Direct Enthalpy Measurements of the Calcium-Dependent Interaction of Annexins V and VI with Phospholipid Vesicles†

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ABSTRACT: Annexins V and VI are two members of the annexin protein family, each of which associate with phospholipid vesicles in a calcium-dependent manner. They may be important intracellular calcium response elements. Titration calorimetry and spectroscopic techniques showed striking features of these interactions. For annexin V and VI, the total heat release from calcium-dependent binding to bilayer vesicles composed of phosphatidylserine/phosphatidylcholine was −25 and −38 kcal/mol, respectively. The enthalpy of association (ΔH_{assoc}) for the respective protein−calcium interactions was about −11 and −5 kcal/mol. The ΔH_{assoc} for the annexin VI−vesicle interaction was largely independent of the headgroup of the anionic phospholipid and of the pH from 7.1 to 8.4. Use of phosphatidylethanolamine as the neutral phospholipid resulted in a slightly more negative ΔH_{assoc}. Enthalpy was either independent of vesicle size (annexin V) or showed a slightly more negative value for large vesicles (annexin VI). The ΔH_{assoc} for annexin VI−membrane interaction was not constant during protein titration but became more exothermic with higher protein density on the membrane. This behavior was surprising because the equilibrium constant showed negative cooperativity with respect to protein density [Bazzi, M. D., & Nelsestuen, G. L. (1991) *Biochemistry 30,* 7970−7977]. Apparently, entropy changes occurred which were large and negative, thereby compensating for the increasingly negative enthalpy but decreasing affinity as protein density was increased. In fact, the exothermic process coincided more closely with a change in the intrinsic tryptophan fluorescence of annexin VI than with actual protein−membrane binding. Circular dichroism detected very small changes in protein secondary structure during these events. The observed ΔH_{assoc} for annexin−membrane interaction appeared to involve contributions from the membrane as well as from the protein. Annexin−membrane binding may exert large effects on the membrane that could serve a regulatory capacity in the cell.

The annexins are a family of highly conserved calcium-dependent membrane-binding proteins which are present in a variety of species and cell types [reviews and texts: Klee (1988), Burgoyne and Geisow (1989), Kaetzel et al. (1990), Creutz (1992), Moss (1993)]. Their conservation and abundance suggest important function(s). Most annexins contain four homologous repeats of approximately 70 amino acids, each of which contains a consensus sequence (Geisow et al., 1986) for calcium binding by the "AB-loop" (Huber et al., 1992; Concha et al., 1993; Weng et al., 1993). Annexin VI contains eight of the 70-amino acid repeats. Calcium binding by the "DE-loops" (Concha et al., 1993; Weng et al., 1993) and, potentially, by other sites (Huber et al., 1992) has also been observed. Both human annexin V and VI contain a single tryptophan in the consensus sequence region of the third 70-amino acid repeat (Huber et al., 1992; Schlaepfer et al., 1992a). This tryptophan becomes exposed to solvent upon calcium binding to annexin V (Concha et al., 1993; Sopkova et al., 1993). The N-terminal portion of the annexins range from 13 to 42 amino acids (Schlaepfer et al., 1992a). For the majority of the annexins, except V and VI, this region contains at least one apparent phosphorylation site (Schlaepfer et al., 1992a). Thus, the N-terminus may regulate the function(s) of the various annexins.

While knowledge of the annexin protein structure is quite extensive (Huber et al., 1992; Concha et al., 1993; Weng et al., 1993), the *in vivo* function(s) of the annexins remain unclear. In general, annexins appear to effect vesicle aggregation and fusion (Meers et al., 1991; Creutz, 1992), they bind many calcium ions (Bazzi & Nelsestuen, 1991a; Evans & Nelsestuen, 1994) and cluster negatively-charged phospholipids upon their association with membranes (Bazzi & Nelsestuen, 1991b). The latter properties are also shared by protein kinase C (Bazzi & Nelsestuen, 1993). Annexin V has also been shown to exhibit calcium channel properties (Rojas et al., 1990; Berendes et al., 1993) and inhibit protein kinase C (Schlaepfer et al., 1992b). Alternatively, annexin VI appears to be required for budding of clathrin-coated pits (Lin et al., 1992) and regulation of the sarcoplasmic reticulum ryanodine-sensitive calcium-release channel (Díaz-Munoz et al., 1990; Hazarika et al., 1991). In the latter case, intact annexin VI was required for calcium channel regulation even though the N- and C-terminal halves retained the ability to bind to membranes in a calcium-dependent manner. While a portion of an annexin's physiological function may reside in its N-terminus, other functions may arise from the manner of its calcium/phospholipid interaction.

Isothermal titration calorimetry (ITC;[1] Wiseman et al., 1989) can help to elucidate the energetics of these interactions. The enthalpy of a binding event can provide informa-

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tion about the type of interaction(s). Previously, ITC has been applied to a limited number of protein/peptide−membrane interactions (Ramsay et al., 1986; Myers et al., 1987; Epand et al., 1990; Beschiaschvili & Seelig, 1992; Plager & Nelsestuen, 1994).

This study utilized ITC to measure the enthalpy associated with calcium-dependent interaction of annexin V and VI with phospholipid vesicles of different size and composition. The enthalpy measurements were complemented by circular dichroism (CD), intrinsic protein fluorescence, and direct calcium-binding measurements. Annexin binding to vesicles involved a large negative enthalpy component, regardless of phospholipid vesicle size or composition. The ΔH_{assoc} for annexin VI−vesicle interaction was not constant during protein titration but became more exothermic as protein density on the membrane increased. Thus, there appeared to be large, compensating changes in entropy as protein was added to the membrane. These large thermodynamic changes were not reflected in protein secondary structure changes measured by CD but may correlate with intrinsic protein fluorescence changes.

EXPERIMENTAL PROCEDURES

Proteins. Annexins V and VI were purified from bovine brain as described by Bazzi and Nelsestuen (1991a). Annexin V appeared as a doublet at 31 and 33 kDa and annexin VI at 64 kDa on a SDS−polyacrylamide gel. Protein concentrations were determined by the Bradford assay (Bradford, 1976) standardized to the appropriate protein. Extinction coefficients at 280 nm were 0.59 and 0.70 (mg/mL)$^{-1}$ cm^{-1} for annexin V and VI, respectively (Evans & Nelsestuen, 1994).

Phospholipid Vesicles. Vesicles were prepared from highly pure phospholipids (≥98%, supplier's estimates). PS (bovine brain) was from Sigma Chemical Co. (St. Louis, MO) or Avanti Polar Lipids, Inc. (Alabaster, AL). PA (from egg lecithin) was from Avanti, and PC (egg yolk) and PE (egg yolk) were from Sigma.

Small unilamellar vesicles (SUVs) were prepared by sonication and gel filtration (Huang, 1969; Nelsestuen & Lim, 1977). Briefly, 80 mg of phospholipid was dried thoroughly with a stream of nitrogen. The phospholipid was suspended in buffer (4 mL of 20 mM Tris or MOPS, 0.1 M NaCl, 1 μM EDTA, pH 7.5) and sonicated by direct probe using the microtip assembly of a Model W-385 Ultrasonic Processor (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). Two-second pulses with 3-s delays were employed for a total of 9 min of sonication. An ice−water bath was used to cool the solution. After centrifugation, the solution was gel-filtered on a S-500-Sephacryl column (1.5 × 50 cm). Fractions were collected, and vesicle size was determined by quasi-elastic light scattering (Bloomfield & Lim, 1978). Fractions containing vesicles with diameters of 25−60 nm were pooled and concentrated by pressure dialysis. The preparations had average vesicle diameters of 40 nm.

