt *c* to liposomes. First, after having partitioned to the liposome surface, they compete with cyt *c* for the deprotonated acidic phospholipid, and, if their affinity is high enough, they detach cyt *c* from the membrane. Second, the association of H1 and the cationic peptides with acidic phospholipids causes charge neutralization. Accordingly, the negative surface charge density of the vesicle diminishes. This, in turn, decreases the extent of protonation of the acidic phospholipid.

Finally, cyt *c* is considered as a paradigm for peripheral membrane proteins. The present results reveal that the affinity of H1 to acidic phospholipids must exceed that of cyt *c* several-fold. Accordingly, perhaps H1 should also be understood as a peripheral membrane protein. It is tempting to speculate that the membrane affinity of H1 may also contribute to its physiological functions in controlling chromatin structure.

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