Direct Enthalpy Measurements of Factor X and Prothrombin Association with Small and Large Unilamellar Vesicles†

Douglas A. Plager and Gary L. Nelsestuen*

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

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ABSTRACT: Isothermal titration calorimetry was used to determine the enthalpy for the calcium-dependent protein conformation change and subsequent interaction of blood clotting factor X and prothrombin with phospholipid vesicles. The effect of vesicle size was also determined. The protein conformation change was accompanied by −12 ± 1 and −7 to −15 kcal/mol for factor X and prothrombin, respectively. The range of values for prothrombin arose from use of different protein preparations and may be due to non-ideal behavior of this protein when calcium was added. The apparent enthalpy of association (ΔH_{assoc}) of both factor X and prothrombin with phosphatidylserine (PS)/phosphatidylcholine (PC) large unilamellar vesicles (LUVs, 120 nm diameter) was shown to be near 0 kcal/mol. In comparison, ΔH_{assoc} for interaction with PS/PC small unilamellar vesicles (SUVs, 40 nm diameter) was −9 ± 3 and −7 ± 2 kcal/mol for factor X and prothrombin, respectively. This difference appeared complementary to ΔH_{assoc} for calcium binding to these vesicles. That is, the interaction of calcium was athermic with SUVs and exothermic with LUVs. While such properties might suggest a considerable difference in the manner of calcium binding to LUVs versus SUVs, little difference in the quantity of calcium bound to SUVs and LUVs was detected by equilibrium dialysis. In any event, the results indicate that protein binding to LUVs was primarily entropy driven whereas binding to SUVs was primarily enthalpy driven. The exothermic process for calcium-dependent factor X or prothrombin binding to SUVs may result from protein-induced changes in the phospholipid packing/calcium interaction, possibly related to changes in how calcium is bound to the phospholipid.

While the energetics of several protein–ligand (Wiseman et al., 1989; Ross & Subramanian, 1981; Eftink & Biltonen, 1980) and protein–protein interactions (Ross & Subramanian, 1981; Klotz et al., 1975) have been studied, less is known about the energetics of peripheral protein–membrane interactions (Ramsay et al., 1986; Epand et al., 1990). Isothermal titration calorimetry (ITC)[1] allows the direct measurement of the enthalpy of a reaction. Unlike van't Hoff enthalpies, ITC measurements are model independent. In contrast, van't Hoff enthalpies are modeled as "two-state" equilibria (Breslauer et al., 1992; Sturtevant, 1987). Thus, comparison of ITC and van't Hoff results can further our understanding of the complexities of macromolecular interactions.

The vitamin K-dependent proteins, factor X and prothrombin, are two of several blood zymogens whose calcium-dependent interaction with membrane surfaces play a vital role in blood coagulation. These two proteins contain 12 and 10 γ-carboxyglutamic acid (Gla) residues in their respective membrane binding domains. It is often proposed that membrane binding involves a calcium bridge between the phospholipid head group and sites on the protein (Nelsestuen, 1988). While the crystal structure of the N-terminal membrane-binding domain of prothrombin, referred to as fragment 1, has recently been solved (Soriano-Garcia et al., 1992), the structure showed minimal availability of calcium coordination sites that might bind simultaneously to the lipid. Nevertheless, earlier studies suggested that the protein–membrane complex may contain calcium ions that are not found on the protein alone. The proposed scheme for the interaction of factor X or prothrombin with membranes shows additional calcium ions binding to the phospholipid (Nelsestuen & Lim, 1977):

$$\begin{aligned} &\mathrm{P} + x\mathrm{Ca} \overset{K_1}{\rightleftharpoons} \mathrm{P}\text{–}x\mathrm{Ca} \overset{K_2}{\rightleftharpoons} \mathrm{P'}\text{–}x\mathrm{Ca} \\ &\qquad\qquad\qquad\qquad + \qquad \overset{K_4}{\rightleftharpoons} \mathrm{P'}\text{–}\mathrm{PL}\text{–}(x+y)\mathrm{Ca} \\ &\qquad\quad \mathrm{PL} + y\mathrm{Ca} \overset{K_3}{\rightleftharpoons} \mathrm{PL}\text{–}y\mathrm{Ca} \end{aligned} \quad (1)$$

In this equation, P is protein, P′ is the protein after undergoing a calcium-dependent conformation change, PL is a protein binding site on the phospholipid membrane, and P′–PL is the protein–phospholipid complex. Previous van't Hoff measurements for prothrombin–membrane interaction at saturating calcium showed a ΔH_{assoc} value of 0 kcal/mol (Resnick & Nelsestuen, 1980).

Several differences for protein binding to SUVs versus LUVs have previously been documented (Abbott & Nelsestuen, 1987; Greenhut et al., 1986; Beschiaschvili & Seelig, 1990; Silversmith & Nelsestuen, 1986). For integral membrane-binding proteins, there is often a substantial selectivity for SUVs (Silversmith & Nelsestuen, 1986; Greenhut et al., 1986). However, the resultant effect(s) of vesicle size on the thermodynamics of peripheral protein–membrane interactions has not been thoroughly addressed (Abbott & Nelsestuen, 1987; Beschiaschvili & Seelig, 1992). Also of interest is the thermodynamic nature of the increased affinity of factor X and prothrombin for membranes containing the neutral

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* Address correspondence to this author at the University of Minnesota, Department of Biochemistry, 1479 Gortner Ave., St. Paul, MN 55108.

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; Gla, γ-carboxyglutamic acid; ITC, isothermal titration calorimetry; fragment 1, N-terminal residues 1–156 of prothrombin; QLS, quasielastic light scattering; SD, standard deviation; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

[2] D. A. Plager and G. L. Nelsestuen, unpublished data.

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phospholipid, phosphatidylethanolamine (PE), in place of PC (Nelsestuen & Broderius, 1977). In fact, the increased affinity for membranes containing PE instead of PC may be a general feature shared by other calcium-dependent peripheral proteins (Bazzi et al., 1992; Plager & Nelsestuen, 1992).

This study involved direct enthalpy measurements of the calcium-dependent interaction of two vitamin K-dependent proteins with vesicles of different size and composition. When titrated at low calcium the results showed a surprising difference between protein binding to LUVs versus SUVs. The former was entirely entropy driven while the latter had a considerable enthalpy component. The difference may arise from the detailed aspects of calcium binding to these different vesicles. Comparison to results from van't Hoff plots suggested that protein–membrane binding consisted of more complex processes than predicted from two-state models.

EXPERIMENTAL PROCEDURES

Proteins. Bovine factor X and prothrombin were purified as described (Nelsestuen, 1984). Protein concentrations were determined by Bradford assay (Bradford, 1976) or by absorbance at 280 nm (extinction coefficients, 1% at 280 nm, of prothrombin = 14.1 (Cox & Hanahan, 1970) and factor X = 12.4 (Jackson, 1972)).