[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; ITC, isothermal titration calorimetry; CD, circular dichroism; LUVs, large unilamellar vesicles; SUVs, small unilamellar vesicles; ΔH_{assoc}, enthalpy of association; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-Morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; aV, annexin V; aVI, annexin VI; λ_{max}, wavelength of maximum fluorescence emission; DTT, dithiothreitol.

Large unilamellar vesicles (LUVs) were prepared by extrusion (Hope et al., 1985). Phospholipids (50 mg) were mixed in organic solvent and dried with a stream of nitrogen. These were suspended in 8 mL of buffer containing excess salt (20 mM Tris or MOPS, 0.15 M NaCl, 1 μM EDTA, pH 7.5). This suspension was frozen and thawed five times using a dry ice/organic solvent solution and a warm water bath. This was followed by six passes through a 0.1-μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA) under 200 psi of nitrogen using an extruder (Lipex Biomembranes, Inc., Vancouver, British Columbia, Canada). The resulting LUV preparation was dialyzed extensively against low-salt buffer (0.1 M NaCl) and concentrated by pressure dialysis. These vesicle preparations gave average diameters of 120 nm. Phospholipid concentrations were determined by assay of organic phosphatee (Chen et al., 1956), assuming a phosphorus:phospholipid weight ratio of 1:25.

Spectroscopic Techniques. Rayleigh light scattering at 90° was used to detect protein−phospholipid vesicle interactions as previously described (Nelsestuen & Lim, 1977). Briefly, for a constant light source and instrumental geometry, the ratio of light scattering for a solution of particles whose diameters are small with respect to the incident wavelength is given by eq 1.

$$I_2/I_1 = (M_2/M_1)^2[(\partial n/\partial c_2)/(\partial n/\partial c_1)]^2 \quad (1)$$

I_2 and I_1 are the light-scattering intensities of the protein−vesicle complex and the vesicles alone, M_2 and M_1 are the weight-average molecular weights of the protein−vesicle complex and the vesicles alone, and $\partial n/\partial c_2$ and $\partial n/\partial c_1$ are the refractive index increments for the protein−vesicle complex and the vesicles, respectively. Values for $\partial n/\partial c$ were taken from Nelsestuen and Lim (1977). Values for I_2 and I_1 were corrected for dilution and background scattering from buffer and unbound protein. These backgrounds were <5% of total scattering, and calcium-induced aggregation of vesicles was not detected at concentrations of ≤4 mM calcium. Excitation and emission were set at identical wavelengths (usually 320 or 600 nm). Light-scattering experiments were performed with a SLM 4800C (SLM-Aminco, Urbana, IL) or SPEX Fluoromax (JY/SPEX Instruments SA, Inc., Edison, NJ) fluorometer.

Intrinsic protein fluorescence measurements were performed with excitation at 295 nm and emission at a single wavelength (e.g., 340 nm) or by scanning from 305 to 405 nm with slits at 3 and 6 nm, respectively. The wavelength of maximum emission (λ_{max}) was determined using the "peak" function provided with the SPEX Fluoromax fluorometer. Light scattering was measured simultaneously during single-wavelength fluorescence experiments. Contributions from Rayleigh and Raman light scattering was <5% of total fluorescence intensity.

Circular dichroism measurements were made using a Jasco J-710 instrument (Japan Spectroscopic Co., Tokyo, Japan). Sample buffer was 20 mM Tris, 0.1 M NaCl, pH 7.5. All measurements were performed using a 1-mm path length cuvette and are the average of three scans (20 nm/min) from 260 to 190 nm. Spectra were recorded at ambient temperature. Estimates of secondary structure were obtained using the self-consistent method of Sreerama and Woody (1993). Mean residue ellipticity was calculated assuming an average molecular weight of 115 Da/residue. Previous CD studies

have been analyzed successfully in vesicular systems (Mao et al., 1982; Walsh et al., 1983). Negligible differences between spectra of protein alone versus that of protein plus vesicles as well as lack of spectral dependence on the sample cell position with respect to the detector confirmed the absence of light-scattering effects on the CD spectra reported in this study.

Isothermal Titration Calorimetry. The MicroCal OMEGA titration calorimeter (MicroCal Inc., Northampton, MA) coupled with a nanovoltmeter preamplifier was used for all calorimetry measurements. The operation and design of the instrument has been previously described by Wiseman et al. (1989). Samples (1.6 mL) were prepared from degassed buffer and concentrated phospholipid and protein solutions. Initially, protein samples were prepared in buffer containing low levels of EDTA (10 μM). The EDTA gave a small exothermic response which was eliminated by addition of an equal amount of calcium prior to calorimetry studies. Later results showed that protein preparations were stable without EDTA, and these preparations gave results that were indistinguishable from those obtained with EDTA-containing protein. The sample cell (volume = 1.4470 mL) was loaded with 1.4 mL of sample, and the system was equilibrated for 20 min. Calcium chloride solutions were prepared by dilution of aqueous 1 M $CaCl_2$ into degassed buffer. Calcium solution was injected over a maximum of 15 s via a 100-μL injection syringe while the sample was stirred at 400 rpm. Experiments were performed at 26 ± 1 °C with the external temperature thermostated at 21 °C. Resultant calorimetry data were analyzed with the software provided with the instrument ("Origin" application). The calorimeter was calibrated electrically.

Calcium Binding. The interaction of calcium with annexin V and VI in the absence of phospholipid was measured by the method of Hummel and Dryer (1962). Briefly, annexin V and VI were dialyzed extensively against 20 mM Tris, 0.1 M NaCl, pH 7.5. A stock calcium chloride solution containing $^{45}CaCl_2$ (New England Nuclear) was added to the protein sample (0.75−1.0 mg of protein) for a final buffer concentration of 36 mM Tris, 180 mM NaCl, pH 7.5, 0.015 μCi of ^{45}Ca/mL, and 50, 200, or 600 μM calcium. This sample (400 μL) was applied to a Sephacryl S-100-HR column (1.0 × 30 cm) which was equilibrated with buffer consisting of 20 mM Tris, 150 mM NaCl, pH 7.5, 0.015 μCi at ^{45}Ca/mL, and the appropriate calcium concentration. Fractions of 0.7 mL were collected. Calcium concentration was estimated by sampling the radioactivity, and protein concentration was estimated by the Bradford assay (1976; see above). The excess calcium concentration in each fraction was determined by the difference between the radioactivity in the test fraction and that of the base line of buffer eluting from the column. Bound calcium was determined for the four or five fractions containing the highest protein concentrations. The moles of excess calcium per mole of protein were calculated for each of these fractions, and a standard deviation for the resulting values was calculated.

RESULTS

Annexin VI Interaction with SUVs. The heat response upon injection of $CaCl_2$ into a solution containing SUVs plus annexin VI is shown in Figure 1A,B. The downward peaks

