Dissociation of peripheral protein–membrane complexes by high pressure

DOUGLAS A. PLAGER AND GARY L. NELSESTUEN

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

(RECEIVED August 27, 1991; REVISED MANUSCRIPT RECEIVED November 21, 1991)

Abstract

The ability of high pressure to dissociate several peripheral protein–membrane complexes was investigated. Three vitamin K-dependent proteins (factor X, protein Z, and prothrombin) dissociated from small unilamellar vesicles (SUVs, 30 nm diameter) composed of 25% phosphatidylserine (PS) and 75% phosphatidylcholine (PC) at comparable pressures (midpoints of 0.3–0.6 kbar). The pressure-induced dissociation curves for the factor X–SUV interaction followed the expected behavior for an interaction with an apparent dissociation equilibrium constant at atmospheric pressure, $K_{D(atm)}$, of 9×10^{-7} M and a change in volume of association, ΔV_a , of 88 mL/mol. Factor X also dissociated from large unilamellar vesicles (LUVs, 100 nm diameter, 25% PS:75% PC) with a midpoint of 0.5 kbar. A second group of calcium-dependent membrane-binding proteins included protein kinase C (PKC), a 64-kDa protein, and a 32-kDa protein. The 32-kDa protein dissociated from SUVs (midpoint of 0.8 kbar), whereas PKC and the 64-kDa protein did not dissociate to a significant degree. The differences in dissociability of these proteins appeared to be a result of the differences in their $K_{D(atm)}$'s (decreased dissociability with decreased $K_{D(atm)}$). This pattern was further demonstrated by the relatively high midpoint of dissociation (1.1–1.4 kbar) of serum amyloid P component (SAP; $K_{D(atm)}$ ca. 10^{-11}) and the limited dissociation of factor V_a light chain ($K_{D(atm)}$ ca. 10^{-11}). Changing the vesicle composition to phosphatidylethanolamine in place of PC gave higher affinity and decreased dissociation of the 32-kDa protein and SAP. Thus, the susceptibility of various peripheral membrane-binding proteins to high pressure dissociation was governed by both the $K_{D(atm)}$ and the ΔV_a of the particular protein–membrane interaction, with the ΔV_a 's of the dissociable proteins being relatively small (<150 mL/mol).

Keywords: annexin; calcium; factor V_a light chain; high pressure; membrane-binding; phospholipid; protein kinase C; serum amyloid P component; vesicle; vitamin K-dependent

The effect of pressure on associated macromolecules is described by the expression

$$K_{D(p)} = K_{D(atm)} \exp(p\Delta V/RT) \quad (1)$$

where $K_{D(p)}$ and $K_{D(atm)}$ represent the dissociation equilibrium constant at pressure p and atmospheric pressure, respectively, R is the gas constant, T is the temperature, and ΔV represents the volume change associated with complex formation (Deckmann et al., 1985; Weber, 1987; Ruan & Weber, 1988). The primary contributors to volume changes are void volumes, which result from the imperfect interfacing of two macromolecular surfaces; electrostriction, the tighter packing of water molecules

around an exposed charge (Heremans, 1982; Weber & Drickamer, 1983); exchange of more tightly packed polar (solvation) for nonpolar interactions; and lipid condensation in cases involving a membrane (Ceuterick et al., 1979; Deckmann et al., 1985; Wong, 1987). Aromatic ring interactions are associated with negative ΔV 's and are strengthened by increased pressure (Harrington & Kegeles, 1973; Heremans, 1982; Weber, 1987; Dannenberg et al., 1990). Changes in protein tertiary structure typically are not observed at pressures less than 2.5 kbar (Heremans, 1982; Weber & Drickamer, 1983). Thus, high pressure can be a useful tool for identifying the type of forces involved in macromolecular interactions.

The majority of previous studies of high pressure have involved the association/dissociation of oligomeric proteins (Payens & Heremans, 1969; Royer et al., 1986; Ruan & Weber, 1988; Silva & Weber, 1988). More re-

Reprint requests to: Gary L. Nelsestuen, Department of Biochemistry, 1479 Gortner Ave., St. Paul, Minnesota 55108.

cently, high pressure has been used for the disruption of protein-DNA (Royer et al., 1990) and protein-membrane interactions (Deckmann et al., 1985; Lester, 1989; Orr et al., 1990). Most studies on protein-membrane dissociation have examined intrinsic membrane proteins and often require addition of an agent, such as benzyl alcohol, to disorder the phospholipid bilayer.

The results described in this study involve several types of peripheral phospholipid-binding proteins. The vitamin K-dependent proteins are often thought to bind to negatively charged phospholipids via a calcium bridge between the phospholipid head group and sites on the protein (Nelsestuen, 1988). The vitamin K-dependent proteins contain 10–13 gamma-carboxyglutamic acids (Glas) within the amino terminal 35 residues, some portion of which are required for membrane binding (Schwalbe et al., 1989). Similarly, a free N-terminus is required for membrane binding (Schwalbe et al., 1989). However, no direct evidence for involvement of Gla residues in a ternary complex with calcium and acidic phospholipid has been shown. Protein kinase C (PKC) (Parker et al., 1986; Ohno et al., 1988), the 32-kDa, and the 64-kDa proteins (Bazzi & Nelsestuen, 1991a) are very different in that they are intracellular proteins and do not contain Gla. These proteins have the added property of binding calcium only when negatively charged phospholipid is present. The 32-kDa and the 64-kDa proteins may be members of the annexin protein family (Smith & Dedman, 1990). Once again, it was suggested that calcium might act as a bridge between the phospholipid head group and a site on the protein for PKC, the 32-kDa, and the 64-kDa protein (Bazzi & Nelsestuen, 1990, 1991a–c). Serum amyloid P component (SAP), a member of the pentraxin protein family, is a third type of calcium-dependent membrane-binding protein, which appears unrelated to either of the other groups (Schwalbe et al., 1990 and references therein). Finally, factor V_a light chain (FV_aLC) is a serum protein whose membrane-binding properties are dependent on negatively charged phospholipids and which can be dissociated from the membrane by high ionic strength (Pusey et al., 1982). Although hydrophobic protein-membrane contact has been proposed (Nesheim et al., 1980), it seems likely that the primary interaction involves positive charges of the FV_aLC protein interacting with negative charges of the membrane (Pusey et al., 1982; Abbott & Nelsestuen, 1987).