Phospholipid Vesicles. Highly pure phospholipids (≥98% purity, suppliers' estimates) were used to prepare all vesicles. PS (bovine brain) was from Sigma Chemical Co. (St. Louis, MO) or Avanti Polar Lipids, Inc. (Alabaster, AL). PC (egg yolk) and PE (egg yolk) were from Sigma. SUVs, approximately 40 nm in diameter, were prepared by sonication and gel filtration (Huang, 1969; Nelsestuen & Lim, 1977). LUVs, approximately 120 nm in diameter, were prepared by the extrusion technique (Hope et al., 1985). These preparations have a very low percentage (≤5%) of vesicles under 70 nm in diameter (Hope et al., 1985). Phospholipid in organic solvent was mixed in the desired ratios (by weight). When appropriate, this solution was divided for separate preparation of SUVs and LUVs of identical composition. The percent phospholipid composition is designated, for example, as PS/PC/PE, 18/32/50. This would represent a phospholipid composition of 18% PS, 32% PC, and 50% PE.

For SUVs, 80 mg of phospholipid was dried thoroughly with a stream of nitrogen and then suspended in 4 mL of Tris buffer (20 mM Tris, 0.1 M NaCl, 1 μM EDTA, pH 7.5). This suspension was then sonicated by direct probe using the microtip assembly of the 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 during sonication. The sonicated solution was then centrifuged and gel filtered on a Sephacryl-500-HR column (1.5 × 50 cm). Fractions were monitored for turbidity at 256 nm and for vesicle size by quasielastic light scattering (QLS) (Bloomfield & Lim, 1978) using a Langley Ford Model LSA2 spectrophotometer coupled to a Model 1096 correlator. Fractions containing vesicles of <60- and >26-nm diameter were pooled and concentrated by pressure dialysis. After concentration the vesicles consistently gave an average diameter of 40 nm.

For LUVs, 50 mg of phospholipid was dried with a stream of nitrogen and were vigorously suspended in 8 mL of Tris buffer with excess salt (20 mM Tris, 0.15 M NaCl, 1 μM EDTA, pH 7.5). This suspension was then frozen and thawed (using a dry ice/organic solvent solution and a warm water bath) five times, 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 LUVs solution was then dialyzed extensively against Tris buffer (20 mM Tris, 0.1 M NaCl, 1 μM EDTA, pH 7.5) and concentrated by pressure dialysis. After concentration these vesicle preparations gave consistent diameter measurement of 120 nm. A yield of 80% of the initial phospholipid was obtained. Final phospholipid concentrations were determined by assay of organic phosphate (Chen et al., 1956), assuming a phosphorus:phospholipid ratio of 1:25.

Light Scattering and Fluorescence. Nelsestuen and Lim (1977) have previously shown the usefulness of 90° Rayleigh light scattering for the detection of protein–phospholipid vesicle interactions. Briefly, for a constant light source and instrumental geometry, the light scattering relationship for a solution of particles whose diameters are small with respect to the incident wavelength is

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

where I_2 and I_1 are the light scattering intensities due to the protein–vesicle complex and the vesicle alone; M_2 and M_1 are the weight-average molecular weights of the protein–vesicle complex and the vesicle alone; and $(\partial n/\partial c_2)$ and $(\partial n/\partial c_1)$ are the changes in refractive index per change in concentration for the protein–vesicle and the vesicle solution, 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 for background scattering from buffer and unbound protein. The background scattering was <5% of total scattering. Calcium-induced aggregation of vesicles was not detected by light scattering at concentrations of ≤4 mM calcium. When measured, replicate titrations gave indistinguishable results. Experiments were performed on the SLM 4800C (SLM-Aminco, Urbana, IL) or SPEX Fluoromax (JY/SPEX instruments SA, Inc., Edison, NJ) fluorometers with excitation and emission set at 600 nm.

Intrinsic protein fluorescence measurements were performed with excitation and emission at 284 and 340 nm using a SPEX Fluoromax fluorometer.

Isothermal Titration Calorimetry. All calorimetry experiments were performed with the MicroCal OMEGA titration calorimeter (MicroCal Inc., Northampton, MA) coupled with a nanovoltmeter preamplifier. The operation and design of the instrument has been previously described by Wiseman et al. (1989). Room-temperature, degassed buffer (20 mM Tris, 0.1 M NaCl, pH 7.5) and concentrated phospholipid and protein solutions were used to prepare 1.6 mL of sample. The volume of phospholipid and protein never exceeded 20% of this sample volume. The sample cell (volume = 1.4470 mL) was loaded with 1.4 mL of sample, and the system was equilibrated while stirring for 20 min. Calcium chloride solutions were prepared by dilution of aqueous 1 M $CaCl_2$ with degassed buffer. Calcium solution (10 μL) was injected over a 15-s time period with stirring at 400 rpm. For experiments involving injection of protein, the protein solutions were warmed to room temperature prior to injection. Experiments were performed at 26 ± 1 °C with the external temperature thermostated at 21 °C. Integration of the resultant calorimetric data was performed using the software provided with the instrument ("Origin" application). The calorimeter was calibrated electrically. The enthalpy for cytidine 2′-monophosphate binding to bovine ribonuclease A was tested as a standard. The results were comparable to those reported by Wiseman et al. (1989).

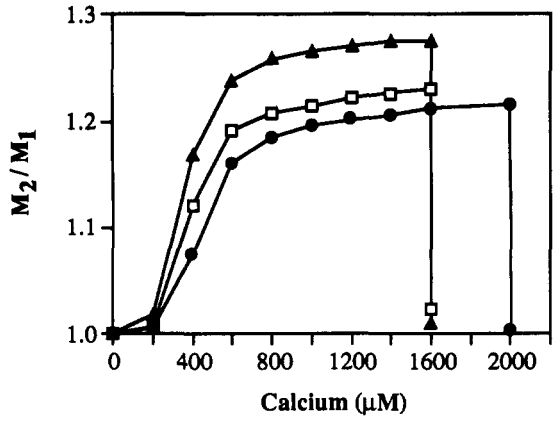

FIGURE 1: Calcium-dependent binding of factor X to vesicles. Samples of 0.4 mL containing 250 μg of factor X plus 750 μg of LUVs (PS/PC, 18/82; – ● –), SUVs (PS/PC, 18/82; – □ –), or LUVs (PS/PC/PE, 18/32/50; – ▲ –) were titrated with $CaCl_2$. Additions (2.92 μL) of 27.4 mM $CaCl_2$ were made to each of the three samples. Addition of excess EDTA after the final $CaCl_2$ addition demonstrated complete reversibility. Light scattering was measured at 600 nm. M_2/M_1 ratios were calculated from light scattering intensities according to eq 2. Data were corrected for dilution and a small background scattering due to protein alone. Buffer composition was 20 mM Tris, 0.1 M NaCl, pH 7.5.

