Interactions of Thionin from *Pyrularia pubera* with Dipalmitoylphosphatidylglycerol Large Unilamellar Vesicles[†]

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ABSTRACT: The peptide toxin thionin from *Pyrularia pubera* binds to dipalmitoylphosphatidylglycerol (DPPG) large unilamellar vesicles as shown by an increase in the intensity and blue-shift of the fluorescence emission spectrum of the single tryptophan residue of the protein. The magnitude of these fluorescence changes increased with temperature near the thermotropic phase transition of DPPG (about 40 °C). Fluorescent probes sensitive to the structure and dynamics of the membrane were used to assess the effect of thionin binding on bilayer properties. The fluorescence emission spectra of Prodan, Patman, and Laurdan all showed spectral changes consistent with an increase in bilayer polarity at temperatures below the DPPG phase transition but a decrease in polarity at higher temperatures. Fluorescence polarization experiments and the ratio of monomer-to-excimer fluorescence of the probe 1,3-bis(1-pyrene)-propane suggested that thionin increases the bilayer order above the transition temperature. Differential scanning calorimetry revealed that thionin broadens the transition and either increases or decreases the melting temperature depending on the concentration of the peptide. Taken together, the data are consistent with at least three distinct interactions of thionin with the bilayer: (1) thionin bound electrostatically to the bilayer surface; (2) tryptophan of the bound thionin inserted into the bilayer; (3) high-order aggregates of thionin-bound vesicles.

An area of considerable interest in membrane biochemistry is the nature of lipid−protein interactions. Such interactions have been pursued from the perspective of both how they are influenced by phospholipid bilayer properties as well as how the presence of the protein alters those properties. One facet that has received considerable attention, both experimentally and theoretically, is the interaction of peripheral proteins with membranes. Examples of proteins so studied include cytochrome *c* (Heimburg & Marsh, 1995), phospholipase A_2 (Bell & Biltonen, 1989), lipase (Smaby et al., 1994), protein kinase C (Newton, 1995), and cardiotoxin (Picard et al., 1996). According to theoretical work involving Monte Carlo simulations, the binding of peripheral proteins should have predictable effects on the thermotropic phase transition of homogeneous bilayers (Heimburg & Biltonen, 1996).

Pyrularia thionin is a small peptide (47 amino acids) isolated from the nuts of the parasitic plant *Pyrularia pubera*. The peptide is strongly basic due to high concentrations of lysine and arginine (Vernon et al., 1985). Thionin exerts a variety of effects on different biological systems. For example, it activates endogenous phospholipase A_2 in cultured fibroblasts (Angerhofer et al., 1990; Evans et al., 1989) and leads to hemolysis of human erythrocytes (Osorio e Castro & Vernon, 1989; Osorio e Castro et al., 1989). It causes depolarization of murine P388 cell membranes and increases Ca^{2+} permeability (Evans et al., 1989). In addition to increased ion flux, thionin also causes leakage of nucleotides and potassium ions from baby hamster kidney cells (Carrasco et al., 1981). In the membranes of S49 lymphoma cells, thionin enhances the activity of forskolin-stimulated adenylate cyclase (Huang et al., 1994). It is thought that many or all of these responses result from specific interactions between thionin and the phospholipid bilayer (Osorio e Castro & Vernon, 1989; Vernon & Rogers, 1992; Thevissen et al., 1996).

Thionin appears to bind to membranes via electrostatic interactions (Vernon & Rogers, 1992; Vernon, 1992). There is also some evidence that the peptide inserts partially into the bilayer after binding (Vernon, 1992). Fluorescence polarization experiments suggest that thionin increases the order of the bilayer in erythrocyte ghosts (Osorio e Castro et al., 1990). Depending on the membrane composition, thionin either increases or decreases the viscosity of liposome membranes according to NMR and EPR experiments (Gasanov et al., 1993). Some of these effects may involve the formation of nonbilayer structures (Gasanov et al., 1993). Lastly, fluorescence digital imaging microscopy has revealed a tendency of thionin to cause aggregation of phosphatidylserine into large domains in erythrocyte membranes (Wang et al., 1993).

In spite of these studies, little is known regarding the details of how thionin alters bilayer properties. In particular, the effects of thionin on the phase properties of artificial membranes remain unexplored. We have used fluorescence spectroscopy and differential scanning calorimetry to study the interactions between *Pyrularia* thionin and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DPPG)[1] large unilamellar vesicles. First, we investigated the effects of the bilayer phase on the interaction of thionin with the membrane. Second, we examined the changes in bilayer physical properties induced by thionin. These properties included the bilayer polarity, the viscosity, and the gel-to-

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liquid-crystalline phase transition. These studies revealed that the interaction of thionin with the bilayer is more complex than simply the binding of a peripheral protein through electrostatics (Heimburg & Biltonen, 1996). Consequently, this peptide is an interesting model for more complex interactions that may occur between peripheral proteins and lipid bilayers.