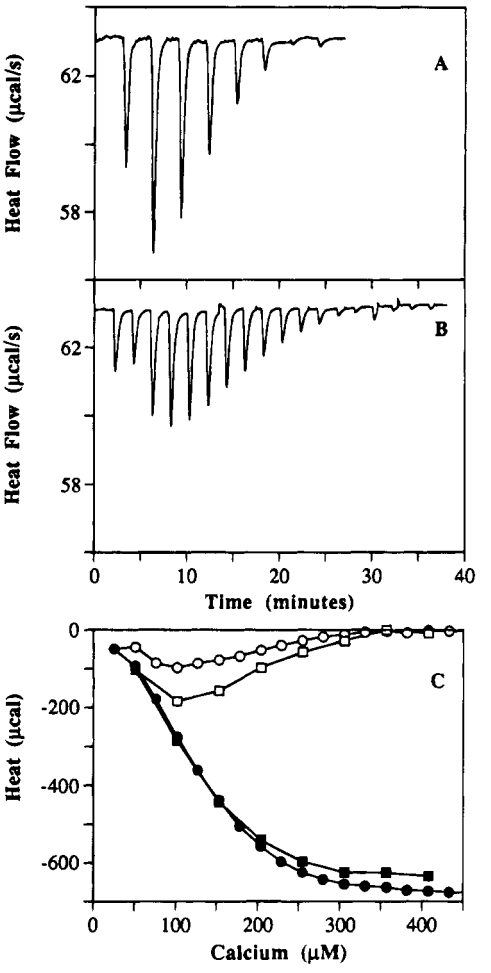

FIGURE 1: Titration calorimetry of annexin VI plus SUVs. Panel A: The differential heat response for a sample (1.4 mL) containing annexin VI (0.84 mg) plus SUVs (PS/PC, 25/75; 2.5 mg). The buffer was 20 mM Tris, 0.1 M NaCl, pH 7.5, 0.5 mM DTT. Injections (10 μL) of 7.2 mM $CaCl_2$ were made over a 15-s duration with a 3-min interval. The downward peaks represent heat released from the sample upon $CaCl_2$ injection. Panel B: The sample and buffer composition were the same as in panel A. Injections (5 μL) of 7.2 mM $CaCl_2$ were made over a 7.5-s duration with a 2-min interval. Panel C: Integrated areas for each peak in panel A (−□−) and panel B (−○−) are shown. Integration was carried out using the "Origin" software provided with the instrument. A running summation of the integrated peak areas from panel A (−■−) and panel B (−●−) is also shown.

represent an exothermic heat response and include contributions from dilution of $CaCl_2$, interaction of $CaCl_2$ with annexin VI or SUVs alone, and subsequent interaction of annexin VI with SUVs. The heat produced was determined from the integrated area of each peak using the "Origin" application provided by the manufacturer. The heat from the individual injections and the running sum are shown in Figure 1C. The coincidence of the two summation curves (Figure 1C) indicated that the enthalpy of the reaction was independent of the magnitude of individual injections.

The cumulative heat responses for titration of SUVs alone, annexin VI alone, and SUVs plus annexin VI are shown in Figure 2. These are expressed as kcal/mol of total annexin VI used in the relevant titrations (13.0 nmol). A small background (≤2 kcal/mol at 400 μM calcium) from injection of $CaCl_2$ into buffer alone was subtracted from each calcium titration. As previously observed (Plager & Nelsestuen, 1994), calcium titration of SUVs resulted in virtually no heat

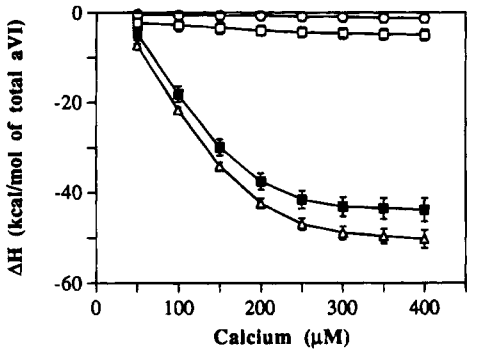

FIGURE 2: ΔH_{assoc} for binding of annexin VI to SUVs. The summation of the heat response, after subtraction of the background heat from injection of $CaCl_2$ into buffer, is shown for samples (1.4 mL) of annexin VI (0.84 mg) plus SUVs (PS/PC, 25/75; 2.5 mg; −△−), annexin VI alone (0.84 mg; −□−), and SUVs alone (PS/PC, 25/75; 2.5 mg; −○−). Error bars represent standard deviations for three or four measurements. Lack of error bars indicates an error that was within the dimension of the symbol. Also shown is the net ΔH_{assoc} for annexin VI binding to SUVs (PS/PC, 25/75; −■−). This was obtained by subtraction of ΔH for protein alone and vesicles alone. The vertical axis shows the cumulative heat divided by the total annexin VI present (13.0 mmol).

response (Figure 2). Annexin VI showed a mild exothermic response which reached a value of −5 kcal/mol at 400 μM calcium (Figure 2). However, the total heat response was nearly −50 kcal/mol when the solution contained both SUVs and annexin VI. The final net, apparent enthalpy for association (ΔH_{assoc}) of annexin VI with SUVs was −44 ± 3(SD) kcal/mol of annexin VI (at 400 μM calcium, Figure 2).

A comparison of the cumulative heat release (from Figure 2) and the binding of annexin VI to SUVs is presented in Figure 3A. Protein−membrane binding, detected by light scattering, was saturable and reversible, as expected (Bazzi & Nelsestuen, 1991c). Simple comparison of these plots (Figure 3A) suggested a considerable discrepancy so that heat release was delayed relative to annexin VI−membrane binding. More direct comparison is provided by the treatment shown in Figure 3B. The "ΔH/mol of bound annexin VI" was calculated by dividing the cumulative ΔH_{assoc} (Figure 3A) by the progress of the M_2/M_1 curve. The plot shown was made with the assumption that the M_2/M_1 ratio at 400 μM calcium represented complete protein−membrane binding (13.0 nmol). This plot showed that ΔH per bound annexin VI varied by nearly 4-fold (−12 to −45 kcal/mol) as the binding progressed. Thus, later binding events produced much more heat than early binding events.

The final M_2/M_1 value (Figure 3A) was less than the maximum theoretical value (1.33) for this mixture. This could arise from any of several factors, none of which should alter, and some of which would enhance, the nature of the conclusions drawn. For example, a portion of the protein may not be bound to the vesicles in the titration. In this case, the ΔH_{assoc} would be even more negative than −44 kcal/mol and the discrepancy between the ΔH and binding curves would remain. Alternatively, eq 1 may not apply strictly to this situation. Equation 1 is based on weight-averaged molecular weights and assumes random protein distribution. Heterogeneity of vesicle size [diameters between 25 and 60 nm for SUVs and 70 and 140 nm for LUVs (Hope et al., 1985)] could produce lower light-scattering changes if the proteins bound with preference for smaller

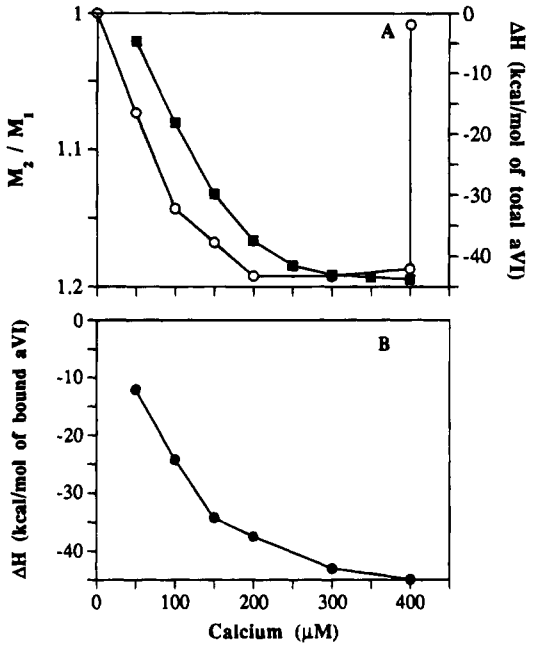

FIGURE 3: Variation of ΔH_{assoc} with annexin VI density on the membrane. Panel A: Calcium was added to a sample (0.6 mL) containing annexin VI (350 μg) and SUVs (PS/PC, 25/75; 1050 μg). Light scattering at 320 nm was used to determine protein−membrane binding, expressed as M_2/M_1 (−○−; eq 1). The scale is inverted to allow comparison to the calorimetry data. The buffer composition was 20 mM Tris, 0.1 M NaCl, pH 7.5, 0.5 mM DTT. The ΔH_{assoc} for annexin VI binding to SUVs (−■−) is taken from Figure 2. Panel B: The ΔH_{assoc} for annexin VI−SUV interaction at each stage of the titration is shown (−●−). This was calculated from the data in panel A by dividing enthalpy change by the amount of membrane-bound protein.

vesicles. However, this should result in underestimation of protein bound in the early stages of the titration. The latter would exacerbate the discrepancy between protein binding and ΔH. Thus, noncoincidence of protein binding and ΔH (Figure 3B) appeared to be a major property of this titration.