Equilibrium Dialysis. The same LUV and SUV preparations that were used in calorimetry experiments were used for equilibrium dialysis to measure calcium binding. Briefly, three 1.5-mL samples of LUVs (10 mg/mL) and three more samples of SUVs (10 mg/mL) were placed in separate Spectra/Por-3 dialysis bags (Spectrum Medical Industries, Inc., Los Angeles, CA) with a molecular weight cutoff of 3500 Da. These were tied at one end and closed with a dialysis clip at the other. The LUV and SUV samples were dialyzed against 4 L of 20 mM Tris, 0.1 M NaCl, 0.01% NaN_3, pH 7.5 for 20 h at room temperature with constant stirring. QLS measurements of vesicle size and inorganic phosphate assays for phospholipid concentration were performed on samples removed from the dialysis bags. Dilution was within acceptable limits, and vesicle size changes due to events such as fusion were not detectable (<10% size change from original samples). The LUV and SUV samples were then placed in 4 L of 20 mM Tris, 0.1 M NaCl, 0.01% NaN3, 0.1 mM $CaCl_2$, pH 7.5 containing 0.2 mCi $^{45}CaCl_2$. Eight h of dialysis was allowed, and samples of the dialysis bag contents were measured for radioactivity and phosphate concentration. ^{45}Ca in the external buffer was measured to determine free calcium concentrations. Nonradioactive $CaCl_2$ was then added to the external buffer to raise the calcium concentration to that needed for the next step, and dialysis was repeated. All dialyses were conducted for 8–16 h. Previous studies indicated that this length of dialysis is sufficient for equilibrium distribution of calcium (Bazzi & Nelsestuen, 1990). Free calcium concentrations were 0.1, 0.2, 0.4, 0.8, and 1.6 mM. Radioactivity and phospholipid concentration of each sample were taken after each dialysis period. Vesicle diameter of the SUV samples was measured after the 0.8 mM $CaCl_2$ dialysis step. Once again, vesicle size was indistinguishable from that of the original sample.

RESULTS

Factor X Interaction with LUVs and SUVs. The binding of blood clotting factor X to three different vesicle preparations is shown in Figure 1. In each case binding was saturable and reversible, and the $CaCl_2$ concentration required for half of maximum binding followed the trend of LUVs > SUVs > LUVs containing 50% PE. The final M_2/M_1 ratios were somewhat variable and slightly lower than the theoretical values expected from eq 2 (M_2/M_1 for quantitative binding should be 1.33), consistent with an equilibrium binding process. The important interpretations presented below are within the limits provided by the variations observed in Figure 1.

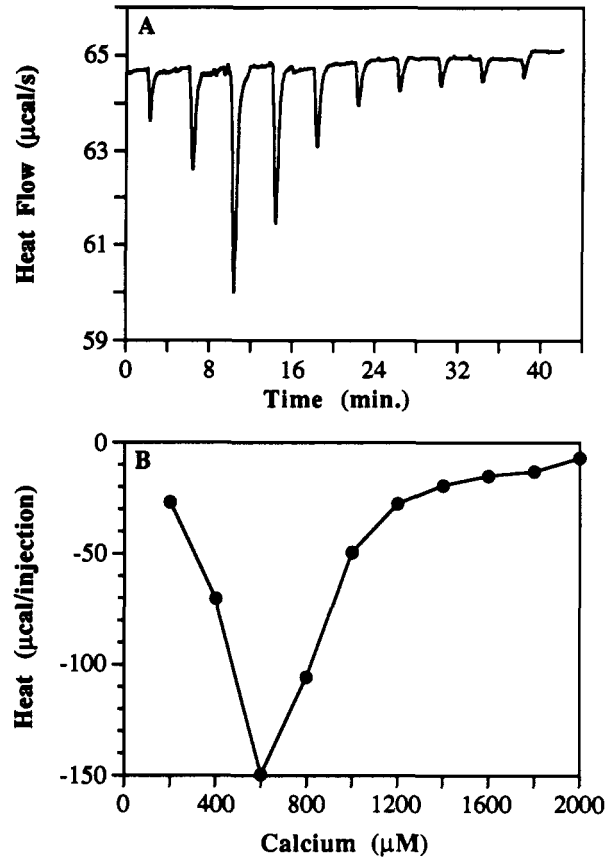

FIGURE 2: Titration calorimetry. Panel A: the differential heat response was measured for a 1.4-mL sample containing LUVs (PS/PC, 18/82; 2.63 mg) plus factor X (0.88 mg). The sample was equilibrated for 20 min to obtain a flat base line. Injections (10 μL) of 27.4 mM $CaCl_2$ were made over a 15-s duration with a 4-min interval. The downward peaks represent heat released from the sample upon $CaCl_2$ injection. Panel B: integrated areas for each peak in Figure 1A are shown. Integration was carried out using the "Origin" software provided with the instrument.

An example of the data obtained from the microcalorimeter is shown in Figure 2A. The downward peaks represent an exothermic heat response upon injection of $CaCl_2$. In this instance, the heat response includes contributions from dilution of the $CaCl_2$, interaction of $CaCl_2$ with factor X or LUVs, and subsequent interaction of factor X with LUVs. The program titled Origin, provided by Microcal Inc., was used to determine the area of each peak. These areas represent the microcalories/injection, and their values are presented in Figure 2B.

To obtain ΔH_{assoc} for protein–membrane binding, four sets of experiments were carried out: injection of $CaCl_2$ into (a) buffer alone, (b) factor X alone, (c) vesicles alone, and (d) factor X plus vesicles. The data for each set were averaged. The titration of buffer alone was subtracted as a background, and the three sets of data were each summed to give a running total, "cumulative μcals". These values were divided by the moles of factor X present (16.2 nmol) in the sample to give kcal/mol of factor X (Figure 3).

The heat response for factor X alone was sigmodial and was slightly endothermic at low calcium but became quite

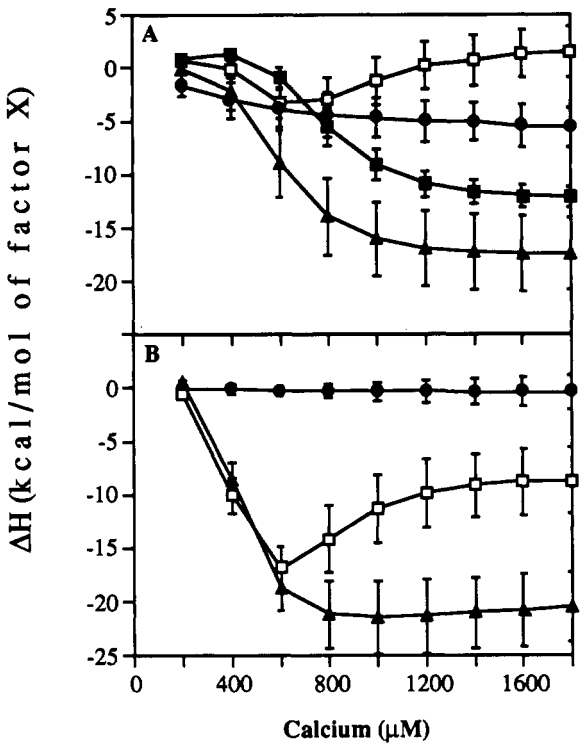

FIGURE 3: ΔH_{assoc} for binding of factor X to LUVs and SUVs. Panel A: the summation of the heat response, after subtraction of background heat for injection of $CaCl_2$ into buffer alone, is shown for 1.4-mL samples of LUVs (PS/PC, 18/82; 2.63 mg) plus factor X (0.88 mg) (– ▲ –), LUVs alone (PS/PC, 18/82; 2.63 mg; – ● –), and factor X alone (0.88 mg; – ■ –). Error bars represent standard deviations for three to five measurements for three separate factor X and LUV preparations. Also shown is the summation of the apparent ΔH_{assoc} for factor X binding to LUVs (PS/PC, 18/82; – □ –). This was calculated as described in the text. The standard deviations represented by the error bars included five measurements using three separate preparations. The vertical axis shows the cumulative heat divided by the total factor X present (16.2 nmol). Panel B: treatment and calculations are as in panel A but for samples of SUVs (PS/PC, 18/82; 2.63 mg) plus factor X (0.88 mg) (– ▲ –) and for SUVs alone (PS/PC, 18/82; 2.63 mg; – ● –). Summation of the apparent ΔH_{assoc} for factor X binding to SUVs is also shown (– □ –). Error bars represent standard deviations for three measurements made with two separate protein and phospholipid preparations.