These membrane-binding proteins were tested to determine the applicability of this method and to detect properties that might suggest the type of interaction involved. The results showed that some of these interactions were subject to pressure dissociation. Unlike most protein-protein complexes, there was no hysteresis between compression and decompression experiments. Both ΔV_a and $K_{D(atm)}$ were important for determining pressure-induced dissociability. Low affinity complexes were dissociated by pressure, whereas high affinity protein-membrane asso-

ciations could not be dissociated. Variation of binding affinity by altering membrane composition converted a dissociable protein to a nondissociable protein.

Results

Typical light scattering and fluorescence energy transfer measurements are presented in Figures 1 and 2 to intro-

Fig. 1. Light scattering data. **A:** Results are shown for three cycles of compression/decompression. Each recorder tracing is separated by a zero reading. The intensities of protein Z (20 μ g) and SUVs (20 μ g of 25% PS:75% PC) in 700 μ L solution were first taken at 0.001 kbar. Buffer was added to a final volume of 1 mL, and the first cycle of compression and decompression was applied (–Ca data). Only a portion of the measurements is shown. The sample was equilibrated at each pressure for 5 min prior to taking the reading. Calcium chloride (2 mM final concentration) was added, and a second series of readings were taken (+Ca data). Finally, EDTA was added (3.6 mM final concentration) and another pressure cycle was applied (+EDTA data). **B:** A more complete summary of the results for compression (closed symbols) and decompression (open symbols) is shown. Data for –Ca ($\bullet$, $\circ$), +Ca ($\blacksquare$, $\square$), and +EDTA ($\blacktriangle$, $\triangle$) are shown. **C:** Results for Tris buffer (1 mL, $\bullet$, $\circ$) and buffer plus protein Z (20 μ g) and calcium ($\blacksquare$, $\square$) are shown.

Fig. 2. Fluorescence energy transfer data. **A:** Fluorescence intensities from a solution of the 32-kDa protein (7 μ g) and SUVs (13.4 μ g of 25% PS:65% PC:10% dansyl-PE) in a volume of 1.0 mL are shown ($-Ca$ data). The sample was equilibrated for 5 min at each pressure before the reading was taken. Calcium chloride was then added (to 1.5 mM), and the sample was subjected to another pressure cycle ($+Ca$ data). EDTA (4 mM final concentration) was then added, and a final pressure cycle was applied ($+EDTA$ data). **B:** Complete results, corrected for background, are shown for compression (closed symbols) and decompression (open symbols) experiments. Data for $-Ca$ ($\bullet$, $\circ$), $+Ca$ ($\blacksquare$, $\square$), $+EDTA$ ($\blacktriangle$, $\triangle$) are shown.

duce the experimental system and demonstrate the nature of the acquired data. This is followed by the results for a variety of peripheral protein-membrane interactions.

Representative light scattering and fluorescence energy transfer data

Typical results for a light scattering experiment are shown in Figure 1A. Measurements were taken during compression and decompression. Although several more intermediate pressure steps were taken, the data tracings are shown for pressures of 0.001, 0.3, 1.2, and 2.5 kbar only. Following the initial pressure cycle, calcium was added and the $+Ca$ data were obtained (Fig. 1A). Finally, excess EDTA was added and a final series of measurements were taken (Fig. 1A, $+EDTA$ data). Other control experiments consisted of signal intensities for buffer only, buffer containing protein only, and buffer containing

small unilamellar vesicles (SUVs) only. The accumulated light scattering data were plotted versus pressure (Fig. 1B, C). The data for SUVs without protein (not shown) were identical to the $-Ca$ data (Fig. 1B).

Although compression and decompression tracings for $+Ca$ data are slightly offset (Fig. 1B), this did not correspond to a typical hysteresis. That is, hysteresis suggests a change in the rate of return to a start point, without a change in the magnitude of the start point. Also, hysteresis should be observed by both light scattering and fluorescence energy transfer. The latter technique showed no evidence of a hysteresis (see below). Rather, the offset shown in Figure 1B was due to a small irreversible event that was observed by light scattering intensity. The increased signal arose during the pressure cycle and was independent of protein. It may have arisen from a low level of vesicle fusion. Rather than attempting to correct for this small irreversible change, the results were analyzed with both values of I_1 (see Equation 2, Materials and methods). Interpretations were limited to conclusions that were compatible with either method of data analysis.

Typical fluorescence energy transfer data are shown in Figure 2A. Once again, fluorescence intensities were collected in the absence ($-Ca$) and presence of calcium ($+Ca$) as well as after addition of excess EDTA ($+EDTA$). Results for Tris buffer alone, buffer with protein, and buffer with SUVs were also collected in separate experiments. Buffer and protein exhibited negligible signals. Increased fluorescence signal from SUVs (25% phosphatidylserine [PS]:65% phosphatidylcholine [PC]:10% dipalmitoyl-*N*-dansyl-L- α -phosphatidylethanolamine [dansyl-PE]) at higher pressure (Fig. 2B) might result from movement of the dansyl moiety to a more hydrophobic environment. The emission maximum was also blue-shifted at higher pressure (data not shown; Campbell & Dwek, 1984). Net fluorescence intensities are plotted in Figure 2B. Once again, data for Tris buffer containing SUVs are not given due to similarity to the $-Ca$ data. The results (Fig. 2B) suggested that high pressure (>1.8 kbar) caused complete dissociation of this protein-membrane complex.