Effect of Phosphatidylethanolamine on ΔH_{assoc} for Annexin VI. Binding of annexin VI to LUVs and LUVs containing 50% PE was saturable and reversible (Figure 4A). As anticipated, less calcium was required for annexin binding to LUVs containing PE (Bazzi & Nelsestuen, 1992). At 400 μM calcium, the ΔH_{assoc}'s for the phospholipid used per mole of protein were −4 ± 2 and −3.5 ± 1 for LUVs and LUVs containing 50% PE, respectively (data not shown). Annexin VI binding to LUVs was highly exothermic with ΔH_{assoc} values of −36 to −43 kcal/mol (Figure 4B, data treatment as in Figure 3B).

The maximum value for M_2/M_1 was less than the theoretical maximum of 1.33 and somewhat different for the two vesicle populations (Figure 4A). Calculation of ΔH_{assoc} from the actual M_2/M_1 ratio rather than from the total amount of protein present provided values of −64 and −62 kcal/mol for LUVs and LUVs containing 50% PE, respectively. Thus, these two methods of data calculation provided different conclusions for the effect of PE, that is, the presence of PE either enhanced the exothermic properties slightly or had no effect. However, the manner of estimating the amount of bound protein had no effect on the observation that early binding events were less exothermic than later binding events.

Effect of pH and Determination of Proton Release. Within the range tested, pH had a small or negligible influence on

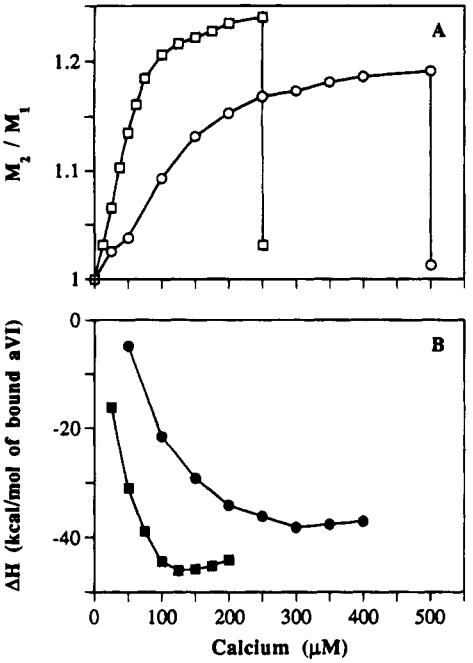

FIGURE 4: Influence of the neutral phospholipid on ΔH_{assoc} of annexin VI. Panel A: Calcium titration of membrane binding for samples (400 μL) containing annexin VI (250 μg) and LUVs (PS/PC, 18/82; 750 μg; −○−; and PS/PC/PE, 18/32/50; 750 μg; −□−). Sequential additions of $CaCl_2$ were made. Addition of excess EDTA resulted in dissociation of the protein as shown by the final measurement. Molecular weight ratios (M_2/M_1) were calculated according to eq 1 from light-scattering intensities at 600 nm. Panel B: Titration calorimetry of a sample (1.4 mL) containing annexin VI (0.86 mg) plus LUVs (PS/PC, 18/82; 2.6 mg; −●−; or PS/PC/PE, 18/32/50; 2.6 mg; −■−). Injections of calcium solution were made over a 15-s duration with a 4-min interval. The resulting enthalpy was calculated as in Figures 1 and 2. The buffer was 20 mM Tris, 0.1 M NaCl, pH 7.5.

annexin VI−membrane binding. The results suggested that ionization of sites on the protein did not occur during annexin−membrane binding. For example, for PS, which does not ionize in the range of pH 7.1−8.4, the calcium requirement (Figure 5A) as well as the enthalpy change (Figure 5C) appeared constant for annexin−membrane binding regardless of the buffer system used.

In contrast, the pK_a for the second phosphate proton of PA is approximately 8.2 (Tocanne & Teissié, 1990), so that annexin binding to PA may involve ionization of this group. Ionization can be detected by use of different buffering agents with different enthalpies of ionization. The enthalpy of ionization (ΔH_b) for Tris is 11.5 kcal/mol, and that for MOPS is 5.3 kcal/mol (Morin & Freire, 1991). The ΔH_b for PA is 2.5 kcal/mol (Blume & Tuchtenhagen, 1992). Therefore, each proton released from PA upon annexin binding should result in an additional −9.0 and −2.8 kcal/mol of annexin VI in Tris and MOPS, respectively. These ΔH_b values were validated by determination of the heat released upon injection of aqueous HCl into Tris or MOPS buffer (data not shown). The presence of LUVs containing PA had no effect. At pH 7.1, the buffers were capable of absorbing at least 10 protons per annexin VI molecules present.

Lack of a detectable buffer-dependent difference in heat release at pH 8.4 (Figure 5B) suggested little or no ionization associated with annexin binding to PA-containing membranes. In contrast, at pH 7.1 the ΔH_{assoc} was more negative in Tris than in MOPS. Furthermore, total ΔH_{assoc} was more

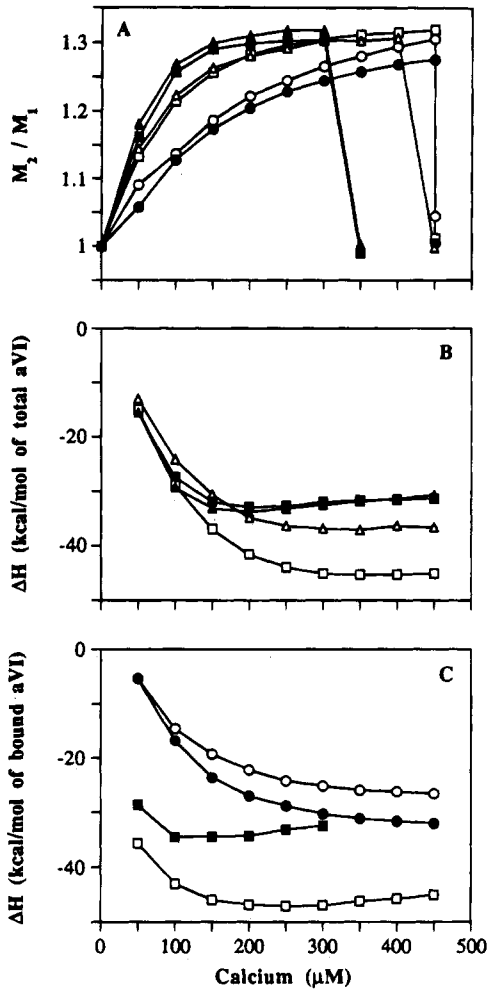

FIGURE 5: Influence of pH and anionic phospholipid on ΔH_{assoc}. Panel A: Calcium titration of membrane binding for samples (400 μL) of annexin VI (215 μg) and LUVs. The vesicles and buffers included PS/PC (18/82; 430 μg in Tris buffer, pH 7.1; −○−), PS/PC (18/82; 430 μg in Tris buffer, pH 8.4; −●−), PA/PC (18/82; 430 μg in Tris buffer, pH 7.1; −□−), PA/PC (18/82; 430 μg in Tris buffer, pH 8.4; −■−), PA/PC (18/82; 430 μg in MOPS buffer, pH 7.1; −△−), and PA/PC (18/82; 430 μg in MOPS buffer, pH 8.4; −▲−). All buffers containing 0.1 M NaCl. Sequential additions of $CaCl_2$ were made and protein−membrane binding is shown by M_2/M_1, calculated from eq 1 and light-scattering intensities at 600 nm. The final experimental point represents the addition of excess EDTA. Panel B: Differential heat response for samples (1.4 mL) containing annexin VI (0.75 mg) plus LUVs (1.5 mg). Injections of $CaCl_2$ were made over a 15-s duration with a 4-min interval, and the data were treated as in Figures 1 and 2. The symbols correspond to the phospholiids and buffers described in panel A. Panel C: ΔH_{assoc} per mole of bound protein as a function of protein density on the membrane. These curves were generated from binding measurements (panel A) and calorimetric measurements (panel B), as described in Figures 3. The symbols represent the phospholipids and buffers described in panel A.