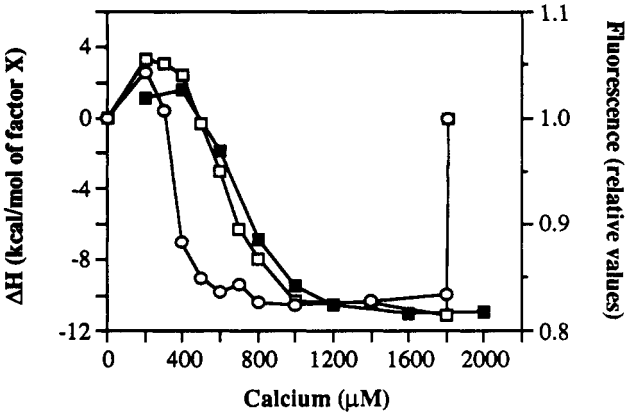

FIGURE 4: ΔH versus fluorescence change for the calcium-induced factor X conformation change. A comparison of the cumulative ΔH_{assoc} for calcium interaction with factor X (0.63 mg/mL; – ■ –) with the calcium-dependent change in fluorescence of factor X alone (0.63 mg/mL; – □ –) and in the presence of LUVs (PS/PC/PE, 18/32/50, 1.89 mg/mL; – ○ –) is shown. For fluorescence, aliquots of a 40 mM $CaCl_2$ solution were added to a single 0.8-mL sample containing factor X. The fluorescence data were normalized to the initial fluorescence intensity, prior to calcium addition, and adjusted for a slight photobleaching that occurred in measurement. Addition of excess EDTA at the completion of the calcium titration demonstrated the reversibility of the fluorescence change. Calorimetry data were obtained as outlined in the legends of Figures 2 and 3.

exothermic with an overall ΔH_{assoc} of –12 ± 1(SD) kcal/mol (Figure 3A). Addition of $CaCl_2$ to the LUVs alone gave an exothermic response (Figure 3A), the magnitude of which was somewhat dependent upon the amount of calcium added per injection, lower calcium per injection resulting in lower cumulative ΔH_{assoc} (data not shown). This appeared to result from accumulative background subtraction errors associated with many small injections. Variation to larger injections (200 μM versus 800 μM $CaCl_2$, presented below) indicated little dependence of ΔH_{assoc} on the calcium per injection. For this reason, the background and experimental measurements were made with large, identical calcium additions.

Figure 3A also shows the apparent ΔH_{assoc} for factor X binding to LUVs. This was obtained by subtracting the contributions of factor X alone and LUVs alone from the heat observed when both factor X and LUVs were present. The final ΔH_{assoc} was 1.5 ± 2.5(SD) kcal/mol. The final standard deviation for factor X binding to LUVs (±2.5 kcal/mol) was less than that for injection of $CaCl_2$ into factor X plus LUVs (±3.5 kcal/mol). This resulted from greater consistency within given protein and vesicle preparations. That is, the total ΔH_{assoc} included an average of several protein and vesicle preparations. In contrast, ΔH_{assoc} for factor X–phospholipid binding was obtained by subtractions within the same preparation. Thus, the difference calculation removed some of the variations produced by different preparations.

Unlike LUVs, the interaction of calcium with SUVs was accompanied by virtually no heat emission (Figure 3B). This was consistent with earlier results for 100% PS SUVs (Portis et al., 1979). After appropriate subtractions the apparent ΔH_{assoc} for factor X binding to SUVs was –9 ± 3(SD) kcal/mol (final value at 1.8 mM calcium, Figure 3B).

Calcium-induced intrinsic fluorescence changes of factor X (Nelsestuen et al., 1976; Persson et al., 1991) were measured to confirm that the factor X–calcium titration enthalpy (Figure 3A) was due to the expected calcium-induced conformation change of factor X (Figure 4). The close coincidence of the enthalpy and fluorescence change suggested that the observed enthalpy change was a result of the calcium-induced factor X conformation change. Although the fluorescence change suggested more complex events, it appeared that virtually all of the ΔH was associated with the major fluorescence quenching event.

Intrinsic fluorescence changes in the presence of vesicles at protein and vesicle concentrations lower than, and equivalent to, those used in calorimetry confirmed the light scattering results of Figure 1. As an example, the fluorescence change in the presence of LUVs containing PE is presented in Figure 4. As expected, the calcium concentration required for the conformation change of factor X in the presence of vesicles was always lower than for factor X alone. In accordance with Le Châtelier's principle (Alberty, 1983; Voet & Voet, 1990), the second factor X–calcium–phospholipid equilibrium effectively reduced the overall calcium requirement (Nelsestuen et al., 1976). The degree to which the calcium requirement was altered by the vesicles followed the trend of LUVs containing 50% PE > SUVs > LUVs.

The ΔH_{assoc} for factor X binding to both LUVs and SUVs showed a trough centered at 600–800 μM $CaCl_2$ (Figure 3A and B, –□–) which probably arose from improper background subtraction. That is, in the presence of phospholipid, there was decreased calcium required for the factor X conformation

