Relationship between Annexin V Tryptophan Exposure, Calcium, and Phospholipid Binding†

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ABSTRACT: Annexin V is a Ca^{2+}-dependent phopholipid-binding protein that may have one or more membrane-related functions, including inhibition of blood coagulation. The fluorescence of the single tryptophan of annexin V was used to monitor Ca^{2+} and/or phospholipid binding in terms of emission wavelength, emission intensity, and susceptibility to acrylamide quenching. In the absence of phospholipid, Ca^{2+} titration showed a strong red shift of the wavelength of maximal emission to approximately 345 nm, where a small increase in intensity occurred and was half maximal at approximately 3 mM Ca^{2+}. The Stern–Volmer quenching constant due to acrylamide was only 5.2 M^{-1} for annexin V alone, indicating limited aqueous exposure of the tryptophan, but 36 M^{-1} for a Ca^{2+}-bound form, indicating full exposure. Binding to both negatively charged and zwitterionic phospholipids was accompanied by a very large increase in fluorescence emission intensity, a red shift, and low exposure to acrylamide. Calculated concentrations of Ca^{2+} near the surface of negatively charged vesicles suggested that the exposure of tryptophan by Ca^{2+} binding to annexin V was sufficient for binding of the protein to all vesicles tested, including those composed of oleic acid and phosphatidylcholine (PC), but not to those composed of pure PC. When binding to PC was monitored, the phenomena associated with phospholipid binding were observed separately, at higher Ca^{2+} concentration, from the red shift and the high exposure to acrylamide due to Ca^{2+} binding alone. There was no observable fluorescence effect of oleic acid alone or a soluble dipropionoyl phosphatidylcholine, but a strict bilayer organization was not necessary for binding-induced fluorescence changes, as they were observed with phosphatidylserine (PS) dispersed in micelles of $C_{12}E_8$, where an approximate requirement of between 6 and 24 phospholipids per micelle was observed. Apparent Ca^{2+}-independent membrane binding of annexin V in the presence of spermine occurred without the characteristic changes in tryptophan fluorescence, demonstrating that these changes are specific for a Ca^{2+}-containing complex of annexin V with phospholipids. Taken together, these data suggest that annexin V–phospholipid binding involves a conformational change that exposes tryptophan 187 to aqueous solvent and allows specific binding to a nonheadgroup part of PS or PC, but that binding to PC alone has an apparently higher intrinsic Ca^{2+} dependence and may involve an intrinsically different mechanism.

Annexin V is a member of the annexin family of Ca^{2+}-dependent phospholipid binding proteins. Most putative functions of the annexins are related to their ability to bind to phospholipids [see Meers (1990)]. Phospholipid-dependent inhibition of blood coagulation has been suggested as a possible role for annexin V on the exterior of the cell (Reutelingsperger et al., 1985; Funakoshi et al., 1987). Roles for annexin V in the cell cytoplasm include a possible interaction with and/or regulation of protein kinase C, another Ca^{2+}-dependent membrane-binding protein (Schlaepfer et al., 1992).

Characterization of Ca^{2+} binding suggests that approximately five Ca^{2+} ions are bound per annexin V monomer and that apparent Ca^{2+} affinity is greatly increased in the presence of acidic phospholipids (Schlaepfer et al., 1987). Annexin V binding to phospholipids appears to be completely and rapidly reversible by Ca^{2+} chelators (Maurer-Fogy et al., 1988; Andree et al., 1990; Meers, 1990; Meers et al., 1991a). At a given Ca^{2+} concentration, there is an apparent preferential affinity for negatively charged phospholipids, where Ca^{2+} dependence rises with increasing mole fractions of zwitterionic phospholipids (Andree et al., 1990).

The sequences of most annexins comprise a unique N-terminal region followed by four 60–70 aminio acid repeated sequences with short linking sequences [see Pepinsky et al. (1988)]. Sequence analysis showed that the Ca^{2+} binding must be novel and not related to the common EF-hand sites (Kretsinger & Creutz, 1986). Removal of a short part of the N-terminus from some of the annexins has shown that Ca^{2+} and phospholipid binding resides in the repeated regions [see discussion in Meers (1990)]. A highly conserved consensus sequence in each repeat has been suggested to be the Ca^{2+} and/or phospholipid binding site (Geisow et al., 1986). The first direct demonstration of contact between a consensus sequence and membrane phospholipids was for tryptophan 187 of annexin V (Meers, 1990; tryptophan 187 incorrectly referred to as 186), located in the third consensus sequence. Subsequent, structural characterization of annexin V, crystallized in the absence of phospholipids (Huber et al., 1990, 1992), showed a four-domain structure with a central open region (or pore). Viewed from the side of the "pore", annexin V has concave and convex surfaces, the latter being the binding site of several incompletely ligand-bound Ca^{2+} ions as well as the area in which tryptophan 187 is located. The apparent Ca^{2+} binding sites are composed of consensus sequences, although no Ca^{2+} ion was observed near tryptophan 187 (Huber et al., 1992). Though no bound phospholipids were observed, a structural similarity of Ca^{2+} binding sites to those of phospholipase A_2 was observed. The location of tryptophan 187 near the membrane surface (Meers, 1990) suggests that the convex surface of the protein makes contact with the

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membrane. This would orient bound Ca^{2+} ions, as found in the crystal structure, such that they could be shared with phospholipid ligands [Figure 7, Meers et al. (1991b)]. High electrotatic potential is also concentrated on the convex side of the protein, in position to interact with negatively charged phospholipid molecules (Huber et al., 1990; Karshikov et al., 1992).

Despite this information, a number of aspects of annexin V binding remain unclear. For instance, the disposition of tryptophan 187 is in question. This residue is buried in the interior of the protein in the Ca^{2+}-bound crystal form and assumed to remain so when bound to phospholipids (Huber et al., 1990, 1992), while other data indicate direct interaction between this tryptophan and phospholipids when annexin V is bound to a membrane (Meers, 1990). Other unresolved points of interest include the structure, structural specificity, and stoichiometry of annexin V phospholipid binding sites and the intrinsic Ca^{2+} dependencies of annexin V binding to various phospholipids.

We have further studied the behavior and dispostion of the single tryptophan in this protein to elucidate the separate effects of Ca^{2+} and phospholipid binding and the aqueous exposure of the residue. The importance of the phospholipid structure, the surface charge, the Ca^{2+} concentration near the membrane surface, and the bilayer organization to the observed conformational changes were also studied.