Factor X interaction with SUVs and large unilamellar vesicles (LUVs)

Figure 3A shows that addition of factor X to SUVs (25% PS:75% PC) in the presence of calcium resulted in reversible protein-vesicle binding. The extent of membrane saturation was dependent on the concentration as well as the ratio of components in solution (Schwalbe et al., 1989). Samples with the same initial degree of vesicle saturation by factor X, but over a 25-fold range of SUV concentration, were chosen for subsequent high pressure experiments (circled points in Fig. 3A). The close overlap of the curves in Figure 3B suggested that the interaction of factor X with phospholipid conforms to a simple equilib-

Fig. 3. Binding of factor X to SUVs at atmospheric pressure. **A:** Factor X was added to 290 μg ($\blacksquare$), 58 μg ($\circ$), and 11.6 μg ($\blacktriangle$) of SUVs (25% PS:75% PC) in 1.5 mL of Tris buffer. The calcium concentration was 2 mM. M_2/M_1 ratios were calculated from light scattering intensities according to Equation 2 (Materials and methods). Circled points indicate the protein/phospholipid ratios used in subsequent high pressure experiments. Addition of excess EDTA after the final addition of factor X demonstrated complete reversibility of membrane binding. **B:** Plots of the free factor X concentration versus the corresponding M_2/M_1 ratio at each addition of factor X are shown. M_2/M_1 ratios and free factor X concentrations were calculated from the light scattering data employing 58 μg ($\circ$) and 11.6 μg ($\blacktriangle$) of SUVs as described in Figure 3A. Free factor X was calculated from total factor X minus bound factor X as described in the Materials and methods.

rium such as that presented in the Materials and methods. Free factor X for the highest phospholipid binding site (PL) concentration is not shown in Figure 3B. In this case, the portion of bound protein is so high that calculation of free protein from total minus bound protein is approximately equal to experimental error. Although saturation of the vesicles was not achieved, previous studies suggested that the results over this range of membrane-bound protein could be described reasonably well with a simple equilibrium constant with a maximum binding capacity of 1.2/1.0 (w/w, factor X/phospholipid) (Nelsen & Broderius, 1977). At higher protein packing density, additional binding parameters may be needed (Wei et al., 1982). The range of membrane-bound protein used in this study was selected to avoid the latter circumstance.

The M_2/M_1 ratios for compression phases with factor X are shown in Figure 4A. Each of the three experiments indicated that the factor X-phospholipid complex was dissociated by high pressure. In agreement with the results in Figures 1 and 2, there was no significant hysteresis between compression versus decompression experiments (data not shown). Data from the various concentrations

Fig. 4. Comparison to theoretical values at a constant ΔV_a . **A:** The compression curves of the +Ca/+EDTA data for samples of 11.6 μg SUVs (25% PS:75% PC) and 129 μg factor X ($\blacksquare$), 58 μg SUVs and 154 μg factor X ($\circ$), and 290 μg SUVs and 308 μg factor X ($\blacktriangle$) in a volume of 1.5 mL are shown. To improve visual comparison with theoretical data, each of the three curves was normalized to a constant M_2/M_1 ratio of 1.0 at 2.0 kbar. This required addition of a nominal value to the three titrations (-0.008 , $+0.029$, $+0.009$, respectively). **B:** Theoretical curves were generated using Equation 3 for the various conditions in A, a $K_{D(atm)}$ of 9×10^{-7} M, and a ΔV_a of 88 mL/mol. For each curve, the total factor X concentration, $C_{o(F.X)}$, was calculated from the known mass of protein, volume of the sample, and a molecular weight of 55 kDa for factor X. For each curve, the total phospholipid binding sites, $C_{o(PL)}$, was calculated from the known mass of phospholipid, volume of the sample, molecular weight of factor X, and the saturation value for factor X binding to SUVs (1.2 μg factor X bound per 1.0 μg of SUVs). For example, the curve generated for the 1.5-mL sample containing 58 μg SUVs and 154 μg factor X used $C_{o(PL)} = 8.44 \times 10^{-7}$ M and $C_{o(F.X)} = 1.87 \times 10^{-6}$ M.

were compared to curves generated using theoretical points calculated from Equation 3 (see Materials and methods) and the parameters given in the legend to Figure 4. Midpoint dissociation pressures of the experimental curves (Fig. 4A) were 0.48, 0.44, and 0.63 kbar for relative SUV concentrations of 1, 5, and 25, respectively. Midpoints of 0.43, 0.40, and 0.58 kbar were predicted by the respective theoretical curves (Fig. 4B). Although an exhaustive search for improved fit (by adjusting $K_{D(atm)}$ and ΔV_a) of experimental and theoretical data was not attempted, moderate adjustments in $K_{D(atm)}$ ($\pm$ one order of magnitude) and ΔV_a (increasing or decreasing by a factor of two), individually and in combination, away from the values indicated in the legend to Figure 4B caused divergence of experimental and theoretical data.

Thus, the general trend of curve shape and differences in midpoints supported the approximate parameters given.

The dissociation of factor X from LUVs was also tested. Reversible and saturable binding of factor X to LUVs at atmospheric pressure was demonstrated (data not shown) and was similar to the results for the factor X-SUV interaction (Fig. 3). The diameters of the LUVs used in these experiments were approximately 100 nm, and the particles should not necessarily conform to Rayleigh light scattering theory. However, approximate binding could be determined from simple changes in signal intensity without application of Equation 2. LUVs were valuable because they provided a surface curvature that differed greatly from SUVs and because the preparation procedure was very mild, with minimal degradation of the phospholipids. These differences had little effect on the pressure needed to dissociate factor X from the vesicles (midpoint of dissociation at 0.5 kbar, Table 1).

Bovine protein Z and prothrombin interactions with SUVs

The effect of pressure on membrane association of bovine protein Z was assayed by both light scattering and fluorescence energy transfer. Both techniques indicated that this vitamin K-dependent protein was susceptible to pressure-induced dissociation (Table 1). The midpoint dissociation pressure for the two pressure cycles varied slightly for fluorescence energy transfer experiments that were conducted at different protein Z concentrations (20 μ g versus 37.4 μ g). However, this difference appeared

to be due to experimental variation, as it was the opposite of expected; the higher protein concentration required lower pressure for dissociation (Table 1). Overall, dissociation of protein Z from the membrane was qualitatively similar to dissociation of factor X.