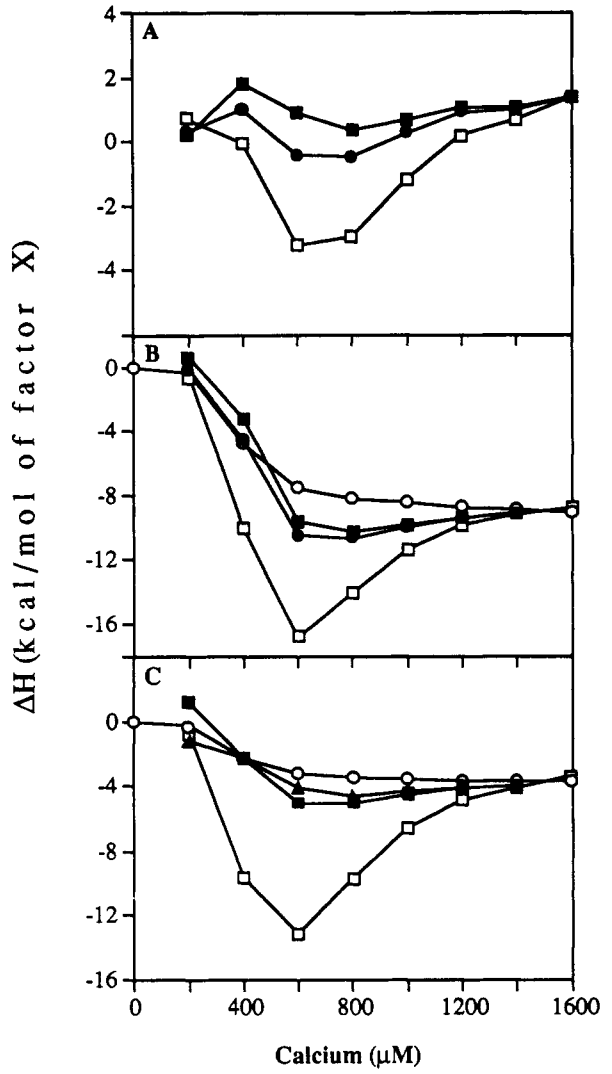

FIGURE 5: Removal of the artificial trough in ΔH_{assoc} Panel A: the cumulative ΔH_{assoc} for binding of factor X to LUVs (PS/PC, 18/82) was calculated as in Figure 3A except that the ΔH_{assoc} for the factor X–calcium titration curve was "offset" by a constant value before it was subtracted from the total ΔH obtained when LUVs plus factor X were present. Results for an offset (decrease) in this titration of 0 (– □ –), 100 (– ● –), and 150 (– ■ –) μM $CaCl_2$ are shown. Panel B: the cumulative ΔH_{assoc} for binding of factor X to SUVs (PS/PC, 18/82) were calculated as described in panel A but for decreases in the calcium required for the factor X conformation change of 0 (– □ –), 250 (– ● –), and 300 (– ■ –) μM. Also shown is an "expected" cumulative ΔH_{assoc} curve (– O –), calculated from the fraction of the total factor X bound at each $CaCl_2$ concentration, determined from light scattering (see Figure 1) and the assumption that the final ΔH_{assoc} (–9 kcal/mol) was directly proportional to the total amount of factor X bound. Panel C: the cumulative ΔH_{assoc} for binding of factor X to LUVs (PS/PC/PE, 18/32/50), calculated as described in panel A, but with constant offsets in calcium titration of factor X conformation change of 0 (– □ –) and 350 (– ■ –) μM. Also shown is the cumulative ΔH_{assoc} (– ▲ –) obtained by subtraction of ΔH for factor X–calcium interaction that was adjusted to directly correspond to the intrinsic fluorescence change for factor X in the presence of LUVs (PS/PC/PE, 18/32/50) (– O – in Figure 4). The expected cumulative ΔH_{assoc} curve (– O –) calculated from binding data (Figure 1) and a constant ΔH_{assoc} of –4 kcal/mol for all steps of the titration is also shown.

change so that subtraction of the factor X–calcium titration data in the manner described in Figure 3 would be in error and could produce an artifactual trough. The plots in Figure 5 were therefore generated by a simple technique that illustrated conditions needed to eliminate the trough. Briefly, the ΔH_{assoc}'s for factor X binding to vesicles were recalculated, assuming a decreased calcium requirement for the factor X conformation change. The factor X–calcium data (Figure 3A, –■–) were simply shifted to the left by a constant value. For instance, a 200 μM decrease meant that the cumulative ΔH value at 400 μM $CaCl_2$ for the factor X–calcium titration (Figure 3A, –■–) became the value for 200 μM $CaCl_2$. Likewise, the 600 μM $CaCl_2$ value became the value for 400 μM $CaCl_2$, and so on. As can be seen, a shift in the factor X–calcium titration of about 125 μM effectively removed the trough in the case of LUVs (Figure 5A). This also produced a constant ΔH_{assoc} for factor X binding of approximately 0.5 kcal/mol throughout the binding curve (Figure 5A). Complete removal of the trough for SUVs required a shift in the factor X–calcium titration of approximately 275 μM (Figure 5B). A shift of approximately 350 μM was needed to eliminate the trough for LUVs containing 50% PE (Figure 5C). These required shifts correlated with the efficacy of these phospholipids to bind factor X at low calcium concentration: that is, LUVs containing 50% PE required less calcium than SUVs, which required less than LUVs (Figure 1). Comparison of these shifts to those observed for the intrinsic fluorescence changes in the presence of LUVs and LUVs containing PE were also made. Slight shape changes in the intrinsic fluorescence curve for factor X occurred when vesicles were present (Figure 4). This complicated exact comparisons of calcium midpoints in the absence and presence of vesicles. However, a decrease in calcium midpoint of 140 and 300 μM for the factor X intrinsic fluorescence change in the presence of LUVs (data not shown) and LUVs containing PE (Figure 4), respectively, were estimated. These approximate shifts compare favorably with those required for removal of the troughs observed in the analogous enthalpy data.

Also presented in Figure 5B and C are the "expected" cumulative ΔH_{assoc} if each factor X–membrane binding event, as determined by light scattering (Figure 1), resulted in constant heat release. These constants corresponded to –9 kcal/mol for SUVs (Figure 5B) and –4 kcal/mol for LUVs containing 50% PE (Figure 5C). The close coincidence between the expected and "corrected" cumulative ΔH_{assoc} suggested that ΔH_{assoc} was constant at the stages of membrane saturation provided by these curves.

Alternative Titration Methods. Earlier studies with prothrombin–membrane binding, analyzed by the van't Hoff equation, suggested that the ΔH_{assoc} for prothrombin–SUV was approximately zero (Resnick & Nelsestuen, 1980). This differed significantly from the values obtained in this study for prothrombin (see below) and factor X binding to SUVs (–9 kcal/mol). However, the experiments were carried out under different conditions which included saturating calcium in the earlier studies versus low calcium here. It was possible that ΔH_{assoc} was zero when binding was measured at saturating calcium concentrations. Therefore, ΔH_{assoc} values for factor X binding to LUVs and SUVs were also measured by injection of a factor X solution which contained high $CaCl_2$ into a sample of vesicles which also contained high $CaCl_2$ (Figure 6).

These experiments presented greater technical difficulties. For example, problems could arise if time-dependent changes in factor X or phospholipid occurred when calcium was present. However, control experiments indicated that this was not a concern. Light scattering changes for LUVs and SUVs in 4 mM and 14 mM $CaCl_2$, respectively, were less than 4% over the time interval of these experiments (data not shown).

Another problem that could not be easily overcome was the manner of background subtraction for calcium dilution. A significant amount of the total calcium was bound by the concentrated protein and phospholipid solutions, and precise

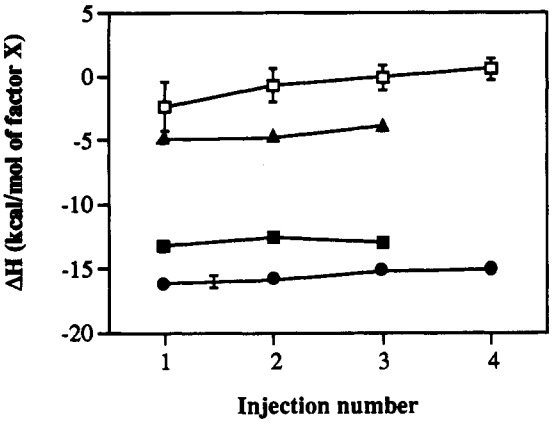

FIGURE 6: Alternative determination of ΔH_{assoc} for binding of factor X to LUVs or SUVs. Factor X (10 μL of 25 mg/mL in 5 mM $CaCl_2$) was injected into 1.4 mL containing 3.0 mg of SUVs (PS/PC, 18/82) and 5 mM $CaCl_2$ (– ● –). The data represent the average of two separate experiments conducted 30 min and 2.5 h after the addition of $CaCl_2$ to the factor X solution. The range for these two experiments is represented by the error bar (less than ±0.5 kcal/mol). A second experiment shows a similar titration but with 10 mM $CaCl_2$ in the factor X solution with injection into either 3.0 mg of SUVs (PS/PC, 18/82) in 5 mM $CaCl_2$ (– ■ –) or 3.0 mg LUVs (PS/PC, 18/82) in 2 mM $CaCl_2$ (– ▲ –). As a control, factor X without $CaCl_2$ was injected into 1.4 mL containing 3.0 mg of SUVs (PS/PC, 18/82; three titrations) or LUVs (PS/PC, 18/82; one titration) without $CaCl_2$. Since the latter gave very small values that were similar to those for SUVs, the average and standard deviation of both sets of data are shown (– □ –).