MATERIALS AND METHODS

3-Palmitoyl-2-[1-pyrenedecanoyl]-L-α-phosphatidylcholine (pyrene-PC)[1] was obtained from Molecular Probes (Eugene, OR). Bovine brain phosphatidylserine (PS) and egg phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Birmingham, AL). EDTA (99.5%) was from Sigma (St. Louis, MO). Oleic acid was from NuChek Prep (Elysian, MN). $C_{12}E_8$ was from Fluka (Ronkonkona, NY). $CaCl_2$ (>99%), KCl (>99%), NaCl (>99%), and TES (98%) were from Fisher (Pittsburgh, PA). Polycarbonate filters were from Nuclepore (Pleasanton, CA).

Vesicle Preparation. All vesicles were prepared by freeze–thawing the aqueous phospholipid dispersion 10 times followed by extrusion 10 times through 0.1-μm-pore polycarbonate membranes (Mayer et al., 1986).

Phospholipid concentrations were determined using a phosphate assay as described in Kingsley and Feigenson (1979) modified from Chen et al. (1956), Bartlett (1959), and Morrison (1964).

Protein Preparation. Recombinant human annexin V (PAP-I, endonexin II) was kindly provided by Dr. R. Blake Pepinsky of Biogen Corporation (Cambridge, MA). This protein was at least 98% pure by single-dimension gel electrophoresis.

Fluorescence Measurements. Fluorescence measurements were made using an SLM 8000C fluorometer (Urbana, IL). Tryptophan excitation was at 280 or 295 nm with emission spectra monitored from 300 to 400 nm. For the pyrene-PC probe, excitation was at 344 nm. Emission was measured at 377 nm for time courses. These measurements were made as described (Meers et al., 1991a).

All experimental samples were maintained at 25 °C in 80 mM NaCl, 50 mM TES, and 0.1 mM EDTA at pH 8.0 (buffer A) or 100 mM NaCl, 10 mM TES, and 0.1 mM EDTA at pH 7.4 (buffer B). Additions of materials to initiate annexin binding, such as Ca^{2+} or acrylamide, were made with small aliquots of a concentrated stock to a stirred cuvette. Corrections were made for volume dilution. No corrections were made for changes in osmolarity, as little change in 90° light scattering indicated little change in vesicle structure. The emission spectrum from 300 to 400 nm was measured in all experiments. Most measurements were made with excitation at both 280 and 295 nm. The 280-nm data (not shown) generally showed similar behavior to the 295-nm data. Both red shifts and large intensity increases (see Results) were observed with 280-nm excitation. Excitation at 280 nm resulted in a peak at approximately 310 nm that was probably due to tyrosine. Annexin V contains several tyrosines. The existence of tyrosinate fluorescence with emission at 345 nm, where many of our tryptophan measurements are made, is considered unlikely because it is generally much weaker than tyrosine fluorescence, and even if all the tyrosines in the protein behaved as tyrosinates, they could not account for the large intensity changes observed at 345 nm.

Stern–Volmer quenching constants were calculated from least squares fits to Stern–Volmer plots. Contributions of static quenching were generally unimportant below approximately 60–80 mM acrylamide as determined by plotting $(F_0/F - 1)/Q$ versus Q, where Q is the quencher concentration (Eftink & Ghiron, 1976, 1981). Lack of upward curvature of plots below 60–80 mM acrylamide supported this assumption. For the low quenching constants (below 6) a downward curvature was often observed for the lowest acrylamide concentrations (below 10–15 mM). This may indicate an equilibrium involving a small amount of an acrylamide-accessible form. No correction was made for these small effects.

Calculations. Estimates of effective surface concentrations of Ca^{2+} for negatively charged vesicles were made as in Nir et al. (1978) by first calculating the surface potential using a seventh-degree polynomial. The unique real positive root for this polynomial was determined by first finding a number close to the root from trial and error and then performing an iteration to minimize the polynomial to less than 10^{-4}. Further iteration to values closer to 0 led to changes in the calculated values of the root that were less than 0.1%. After the surface potential was found, the Boltzmann relation was used to determine the Ca^{2+} concentration at that potential. The same procedure was used for estimating the value of the effective Ca^{2+} concentration for PC vesicles, except that the methods outlined in McLaughlin et al. (1978) were used. This leads to a fourth-degree polynomial, which was solved as above to give the surface potential, from which the surface Ca^{2+} concentration was estimated. Activity coefficients for Ca^{2+} were not included.

RESULTS

Solvent Exposure of Tryptophan 187 in the Ca^{2+}-Bound Form of Annexin V. The effect of Ca^{2+} binding to annexin V was first investigated by observation of tryptophan fluorescence in the absence of phospholipids. A large red shift of the emission peak was induced by Ca^{2+} with a no increase in total intensity (Figure 1), suggesting exposure of this tryptophan to the aqueous solution. When annexin V was titrated with Ca^{2+}, the red shift was manifested as a small increase (approximately 20–25%) in emission intensity at 345 nm. This increase was half maximal at approximately 3 mM

[1] Abbreviations: pyrene-PC and pPC, 3-palmitoyl-2-[1-pyrenedecanoyl]-L-α-phosphatidylcholine; PS, bovine brain phosphatidylserine; PC, egg phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; $C_{12}E_8$, octaethylene glycol monododecyl ether; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

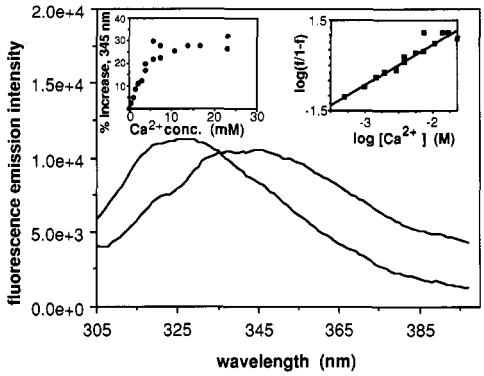

FIGURE 1: Effect of Ca^{2+} on the tryptophan fluorescence of annexin V. The emission spectra for 6 μg/mL annexin V in buffer A in the absence (curve on left) or presence (curve on right, higher wavelengths) of 23 mM $CaCl_2$ at 25 °C are shown. Excitation was at 295 nm. Spectra are corrected for background by subtraction of blank spectra. The effect of titration of Ca^{2+}, under the same conditions, on the fluorescence intensity at 345 nm is shown in the inset at upper left. The linear part of a Hill plot of this data is shown in the inset at upper right.