MATERIALS AND METHODS

Materials. Thionin was purified as described (Vernon et al., 1985). DPPG was purchased from Avanti Polar-lipids, Inc. (Alabaster, AL). 6-Propionyl-2-(dimethylamino)naphthalene (Prodan), 6-lauroyl-2-(dimethylamino)naphthalene (Laurdan), 6-palmitoyl-2-[[[2-(trimethylammonio)ethyl]methyl]amino]naphthalene chloride (Patman), and 1,3-bis-(1-pyrene)propane (bis-pyrene) were obtained from Molecular Probes, Inc. (Eugene, OR).

Phospholipid Vesicles. Large unilamellar vesicles were prepared from DPPG by extrusion (Hope et al., 1985) as described (Bell et al., 1996). The DPPG concentration was determined by assay of the phosphate content (Bartlett, 1959). Prodan, Laurdan, and Patman were mixed with DPPG in chloroform prior to vesicle preparation. Bis-pyrene (dissolved in dimethylformamide) was mixed and equilibrated with preformed vesicles. The ratio of bis-pyrene to DPPG was 1:1000. The ratio of the other probes varied between about 1:400 and 1:300.

Fluorescence Measurements. Fluorescence excitation and emission spectra of DPPG vesicles were obtained with a FluoroMax spectrofluorometer using a xenon arc lamp (SPEX Industries, Inc., Edison, NJ). The excitation and emission band-passes of the monochromators were 3.4−4.25 nm depending on the intensity of the probe. Temperature was controlled with a circulating water bath, and sample homogeneity was maintained by continuous magnetic stirring. Sample temperature was equilibrated prior to mixing of vesicles and thionin. DPPG vesicles and thionin were diluted with buffer containing 50 mM KCl, 10 mM sodium borate, and 3 mM NaN_3, pH 8. The temperature and final concentrations of DPPG and thionin are indicated in the figure legends. The anisotropy of Prodan, Patman, and Laurdan was determined under the same conditions by steady-state polarization experiments with the exception that the band-pass was increased to 8 nm (Bell et al., 1996). Some of the fluorescence emission data presented represent intensity measurements obtained during anisotropy experiments. All spectra are technical spectra.

Differential Scanning Calorimetry (DSC). The DPPG phase transition was examined by heat-conduction scanning calorimetry (Model 7707; Hart Scientific, Pleasant Grove, UT). Temperature was determined using an NIST-traceable thermometer in the calorimeter. Samples (approximately 500 μL) contained vesicles (10 mM DPPG) in the same buffer described above for fluorescence experiments. Various concentrations of thionin (0.07 mM−1.5 mM) were added to separate samples. Three samples and one reference (buffer) were scanned simultaneously in each experiment.

[1] Abbreviations: DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-(phospho-*rac*-(1-glycerol)]; Prodan, 6-propionyl-2-(dimethylamino)naphthalene; Laurdan, 6-lauroyl-2-(dimethylamino)naphthalene; Patman, 6-palmitoyl-2-[[[2-(trimethylammonio)ethyl]methyl]amino]naphthalene chloride; bis-pyrene, 1,3-bis(1-pyrene)propane; DSC, differential scanning calorimetry; GP, generalized polarization.

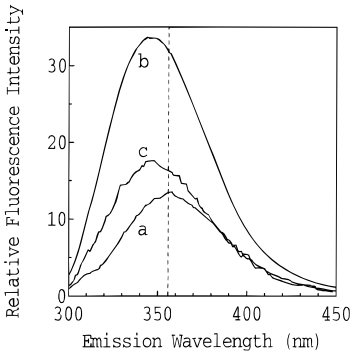

FIGURE 1: Emission spectra of thionin. Tryptophan emission spectra of 6 μM thionin were obtained without (curve a) and immediately following (curve b) the addition of 30 μM DPPG at 42 °C (excitation wavelength = 280 nm). The spectrum was also obtained 1000 s after DPPG addition (curve c). The dashed line marks the maximum in the spectrum prior to addition of vesicles.

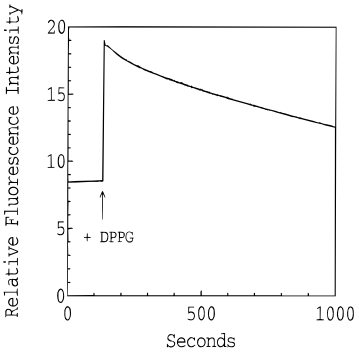

FIGURE 2: Time dependence of the thionin fluorescence intensity upon the addition of DPPG vesicles. 6 μM thionin was incubated in the sample buffer for 30 min at 42 °C before the fluorescence was recorded. 30 μM DPPG vesicles were added into the sample at the time indicated. Emission = 340 nm, excitation = 280 nm.

Data were obtained for each sample during two heating−cooling cycles, i.e., from 20 to 60 °C, then cooling back to 20 °C and then repeating the process. The scan rate was 5 °C/h in each direction.