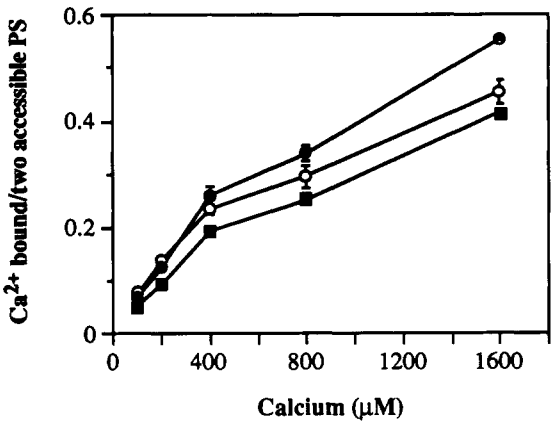

FIGURE 7: Calcium binding to LUVs and SUVs. The vesicles used to generate the calorimetry data in Figure 6 were tested for Ca^{2+} binding by equilibrium dialysis. The ratios of Ca^{2+} bound per two accessible PS molecules (assuming 50% accessibility of PS) for SUVs (PS/PC, 18/82; – ● –) and LUVs (PS/PC, 18/82; – ○ –) are shown. The same results for SUVs were recalculated but with the assumption of 67% accessible PS (– ■ –). Dialysis was at room temperature against buffer containing 20 mM Tris, 0.1 M NaCl, 0.01% NaN_3, pH 7.5 and the indicated calcium concentration. Error bars represent standard deviations for three separate determinations. Lack of error bars indicates an error that was within the dimension of the symbol.

corrections appeared difficult. The values in Figure 6 therefore do not have correct calcium background subtraction, and the absolute values for ΔH have an unknown error. Nevertheless, this should be similar for all titrations so the results should be valuable for comparisons.

The apparent ΔH_{assoc} for factor X binding to SUVs appeared more exothermic (Figure 6) than when calcium was added as the titrant (Figure 3B). However, the background problem may have contributed to this difference. Furthermore, 5 mM $CaCl_2$ provided only about 10 Ca^{2+} ions per factor X molecule in the protein solution which may not be adequate for the conformation change (Henriksen & Jackson, 1975). Therefore, the larger ΔH_{assoc} observed in this experiment may have also included contribution from the factor X conformation change. Experiments with 10 mM $CaCl_2$ gave apparent ΔH_{assoc} values that were 3 kcal/mol less exothermic (Figure 6). This ΔH_{assoc} was still somewhat more exothermic than when calcium was added as titrant. The apparent ΔH_{assoc} for LUV–factor X titration (Figure 6) was also more exothermic than that obtained when calcium was the titrant (Figure 3). The important point obtained from these measurements was the comparison value which gave a difference between LUVs and SUVs of 8–9 kcal/mol, the same as that obtained when calcium was the titrant (Figure 3). This suggested that, under all conditions, the binding of factor X to SUVs was 8–9 kcal/mol more exothermic than its binding to LUVs.

Calcium Binding to LUVs and SUVs. It appeared possible that the difference between ΔH_{assoc}'s for factor X binding to LUVs versus SUVs reflected different calcium binding properties of these vesicles. Therefore, calcium binding to the LUV and SUV preparations described in Figure 6 was measured by equilibrium dialysis (Figure 7). Due to the greater surface curvature, a SUV with a mean radius of 15 nm and bilayer thickness of 5 nm presents 67% of its total phospholipid on the outer surface. Thus, SUVs may provide a greater number of calcium and protein binding sites per weight of phospholipid than LUVs, if the PS is distributed homogeneously between the inner and outer membrane leaflets. However, this estimated accessible PS may be too high by about 15% (Berden et al., 1975). Therefore, equilibrium dialysis data for SUVs are shown assuming 50% or 67% accessible PS (Figure 7). This should represent the maximum range of accessible PS. Regardless of the assumed accessible PS, SUVs bound almost the same amounts of Ca^{2+} as LUVs. Therefore, the basis for the difference in ΔH_{assoc} for Ca^{2+} addition to these vesicles was apparently not due to a gross difference in Ca^{2+} binding behavior.

Prothrombin Interaction with LUVs and SUVs. Calcium-dependent binding of prothrombin to vesicles, as detected by light scattering, was saturable and reversible (data not shown, but see Nelsestuen & Broderius, 1977). Direct enthalpy measurements for prothrombin binding to LUVs and SUVs are shown in Figure 8. Experiments involving prothrombin have the complicating factor of a slow calcium-induced conformation change (Nelsestuen, 1976). Results utilizing a single prothrombin preparation were treated as previously described for factor X in Figure 3. Final ΔH_{assoc} values of approximately 0 and –7 kcal/mol were determined for prothrombin binding to LUVs (Figure 8A) and SUVs (Figure 8B), respectively. Once again, calcium titration of LUVs alone (Figure 8A) and SUVs alone (Figure 8B) gave exothermic and athermic heat responses, respectively. While the variability in the ΔH_{assoc} for calcium titrations of prothrombin alone (Figure 8A) was satisfactory, –7 ± 2 kcal/mol, a larger discrepancy was obtained when different preparations of prothrombin were compared. The apparent ΔH_{assoc} ranged from –7 (Figure 8A) to –15 kcal/mol (Figure 8C). The cause for this wide range is unknown but may involve differences in the amount of calcium-dependent protein aggregation (Nelsestuen et al., 1981; Jackson et al., 1979) and time-dependent conversion of prothrombin to prethrombin 1 and fragment 1. However, this variation in ΔH_{assoc} for prothrombin–calcium interaction did not appear to greatly influence the value for prothrombin–SUV binding which was constant within the different preparations. As for factor X, the variations in individual preparations were eliminated by subtraction of ΔH_{assoc} for calcium–prothrombin binding from the total ΔH_{assoc} utilizing the same prothrombin preparation.