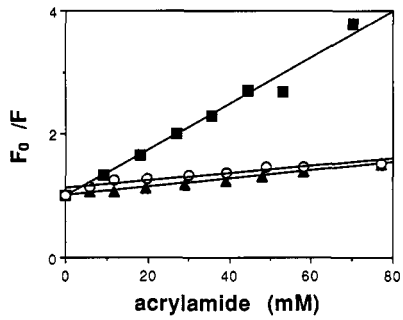

FIGURE 2: Acrylamide quenching of annexin V tryptophan. Samples contained 10 μg/mL annexin V alone (open circles; K_{sv} = 5.2 M^{-1}); 6 μg/mL annexin V and 91 mM Ca^{2+} (filled squares; K_{sv} = 36 M^{-1}); or 10 μg/mL annexin V, 200 μM PS/PC, 1:3, and 0.5 mM Ca^{2+} (filled triangles; K_{sv} = 6.7 M^{-1}). All experiments were performed in buffer B at 25 °C.

Ca^{2+} (Figure 1, inset). The Hill coefficient was 1.3 (Figure 1, inset) under the conditions of the experiment, indicating little if any Ca^{2+} binding site cooperativity leading to the observed change in tryptophan fluorescence.

The exposure of the tryptophan residue as a result of Ca^{2+} binding was further measured by acrylamide quenching (Figure 2). In the absence of Ca^{2+}, quenching was minimal, with a Stern–Volmer quenching constant of 5.2 M^{-1}. When the full red shift in tryptophan fluorescence was induced by high Ca^{2+} concentration, tryptophan 187 was very accessible to acrylamide, with a quenching constant of 36 M^{-1} or more. Therefore, excited-state tryptophan 187 is exposed to the solvent in a Ca^{2+}-bound form of annexin V, but not in uncomplexed annexin V.

Binding to Phospholipids. Binding to phospholipid vesicles resulted in a large increase in the emission intensity of the tryptophan (4–5-fold). For example, binding to vesicles composed of PS and PC (1:3) led to an increase of over 4-fold in the emission intensity (Figure 3A) that was half maximal at approximately 130 μM Ca^{2+}. This increase in emission intensity was accompanied by a red shift giving a maximum intensity at approximately the same wavelength (345 nm) as the Ca^{2+}-bound form of the protein in the absence of phospholipids. A decrease in intensity at higher Ca^{2+} concentrations, observed only for PS/PC vesicles, has not been further investigated. When annexin V was bound to phospholipids, the tryptophan was also protected from acrylamide

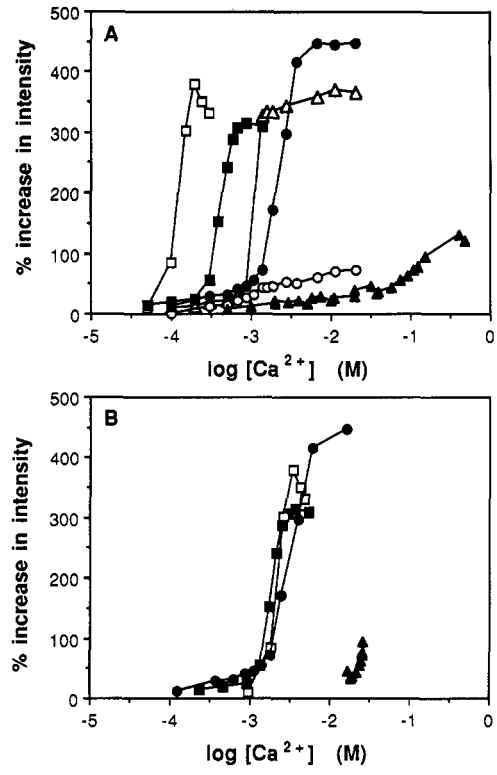

FIGURE 3: Ca^{2+} dependence of tryptophan emission intensity changes. Ca^{2+} concentrations were bulk (A) or calculated surface concentrations (B). All samples contained 6 μg/mL annexin V in buffer A at 25 °C, except for OA/PC at pH 6.6 which contained 100 mM NaCl, 10 mM PIPES, and 0.1 mM EDTA. Samples also contained PS/PC (1:3), 100 μM total phospholipid (open squares); OA/PC (1:3), pH 8, 100 μM total phospholipid (filled squares); OA/PC (1:3), pH 6.6, 100 μM total phospholipid (filled circles); PC, 100 μM total phospholipid (filled triangles); PS in 1.1 mM $C_{12}E_8$, 200 μM total phospholipid (open triangles); and PS in 1.1 mM $C_{12}E_8$, 50 μM total phospholipid (open circles). For 400 μM OA alone (up to 5 mM Ca^{2+}) and 25 μM OA alone (up to 20 mM Ca^{2+}) no intensity increase above approximately 20% was observed. Data for 400 μM oleic acid were corrected for an increased absorbance at 295 nm generated by titration with Ca^{2+} in the absence of annexin V. The intensity of the water Raman peak was used as an internal reference for this correction, which increased the values. In panel B, maximal effective surface concentrations of Ca^{2+} were calculated as in Table III, except for PC vesicles. Effective surface concentrations of Ca^{2+} for PC vesicles were calculated as in McLaughlin et al. (1978), using 1 M^{-1} as the binding constant for Ca^{2+} to PC. Binding to PC between bulk Ca^{2+} concentrations of 30 and 160 mM only is plotted, as the calculated surface concentration of Ca^{2+} actually decreased somewhat at very high Ca^{2+} (last two points) due to the positive surface potential from PC-bound Ca^{2+}.

quenching. For PS/PC (1:3), the quenching constant was only 6.7 M^{-1} (Figure 2, Table I). Therefore, phospholipid binding is characterized by a red shift, a large intensity increase, and a lack of exposure of the tryptophan, while Ca^{2+} binding alone to some or all of the annexin V sites, without phospholipids, results in only a red shift and exposure of the tryptophan to the aqueous environment.