RESULTS

Thionin Fluorescence. The addition of thionin to DPPG large unilamellar vesicles at 42 °C caused an immediate 2-fold increase in the intensity of the fluorescence of the single tryptophan residue of the protein (curve b, Figure 1). This increase in intensity was accompanied by a 10 nm blue shift in the emission maximum.

The immediate enhancement of thionin fluorescence was followed by a slow decrease in the emission intensity (Figure 2). The rate of this decay increased with increasing temperature (31−45 °C) and concentration of thionin and vesicles (not shown). As shown in Figure 1, this decay in tryptophan intensity was not accompanied by a reversal of the 10 nm shift in the emission spectrum (curve c). Control experiments in which the time of exposure of the sample to UV light from the fluorometer lamp was varied demonstrated that the decrease in fluorescence intensity was not due to photobleaching. Further investigation using fluorescent probes located in the vesicle bilayer revealed that the decay in thionin fluorescence intensity was accompanied by a similar wavelength-independent decrease in probe intensity. The correlation between thionin and probe fluorescence decay was quantitative and reproducible, suggesting that the reduction in intensity was probably due to adsorption of the

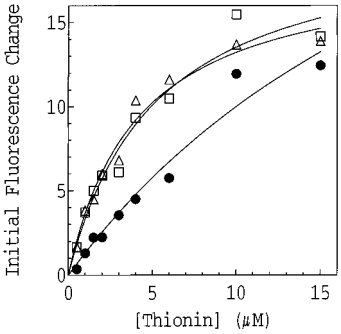

FIGURE 3: Thionin concentration dependence of the initial thionin fluorescence change in the presence of DPPG. The tryptophan emission spectrum was obtained at the indicated concentrations of thionin before and immediately after the addition of vesicles (15 μM DPPG) at 37 (circles), 42 (squares), or 45 °C (triangles). The fluorescence change was calculated as the difference in intensity upon the addition of vesicles and corrected for the amount of light scattered by the vesicles.

vesicle/thionin complex to the walls of the sample cuvette. Consequently, all fluorescence data were gathered immediately after mixing thionin and vesicles and then compared to those obtained several hundred seconds later to verify that observations pertained to steady state. Likewise, analyses based on spectrum shape and independent of total fluorescence intensity were used as much as possible.

The initial elevation of thionin fluorescence after mixing with vesicles increased monotonically with increasing concentration of protein (Figure 3). Furthermore, as shown in Figure 3, the magnitude of the fluorescence change increased as the temperature approached the main thermotropic phase transition of DPPG (about 40 °C). This temperature dependence was likewise observed in comparisons of the emission spectrum wavelengths. For example, at 3 μM thionin and 15 μM DPPG, the thionin emission maximum shifted from 356 to 346 nm at 45 °C, from 357 to 348 nm at 42 °C, and from 357 to 350 nm at 37 °C. Similar results were obtained when the concentration of vesicles was varied (not shown). These data suggest that thionin interacts preferentially with the liquid-crystalline phase of the lipid. In an attempt to distinguish whether the preference was due to kinetics or thermodynamics, we incubated thionin with DPPG vesicles at low temperature ($\leq$35 °C) and then raised the temperature to above the lipid phase transition. As expected from the data of Figure 3, the fluorescence intensity and the blue-shift of the emission spectrum both increased as the temperature was raised. However, when the temperature was lowered, the thionin fluorescence change (both intensity and wavelength maximum) did not reverse to the initial state (not shown). This lack of reversibility indicated that at least some of the effect of temperature was due to the kinetics of the interaction rather than solely the thermodynamics.

Effects of Thionin on Bilayer Polarity. Fluorescent probes sensitive to the polarity of the membrane were used to observe the effect of thionin binding on the properties of the bilayer. The emission spectrum of DPPG vesicles labeled with Laurdan was obtained at 45 °C (Figure 4). The addition of thionin shifted the spectral maximum to shorter wavelength and decreased the intensity. The blue-shift suggested that the addition of thionin resulted in either a less polar environment or a decrease in the rate of dipolar relaxation (i.e., decreased mobility of local water molecules) (Parasassi et al., 1991). Similar results were obtained with the related probes Patman and Prodan (not shown).

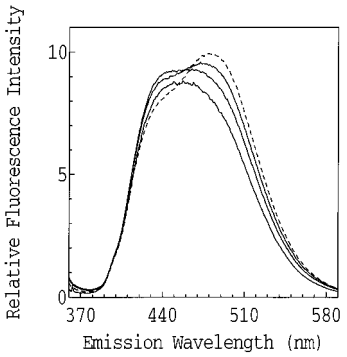

FIGURE 4: Effect of thionin concentration on Laurdan emission spectra. 100 μM DPPG vesicles containing 0.3 μM Laurdan were equilibrated in the fluorometer for 15 min at 45 °C. Various concentrations of thionin were then added, and the emission spectrum was recorded about 100 s later (0, 3, 8, 15 μM from top to bottom at 490 nm; the dashed curve represents 0 thionin). (Excitation wavelength = 350 nm.)