negative at pH 7.1 than at pH 8.4. Both observations were consistent with release of protons from PA at pH 7.1. A difference of −14 kcal/mol in Tris at pH 7.1 versus 8.4 (at 450 μM calcium, Figure 5B) would correspond to the release of 1.6 protons per annexin VI. A corresponding difference of −5.5 kcal/mol in MOPS buffer correlated to the release of 2.0 protons per mol of annexin. Use of the actual M_2/M_1 value (1.30; Figure 5A), rather than total protein, to calculate proton stoichiometry would give about three protons released per annexin VI molecule. Therefore, it appeared that 2 ± 1

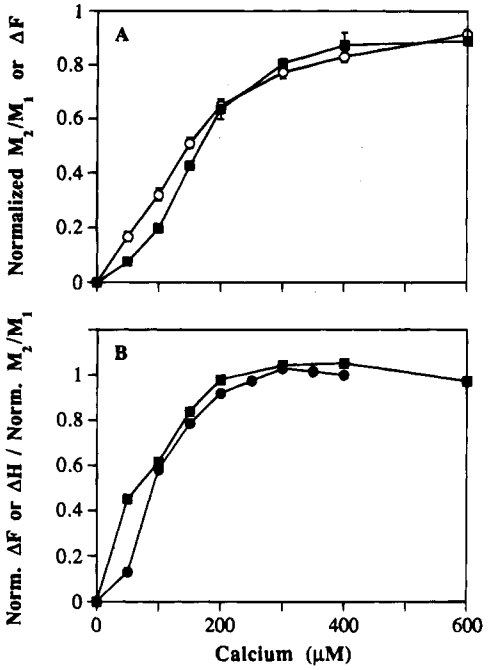

FIGURE 6: Change in tryptophan fluorescence (ΔF) relative to membrane binding of annexin VI. Panel A: Membrane binding (−○−) and fluorescence (−■−) of annexin VI (375 μg) in the presence of LUVs (PS/PC, 18/82; 1125 μg). The sample volume was 0.6 mL. Membrane binding, expressed as M_2/M_1, was estimated from light scattering at 650 nm using eq 1. Fluorescence used excitation and emission at 295 and 340 nm, respectively. These measurements were carried out simultaneously on the same sample using the multiwavelength scanning program on the Fluoromax. The plots show values that were normalized to a maximum of 1.0 to allow more direct comparisons. The maximum M_2/M_1 value, at 1000 μM calcium, was 1.35, and the maximum fluorescence was 1.3 times the calcium-free protein fluorescence intensity. The average values from two experiments are shown, and the error bars represent the range of the data. Panel B: Fluorescence intensity and enthalpy as a function of membrane-bound annexin VI. The fluorescence relationship (−■−) was generated by dividing the normalized value of ΔF (panel A) by the normalized value of M_2/M_1 (also from panel A). The ΔH_{assoc} per protein at varying levels of bound annexin VI is also shown for annexin VI binding to LUVs (PS/PC, 18/82; −●−). This is the data from Figure 4B which was normalized to a value of 1.0 for the final ΔH (actual value of −36 kcal/mol at 400 μM calcium).

protons were released per annexin VI upon binding to PA at pH 7.1.

The results for LUVs containing PS in Tris (Figure 5C) and MOPS (data not shown) resulted in comparable final ΔH_{assoc}'s of −29 ± 3 and −29 ± 1 kcal/mol of annexin VI, respectively. These values were also similar to those obtained for binding to LUVs containing PA at pH 8.4. Thus, the headgroup of the anionic phospholipid appeared to have little effect on the ΔH_{assoc} for annexin VI−membrane interaction, except in those cases where proton release occurred.

While early binding events for vesicles containing PS were less exothermic than later events (Figure 5C), binding to LUVs containing PA showed relatively little of this behavior (Figure 5C). However, a relatively high percentage of annexin VI was bound upon the first calcium addition (50 μM) so that the first half of the titration was bypassed. The early portions of the binding curves for PA-containing membranes were therefore not investigated.

Relationship between ΔH and Intrinsic Fluorescence Change. The environment of the tryptophan of rat and human annexin V changes upon calcium and calcium−phospholipid binding (Concha et al., 1993; Meers, 1990). The high sequence homology of these annexins (Barton et al., 1991; Hamman et al., 1988; Schlaepfer et al., 1992a) with bovine annexin VI (Creutz et al., 1992), together with

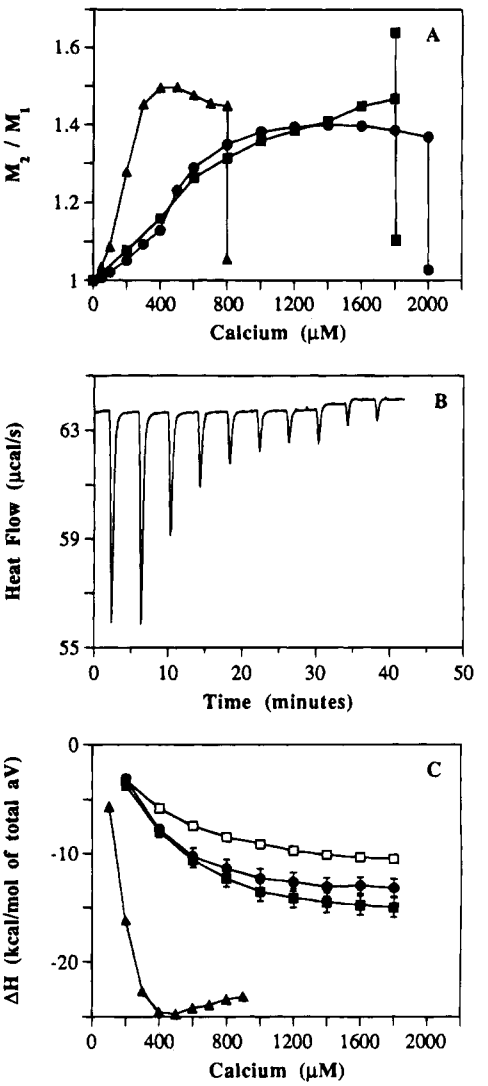

FIGURE 7: Titration calorimetry of annexin V. Panel A: Calcium titration of membrane binding. The samples (400 μL) contained annexin V (250 μg) plus LUVs containing PS/PC (18/82; 750 μg; −●−), LUVs containing PS/PC/PE (18/32/50; 750 μg; −▲−), or SUVs containing PS/PC (18/82; 750 μg; −■−). Additions of $CaCl_2$ were made, and light scattering at 600 nm was used to estimate protein−membrane binding, expressed as M_2/M_1 (eq 1). The final point represents addition of excess EDTA. At high calcium concentrations, there was a slow increase in light scattering. For example, the higher value for M_2/M_1 at 1800 μM calcium (■) was measured after a 5-min incubation. Such time-dependent changes in light scattering were minimal below 1000 μM calcium. Buffer composition was 20 mM Tris, 0.1 M NaCl, pH 7.5. Panel B: Differential heat response for a sample (1.4 mL) containing annexin V (0.86 mg) plus LUVs (PS/PC, 18/82; 2.6 mg). Injections of $CaCl_2$ were made over 15-s duration with a 4-min interval. Panel C: Enthalpy changes for Annexin V. The ΔH_{assoc} for annexin V alone (0.86 mg/mL; −□−) is shown. The net enthalpy for annexin V plus LUVs containing PS/PC (18/82; 750 μg; −●−), LUVs containing PS/PC/PE (18/32/50; 750 μg; −▲−), or SUVs containing PS/PC (18/82; −■−) were calculated as in Figure 2 (−■−). The error bars for titrations of SUVs and LUVs represent the range of two experiments. A single measurement for LUVs containing PE was made. The vertical axis shows the cumulative heat divided by the total annexin V present (26.8 nmol).

similar fluorescence properties [compare Meers (1990) with studies below], suggests a similar tryptophan location and exposure in bovine annexin V and VI.