Binding to Phosphatidylcholine. Binding to phosphatidylcholine (PC) vesicles was studied next for comparison with negatively charged vesicles. When a mixture of annexin V and PC vesicles was titrated with Ca^{2+}, the initial red shift was observed and manifested as a small increase in emission intensity at 345 nm almost identical to that in the absence of phospholipids (Figures 3A and 4A). Fluorescence emission spectra at 37 mM Ca^{2+} with excitation at either 280 or 295 nm (not shown) were nearly identical to those obtained in high Ca^{2+} and the absence of membranes (Figure 1, spectrum on right). However, very high concentrations of Ca^{2+}, in the

Table I: Stern–Volmer Quenching Constants[a]

lipid (concn)	Ca^{2+} (mM)	K_{sv} (M^{-1})
PC (100 μM)	37	49
PC (100 μM)	152	5.9
PC (100 μM)	462	5.7
OA/PC, 1:3, pH 8 (133 μM)	1.5	8.8
OA/PC, pH 6.6 (100 μM)	20	5.4
OA (25 μM)	20	45
PS/$C_{12}E_8$ (200 μM/1.1 mM)	20	6.4
PS/$C_{12}E_8$ (50 μM/1.1 mM)	20	24

[a] Experiments were in buffer A with 6 μg/mL annexin V at 25 °C as described in Materials and Methods.

presence of PC, resulted in a large increase in emission intensity at 345 nm (Figures 3A and 4B), similar to that observed for binding to negatively charged phospholipids. The apparent cooperativity of binding to PC was much lower than that of binding to PS/PC. The fluorescence change had apparently reached maximal intensity at less that 400 mM Ca^{2+}, and the bulk Ca^{2+} concentration required for apparently half-maximal intensity increase in the presence of PC was approximately 100 mM (see discussion of surface concentrations of Ca^{2+} below).

The fact that a red shift was observed at lower Ca^{2+} concentrations than required for the large intensity increase suggested that exposure of the tryptophan is a separate step from binding to PC. Solvent exposure in the presence of PC at intermediate Ca^{2+} concentrations was tested by acrylamide quenching at 37 mM Ca^{2+} in the presence of PC (Table I). Under these conditions, the red shift of fluorescence was complete (Figure 4A), but none of the large intensity increase associated with higher Ca^{2+} concentrations had occurred. The results (Table I) show that, even in the presence of PC, the tryptophan was exposed until titration to higher Ca^{2+} concentrations, where, at 152 and 462 mM Ca^{2+}, annexin V was presumably PC-bound and the tryptophan showed a relatively low exposure to acrylamide, with quenching constants similar to annexin V bound to PS/PC (Table I). Therefore, binding to pure PC vesicles appears to occur only at higher Ca^{2+} concentrations than tryptophan exposure, suggesting a possible mechanism of at least two steps, where PC may only participate in the second step.

No intensity increase could be observed when annexin V (6 μg/mL), in the presence of 400 mM Ca^{2+}, was titrated with the water-soluble dipropionoyl PC up to a concentration of 2.6 mM, nor did 2.6 mM dipropionoyl PC inhibit the intensity increase due to annexin V binding to PC vesicles at up to 400 mM Ca^{2+} (data not shown). Therefore there is a requirement for either long acyl chains or (more likely) a bilayer or aggregate organization of the phospholipids where the effective local phospholipid concentration is very high.

Binding to Phosphatidylcholine Mixed with Other Phospholipids. Since Ca^{2+}-dependent annexin V binding to PC was confirmed, it was of interest to determine if direct binding to PC could occur in the presence of negatively charged lipids, by studying binding of annexin V to vesicles composed of PC mixed with oleic acid in a 3:1 ratio. Binding to these vesicles was measured by Ca^{2+} titration of tryptophan fluorescence at pH 6.6 and 8, where approximately 9 and 72%, respectively,

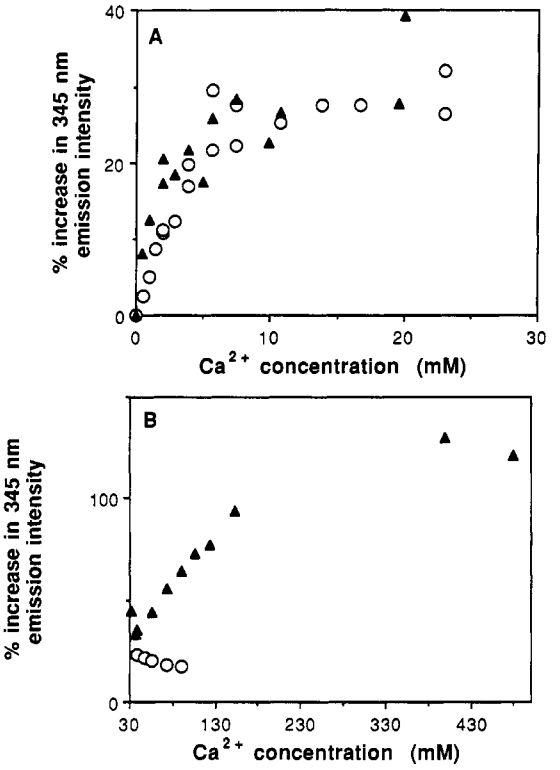

FIGURE 4: Effect of annexin V binding to PC on fluorescence emission intensity at 345 nm. Samples contained 6 μg/mL annexin V in buffer A and 100 μM PC (triangles) or no other additions (circles). Plots A and B represent two different scales for the same data. All experiments were performed at 25 °C.

of the oleic acid (OA) is charged, based on an effective pK of 7.6 (Hamilton & Cistola, 1986). Binding occurred at a much lower Ca^{2+} concentration than that required for binding to pure PC vesicles, even though no binding directly to OA alone at high or low concentrations (or in mixed micelles; see below) could be observed, as reported by tryptophan fluorescence under these conditions (Figure 3A). Therefore, the presence of negative charge was sufficient to greatly lower the Ca^{2+} requirement for binding to PC. This was at least partly due to the increased concentration of Ca^{2+} near the surface and possibly also increased annexin V concentration near the surface, as a result of the negative surface potential imparted by OA (see below).