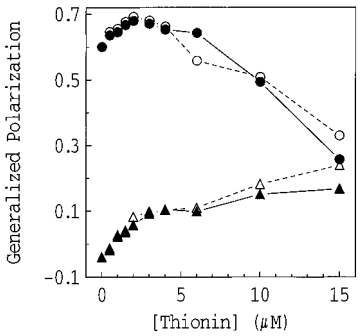

FIGURE 5: Laurdan GP as a function of thionin concentration at 45 °C (triangles) and at 37 °C (circles). GP was calculated as indicated in the text from Laurdan emission spectra (excitation wavelength = 312 nm). Vesicle concentration was 50 μM DPPG. The solid symbols represent values obtained 100 s after mixing thionin and vesicles. The open symbols were obtained 1000 s later.

The effects of thionin concentration on the spectra of these probes were quantified by calculation of the generalized polarization (GP) for each emission spectrum with the relationship:

$$\mathrm{GP} = \frac{I_{435} - I_{500}}{I_{435} + I_{500}} \quad (1)$$

GP is useful for this purpose since the values of GP (in contrast to ratios) follow the additivity rule and are thus directly proportional to changes in the spectrum (Parasassi et al., 1991). A decrease in the value of GP signifies an increase in the interactions between Laurdan and water molecules in the bilayer (Parasassi et al., 1994). As shown in Figure 5, the value of Laurdan GP increased with increasing thionin concentration at 45 °C (triangles), consistent with the spectral changes shown in Figure 4. At a temperature below the phase transition (37 °C), the value of GP in the absence of thionin was much higher than at 45 °C, reflecting the probe's environment in the gel phase (circles). Interestingly, in contrast to the effect in the liquid-crystalline phase, thionin tended to reduce the value of GP in the gel phase. (In some experiments at 37 °C, thionin increased the value of GP at the lowest concentrations and then decreased it at higher concentrations.) Intermediate effects occurred at intermediate temperatures. Similar data were also obtained with Prodan and Patman (not shown).

Because of the time dependence displayed by the thionin fluorescence (Figures 1 and 2), it was necessary to ascertain

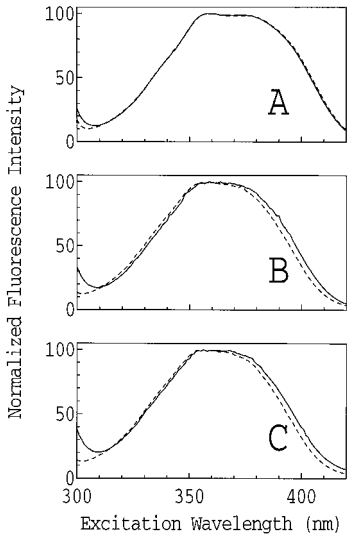

FIGURE 6: Effect of thionin concentration on Laurdan excitation spectra. 100 μM DPPG vesicles containing 0.3 μM Laurdan were mixed with different concentrations of thionin at 37 °C (panel A), 42 °C (panel B), and 45 °C (panel C) as described for Figure 4, and the excitation spectrum was obtained 100 s later. Thionin concentrations were 0 (dashed curve) and 15 μM (solid curve). The spectra were normalized to the peak intensity to emphasize the shifts. (Emission wavelength = 435 nm.)

whether the spectral observations with Laurdan, Patman, and Prodan were stable. Accordingly, the experiment of Figure 5 was repeated for each probe as a function of time (compare open symbols to solid symbols in Figure 5). No time dependence was observed in the value of GP when the ratio of thionin to DPPG was low (<1:4 at 37 °C, <1:9 at 45 °C; the highest ratio in Figure 5 is 1:3.3). At higher thionin-to-DPPG ratios, the value of GP changed with time depending on the probe (increase with Laurdan and Patman, decrease with Prodan).

Additional detail was obtained by investigating the influence of thionin concentration on the excitation spectrum of each probe. Figure 6 demonstrates the effect of thionin on the excitation spectrum of Laurdan in DPPG vesicles. (The data are normalized to emphasize the small spectral shifts.) At temperatures above the DPPG phase transition, the excitation spectra shifted to longer wavelengths (i.e., solid compared to dashed curves, panels B and C). Below the transition temperature, no consistent shift in the spectrum was observed (panel A). Similar results were obtained with Prodan and Patman (not shown).