A third vitamin K-dependent membrane-binding protein, prothrombin, was also studied. As indicated in Table 1, prothrombin dissociated from SUVs (25% PS:75% PC) with a midpoint pressure of 0.3–0.4 kbar. Again, this midpoint dissociation pressure, as well as the dissociation curve shape (not shown), were comparable to the other vitamin K-dependent proteins.

Effect of pressure on other calcium-dependent membrane-binding proteins

Light scattering measurements at atmospheric pressure (data not shown) indicated that the 32-kDa protein bound to SUVs in a saturable, calcium-dependent manner as reported by Bazzi and Nelsestuen (1991a–c). Figure 5A shows the results of an experiment in which light scattering was used to monitor membrane binding of the 32-kDa protein to SUVs (25% PS:75% PC) under increased pressure. In separate experiments (Fig. 5B), fluorescence energy transfer was also employed with vesicles of similar composition. Both data sets suggested that complete dissociation of the 32-kDa protein occurred with a midpoint dissociation pressure of 0.7–0.8 kbar (Table 1). The effects of pressure on the association of the 32-kDa protein with SUVs composed of 25% PS:15% PC:50% phosphatidylethanolamine (PE):10% dansyl-PE is also

Table 1. Summary of high pressure effects

Protein	Vesicle (PS:PC:dansyl-PE:PE)	Protein/phospholipid (in μ M)	$K_{D(atm)}$ ^a (M)	$p_{0.5}$ ^b
Vitamin K-dependent				
Factor X	(25:75)	1.6–3.7/9.7–242	10^{-7}	0.4–0.7
	(25:75) ^c	1.9/15		0.5
Protein Z	(25:75)	0.40/25	10^{-7}	0.5–0.6
	(50:40:10)	0.40–0.75/25		0.5–0.9
Prothrombin	(25:75)	0.28/25	10^{-7}	0.3–0.4
PL-dependent calcium-binding				
32 kDa	(25:75 or 25:65:10)	0.25/25	$<10^{-14}$	0.7–0.8
	(25:15:10:50)	0.25/25	$<10^{-14}$	>2.5
64 kDa	(25:75 or 25:65:10)	0.15/25	$<10^{-14}$	>2.5
PKC	(25:65:10)	0.22/17	$<10^{-14}$	>2.5
SAP	(25:75 or 25:65:10)	0.13/25	10^{-11}	1.0–1.4
	(25:15:10:50)	0.13/25	$<10^{-11}$	>2.5
FV _a LC	(25:75 or 25:65:10)	0.07/9.3	10^{-11d}	>2.7

^a Data from: factor X and prothrombin, Nelsestuen and Broderius (1977); protein Z and SAP, Nelsestuen and Schwalbe (pers. comm.); 32 kDa, 64 kDa, and PKC, Bazzi and Nelsestuen (1991b,c); FV_aLC, Pusey et al. (1982). All interactions, except FV_aLC, were carried out in the presence of 0.8–2.0 mM calcium.

^b Pressure, in kbar, at half dissociation.

^c LUV preparation. All other data are for SUV.

^d The only protein-membrane interaction that was calcium independent.

Fig. 5. Dissociation of the 32-kDa protein from SUVs at elevated pressure. **A:** Binding of the 32-kDa protein (8.0 μ g) and SUVs (20 μ g of 25% PS:75% PC) at increased pressure is shown. M_2/M_1 ratios were calculated from light scattering intensities for compression (closed symbols) and decompression (open symbols) phases using I_2/I_1 values from +Ca and -Ca data ($\bullet$, $\circ$) and separately from +Ca and +EDTA data ($\blacksquare$, $\square$). **B:** Results for binding of the 32-kDa protein to SUVs of different composition are shown. Data for the 32-kDa protein (7.0 μ g) and SUVs (13.4 μ g of 25% PS:65% PC:10% dansyl-PE) during compression ($\bullet$) and decompression ($\circ$) phases are shown. Percent change in fluorescence was calculated from +Ca and -Ca data. Also shown in B are results for the 32-kDa protein (7.9 μ g) and SUVs containing PE (19.9 μ g of 25% PS:15% PC:50% PE:10% dansyl-PE). Percent change in fluorescence for compression ($\blacksquare$) and decompression ($\square$) phases was calculated from +Ca and +EDTA data. I is the fluorescence intensity for the protein-vesicle complex, and I_0 is the fluorescence intensity of the vesicle alone. In all cases, the calcium chloride concentration was 1.5 mM.

shown in Figure 5B. An important difference was that only a portion of the 32-kDa protein appeared to dissociate from vesicles containing PE.

A third type of calcium-dependent membrane-binding protein, SAP, was examined. Experiments utilizing SUVs composed of 25% PS:65% PC:10% dansyl-PE and monitored by fluorescence energy transfer suggested that SAP dissociated completely from the vesicles with a midpoint dissociation pressure of 1.1–1.4 kbar (Fig. 6A). Changing to a vesicle composition with 50% PE, instead of PC, resulted in little pressure-induced dissociation of SAP from the membrane (Fig. 6B). Recent studies suggest that, in general, proteins that interact with phospholipids show much greater affinity for membranes containing PE, rather than PC, as the neutral phospholipid (Bazzi et al., 1992).

Two other phospholipid-dependent calcium-binding proteins, similar to the 32-kDa protein, were also studied. Binding of the 64-kDa protein to SUVs (25% PS:75% PC) was shown to be saturable and reversible (Bazzi & Nelsestuen, 1991a–c). Both fluorescence energy

Fig. 6. Dissociation of SAP from SUVs at elevated pressure. **A:** Results for binding of SAP (29.7 μ g) to SUVs (20 μ g of 25% PS:65% PC:10% dansyl-PE) are shown for compression ($\blacksquare$) and decompression ($\square$) phases. **B:** A sample of SUVs containing PE (20 μ g of 25% PS:15% PC:50% PE:10% dansyl-PE) in the presence of SAP (29.7 μ g) was studied. Data for compression ($\blacksquare$) and decompression ($\square$) phases are shown. Data in both panels were calculated from +Ca and +EDTA data. The calcium chloride concentration was 2 mM.

transfer (Fig. 7A) and light scattering (not shown) indicated that only a small fraction (<20%) of this protein was dissociated by pressure. Comparable results were obtained for PKC (Table 1). Both the 64-kDa protein and PKC were completely dissociated from the membrane by EDTA addition after the pressure cycles. The results therefore represent calcium-dependent protein-membrane complexes.