Protein fluorescence and protein−membrane binding were monitored simultaneously (Figure 6A). The results of two separate experiments were normalized to a maximum value of 1.0, and the averages are shown in Figure 6A. The most unusual feature of this comparison was a displacement of the fluorescence change (ΔF) relative to membrane binding. In fact, this displacement appeared similar to that observed for ΔH_{assoc} versus membrane binding (above). Figure 6B shows the ratios of normalized data, fluorescence/binding and enthalpy/binding. If fluorescence change and ΔH were coincident with binding, these curves would be horizontal and have a value of 1.0. Thus, these plots showed, once again, that both fluorescence change and ΔH were offset relative to membrane binding. Furthermore, their close relationship to each other suggested that ΔH and fluorescence change may arise from the same event.

Studies with Annexin V. The interaction of annexin V with SUVs, LUVs, and LUVs containing 50% PE was also studied. One notable complication with annexin V was an apparent protein-induced aggregation. This was indicated by greater than theoretical (1.33) M_2/M_1 values (Figure 7A). This problem was somewhat unexpected since most previous reports indicated that annexin V did not cause aggregation (Meers et al., 1991; Bazzi & Nelsestuen, 1991a). Small amounts of vesicle fusion may also have occurred as suggested by incomplete signal reversal upon addition of excess EDTA.

However, enthalpy contributions due to vesicle aggregation (Ramsay et al., 1986) and fusion (Myers et al., 1987) were probably small. For example, each ITC peak was essentially complete within 1 min (Figure 7B), similar to the time course for annexin VI. In contrast, aggregation and fusion occurred over long time periods as suggested by a slow increase in light-scattering intensity (Figure 7A, −■−) and should produce an extended absorption or release of heat as reported previously (Ramsay et al., 1986; Myers et al., 1987). The apparent saturation of ΔH (Figure 7C) would not be expected if aggregation/fusion provided a significant heat component. The slow time-dependent changes in light scattering were minimal until the density of annexin V on the membrane became high, and so these events should have no influence on the earlier time points at all. Thus, aggregation and fusion probably did not constitute major contributors to heat flow in these experiments.

The apparent ΔH_{assoc} for interaction of annexin V with calcium was −6 kcal/mol at 450 μM (Figure 7C), comparable to the value of −5 ± 1 kcal/mol for annexin VI (Figure 2). This similarity for the free proteins contrasted with the fact that annexin VI has twice as many repeating units and required much less calcium to support membrane binding.

Subtraction of ΔH_{assoc} for annexin V−calcium interaction provided comparable net enthalpies for binding to SUVs and LUVs, −15 ± 1 and −13 ± 1 kcal/mol, respectively. Interaction of annexin V with LUVs containing 50% PE was more exothermic and required less calcium (Figure 7C). This was similar to the effects of PE on annexin VI−membrane binding (Figure 4). A summary of the observed enthalpies for protein−calcium and protein−calcium−vesicle association are presented in Table 1.

Table 1: Enthalpies for Annexin Interactions

	annexin VI (ΔH, kcal/mol of total protein)	annexin V (ΔH, kcal/mol of total protein)
protein alone:		
450 μM calcium	−5 ± 1 (6)[a]	−6
1800 μM calcium		−11
protein + SUVs:[b]		
PS/PC (25/75) (3:1)[c]	−49 ± 3 (4)	
PS/PC (18/82) (3:1)	−34[d]	−26 ± 1 (2)[d]
protein + LUVs:[b]		
PS/PC (18/82) (3:1)	−41	−24 ± 1 (2)[d]
PS/PC (18/82) (2:1)	−34 ± 3 (4) (pH 7.1 and 8.4)	
PA/PC (18/82) (2:1)	−36 ± 1 (2) (pH 8.4, MOPS and Tris)	
	−41 (pH 7.1, MOPS)	
	−50 (pH 7.1, Tris)	
PS/PC/PE (18/32/50) (3:1)	−48	−34[d]

[a] Number of experiments conducted to obtain the standard deviation shown. [b] Enthalpy values do not include subtraction of ΔH for protein−calcium titration. [c] Phospholipid/protein ratio (wt/wt) in the titration. [d] Some aggregation/fusion.

Calcium-Binding Measurements. While calcium-binding properties of some annexins have been reported (Klee, 1988), previous studies with these specific annexins failed to detect binding at 20−50 μM calcium (Bazzi & Nelsestuen, 1991a). Thus, calcium binding was measured at the higher calcium concentrations used in this study. To improve this comparison, the maximum protein concentration in the column eluate was at least half of that used for the calorimetry experiments.

An example of calcium binding measured by the Hummel−Dreyer technique is presented in Figure 8A. The small positive peak at fractions 27−28 was consistently observed and appeared to result from residual EDTA that was not removed by extensive dialysis. Calcium-binding stoichiometry was therefore determined from the positive peak of bound calcium. In agreement with the enthalpy data (Table 1), less calcium was bound to annexin VI (Figure 8B), even though it contained twice as many repeating units as annexin V. This behavior, less calcium bound to a protein with twice as many potential binding sites, has also been observed for lipocortin 85 and annexin II (Evans & Nelsestuen, 1994). The first half-equivalent of calcium was bound at approximately 30 and 140 μM calcium for annexin V and VI, respectively. These values were similar to previous reports for other annexins (Klee, 1988).

Calcium titration of the annexin V fluorescence change in the absence of phospholipid (Figure 8C) was compared to the ΔH_{assoc} for annexin−calcium interaction. The fluorescence change of annexin V appeared to involve at least two processes, an initial fluorescence increase followed by a change in the wavelength of emission at higher calcium. Addition of 0.6 mM calcium caused most of the fluoresence intensity increase but was not accompanied by a shift in λ_{max} (Figure 8C, compare −△− and −●−). At 2 mM calcium, the intensity change was complete as was heat release (Figure 7C), suggesting that they may arise from related events. This correlation was tentative since detailed interpretation of fluorescence intensity changes can be complex if they are accompanied by a change in λ_{max}. This is demonstrated by the calcium dependence of intensity change at different wavelengths (345 and 360 nm) as shown in Figure 8C. In any event, the red shift λ_{max} required much higher calcium