On the basis of the indication that annexin V could bind to PC in the presence of negatively charged lipids, it was of interest to reassess the previously observed decrease in the excimer:monomer ratio of a pyrene derivative of PC mixed at low percentage with PS (Meers et al., 1991a). Associated with this ratio change was an increase in the monomer peak intensity (as measured by peak height, Figure 5) that was relatively linear with bound annexin V concentration. To investigate whether the pyrene-PC response could be due to direct interaction of annexin V with this probe, pyrene fluorescence in pyrene-PC/PC vesicles was measured at a Ca^{2+} concentration that was high enough for PC binding, as measured by the tryptophan fluorescence intensity increase. As the data in Table II show, a similar change in the excimer:monomer ratio as that observed in PS vesicles was observed if 286 mM Ca^{2+} was added to PC vesicles in the presence of annexin V. This suggested that annexin V could directly bind to PC even in the presence of excess PS and, furthermore, that the response of the pyrene probe may be due at least partly in this case to direct binding to PC, rather than solely an

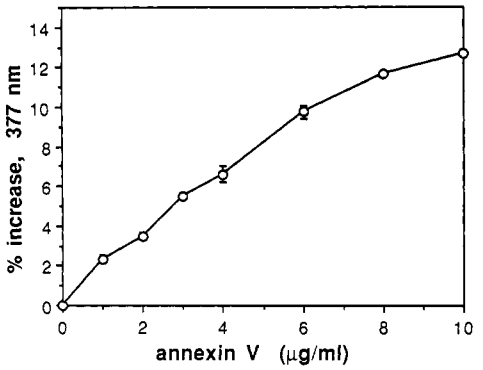

FIGURE 5: Effect of annexin V binding on the peak intensity of pyrene-PC monomer emission at 377 nm. Samples contained vesicles composed of 95 mol % PS and 5 mol % pyrene-PC (20 μM total phospholipid) in buffer B. The indicated amounts of annexin V were added to separate samples. A final concentration of 1 mM Ca^{2+} was added to each sample, and the change in fluorescence intensity was read after approximately 20–30 s. Error bars represent ±SD for duplicates.

Table II: Effect of Annexin V Binding on the Excimer:Monomer Ratio of Pyrene-PC

sample[a]	Ca^{2+} concn (mM)	normalized excimer:monomer[b] ratio ± *sd*
pPC/PS	0	100 ± 1.9
pPC/PS	0.2	83.6 ± 0.93
pPC/PC	0	100 ± 0.58
pPC/PC	0.2	100 ± 0.37
pPC/PC	286	88.3 ± 1.5

[a] Samples contained 10 μM total phospholipid, with pyrene-PC comprising 5 mol %, and 3 μg/mL annexin V at 25 °C. [b] Excimer: monomer ratios were measured by adding Ca^{2+} and, after 30 s, reading the intenisty both at 377 nm (monomer) and at 480 nm (excimer) within a few seconds. Ratios were normalized by setting the ratio in 0 Ca^{2+} to 100%.

indirect decrease of the effective lateral mobility of the probe (Meers et al., 1991a).

These data demonstrate the importance of knowing the local Ca^{2+} concentration at which annexin V binds to vesicles containing negatively charged phospholipids for comparison with other phenomena that occur at known Ca^{2+} concentrations. To bind to membrane phospholipids, presumably annexin V must pass through a region where the Ca^{2+} activity is either enhanced or reduced by the existence of a membrane surface potential. In Table III, the highest Ca^{2+} concentration expected near the surface of PS and PS/PC vesicles was calculated, based on a simple Guoy–Chapman model with correction for ion binding and screening (Nir et al., 1978). Several simplifying assumptions are used for these calculations, with the most tenuous being those for OA/PC vesicles. Nonetheless, they should provide a reasonable estimate for effective surface concentrations of Ca^{2+}, as demonstrated by their ability to predict Ca^{2+}–phospholipid binding isotherms and electrophoretic vesicle mobility. As can be seen, the maximal interfacial concentration of Ca^{2+} would be expected to reach several millimolar at the bulk concentrations used to elicit half-maximal binding of annexin V to vesicles of several different PS/PC compositions. It should be noted that this concentration is near that required for exposure of tryptophan 187 in the absence of phospholipids, suggesting that this conformational change is sufficient for binding to negatively charged membranes. Similar corrections were made for Ca^{2+} binding to pure PC vesicles using the data and methods of McLaughlin et al. (1978). In Figure 3B, corrected binding data for PC vesicles and those of several other compostions

Table III: Calculated Effective Surface Concentrations of Ca^{2+} for Annexin V Binding

%PS	$[Ca^{2+}]$ (mM), half-max binding	Ψ (mV)[a]	$[Ca^{2+}]_s$[b] (mM)
100[c]	0.036	−66	7
25[d]	0.13	−36	2.3
20[c]	0.22	−31	2.7
5[c]	1.5	−10	3.1
	OA/PC[d,e] effective % charge		
10 (20), pH 8	0.4	−19 (−31)	1.8 (4.7)
1.3 (2.5), pH 6.6	2.3	−4 (−6)	3.1 (3.8)

[a] Surface potentials were calculated as in Nir et al. (1978) using intrinsic binding constants for Na^+ and Ca^{2+} to PS of 0.8 and 35 M^{-1}, respectively. Ca^{2+} binding to PC was considered negligible under these conditions. These calculations are not applicable where surface charge reversal occurs, i.e., high bulk Ca^{2+} and low mol % negatively charged lipid. [b] Subscript s denotes the effective Ca^{2+} concentration at the membrane surface assuming nondiscrete charges and a continuous gradient of Ca^{2+} from the surface based on the calculated value of Ψ_0 and a simple Boltzmann distribution as in Nir et al. (1978). [c] Data from Andree et al. (1990); % denotes percentage of total phospholipid, with the rest composed of PC. [d] Data from this study. [e] Calculated as above on the assumptions that two oleic acid molecules are equivalent in surface area to one phospholipid, that the effective p*K* for oleic acid is 7.6, that the binding parameters for Ca^{2+}-oleic acid are the same as for Ca^{2+}-PS, and that oleic acid contributes half the expected effective surface charge (Hauser et al., 1979). Numbers in parentheses were calculated without the last assumption.

are shown. The corrections affect apparent cooperativity as well as Ca^{2+} dependence. Hill coefficients for the corrected binding isotherms in Figure 3B range from 3 to 5.4. Lower numbers seem to be associated with lower percentages of negatively charged lipid, but the data do not allow these coefficients to be unequivocally distinguished within the likely error. The maximal intensity increase for pure PC may not be attainable, as the calculated surface concentration of Ca^{2+} does not increase much at higher bulk Ca^{2+} concentrations due to the positive surface charge imparted by PC-bound Ca^{2+}. However, it is clear that, even with correction for surface concentrations, more Ca^{2+} is required for binding to pure PC vesicles than for negatively charged vesicles. On the other hand, when PC is mixed with PS or OA, the intrinsic Ca^{2+} dependence for binding directly to PC is approximately the same as for PS and is near that needed for tryptophan exposure.