FV_aLC interaction with SUVs

Unlike the other proteins used in this study, FV_aLC interaction with membrane does not require calcium. Binding of FV_aLC to SUVs (25% PS:75% PC) at atmospheric pressure was saturable and reversed by NaCl, in a manner similar to that reported earlier (Abbott & Nelsestuen, 1987). The FV_aLC complex with SUVs (25% PS:75% PC) was not dissociated by pressures of up to 2.7 kbar (Fig. 7B). Similar results were seen for fluorescence energy transfer experiments (Table 1).

Discussion

High pressure often causes dissociation of macromolecular assemblies. For example, Deckmann and coworkers (1985) used high pressure for the selective “shedding” of erythrocyte integral membrane proteins. Lester (1989) applied a similar technique to extract membrane-associated

Fig. 7. Binding of the 64-kDa protein or FVaLC to SUVs at elevated pressure. **A:** Data for the 64-kDa protein (9.5 μ g) and SUVs (20 μ g of 25% PS:65% PC:10% dansyl-PE) are shown. A concentration of 1.5 mM calcium chloride was used. Change in fluorescence is shown for compression (closed symbols) and decompression (open symbols) phases using data from +Ca and -Ca titrations (●, ○) and separately from +Ca and +EDTA titrations (■, □). **B:** The M_2/M_1 ratios for a 1-mL sample containing 7.4 μ g SUVs (25% PS:75% PC) and 5.6 μ g FVaLC are shown for compression (▲) and decompression (△).

PKC from rat brain membranes. PKC forms two distinct membrane-bound states, a reversible calcium-dependent complex and an irreversible integral membrane protein (Bazzi & Nelsestuen, 1988). The studies of Lester appear to involve the latter form, where dissociation required benzyl alcohol and EDTA. This study applied high pressure to an array of peripheral membrane-binding proteins in an attempt to determine the applicability of the approach and whether the types of forces involved in protein-membrane binding could be elucidated by this technique. The studies on PKC dealt exclusively with the reversible membrane-bound form.

All of the vitamin K-dependent proteins were susceptible to pressure-induced dissociation from membranes. The results were consistent with pressure-induced dissociation as the result of alterations in K_D according to Equation 1. The relatively high $K_{D(atm)}$ (10^6 – 10^7 M, Nelsestuen & Broderius, 1977) for the factor X-membrane complex was consistent with a relatively low midpoint dissociation pressure. Experimental data over a 25-fold range of SUV concentration were also consistent with Equation 1 (see Fig. 4). Fitting of experimental data to Equation 1 indicated that the volume change upon factor X association with the membrane surface was small, in the range of about 90 mL/mol. This can be compared to the ΔV_a for dimerization of tryptophan synthase β_2 (162 mL/mol [Silva et al., 1986]) or yeast enolase (60

mL/mol [Paladini & Weber, 1981b]). Previous studies indicate that electrostriction decreases the volume of a system (Heremans, 1982; Weber & Drickamer, 1983). This would suggest that a binding process involving a calcium bridge between two anionic components, as proposed for vitamin K-dependent proteins with membranes, would be reversed as pressure is increased because of the negative change in volume due to electrostriction. Although dissociation of the vitamin K-dependent proteins by pressure was consistent with this popular model, this conclusion is not unique, as other types of interactions can also produce significant volume changes.

Dissociation due to factors other than ΔV of contact, such as destruction of binding sites by high pressure (Ceuterick et al., 1979; Deckmann et al., 1985; Wong, 1987), could not be entirely ruled out, but seemed less consistent with the results. For example, dissociation due to destruction of binding sites should show no shift in the dissociation pressure due to concentration. Although small, the shifts due to concentration (Fig. 4) were reproducible and correlated with Equation 1. In addition, membrane surface curvature (SUVs versus LUVs) had little impact on dissociation pressure despite large differences in the interhead group distances for these two types of lipids. This result suggested that pressure-induced alterations in membrane structure were less relevant to dissociation than were factor X-membrane interfacial volume changes.

Important properties of these interactions, that were independent of the basis for pressure-induced dissociation, included reversibility and equilibrium characteristics. That is, dissociation and reassociation showed no indication of the hysteresis that is displayed by many protein-protein interactions (Ruan & Weber, 1988, 1989). Thus, dissociation from membranes appeared to be an equilibrium process. This might be possible due to the more dynamic nature of a membrane versus protein structure. Membranes may be able to compensate for slight changes in protein structure or conformational drift (King & Weber, 1986; Silva et al., 1986; Ruan & Weber, 1989) upon dissociation of the protein.

This study showed that $K_{D(atm)}$ was a critical factor for determining dissociability by pressure. For example, the 32-kDa protein was dissociable but required higher pressures than the vitamin K-dependent proteins. It also bound to the PS/PC membranes with higher affinity. Replacement of PC with PE, which enhances the affinity of this protein for membranes (Bazzi et al., 1992), increased the dissociation pressure to >2.5 kbar. Similar behavior, pressure dissociation for lower affinity interaction but not for higher affinity interaction, was also exhibited by SAP. Finally, PKC and the 64-kDa protein, which both display higher membrane-binding affinity than the 32-kDa protein (Bazzi & Nelsestuen, 1991b), were not dissociated by high pressure. Thus, the pressure-induced dissociability of a given protein-membrane interaction appeared to follow an inverse relationship with the affinity of the inter-

action. This agreed with the theoretical expectations (Equation 1).