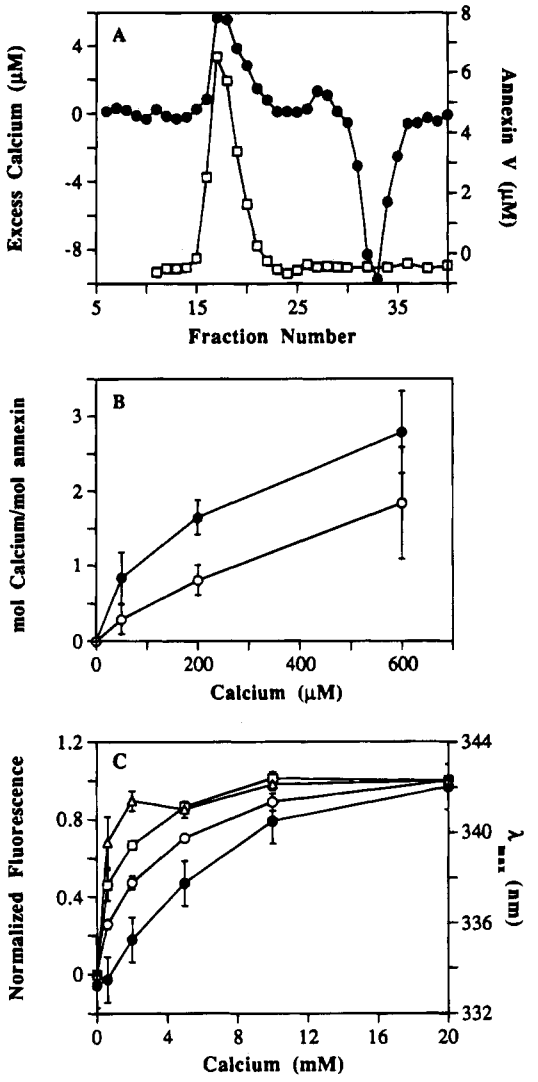

FIGURE 8: Calcium binding by annexin V and VI. Panel A: Elution profile for annexin V (750 μg) at 50 μM calcium. The annexin was mixed with buffer containing 50 μM $^{45}CaCl_2$ (400 μL) and applied to a Sephacryl S-100-HR column that was equilibrated in 20 mM Tris, 0.15 M NaCl, pH 7.5, 50 μM $^{45}CaCl_2$. Fractions (0.7 mL) were collected and monitored for radioactivity (−●−) and protein concentrations (−□−). Panel B: Calcium bound per mole of annexin V (−●−) and annexin VI (−○−). The error bars represent the standard deviation of stoichiometries obtained from fractions which contained the highest protein concentrations, as described in Experimental Procedures. Panel C: Calcium-induced change in fluorescence of annexin V (360 μg in 0.6 mL) in the absence of phospholipid. Excitation was at 295 nm, and emission was scanned from 305 to 405 nm. Fluorescence intensity at the λ_{max} (−△−), as well as at 345 nm (−□−) and 360 nm (−○−), is shown. The λ_{max} (−●−), obtained as described in Experimental Procedures, is also shown. Error bars represent the range of two measurements. Fluorescence was corrected for photobleaching of a sample that was titrated with buffer that did not contain calcium and for dilution.

and did not appear to be associated with the event that produced the major enthalpy change for annexin V alone.

Circular Dichroism Spectral Changes. Calcium-dependent tryptophan fluorescence changes (Figures 6A and 8C; Meers & Mealy, 1993) and large enthalpies for annexin−membrane binding (Table 1) could result from protein conformation changes that might be detected by CD spectra. The measurements for annexin V (Figure 9) and VI (data not shown) were quite similar to one another and to those previously observed for annexin V in the absence of

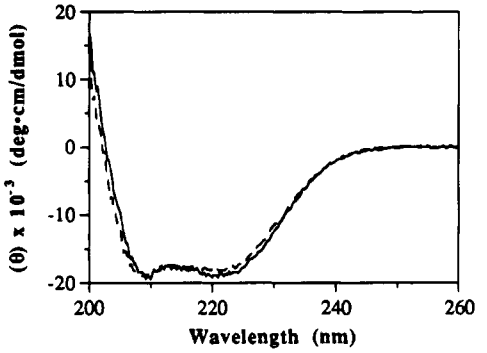

FIGURE 9: Circular dichroism of annexin V in the presence of SUVs. Far-UV CD spectra of annexin V (20 μg) in the presence of SUVs (PS/PC, 25/75; 80 μg) without calcium (− − −) and plus 2.0 mM $CaCl_2$ (−) are shown. The sample volume was 200 μL with a path length of 0.1 cm. The buffer was 20 mM Tris, 0.1 M NaCl, pH 7.5. Mean residue ellipticity is presented on the vertical axis.

Table 2: Percentage of Annexin Secondary Structure ± Calcium[a]

	α-helix	β-sheet	turn	unordered
annexin V:				
protein alone	57 ± 1[c]	8 ± 1	21 ± 1	14 ± 1
protein + PL[b]	59 ± 1	7 ± 1	19 ± 1	13 ± 1
annexin VI:				
protein alone	63 ± 2	6 ± 1	19 ± 1	12 ± 2
protein + PL[b]	66 ± 3	4 ± 2	19 ± 1	10 ± 1

[a] Using the self-consistent model of Sreerama and Woody (1993). [b] SUVs (PS/PC, 25/75). [c] Average and standard deviation of three measurements: (1) prior to calcium addition, (2) plus calcium, and (3) plus excess EDTA.

phospholipid (Fritsche et al., 1988; Sopkova et al., 1994). Very minor, but highly reproducible, calcium-dependent changes in the shape of the CD spectra occurred. These changes (Figure 9) included an increase in negative ellipticity at 222 nm versus 209 nm and a cross-over to positive ellipticity at a slightly greater wavelength (near 203 nm). Calcium (2 mM) induced the CD change of annexin V, while the presence of SUVs plus 0.6 mM calcium was required to generate the CD change of annexin VI (data not shown). These changes were reversed by addition of excess EDTA. Spectra taken at different cell positions relative to the detector ensured that light scattering did not influence the spectra to a significant extent. The changes were insufficient to give a change in secondary structure as calculated by the program used (Table 2). Therefore, the values shown in Table 2 are the average and standard deviations of spectra taken (1) prior to calcium addition, (2) after calcium addition, and (3) after addition of excess EDTA. The values in Table 2 were consistent with those indicated by the annexin V crystal structure (Huber et al., 1992). The very small changes in secondary structure were consistent with two-dimensional electron microscopy results from Brisson et al. (1991). Overall, it appeared that very minor secondary structure changes occurred for annexin V and VI upon calcium and membrane binding.

DISCUSSION

A popular model for annexin−membrane interaction depicts calcium ion bridges between sites on the annexin and the phospholipid vesicle. The ability to form such interactions was suggested by the X-ray crystal structures of annexin−calcium complexes (Huber et al., 1992; Weng

et al., 1993). If these interactions were entirely ionic in nature, the ΔH for membrane binding would be expected to be very low (Ross & Subramanian, 1981; Eftink & Biltonen, 1980). However, the ΔH_{assoc} for annexin binding to phospholipid vesicles was found to be highly exothermic. To illustrate the magnitude of the ΔH observed, a comparable free energy of association ($\Delta G_{assoc} = -20$ to -40 kcal/mol) would correspond to an apparent association equilibrium constant of $10^{15}-10^{29}$ M^{-1}. The ΔH for unfolding of a globular protein of about 25 kDa is typically 50−100 kcal/mol (Eftink & Biltonen, 1980). The magnitude of these thermodynamic changes may suggest an importance for annexin function in the cell.

An unexpected property was that enthalpy became more negative at higher protein density on the membrane. This seemed inconsistent with reports that later binding events required higher calcium concentration and would be expected to have lower affinity (Bazzi & Nelsestuen, 1991c). These properties would be compatible if there was an overcompensating decrease in entropy as the protein density on the membrane was increased. The source(s) of such large entropy changes are not known but could arise from factors such as increased membrane rigidity as the surface became occupied by protein. The recent report of changes in the fluid-phase structure and the overall fluid-mechanical properties of the lipid bilayer upon binding of annexin IV (Gilmanshin et al., 1994) would appear to be consistent with this idea.