Annexin V Binding to Micelles. Binding of annexin V to phospholipids in a nonbilayer configuration was also monitored to investigate the importance of the bilayer structure and to allow experiments under conditions where the effective surface potential may be reduced due to the high curvature of the micelle. Behavior similar to that observed for binding to bilayers was observed when Ca^{2+} was added to annexin V and $C_{12}E_8$ micelles with approximately 20 mol % PS. Approximately 1 mM Ca^{2+} was required for a half-maximal increase in tryptophan emission intensity, ultimately reaching an over 4-fold increase (Figure 3A). The increased intensity was also accompanied by a low Stern–Volmer quenching constant of 6.4 M^{-1} (Table I). Therefore, annexin V forms the same type of complex with PS in micelles as in membranes. When micelles contained an average of 5 mol % PS, only a very minor increase in fluorescence was observed up to 20 mM Ca^{2+}. This small increase may represent a small amount of bound annexin V, as acrylamide quenching showed a Stern–Volmer constant somewhat lower than for phospholipid-free annexin V (Table I). Binding to oleic acid (333 μM) in $C_{12}E_8$ (1.1 mM) micelles at pH 8 was also tested (not shown) and showed no increase in tryptophan fluorescence up to 1.5 mM Ca^{2+}, beyond which aggregation of micelles to larger structures made measurements impossible.

$C_{12}E_8$ forms micelles of approximately 120 molecules (Tanford et al., 1977) and has a critical micelle concentration of approximately 87 μM. The approximate critical micelle concentration and the estimated number of phospholipids per micelle were confirmed under the conditions of our experiments by monitoring the excimer:monomer ratio of 20 mol % pyrene-PC dispersed in this detergent (data not shown). Only small light scattering increases were observed when phospholipid was incorporated up to 30 mol %, suggesting that the micelles do not change greatly in size when the phospholipids are present. Assuming that micelles of 120 monomers each contain a proportional amount of added phospholipid, annexin V binding appears to require between 6 and 24 phospholipids per micelle as measured by tryptophan fluorescence. This calculation is not greatly dependent on a precise measurement of protein concentration and should be useful for a more precise determination of binding stoichiometry for PS and other phospholipids.

The ability of micellar PS to mediate the 4–5-fold increase in tryptophan emission intensity further supported the idea that this phenomenon is induced by a specific complex with the phospholipids, rather than being a general effect of membrane localization. A model peptide, lysyltryptophyllysine, was used to test whether a nonspecific adsorption to PS in micelles could give similar results. Since the tryptophan in this peptide is expected to localize near the PS headgroups, it could serve as a model for similar localization of tryptophan 187 in annexin V. When 400 μM PS in 2.2 mM $C_{12}E_8$ was added to 2 μg/mL peptide, no significant change in the tryptophan emission spectrum was observed, but the acrylamide quenching constant dropped from 16 to 10 M^{-1}, indicating that binding to micellar PS can at least partially reduce accessibility to acrylamide, as in the case of annexin V. Clearly, localization of the tryptophan near the PS headgroups is insufficient to induce the large increase in intensity observed for annexin V.

Vesicle Binding Not Associated with Tryptophan Intensity Changes. Spermine, because of its polycationic nature, is able to efficiently aggregate negatively charged phospholipid vesicles. In the presence of annexin V, in amounts that are insignificant compared to spermine, aggregation of vesicles was strongly inhibited. Spermine (2.8 mM) caused the percent transmittance at 400 nm of a solution of PS/PC (1:3) vesicles (100 μM total phospholipid) to decrease to 73% after 10 min, while the same mixture in the presence of 6 μg/mL annexin V decreased to only 99% transmittance. The most likely explanation for this effect is that annexin V binds to the surface of negatively charged phospholipids in the presence of spermine. Annexin V was titrated with spermine up to 2.8 mM to try to detect any red shift in fluorescence analogous to that due to Ca^{2+} binding. No change was observed (not shown). Ca^{2+} binding by annexin V, as monitored by the red shift, was also unaffected by the presence of 2 mM spermine (not shown). Therefore, spermine may bind to a site on annexin V away from the Ca^{2+} binding sites to mediate binding to phospholipids. This may be a relatively nonspecific electrostatic binding. A much less likely alternative is that sufficiently inhibitory amounts of annexin V may adsorb to the surface of the vesicles whether or not spermine is present. These data suggest that fluorescence changes associated with binding are specific for Ca^{2+}-dependent interaction with phospholipids.

DISCUSSION

Exposure of Tryptophan 187. The relatively high exposure of tryptophan 187 to solvent in the Ca^{2+}-bound form of the protein is particularly interesting. Exposure of this tryptophan was previously suggested on the basis of an absorbance difference spectrum in 30 μM Ca^{2+} (Schlaepfer et al., 1987). This would appear to correspond to approximately 0.2 Ca^{2+} ion bound per annexin V according to equilibrium dialysis data in the same study. A much higher concentration of Ca^{2+} was necessary for the exposure of Ca^{2+} as determined by fluorescence, corresponding to greater than 2, and possibly as many as 4 to 5, Ca^{2+} per annexin V, extrapolating the data (Schlaepfer et al., 1987). No explanation for this discrepancy is clear at this time. However, it does seem likely that at least one Ca^{2+} ion would need to be bound for a complete conformational change.

Previously presented data suggested that tryptophan 187 can make direct contact with phospholipids in the membrane-bound form of annexin V (Meers, 1990). On the other hand, diffraction data from crystalline annexin V in the absence of phospholipids has suggested that this residue is buried in the interior of the protein, in both the Ca^{2+}-bound and the free form (Huber et al., 1992). The data presented here suggest that this is not the case in either the soluble form or the membrane-bound form of the protein when Ca^{2+} is bound. One possible explanation for this discrepancy is the fact that the Ca^{2+}-bound crystal structure was obtained by adding Ca^{2+} to preformed crystals. These crystals cracked and reannealed, suggesting that the Ca^{2+}-induced conformational change of the protein may have been inhibited by lattice contacts (Huber et al., 1990). Determination of the feasibility of a structural change of the crystal form that would expose tryptophan 187 awaits the coordinates from the crystal structure. It is also possible that the data presented here represent only the excited state of tryptophan. However, excitation alone does not cause exposure of the tryptophan in Ca^{2+}-free annexin V. Furthermore, the exposure of tryptophan 187 in the Ca^{2+}-bound form of annexin V is consistent with the increased radius of gyration observed by neutron diffraction (Ravanat et al., 1992) and the previous suggestion that the Ca^{2+}-bound form has a more open structure (Meers, 1990).