This study indicated that the maximum utility of high pressure for investigation of protein-membrane binding will require lower affinity interactions or high affinity interactions with large values of ΔV_a . As a minimum, $K_{D(atm)}$ and pressure dissociation can be used to estimate upper limits for ΔV . For example, a dissociation pressure for FV_aLC of >2.7 kbar and a dissociation constant of 10^{-11} M require a ΔV_a for interaction of <100 mL/mol. Qualitatively, these results suggest that volume changes associated with most of these protein-membrane interactions are relatively small and on the order of that for protein-protein interactions. More detailed investigations of some of these protein-membrane interactions will require use of situations that provide reduced affinity. This can be achieved by altering the membrane composition, especially by using PC as the neutral phospholipid and reducing the membrane content of PS. Determination of the types of forces involved in these protein-membrane interactions by pressure dissociation may then be possible by use of high pressure in conjunction with variations of additional parameters such as temperature, pH, and ionic strength.

Materials and methods

Proteins

Bovine factor X, prothrombin, protein Z (Nelsestuen, 1984), bovine PKC (Bazzi & Nelsestuen, 1987), and the 64-kDa and 32-kDa proteins (Bazzi & Nelsestuen, 1991a) were purified as described. SAP component was purchased from Sigma Chemical Co. (St. Louis, Missouri). FV_aLC was prepared as described by Lindhout et al. (1982) and Pusey et al. (1982).

Phospholipid vesicles

Highly pure phospholipids ($\geq 98\%$ purity, suppliers estimates) were used to prepare all vesicles. PS (bovine brain), PC (egg yolk), and dansyl-PE were all purchased from Sigma. SUVs, approximately 30 nm in diameter, were prepared by sonication and gel filtration (Huang, 1969; Nelsestuen & Lim, 1977). Briefly, 20 mg of phospholipid were dried with a stream of nitrogen and then resuspended in 3 mL Tris buffer (0.05 M Tris, 0.1 M NaCl, pH 7.5). This suspension was sonicated by direct probe using the microtip assembly of the model W-385 Ultrasonic Processor, Heat Systems—Ultrasonics, Inc. (Farmingdale, New York). Two-second pulses with 3-s delays were employed for a total of 5 min of sonication. The solution was cooled in an ice-water bath during sonication. The sonicated solution was then centrifuged and gel filtered on a Sepharose 4B column (1.5 $\times$ 25 cm).

Fractions were monitored for turbidity at 256 nm, and fractions containing SUVs were pooled.

LUVs of 100 nm diameter were prepared by the extrusion technique (Hope et al., 1985). Twenty milligrams of phospholipid were dried with a stream of nitrogen, vigorously resuspended in 4 mL Tris buffer, frozen and thawed (using dry ice and a warm water bath) five times, then forced through a 0.4 μ m polycarbonate filter (Nucleopore Corp., Pleasanton, California) three times, followed by six passes through a 0.1- μ m filter under 40 psi of nitrogen using an Amicon ultrafiltration cell. Final phospholipid concentrations of SUV and LUV preparations were determined by assay of organic phosphate (Chen et al., 1956), assuming a phosphorus:phospholipid ratio of 1:25.

Light scattering

The HPSC-3K high pressure spectroscopy cell (essentially as described by Paladini & Weber, 1981a) and system (SLM Instruments, Inc., Urbana, Illinois) were used in conjunction with the SLM model 4800C spectrofluorometer (SLM Instruments) to acquire 90° Rayleigh light scattering and fluorescence energy transfer data at pressures of up to 2.7 kbar. A Perkin-Elmer-Hitachi model MPF-44A fluorescence spectrophotometer was also used for light scattering experiments at atmospheric pressure.

The usefulness of 90° Rayleigh light scattering measurements as a means to detect protein-phospholipid vesicle interactions is described in detail by Nelsestuen and Lim (1977). Briefly, for a constant light source and instrumental geometry, a solution of particles that are much smaller than the incident wavelength of light produces a ratio of light scattering intensity due to protein-vesicle complexes (I_2) to that of vesicles alone (I_1), which follows the relationship

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

where 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 solution 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 for free protein was usually negligible.

The general procedure for high pressure data acquisition involved loading of sample into a 1-mL cylindrical, bottle-shaped cuvette, which was then capped with a polyethylene, collapsible cap that had either been filled with sample or with Tris buffer. The cuvette was placed in the high pressure spectroscopy cell and measurements at various pressures (0.001–2.7 kbar) were taken. The

sample was maintained at each pressure step for 5 min prior to taking the measurement. Measurements were taken during both compression and decompression phases. The term "pressure cycle" refers to the procedure of applying one cycle of compression and decompression. The sample was maintained at a temperature of 23.7 ± 0.4 °C. Excitation and emission wavelengths were both set at 320 nm, excitation slits were 4 nm, and emission slits were 8 nm.

For proteins requiring calcium for membrane binding, a sample containing protein and vesicles in the absence of calcium is referred to as the $-Ca$ sample. After membrane binding (i.e., $CaCl_2$ added) and a second cycle of pressure (referred to as $+Ca$ data), excess EDTA (2–3 times the $CaCl_2$) was added and another cycle of pressure (referred to as EDTA data) was run. The slight differences in the $-Ca$ and $+EDTA$ data gave two sets of I_1 values, which, when used with the $+Ca$ data (I_2 values), represented the maximum probable range of experimental variation of M_2/M_1 .

For protein that did not require calcium for membrane binding, separate samples containing vesicles in the presence and absence of protein were each subjected to high pressure, and a single set of M_2/M_1 ratios was obtained.

Fluorescence energy transfer

Nonradiative fluorescence energy transfer is proportional to the inverse sixth power of the distance separating the given fluorophore donor and acceptor. Protein-membrane binding decreases the distance between the donor (in this case, protein tryptophan, excitation at 284 nm) and acceptor (dansyl-PE, emission at 520 nm) (Fairclough & Cantor, 1977; Stryer, 1978). A 500-nm cut-off filter (LL-500 filter, Corion Corp., Holliston, Massachusetts) was placed in front of the emission monochromator. All other instrument settings and procedures remained the same as in the light scattering experiments. Fluorescence energy transfer data are presented as a percent change in emission intensity, $[(I - I_0)/I_0] \times 100$, where I is the signal for the protein-vesicle complexes and I_0 is the signal resulting from direct excitation of the dansyl group in the absence of protein. Background signals for buffer and unbound protein were negligible. All experiments were carried out in Tris buffer (0.05 M Tris, 0.1 M NaCl, pH 7.5). Tris provides a low pressure dependence of ionization (Neuman et al., 1973).