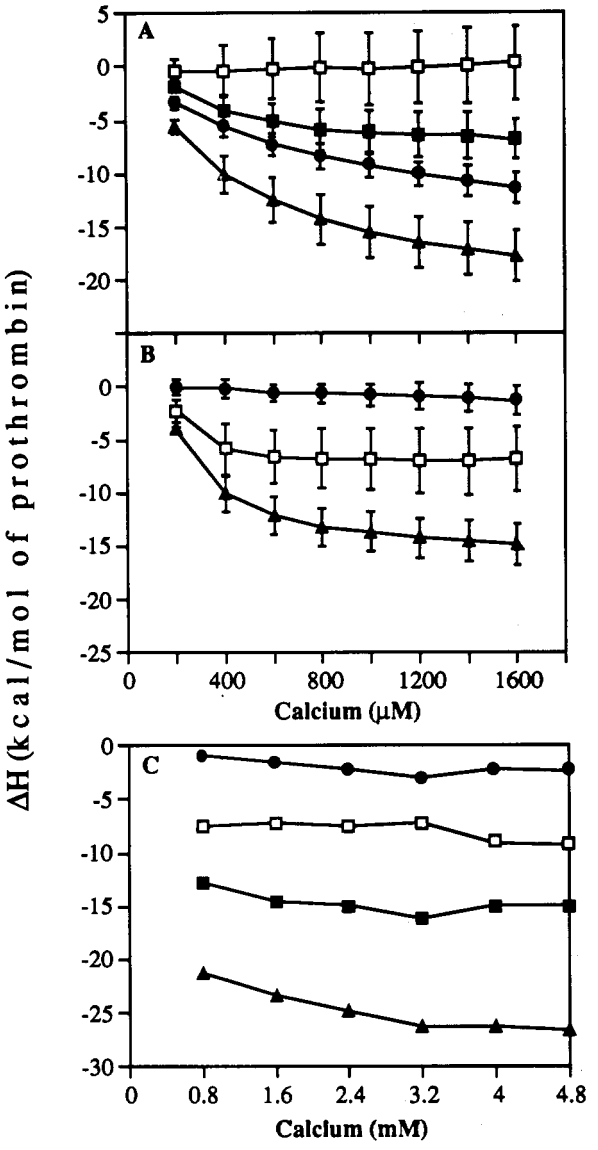

FIGURE 8: ΔH_{assoc} for binding of prothrombin to LUVs and SUVs. Panel A: cumulative values of ΔH for 1.4-mL samples of prothrombin alone (0.93 mg, – ■ –), LUVs alone (3.73 mg of PS/PC, 25/75; – ● –), and 3.73 mg of LUVs (PS/PC, 25/75; 3.73 mg) plus 0.93 mg prothrombin (– ▲ –) are shown. Fifteen min were allowed after the first and second calcium injections, followed by 4-min intervals for subsequent injections. The calculated ΔH_{assoc} for prothrombin binding to LUVs is also presented (– □ –). Panel B: cumulative ΔH for 1.4-mL samples containing SUVs alone (PS/PC, 25/75; 3.26 mg; – ● –) and SUVs (PS/PC, 25/75; 3.26 mg) plus prothrombin (0.93 mg; – ▲ –) are shown. ΔH_{assoc} for prothrombin binding to SUVs is also presented (– □ –). The error bars in panels A and B represent the standard deviation for three measurements from a single prothrombin, LUV, and SUV preparation. Panel C: the summation of the heat response for samples with larger calcium additions and with different prothrombin and SUVs preparations are presented. Results are shown for 1.45-mL samples of SUVs (PS/PC, 25/75; 2.75 mg; – ● –), prothrombin (0.92 mg; – ■ –), and SUVs (PS/PC, 25/75; 2.75 mg) plus prothrombin (0.92 mg) (– ▲ –). Single measurements were made. The calculated ΔH_{assoc} for the prothrombin–SUV interaction is also shown (– □ –). Injections (10 μL) of 116 mM $CaCl_2$ were made over a 15-s duration with a 10-min interval. The ΔH values were determined as described in Figures 2 and 3.

The final ΔH_{assoc} for prothrombin–SUV interaction were –7 and approximately –8 kcal/mol, respectively (Figure 8B and C). These results also showed that the calcium added per injection did not greatly influence the ΔH_{assoc} for prothrombin–SUV interaction (compare Figure 8B and C). Furthermore, both Figure 8B and C show that the enthalpy associated with membrane binding was complete by 0.8 mM calcium, as expected from light-scattering measurements (data not shown). Thus, it appeared that the ΔH_{assoc} for membrane binding of prothrombin, like for factor X, was primarily entropy driven for LUVs ($\Delta H_{assoc} \approx 0$ kcal/mol, Figure 8A) but had a large enthalpy component for SUVs ($\Delta H_{assoc} \approx -7$ kcal/mol, Figure 8B and C).

DISCUSSION

Several previous applications of direct calorimetric studies have examined the interaction of organic amphiphiles, peptides, and calcium-independent peripheral membrane proteins with membranes (Bäuerle & Seelig, 1991; Myers et al., 1987; Epand et al., 1990; Ramsay et al., 1986). This study applied this approach to a novel type of calcium-dependent protein–membrane interaction. An important advantage of direct enthalpy measurement over van't Hoff energy measurements is that the former is not model dependent (Breslauer et al., 1992).

One of the most striking properties observed was the influence of vesicle size on two parameters, in nearly equal and opposite directions. The first was the enthalpy for protein binding to the phospholipid and the second was calcium binding to the phospholipid. For factor X and prothrombin, ΔH_{assoc} for binding was near 0 kcal/mol for LUVs and was –9 and –7 kcal/mol, respectively, for SUVs. For comparison, the apparent association equilibrium constant (K_a) for the membrane binding reactions described in this study are in the range of 10^6 to 10^7 M^{-1} (Nelsestuen & Broderius, 1977) which corresponds to a free energy of association (ΔG_{assoc}) of –8.2 to –9.5 kcal/mol. Other studies have shown similar differences for protein/peptide binding to LUVs versus SUVs. The binding of factor V_a light chain to SUVs and to LUVs gave ΔH_{assoc} values of about –16 and 0 kcal/mol, respectively (Abbott & Nelsestuen, 1987). Likewise, melittin and a somatostatin analogue each showed an increased enthalpy component (19 and 8 kcal/mol, respectively) for binding to SUVs as opposed to LUVs (Beschiaschvili & Seelig, 1992). Previous studies involving vitamin K-dependent proteins indicated little dependence of phospholipid binding on vesicle size (Nelsestuen & Lim, 1977) or monolayer surface pressure (Mayer et al., 1983). However, the calcium concentration needed for protein–membrane interaction (Figure 1) suggested a slight preference of factor X for SUVs. This preference for SUVs could be a result of an increased number of binding sites per weight of phospholipid (see text accompanying Figure 7) and/or an increased exposure of the hydrophobic core of the bilayer (see below). Factor V_a light chain (Abbott & Nelsestuen, 1987), melittin, and the somatostatin analogue also showed a preference for binding to SUVs (Beschiaschvili & Seelig, 1992). Thus, in all cases, it appeared that the binding of peripheral proteins to LUVs was primarily entropy-driven whereas binding to SUVs contained a large enthalpy component.

The lower calcium requirement for binding to membranes containing PE instead of PC (Figure 1 and Figure 5A and C) demonstrated a preference for PE as the neutral phospholipid. This enhanced binding to PE-containing vesicles could also result from the more favorable enthalpy contribution to binding (compare final ΔH_{assoc}, Figure 5A and C).