Effects of Thionin on Bilayer Viscosity. A possible explanation for the effects of thionin on the bilayer polarity could be that thionin alters the order of the bilayer lipids (Gasanov et al., 1993). Bis-pyrene fluorescence is sensitive to the mobility of molecules in the region of the hydrophobic core of the bilayer (Melnick et al., 1981). The interaction between adjacent pyrene molecules depends on their ability to diffuse through the surrounding environment. The frequency of pyrene/pyrene interactions can be detected experimentally by comparing the intensities of monomer (377 nm) and excimer (484 nm) fluorescence. Generally, changes in excimer intensity are accompanied by an inverse change in monomer fluorescence. Addition of thionin caused the ratio of monomer-to-excimer fluorescence of bis-pyrene to increase at temperatures above 42 °C (solid circles, Figure 7). (Traditionally, pyrene data are expressed as the excimer-to-monomer ratio. We have chosen to show the inverse for ease of comparison to the GP and anisotropy data in Figures 5 and 8.) Below the phase transition, the effect of thionin was complex (open circles, Figure 7). At low thionin concentrations, thionin caused an increase in the monomer-to-excimer ratio. At higher thionin concentration, the ratio decreased. This decrease was followed by another increase at the highest concentrations. Notably, the values of the monomer-to-excimer ratio at 37 and 45 °C became similar to each other at the highest thionin concentrations. The complexity in the effect of thionin concentration at 37 °C clearly depended on the density of thionin molecules on the vesicle surface. As shown in Figure 7B, the behavior seen at the lower thionin concentration range was expanded when the experiment was repeated at higher vesicle concentration (70 μM DPPG compared to 15 μM).

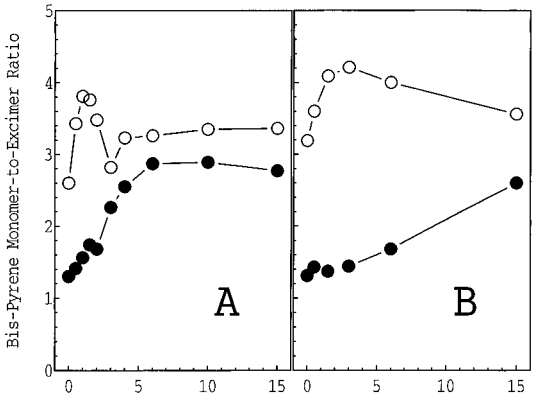

FIGURE 7: Effect of thionin concentration on the ratio of bis-pyrene monomer-to-excimer fluorescence at 15 μM (panel A) or 70 μM DPPG (panel B). The ratio of bis-pyrene (contained in the vesicle bilayers at a mole fraction of 0.001) fluorescence intensity at 377 nm (monomer) to that at 484 nm (excimer) was determined (excitation wavelength = 344 nm) at the indicated thionin concentrations 900 s after addition of vesicles to the equilibrated thionin sample (or at time ≥150 s in the absence of thionin). Temperatures were 37 °C (open circles) and 45 °C (solid circles).

To test whether these effects of thionin were time-dependent, we compared the monomer-to-excimer ratio at various thionin concentrations (i.e., as in Figure 7) immediately after addition of vesicles to the sample and following 900 s of incubation. Although the absolute value of the monomer-to-excimer ratio increased with time, presumably due to probe reequilibration following dilution of the vesicle stock, the effect of thionin on the ratio was the same regardless of incubation time. The data in Figure 7 represent the data obtained after the probe fluorescence appeared stabilized.

These apparent effects of thionin on the bilayer order were examined further by evaluating the anisotropy of Prodan, Patman, and Laurdan as a function of thionin concentration ([DPPG] = 100 μM). Thionin increased the anisotropy of the three probes at temperatures above the phase transition (solid circles, Figure 8). Below the transition, the effects of thionin on probe anisotropy were minimal (open circles).

Phase Transition. The effect of thionin on the phase transition of the membrane was investigated by DSC. As illustrated in Figure 9, the phase transition of pure DPPG vesicle occurred around 39.5 °C. Addition of thionin increased the melting temperature by about 1 °C. The width of the main transition was narrowed between 0.07 and 0.2 mM thionin. At the same time, a broad shoulder was observed at the lower-temperature side of each individual peak. The temperature at which the phase transition occurred remained constant until above 0.2 mM thionin where the

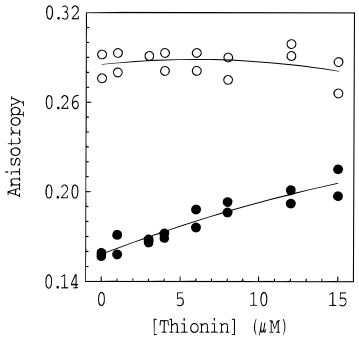

FIGURE 8: Effect of thionin concentration on the anisotropy of Laurdan. The indicated concentrations of thionin were mixed with 100 μM DPPG vesicles containing Laurdan as described for Figure 4 at 36.5 (open circles) and 45.7 °C (solid circles). The anisotropy value of Laurdan was measured at excitation = 350 nm, emission = 435 nm.