Theoretical curves

For a simplified dissociation of a factor X-phospholipid complex at a constant calcium concentration of 2 mM (Nelsestuen & Broderius, 1977; Nelsestuen & Lim, 1977), an expression for the apparent dissociation equilibrium constant between factor X and a corresponding phospholipid binding site (PL) at pressure p would be

$$K_{D(p)} = \frac{[\text{factor X}][\text{PL}]}{[\text{factor X} * \text{PL}]}$$

With $C_{o(F,X)}$ = total factor X concentration, $C_{o(PL)}$ = total concentration of protein binding sites on the phospholipid, and y = fraction of total protein bound, then

$$K_{D(p)} = \frac{[C_{o(F,X)} - (y)C_{o(F,X)}][C_{o(PL)} - (y)C_{o(F,X)}]}{[(y)C_{o(F,X)}]}$$

Simplifying this expression and substituting into Equation 1, gives the following quadratic (Equation 3),

$$(C_{o(F,X)})(y^2) - [(K_{D(atm)}[\exp(100p\Delta V/RT)] + C_{o(F,X)} + C_{o(PL)})(y) + C_{o(PL)}] = 0.$$

Thus for a given $K_{D(atm)}$ (in molarity) and ΔV (in mL/mol), and for known protein concentrations ($C_{o(F,X)}$) and known concentrations of protein binding sites ($C_{o(PL)}$) (in molarity), a curve of the fraction of total protein bound (y) versus the applied pressure p (in kbar) was generated. The molar concentrations of protein binding sites were calculated from the protein to phospholipid (w/w) ratio at binding saturation and the molecular weight of the protein.

Note added in proof

Recent findings show that the 32-kDa protein corresponds to annexin V and the 64-kDa protein to annexin VI (Bazzi et al., 1992).

Acknowledgments

We thank Ruth A. Schwalbe (bovine factor X and protein Z), Katherine M. Walstrom (prothrombin), Mohammad D. Bazzi (PKC, 64-kDa protein, and 32-kDa protein), and Alan Abbott (FV_aLC) for providing the proteins used in these studies. This work was supported by grant HL15728 and GM38819 from the National Institutes of Health. D.A.P. is a predoctoral trainee supported by the National Institutes of Health Molecular Biophysics Training Grant GM08277-04.

References

- Abbott, A.J. & Nelsestuen, G.L. (1987). Association of a protein with membrane vesicles at the collisional limit: Studies with blood coagulation factor V_a light chain also suggest major differences between small and large unilamellar vesicles. *Biochemistry* 26, 7994–8003.
- Bazzi, M.D. & Nelsestuen, G.L. (1987). Association of protein kinase C with phospholipid vesicles. *Biochemistry* 26, 115–122.
- Bazzi, M.D. & Nelsestuen, G.L. (1988). Properties of membrane-inserted protein kinase C. *Biochemistry* 27, 7589–7593.
- Bazzi, M.D. & Nelsestuen, G.L. (1990). Protein kinase C interaction with calcium: A phospholipid-dependent process. *Biochemistry* 29, 7624–7630.
- Bazzi, M.D. & Nelsestuen, G.L. (1991a). Proteins that bind calcium in a phospholipid-dependent manner. *Biochemistry* 30, 971–979.