It is important to note that the exposure of tryptophan 187 is probably not part of a hydrophobic binding mechanism. Annexin V does not appear to self-aggregate upon Ca^{2+}-dependent exposure of this residue. Furthermore, this tryptophan clearly has a membrane localization that is near the polar headgroups or glycerol backbone region of the phospholipid molecules (Meers, 1990). The relatively high wavelength of maximal emission intensity (345 nm) when annexin V is bound to the membrane, characteristic of polar solvents, also supports this conclusion. If, in fact, annexin V has phospholipid binding pockets similar to phospholipase A_2 (Scott et al., 1990), tryptophan 187 may make contact with only a single phospholipid abstracted from the bilayer, rather than inserting into the hydrophobic acyl chain region.

Ca^{2+}-Dependence of Phospholipid Binding. In general, the bulk concentrations of Ca^{2+} required for annexin V binding to PS/PC and PC that have been measured here correspond quite well to those measured previously by Andree et al. (1990). This supports the assumption that the large emission intensity increase and the lack of exposure of tryptophan 187 are direct indications of binding. The fact that annexin V can bind to PC in the presence of PS is also consistent with the observation (Andree et al., 1990) that the maximal amount of binding of annexin V did not vary with different percentages of PS and PC. The suggestion of equivalence of PS and PC has been further supported here by showing that the *intrinsic* Ca^{2+} dependence for binding to PS and PC in many situations is

approximately the same. One case that seems contrary is the 8.6 mM bulk Ca^{2+} dependence for binding to 1 mol % PS in PC (Andree et al., 1990). Our calculations suggest that charge reversal may occur in the Ca^{2+} concentration range necessary for binding to this composition, such that the concentration of Ca^{2+} near the surface may actually be lower than the bulk, despite the presence of PS. This situation cannot be treated by the formalism used for calculations at higher percentages of PS.

By estimation of the concentration of Ca^{2+} near the surface, it was further shown that this intinsic Ca^{2+} dependence of PS and PC mixed with other lipids corresponds closely to a well-defined conformational change of a specific part of the annexin V molecule, exposure of tryptophan 187. This also more strongly supports the suggestion that the covalent structure of the headgroup is not important for phospholipid binding (Andree et al., 1990). In fact, the relatively small differences for bulk Ca^{2+} dependence of binding to other phospholipids may be due simply to differences in the surface structures that change the effective Ca^{2+} concentration near the groups specifically bound.

These data also suggest that exposure of the tryptophan is not sufficient for binding to pure PC bilayers, since annexin V binding to PC required higher Ca^{2+} than tryptophan exposure. There are three plausible reasons for this observation. One is that binding to PC is exactly the same as to any other phospholipid in terms of the annexin V molecule, but that higher Ca^{2+} is required because of some feature of the PC bilayer surface, perhaps ionic interaction between adjacent phospholipids. This would not be the result of the covalent structure of a single PC molecule, as there are clearly situations where it can behave like any other phospholipid. A second possibility is that binding to pure PC bilayers does not involve as many specific phospholipid binding sites on the annexin V molecule. The Ca^{2+} binding site(s) minimally necessary for exposure of tryptophan may not be used in binding to pure PC vesicles. Negative charge may induce a change in the protein that allows added binding sites. Binding to oleic acid by other annexins has been observed (Edwards & Crumpton, 1991), and oleic acid binding may affect annexin V in a manner that does not cause the observed increase in tryptophan fluorescence, but does enhance binding to PC. A change in the number of phospholipid sites would be consistent with the preliminary cooperativity measurements above that suggest somewhat lower cooperativity for PC alone. But further cooperativity and stoichiometry measurements will be necessary to draw this conclusion. A third possibility is that the same number of specific phospholipid sites is involved but the negatively charged membranes allow a nonspecific electrostatic interaction with the positively charged convex side of the annexin V molecule (Huber et al., 1990; Karshikov et al., 1992), increasing the local annexin V concentration and leading to binding at lower Ca^{2+} concentrations. This would also lead to increased apparent cooperativity at higher negative surface charge in a manner similar to that seen for pentalysine binding to acidic phospholipids (Kim et al., 1991). In this case the existence of multiple binding sites as well as the electrostatic effect of partitioning the peptide to the bilayer surface leads to an apparent cooperativity. The detergent stoichiometry data showing a relatively large number of phospholipid binding sites (6–24) indicate abundant possibility for cooperativity and changes therein.

Phospholipid-Induced Fluorescence Changes. Several observations presented here help to further define the fluorescence changes observed when annexin V binds to phospholipids in the presence of Ca^{2+}. First, there is evidence that the observed intensity increase and lack of tryptophan exposure (compared to the Ca^{2+}-bound form) are related directly to phospholipid binding and possibly a specific complex involving Ca^{2+}-mediated tryptophan–phospholipid contact. Experiments with spermine indicated that apparent Ca^{2+}-independent association with the membrane is not sufficient to induce the large intensity increase. A negative charge is not required, as a substantial intensity increase accompanied by lack of tryptophan exposure was observed for binding to PC alone (although we cannot rule out trace amounts of fatty acid in the PC vesicles). Negative charge is also not sufficient, as oleic acid in mixed micelles, in homogeneous micelles, or as monomers does not substitute for phospholipids to give the observed effect on tryptophan fluorescence. Self-association of annexin V (Andree et al., 1992) is probably not required for the observed fluorescence changes, as both the full intensity increase and the lack of tryptophan exposure were observed for PS dispersed in $C_{12}E_8$ detergent micelles, even though the micelle:protein ratio was greater than 20:1.