The basis for the difference in binding of factor X and prothrombin to LUVs versus SUVs may be seen in Scheme 1. Although equilibriums 1 and 2 were associated with significant heat release, they involved only protein. For prothrombin, this exothermic process may correspond to the

calcium-induced increase in stability observed by Plopis et al. (1981). The difference may involve equilibrium 3, calcium binding to the phospholipids. In this case, calcium binding to LUVs was always exothermic (Figure 3A and 8A) while that for SUVs was nearly zero (Figure 3B and 8B). Thus, while equilibrium dialysis (Figure 7) indicated that similar amounts of calcium were bound to LUVs and SUVs, the calorimetry data suggested that the complexes were not energetically equivalent. This may result from differing degrees of actual calcium–phospholipid association versus electrostatic double layer association (McLaughlin, 1989) for LUVs versus SUVs. An attractive interpretation of these results is that protein binding (equilibrium 4, eq 1) to SUVs simply produced calcium–phospholipid contacts that were similar to those in LUVs.

It is doubtful that the thermodynamic differences were due to calcium–phospholipid binding *per se*. In general, purely ionic interactions are proposed to have little or no enthalpy component (Ross & Subramanian, 1981; Eftink & Biltonen, 1980). While chelation of Ca^{2+} by $EDTA^{4-}$ is quite exothermic (−6.5 kcal/mol; Martell & Smith, 1974) it is possible that this arises from structural rearrangement of the EDTA molecule in response to calcium binding rather than to calcium binding *per se*. Structural reorganization may also explain the large enthalpy associated with calcium binding to factor X and prothrombin in this study. In contrast, the interaction of Ca^{2+} with adenosine triphosphate (ATP^{4-}; Wilson & Chin, 1991) and Mg^{2+} with adenine monophosphate (AMP^{2-}; Belaich & Sari, 1969) have ΔH_{assoc} values of 2.1 and 1.8 kcal/mol, respectively. The relatively low affinity interaction of calcium with the phosphate moiety of PS (Dluhy et al., 1983) may suggest the latter type of binding. Also, the fact that calcium-independent peripheral proteins (Abbott & Nelsestuen, 1987; Ramsay et al., 1986) showed similar difference in enthalpy for SUVs versus LUVs may indicate that calcium–phospholipid contact was not the major source of the exothermic enthalpies observed in this study.

The most likely candidate for an exothermic protein–SUV enthalpy was a change in the packing of the phospholipids. Calorimetry studies for myelin basic protein association with vesicles in the gel versus fluid phase (Ramsay et al., 1986), binding of amphiphilic molecules to LUVs versus SUVs (Beschiaschvili & Seelig, 1992), and peptide-induced fusion of SUVs (Myers et al., 1987) all suggest that an increase in the internal tension of a phospholipid bilayer is accompanied by a corresponding release of heat. For the system studied here, the changes in phospholipid packing may be a result of a "pulling together" of the SUV phospholipid headgroups and a relaxation of the strained nature of phospholipids in SUVs. Such changes in phospholipid packing upon prothrombin fragment 1 binding have been suggested (Lentz et al., 1985). However, the latter changes were observed without calcium present and may not be related. Structural differences between LUVs and SUVs (Gennis, 1989) may allow calcium to alter phospholipid packing of LUVs but not SUVs. Changes in phospholipid packing upon interaction with calcium has been demonstrated by infrared spectroscopy (Dluhy et al., 1983), differential scanning calorimetry, and X-ray diffraction (Hauser & Shipley, 1984; Jacobson & Papahadjopoulos, 1975).

A calcium bridging ionic/chelation interaction for factor X and prothrombin binding to vesicles is consistent with these results, and most information indicates this type of interaction for factor X and prothrombin (for a review see Nelsestuen (1988)). Electrostatic potential calculations indicate that the N-terminal two-thirds of the Gla domain is highly electronegative within 2–3 Å of its surface, even after compensating for seven Ca^{2+} ions (Soriano-Garcia et al., 1992). These authors proposed that intercalation of additional bridging calciums could alleviate the unfavorable interaction with the negatively charged membrane surface. Direct calcium binding measurements also suggested additional calcium in the complex (Nelsestuen and Lim, 1977). Direct interaction of positively charged residues (such as arginine 10) with negatively charged PS may also occur. A similar model of nonspecific adsorption followed by association of acidic lipids with specific protein sites has been proposed by Cutsforth et al. (1989). The additional bridging calciums may correspond to calcium already "bound" to the phospholipid surface.

However, the possibility of hydrophobic protein–membrane interaction(s) cannot be eliminated (Madar et al., 1982; Lentz et al., 1985; Beschiaschvili & Seelig, 1992). The X-ray crystal structure of the Gla domain of calcium–prothrombin fragment 1 (Soriano-Garcia et al., 1992) shows an exposed hydrophobic area comprised of phenylalanine 5, leucine 6, and valine 9 that is conserved in factor X (Enfield et al., 1980). This region extends approximately 15 Å perpendicularly from the linear array of bound calciums (Soriano-Garcia et al., 1992) and could penetrate the bilayer interface. This hydrophobic region may produce the enthalpy changes and slight preference for SUVs similar to those cited above for the somatostatin analogue (Beschiaschvili and Seelig, 1992). Beschiaschvili and Seelig (1992) proposed that these properties arose from a relaxation of the packing constraints and an increase in the internal tension of the phospholipids as the membrane curvature decreased. Therefore, the specific geometry of protein–phospholipid complexes (via bridging calcium or direct ionic interactions) and/or the membrane insertion of a small hydrophobic region of the protein could be the primary molecular event(s) that generate the observed enthalpies.

An earlier study of prothrombin binding to SUVs indicated a $\Delta H_{van't\ Hoff(vH)}$ of approximately 0 kcal/mol (Resnick & Nelsestuen, 1980) which did not compare well with the ΔH_{cal} value of about −7 kcal/mol obtained here. However, the earlier study was conducted at saturating calcium (>10 mM) while this study utilized low calcium (Figure 1, <1.8 mM calcium). It was therefore possible that high calcium produced a calcium–SUV with thermodynamic behavior that was similar to that obtained with LUV at low calcium. Attempts to study ΔH_{cal} for prothrombin–membrane interaction at high calcium (Figure 8) could not be interpreted quantitatively due to uncertainties in background subtraction. Thus, the basis for the difference in the two studies could not be determined. Situations where $\Delta H_{vH} > \Delta H_{calorimetric(cal)}$ can arise due to intermolecular cooperation (Sturtevant, 1987), and these results may suggest that prothrombin interaction with membranes was more complex than the two-state model assumed by the van't Hoff measurement. However, further studies will be needed to clarify this matter.

Similar titration calorimetry experiments utilizing a second class of calcium-dependent phospholipid-binding proteins (annexins V and VI) are being performed (D. A. Plager and G. L. Nelsestuen, unpublished data). While a calcium bridging model may be applicable to both the vitamin K-dependent and the annexin proteins, the enthalpies appear to be widely different with values of approximately −14 kcal/mol and −30 to −40 kcal/mol for annexin V and VI, respectively, regardless of vesicle size.[2] Thus, thermodynamic behavior may be very different, despite modes of interaction that may appear to be similar.

Overall, this study has revealed basic thermodynamic differences in the manner of association of vitamin K-dependent proteins with LUVs versus SUVs. While the results suggest that this might reflect, entirely, a difference in the manner of calcium binding to these different forms of phospholipids, further work will be needed to determine the actual basis for this behavior.

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