Another unusual property was an inverse relationship between calcium binding to the free annexin and the calcium concentration required for membrane interaction (Bazzi & Nelsestuen, 1991c), that is, while free annexin VI bound less calcium than free annexin V, it required a lower calcium concentration for membrane binding. A similar relationship was observed for lipocortin 85 and annexin II (Evans & Nelsestuen, 1994). Thus, calcium binding to the free annexin apparently did not presage subsequent membrane binding.

Protein Contributions to ΔH. The high exothermic ΔH_{assoc} for annexin−vesicle interaction, regardless of vesicle composition or size (Table 1), suggested that the enthalpy was at least in part due to changes in the protein or general changes in bilayer structure. High negative enthalpies have been reported for other ion-induced protein conformation changes (Kuriki et al., 1976; Lovrien & Sturtevant, 1971).

The enthalpy per calcium ion bound to free annexin was relatively high. ITC for annexin V alone (Figure 7C, Table 1) suggested that binding of the first three calciums liberated about 7.5 kcal/mol of annexin V. These events appeared to precede exposure of the single tryptophan which occurred above 0.6 mM calcium. This relationship was consistent with the proposed number of calciums bound prior to tryptophan exposure (Meers & Mealy, 1993). A minor source of heat release from the protein may be the formation of a hydrogen bond between the conserved consensus sequence Thr and Asp/Glu of the calcium binding AB-loop (Weng et al., 1993).

A value of roughly -3 ± 1 kcal/mol for each calcium bound was also obtained for annexin VI (values at 450 μM calcium; Table 1, Figure 8B). If ΔH per bound calcium ion was constant, the total enthalpy change for annexin VI binding to membranes would be provided by 11−16 calcium ions. Eight to eleven calcium ions would be required for annexin V. While large, these stoichiometries were comparable to values reported in several studies (Evans & Nelsestuen, 1994; Bazzi & Nelsestuen, 1991a; Concha et al., 1993; Weng et al., 1993).

Unfortunately, a constant heat release per calcium ion would not explain the variation of heat release with protein density on the membrane. Thus, major heat release may result from subsequent events, and several sources can be considered. For example, heat release appeared to be closely coincident with tryptophan fluorescence change (Figure 6). Therefore, initial membrane binding may occur via low-exothermic, high-affinity sites that involve only the AB-loops (Karshikov et al., 1992) which do not contain tryptophan. Subsequent interactions of the tryptophan-containing site might generate most of the heat. This would require two other phenomena: the secondary calcium-dependent process would have to involve a small portion of the total calcium sites since membrane-bound annexins bind about the same number of calcium ions regardless of external calcium concentration or protein density on the membrane (Evans & Nelsestuen, 1994). Furthermore, an even more entropically unfavorable process would have to occur since later binding events require higher calcium and appear to be of lower affinity (Bazzi & Nelsestuen, 1991c).

Binding of annexin via widely-distributed sites on the convex face of the protein (Huber et al., 1992; Weng et al., 1993; Meers et al., 1991) to the apposed convex surface of the vesicle would appear to require flattening of either surface. Such a change in protein structure may not involve significant changes in secondary structure. Alternatively, the annexin structure change may involve a "hinged" motion of one or more of the 70-amino acid repeats relative to the other repeats [see Figure 7 of Weng et al. (1993); Karshikov et al. (1992)].

Annexin self-association (Klotz et al., 1975) is another potential source for the increased heat release at higher protein density on the membrane. Electron microscopy demonstrated the formation of two-dimensional crystals of annexin IV, V, and VI on monolayers (Newman et al., 1989, 1991; Brisson et al., 1991). Zaks and Cruetz (1991) have also presented evidence for apparently cooperative self-association of annexin IV, VI, and VII on membranes. While protein clustering should be a positively cooperative process, it could be offset by unfavorable entropy changes associated with protein clustering.

Other Potential Contributors to ΔH. Processes other than protein structure changes may also contribute to the large heat release. Again, a major requirement would be that these events become more dramatic at higher protein density. For example, the interaction of the convex surfaces of the annexin and the vesicle could cause flattening of the bilayer surface (Andree et al., 1992) which could become enhanced at higher protein density. Clustering of negatively-charged phospholipids (Bazzi & Nelsestuen, 1991b) may also have an enthalpy component that becomes more dramatic as segregation becomes more complete.

Other factors may make important contributions to enthalpy. Changes in monolayer surface pressure (Newman et al., 1989; Huber et al., 1992) and lateral lipid diffusion (Gilmanshin et al., 1994) and ion channel formation by annexin V (Rojas et al., 1990; Karshikov et al., 1992) each suggest changes in bilayer structure. The near identical structure of the annexin AB-loop calcium-binding sites to

that of phospholipase A_2 suggests that bilayer phospholipids may be bound in the manner described by Scott et al. (1990). Such binding could potentially alter phospholipid−phospholipid interactions for both bound and nearby phospholipids so that changes could become more intense with protein density.

Extrapolations of properties of protein−calcium complexes to protein−phospholipid complexes must be made with caution. An example may be provided by the pentraxin family of proteins which also bind to phosphorylated compounds through calcium ions. In this case, the X-ray crystal structure shows that interaction with phosphate causes some displacement of calcium-coordinated protein ligands (Emsley et al., 1994). By analogy, the number of protein−calcium ligands in the annexin−calcium structure may differ from those in the protein−membrane complex. Thus, the ΔH_{assoc} for calcium interaction with free protein may not be an appropriate background to subtract from total ΔH_{assoc} for protein−membrane association. In this event, the total ΔH_{assoc} for titration of protein−calcium−membrane interaction may be a more appropriate estimate of the ΔH of protein−membrane binding. These values are −24 to −34 and −34 to −49 kcal/mol for annexin V and VI, respectively (Table 1).

No ionization of protein or PS appeared to occur during annexin−PS interaction. In contrast, at pH 7.1 a small number of protons (<4) appeared to be released upon annexin VI binding to vesicles containing PA. The ionization probably corresponded to the second proton of the phosphate, an ionization site that is not present in PS. However, the relatively constant ΔH_{assoc} for annexin VI−membrane to PS or PA membranes at pH 8.4 suggested that enthalpy was relatively independent of other portions of the phospholipid headgroups, such as the serine of PS. The phosphate group appeared to be the major site of contact for the annexins.

This study of annexins provided a strong contrast to the relatively low or nonexothermic membrane-binding reaction of two vitamin K-dependent proteins (Plager & Nelsestuen, 1994). The vitamin K-dependent proteins also gave relatively constant heat release with respect to protein density on the membrane. Furthermore, for the vitamin K-dependent proteins, vesicle size was an important factor and dictated whether the membrane-binding reaction was entropy- or enthalpy-driven (Plager & Nelsestuen, 1994). For the annexins, the total enthalpy was so large that the small differences provided by membranes with different surface curvature became minor or insignificant factors. Thus, although both protein families may use a calcium-bridging mechanism for membrane binding, the thermodynamic features of their interactions are drastically different. A slightly more favorable enthalpy and increased affinity for both the annexins (Table 1) and vitamin K-dependent proteins (Plager & Nelsestuen, 1994) resulted from use of membranes containing PE as the neutral phospholipid. The cause for this effect of PE is unknown.

Overall, this study has shown some rather striking thermodynamic behaviors for the annexin proteins. While detailed interpretation of the structural events from thermodynamic behavior remains difficult, the magnitude of the thermodynamic events provides interesting potential relationships for annexin protein and membrane bilayer structure. Furthermore, nonlinear heat response and an inverse relationship between membrane-binding affinity and calcium binding by the free annexins further suggest a complex process for the annexin−calcium−phospholipid interaction. Further study will be needed to determine the relationship between protein conformation changes and/or membrane structural changes that account for the observed enthalpies.

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