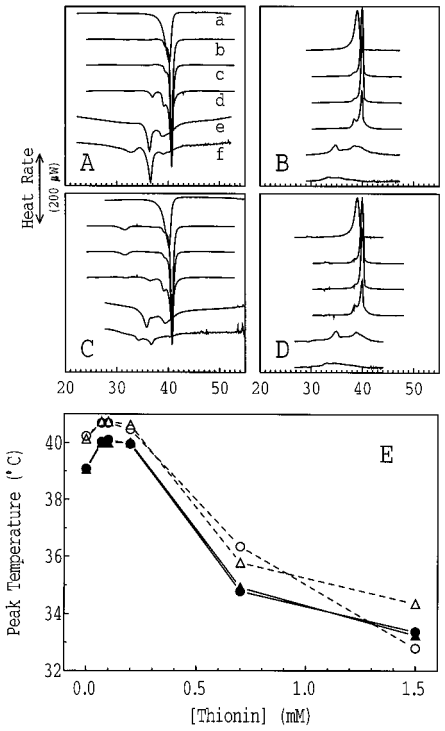

FIGURE 9: Effect of thionin on the phase transition of DPPG vesicles as measured by DSC. Panel A: various concentrations of thionin were mixed with 10 mM DPPG, and the rate of heat absorbed relative to a buffer blank was measured as temperature was raised at a rate of 5 °C/h. The procedure was repeated with the same sample while the temperature was lowered (panel B) and then raised (panel C) and lowered a second time (panel D). The data are displaced along the ordinate to facilitate viewing of the results. Thionin concentrations were 0 (a), 0.07 (b), 0.1 (c), 0.2 (d), 0.7 (e), and 1.5 mM (f). The double arrow indicates the scale. Panel E represents the temperature of the midpoint of the main peak in the data at 0−0.2 mM thionin and the lowest temperature peaks at 0.7 and 1.5 mM thionin for panel A (open circles), panel B (solid circles), panel C (open triangles), and panel D (solid triangles).

sharp peak was abolished almost completely. As the thionin concentration was increased further, only the broad shoulder remained.

We tested for possible kinetic effects by repeating the calorimetric measurements during two successive heating and cooling cycles. A large peak at about 36.5 °C was apparent at the two highest thionin concentrations in the first heating cycle (panel A) but was absent in subsequent cooling and heating scans (panels B−D). Furthermore, this peak appeared to correspond to samples that also displayed substantial visible precipitation at the end of the experiment. Thus, we conclude that the transient peak may represent the heat of vesicle aggregation. Otherwise, the samples appeared stabilized after the first heating cycle since the subsequent cooling scan (panel B) was identical to that obtained after a second heating cycle (panel D). The small difference in melting temperature between the up and down scans was due to the instrument response time since the relative positions of the various peaks did not change after the first up scan (Figure 9E).

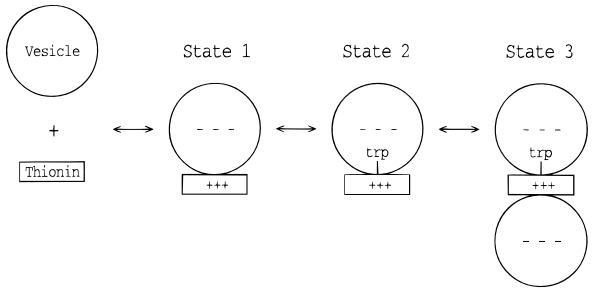

FIGURE 10: Hypothetical states of thionin bound to DPPG vesicles. The plus and minus symbols represent qualitatively the charges on the protein and the vesicles. No stoichiometries are implied by the diagram. See the text for explanations of each state.

DISCUSSION

The results of this study identified at least three states of thionin bound to DPPG vesicles. These states are summarized by the scheme shown in Figure 10. The three proposed states are: (1) thionin bound electrostatically to the bilayer surface; (2) tryptophan of the bound thionin inserted into the bilayer; (3) high-order aggregates of thionin-bound vesicles. Whether these states occur sequentially as implied by the scheme or whether state 3 is independent of state 2 cannot be fully resolved with the current data. The correlation between the experimental results and these proposed states is now summarized.

Thionin Fluorescence. The thionin molecule contains a single tryptophan residue at position 8. Chemical modification studies have suggested that this tryptophan is responsible for at least some of the biological actions of the peptide (Fracki et al., 1992). By comparison to the published X-ray crystal structures of related peptides, it has been suggested that this tryptophan is located on a hydrophobic α-helix face of the peptide that may insert into the membrane during the binding of the peptide to the bilayer surface (Vernon, 1992). The increase in intensity and blue-shift of the tryptophan emission spectrum (Figures 1 and 3) support the idea that the interaction of thionin with the bilayer results in a reduction of the exposure of Trp-8 to water consistent with insertion into the bilayer. The temperature dependence of these fluorescence changes (Figure 3) implies that the process of inserting Trp-8 into the bilayer prefers the liquid-crystalline phase. Since the calorimetry data revealed that low concentrations of thionin stabilize the gel phase of the bilayer (i.e., raise the melting temperature) regardless of whether the sample had been previously exposed to higher temperature (see Figure 9), it appeared that thionin exerted other effects on the bilayer distinguishable from the insertion of Trp-8. Hence, we propose the existence of both states 1 (initial adsorption) and 2 (insertion of Trp-8) in Figure 10.