- Bazzi, M.D. & Nelsestuen, G.L. (1991b). Extensive segregation of acidic phospholipids in membranes induced by protein kinase C and related proteins. *Biochemistry* 30, 7961-7969.
- Bazzi, M.D. & Nelsestuen, G.L. (1991c). Highly sequential binding of protein kinase C and related proteins to membranes. *Biochemistry* 30, 7970-7977.
- Bazzi, M.D., Youakim, M.A., & Nelsestuen, G.L. (1992). Importance of phosphatidylethanolamine for association of protein kinase C and other cytosolic proteins with membranes. *Biochemistry* 31, 1125-1134.
- Campbell, I.D. & Dwek, R.A. (1984). Fluorescence. In *Biological Spectroscopy* (Elias, P., Ed.), pp. 91-125. Benjamin/Cummings Publishing Company, Inc., Menlo Park, California.
- Ceuterick, F., Heremans, K., De Smedt, H., Nieuwenhuysen, P., & Clauwaert, J. (1979). Dynamic light scattering measurements on the phase transitions of phospholipid vesicles. *Chem. Phys. Lett.* 62, 341-343.
- Chen, P.S., Toribara, T.Y., & Warner, H. (1956). Microdetermination of phosphorus. *Anal. Chem.* 28, 1756-1758.
- Dannenberg, A.J., Kavcansky, J., Scarlata, S., & Zakim, D. (1990). Organization of microsomal UDP-glucuronosyltransferase. Activation by treatment at high pressure. *Biochemistry* 29, 5961-5967.
- Deckmann, M., Haimovitz, R., & Shinitzky, M. (1985). Selective release of integral proteins from human erythrocyte membranes by hydrostatic pressure. *Biochim. Biophys. Acta* 821, 334-340.
- Fairclough, R.H. & Cantor, C.R. (1977). The use of singlet-singlet energy transfer to study macromolecular assemblies. *Methods Enzymol.* 48, 347-379.
- Harrington, W.F. & Kegeles, G. (1973). Pressure effects in ultracentrifugation of interacting systems. *Methods Enzymol.* 27, 306-345.
- Heremans, K. (1982). High pressure effects on proteins and other biomolecules. *Annu. Rev. Biophys. Bioeng.* 11, 1-21.
- Hope, M.J., Bally, M.B., Webb, G., & Cullis, P.R. (1985). Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* 812, 55-65.
- Huang, C. (1969). Studies on phosphatidylcholine vesicles. Formation and physical characteristics. *Biochemistry* 8, 344-352.
- King, L. & Weber, G. (1986). Conformational drift of dissociated lactate dehydrogenases. *Biochemistry* 25, 3632-3637.
- Lester, D.S. (1989). High-pressure extraction of membrane-associated protein kinase C from rat brain. *J. Neurochem.* 52, 1950-1953.
- Lindhout, T., Grovers-Riemsag, J.W.P., van de Waart, P., Hemker, H.C., & Rosing, J. (1982). Factor V_a-factor X_a interaction. Effects of phospholipid vesicles of varying composition. *Biochemistry* 21, 5494-5502.
- Nelsestuen, G.L. (1984). Vitamin K-dependent plasma protein. *Methods Enzymol.* 107, 507-516.
- Nelsestuen, G.L. (1988). Basis for prothrombin-membrane binding. In *Current Advances in Vitamin K Research* (Suttie, J.W., Ed.), pp. 335-339. Elsevier Scientific Publishing Co., Inc., New York.
- Nelsestuen, G.L. & Broderius, M. (1977). Interaction of prothrombin and blood-clotting factor X with membranes of varying composition. *Biochemistry* 16, 4172-4177.
- Nelsestuen, G.L. & Lim, T.K. (1977). Equilibria involved in prothrombin- and blood-clotting factor X-membrane binding. *Biochemistry* 16, 4164-4171.
- Nesheim, M.E., Hibbard, L.S., Tracy, P.B., Bloom, J.W., Myrmel, K.H., & Mann, K.G. (1980). Participation of factor V_a in prothrombinase. In *The Regulation of Coagulation* (Mann, K.G. & Taylor, F.B., Jr., Eds.), pp. 145-149. Elsevier, New York.
- Neuman, R.C., Jr., Kauzmann, W., & Zipp, A. (1973). Pressure dependence of weak acid ionization in aqueous buffers. *J. Phys. Chem.* 77, 2687-2691.
- Ohno, S., Akita, Y., Konno, Y., Imajoh, S., & Suzuki, K. (1988). A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. *Cell* 53, 731-741.
- Orr, N., Yavin, E., Shinitzky, M., & Lester, D.S. (1990). Application of high-pressure to subfractionate membrane protein-lipid complexes: A case study of protein kinase C. *Anal. Biochem.* 191, 80-85.
- Paladini, A.A. & Weber, G. (1981a). Absolute measurements of fluorescence polarization at high pressures. *Rev. Sci. Instrum.* 52, 419-427.
- Paladini, A.A. & Weber, G. (1981b). Pressure-induced reversible dissociation of Enolase. *Biochemistry* 20, 2587-2593.
- Parker, P.J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stable, S., Waterfield, M.D., & Ullrich, A. (1986). The complete primary structure of protein kinase C—The major phorbol ester receptor. *Science* 233, 853-859.
- Payens, T.A.J. & Heremans, K. (1969). Effect of pressure on the temperature-dependent association of β -casein. *Biopolymers* 8, 335-345.
- Pusey, M.L., Mayer, L.D., Wei, G.J., Bloomfield, V.A., & Nelsestuen, G.L. (1982). Kinetic and hydrodynamic analysis of blood clotting factor V-membrane binding. *Biochemistry* 21, 5262-5268.
- Royer, C.A., Chakerian, A.E., & Matthews, K.S. (1990). Macromolecular binding equilibria in the lac repressor system: Studies using high-pressure fluorescence spectroscopy. *Biochemistry* 29, 4959-4966.
- Royer, C.A., Weber, G., Daly, T.J., & Matthews, K.S. (1986). Dissociation of the lactose repressor protein tetramer using high hydrostatic pressure. *Biochemistry* 25, 8308-8315.
- Ruan, K. & Weber, G. (1988). Dissociation of yeast hexokinase by hydrostatic pressure. *Biochemistry* 27, 3295-3301.
- Ruan, K. & Weber, G. (1989). Hysteresis and conformational drift of pressure-dissociated glyceraldehyde phosphate dehydrogenases. *Biochemistry* 28, 2144-2153.
- Schwalbe, R.A., Dahlbäck, B., & Nelsestuen, G.L. (1990). Independent association of serum amyloid P component, protein S, and complement C4b with complement C4b-binding protein and subsequent association of the complex with membranes. *J. Biol. Chem.* 265, 21749-21757.
- Schwalbe, R.A., Ryan, J., Stern, D.M., Kisiel, W., Dahlbäck, B., & Nelsestuen, G.L. (1989). Protein structural requirements and properties of membrane binding by gamma-carboxyglutamic acid-containing plasma proteins and peptides. *J. Biol. Chem.* 264, 20288-20296.
- Silva, J.L., Miles, E.W., & Weber, G. (1986). Pressure dissociation and conformational drift of the β -dimer of tryptophan synthase. *Biochemistry* 25, 5780-5786.
- Silva, J.L. & Weber, G. (1988). Pressure-induced dissociation of brome mosaic virus. *J. Mol. Biol.* 199, 149-159.
- Smith, V.L. & Dedman, J.R. (1990). The role of intracellular calcium-binding proteins in stimulus-response coupling. In *Stimulus Response Coupling: The Role of Intracellular Calcium-Binding Proteins* (Smith, V.L. & Dedman, J.R., Eds.), pp. 1-20. CRC Press, Inc., Boca Raton, Florida.
- Stryer, L. (1978). Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* 47, 819-846.
- Weber, G. (1987). Dissociation of oligomeric proteins by hydrostatic pressure. In *High Pressure Chemistry and Biochemistry* (van Eldik, R. & Jonas, J., Eds.), pp. 401-420. D. Reidel Publishing Company, Norwell, Massachusetts.
- Weber, G. & Drickamer, H.G. (1983). The effect of high pressure upon proteins and other biomolecules. *Q. Rev. Biophys.* 16, 89-112.
- Wei, G.J., Bloomfield, V.A., Resnick, R.M., & Nelsestuen, G.L. (1982). Kinetic and mechanistic analysis of prothrombin-membrane binding by stopped-flow light scattering. *Biochemistry* 21, 1949-1959.
- Wong, P.T.T. (1987). High-pressure studies of biomembranes by vibrational spectroscopy. In *High Pressure Chemistry and Biochemistry* (van Eldik, R. & Jonas, J., Eds.), pp. 381-400. D. Reidel Publishing Company, Norwell, Massachusetts.