Although time dependence was observed in the thionin fluorescence intensity upon mixing of the peptide with the

vesicles (Figure 2), this time dependence did not appear to be a reflection of slow processes governing the interaction of the peptide with the bilayer (at modest thionin-to-DPPG ratios, see below). Two lines of evidence confirmed this point. First, in spite of the decay of the tryptophan fluorescence intensity, the blue-shift of the emission spectrum was stable over time. Second, the changes in bilayer probe fluorescence spectra evoked by thionin (i.e., bis-pyrene, Prodan, Patman, Laurdan) were also stable with time.

At very high thionin-to-phospholipid ratios (i.e., $\geq$1:3), a reversal of the tryptophan spectral shift was observed, suggesting the possibility of decreasing interaction of thionin with the membrane under those conditions. In addition, time-dependent changes in the fluorescence intensity and value of GP for Prodan, Patman, and Laurdan were detected at similar high thionin-to-phospholipid ratios. The fluorescence data also became very noisy under such conditions, indicating random light scattering events resulting from the stirring of large particles. These results suggested that the time dependence at high vesicle and thionin concentrations reflected the slow formation of vesicle aggregates (state 3, Figure 10).

Bilayer Polarity. The emission spectra maxima of Prodan, Patman, and Laurdan are modified by both the polarity and the dipolar relaxation of molecules surrounding the fluorophore (Weber & Farris, 1979; Parasassi et al., 1991, 1994). The most likely molecule to thus affect the probe environment in the bilayer is water (Parasassi et al., 1991, 1994). Thus, the spectral shifts observed (Figure 4) and quantified by GP (Figure 5) could reflect changes in the bilayer polarity (presumably water content) and/or changes in dipolar relaxation (presumably the mobility of adjacent water molecules). These possibilities can be distinguished by examining the fluorescence excitation spectra of the probes. Changes in environment polarity cause both the emission and excitation spectra to shift in the same direction (Parasassi et al., 1994). The emission and excitation spectra shift in opposite directions when dipolar relaxation is altered. Comparison of the excitation spectra in Figure 6 to the effect of thionin on the emission spectrum (Figures 5 and 6) reveals that thionin caused the spectra to shift in opposite directions above the DPPG phase transition temperature. Thus, thionin appeared to reduce the mobility of water molecules in the bilayer in the liquid-crystalline phase. Below the phase transition temperature, shifts in the excitation spectrum were small and variable, suggesting that thionin may affect both the amount and mobility of water molecules in the bilayer at the region occupied by these probes.

Bilayer Viscosity. The data obtained with bis-pyrene and the Prodan, Patman and Laurdan anisotropies were consistent with the effects of thionin on bilayer polarity and dipolar relaxation. This consistency was especially true at the higher temperatures. In this case, thionin appeared to increase the order of the bilayer. Increased order would be expected to decrease the mobility of water molecules as was observed according to the Prodan, Patman, and Laurdan spectra. The data at 37 °C were more complex with both increases and decreases in apparent viscosity of the bilayer monitored by bis-pyrene. This complexity was also observed with the emission spectra of Prodan, Patman, and Laurdan with an overall trend toward an increase in the water content and/or water mobility in the bilayer. One would expect the water content and mobility to increase if the bilayer became less ordered. However, the effect of thionin on the bilayer polarity appeared to be of greater magnitude than the effect on the values of anisotropy or the monomer-to-excimer ratio of bis-pyrene. Therefore, some of the ability of thionin to introduce water into the bilayer seems not to reflect simply a change in the overall bilayer viscosity. One possibility is that the interaction with the thionin molecule disrupts the bilayer surface sufficiently to allow increased water accessibility in the gel phase (Vernon, 1992).

Phase Transition. Recently, Heimburg and Biltonen published a theoretical study predicting the effects of the binding of peptides to phospholipid bilayers on the gel-to-liquid-crystalline phase transition (Heimburg & Biltonen, 1996). Their study identified distinctive behaviors of the transition depending on whether the peptide in question was a peripheral protein or an integral membrane protein. In each case, the protein should shift the phase transition temperature depending on whether it interacts more strongly with the gel or fluid phase of the bilayer. The binding of the proteins was also predicted to cause asymmetric broadening of the transition profile. These theoretical predictions were furthermore shown to be in good agreement with experimental data obtained from selected proteins.

The calorimetric data on the phase transition obtained here with thionin were more complex than the simple behavior described by Heimburg and Biltonen. The fact that the transition was shifted toward higher temperature and then lower temperature as thionin concentration was increased suggests the likelihood of multiple types of interactions between thionin and the bilayer. At low concentrations of thionin, the elevation of the melting temperature is consistent with the preference of the protein for the gel phase which would be likely for a pure electrostatic interaction between the peptide and the bilayer surface. The subsequent lowering of the melting temperature and asymmetric broadening are also consistent with the binding of a peripheral protein, but with preference for the liquid-crystalline phase (Heimburg & Biltonen, 1996). As introduced above in the discussion on thionin fluorescence, these two effects on the phase transition may coincide with two of the proposed states of bound thionin (states 1 and 2, Figure 10). The electrostatic binding of the peptide to the bilayer surface corresponds to the initial binding of thionin to the vesicle surface without insertion of Trp-8. The second process dominating at the higher thionin concentrations could represent a hydrophobic interaction between the peptide and the bilayer and thus may relate to the postulated penetration of the α-helix containing Trp-8 into the bilayer surface (Vernon, 1992). If the latter were true, one would expect there to be differences between the first and subsequent calorimetry scans since the thionin fluorescence data suggested that Trp-8 penetration occurs best in the liquid-crystalline phase and does not reverse upon return to the gel phase. Comparison of panel A with panels B−D of Figure 9 reveals that such differences did occur. The DSC results also explain the complex behavior of the membrane probe fluorescence at 37 °C (Figures 5, 7, 8), and those data support the conclusion of multiple binding modes of thionin. Lastly, examination of the area under the peaks in Figure 9 reveals that thionin reduced the apparent enthalpy of the DPPG phase transition from about 8 kcal/mol to about 4 kcal/mol at the highest concentration. This result supports the fluorescence observations that the properties of the gel and liquid-crystalline phases are less distinct in the presence of thionin.

Interestingly, low concentrations of thionin appeared to make the phase transition more narrow (i.e., more cooperative) rather than broader. This result is unusual for experiments in which a guest molecule is introduced into a homogeneous phospholipid bilayer and is not predicted theoretically for simple interactions (Heimburg & Biltonen, 1996). We do not have a definite explanation for this unexpected result. One possibility is that these concentrations of thionin induce vesicle−vesicle interactions that couple the melting of multiple bilayers such as occurs in multilamellar vesicles. In support of this possibility, we note the presence of an apparent pretransition peak at about 32 °C at 0.07, 0.1, and 0.2 mM thionin in Figure 9. Such pretransitions are visible in DSC scans of multilamellar vesicles but are generally absent with less cooperative systems such as unilamellar vesicles (Wong et al., 1982).

Interpretation of the calorimetry data is complicated by the multiple peaks in the data at high thionin concentrations (Figure 9). These data suggest the likelihood of multiple phases and/or multiple events that result in the absorption of heat. As stated above in the text with Figure 9, one or more of these peaks probably relates to aggregation or fusion of vesicles at high thionin and vesicle concentrations (state 3, Figure 10). We note the discussion above that evidence from both the thionin and probe fluorescence at high thionin concentrations was consistent with the possibility of such a transition in the overall state of the sample. Furthermore, the presence of lipid aggregates at the highest vesicle and thionin concentrations could be observed visually as precipitates at the conclusion of experiments.

Concluding Remarks. Our results complement other attempts to identify the effects of thionin on bilayer properties. Previous work has suggested that thionin increases the order of the bilayer (Osorio e Castro et al., 1990). Furthermore, depending on the composition of the bilayer, thionin appears capable of increasing or decreasing the viscosity of the membrane and of inducing the formation of nonlamellar structures (Gasanov et al., 1993). In this study, we have focused on a single-component bilayer with well-defined thermodynamic properties. We have used this system to reveal the effects of thionin on the bilayer phase transition as well as on certain physical properties of the gel and liquid-crystalline phases. Consequently, we can now explain the complexity of prior observations regarding effects of thionin on bilayer viscosity. For example, our results demonstrate that either increases or decreases in bilayer order in the presence of thionin are possible depending on the thionin and phospholipid concentrations and depending on the initial state of the bilayer.

In a more general sense, our results indicate that the interaction of the peptide with the bilayer is complex and involves multiple processes. Specifically, three distinct interactions involving thionin and the DPPG bilayer were identified:

(1) Thionin bound to the bilayer surface. This interaction is presumably electrostatic and prefers the gel phase of the bilayer. It appeared to be rapid and stable based on the data from the fluorescent probes.

(2) A state represented by the elevated thionin fluorescence and blue-shift of the emission maximum, a separate hydrophobic interaction between thionin and the bilayer probably involving the α-helix that contains Trp-8. This state may be responsible for the lowering of the DPPC melting temperature and broadening of the transition at high thionin concentrations. Furthermore, access to this state appears to be facilitated by the liquid-crystalline phase of the bilayer.

(3) Aggregation of the thionin/vesicle complex. These aggregates appeared to occur at the highest vesicle and thionin concentrations. Both the fluorescence and calorimetric experiments suggested that this state also accumulates slowly.

Based on structural similarities, it is likely that these results and conclusions are relevant to a wide variety of basic peptides including the purothionins, crambin and viscotoxins (Florack & Stiekema, 1994).

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