While internal quenching may explain the relative low intensity of the annexin V tryptophan in the absence of Ca^{2+} and phospholipids (Meers, 1990), other mechanisms may be responsible for the difference between Ca^{2+}-annexin V and Ca^{2+}-annexin V bound to phospholipids. One possible reason for the large observed intensity increase for the latter may be partial immobilization of the tryptophan residue and/or surrounding solvent molecules due to increased microviscosity or orientation of excited-state dipoles at negatively charged membrane surfaces or near the negative charge of phospholipid phosphate groups. Slow solvent reorientation leads to a large increase in the quantum yields of indole derivatives without the blue shift of the emission maximum (Eisinger & Navon, 1969). It is suggested that activated quenching results from solvent reorientation that leads to tunneling between solvent-relaxed excited and ground states (Eisinger & Navon, 1969). Another mechanism by which exposure to water may decrease quantum yield is by electron ejection from the excited state (Feitelson, 1971). Ejection of electrons (and hence quenching) is enhanced by disruption of water hydrogen bonds at high temperatures and inhibited by the absence of water, suggesting that solvation of electrons is important (Feitelson, 1971). Strongly immobilized and hydrogen-bonded water at the membrane interface probably would not efficiently mediate electron solvation, decreasing the rate of quenching. Further experiments will be required to test the various factors potentially responsible.

Structure–Function Correlations. Though the actual physiological functions of the annexins are not well established, Ca^{2+}-dependent binding to phospholipids is probably an integral part of their actions, as discussed above. The mechanism by which they bind to phospholipids is therefore important to their functions, as we know them. Yet, there is little direct information on the structure of the annexin phospholipid binding sites. Determination of the structure of these binding sites by conventional diffraction methods has been difficult because monomeric phospholipid-like ligands do not bind well to annexins (Tait et al., 1989; this paper). Furthermore, some annexins even mediate contact between more than one membrane and may exist in a different conformation to participate in this event (Zaks & Creutz, 1991; Meers et al., 1992). Using intrinsic protein fluorescence, we have identified a Ca^{2+}-dependent conformational change of part of the annexin V molecule that is sufficient for binding to most phospholipid membranes and probably necessary for

all membrane binding. Annexin V binding to phospholipids probably first involves a conformational change that exposes tryptophan 187 to the solvent, followed by (or simultaneous with) binding to maximally 6–24 phospholipids at the membrane surface, where the tryptophan is protected from aqueous quenchers like acrylamide. These data warrant studies to determine whether the third consensus sequence of annexin V constitutes part of a phospholipid binding site. Further studies on annexin mutants with tryptophan inserted into various sites, along with information from crystal structures, may allow a more detailed analysis of the various conformations these proteins assume and how they relate to membrane-associated functions.

NOTE ADDED IN PROOF

Preliminary data from diffraction studies of the structure of rat annexin V crystallized in the presence of Ca^{2+} (Concha and Seaton, manuscript in preparation) indicate that tryptophan 187 is exposed to solvent, in agreement with the fluorescence data.

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REFERENCES

Andree, H. A. M., Reutelingsperger, C. P. M., Hauptmann, R., Hemker, H. C., Hermens, W. T., & Willems, G. M. (1990) *J. Biol. Chem. 265*, 4923–4928.

Andree, H. A. M., Stuart, M. C. A., Hermens, W. T., Reutelingsperger, C. P. M., Hemker, H. C., Frederik, P. M., & Willems, G. M. (1992) *J. Biol. Chem. 267*, 17907–17912.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Chen, P. S., Jr., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Edwards, H. C., & Crumpton, M. J. (1991) *Eur. J. Biochem. 198*, 121–129.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–680.

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.

Eisinger, J., & Navon, G. (1969) *J. Chem. Phys. 50*, 2069–2077.

Feitelson, J. (1971) *Photochem. Photobiol. 13*, 87–96.

Funakoshi, T., Heimark, R. L., Hendrickson, L. E., McMullen, B. A., & Fujikawa, K. (1987) *Biochemistry 26*, 5572–5578.

Geisow, M. J., Fritsche, U., Hexham, J. M., Dash, B., & Johnson, T. (1986) *Nature 320*, 636–638.

Hamilton, J. A., & Cistola, D. P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 82–86.

Huber, R., Schneider, M., Mayr, I., Römisch, J., & Paques, E.-P. (1990) *FEBS Lett. 275*, 15–21.

Huber, R., Berendes, R., Burger, A., Schneider, M., Karshikov, A., Luecke, H., Römisches, J., & Paques, E. (1992) *J. Mol. Biol. 223*, 683–704.

Karshikov, A., Berendes, R., Burger, A., Cavalié, A., Lux, H.-D., & Huber, R. (1992) *Eur. Biophys. J. 20*, 337–344.

Kim, J., Mosior, M., Chung, L. A., Wu, H., & McLaughlin, S. (1991) *Biophys. J. 60*, 135–148.

Kingsley, P. B., & Feigenson, G. W. (1979) *Chem. Phys. Lipids 24*, 135–147.

Kretsinger, H., & Creutz, C. E. (1986) *Nature (London) 320*, 573.

Maurer-Fogy, I., Reutelingsperger, C. P. M., Pieters, J., Bodo, G., Stratowa, C., & Hauptmann, R. (1988) *Eur. J. Biochem. 174*, 585–592.

Mayer, L. D., Hope, M. J., & Cullis, P. R. (1986) *Biochim. Biophys. Acta 858*, 161–168.

McLaughlin, A., Grathwohl, C., & McLaughlin, S. (1978) *Biochim. Biophys. Acta 513*, 338–357.

Meers, P. (1990) *Biochemistry 29*, 3325–3330.

Meers, P., Daleke, D., Hong, K., & Papahadjopoulos, D. (1991) *Biochemistry 30*, 2903–2908.

Meers, P., Hong, K., & Papahadjopoulos, D. (1991b) in *Cell and Model Membrane Interactions* (Ohki, S., Ed.) pp 115–134, Plenum Press, New York.

Meers, P., Mealy, T. R., & Tauber, A. I. (1992) *Biochemistry 31*, 6372–6382.

Morrison, W. R. (1964) *Anal. Biochem. 7*, 218–224.

Nir, S., Newton, C., & Papahadjopoulos, D. (1978) *Bioelectrochem. Bioenerg. 5*, 116–133.

Pepinsky, R. B., Tizard, R., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, J. L., Chow, E. P., Burne, C., Huang, K.-S., Pratt, D., Wachter, L., Hession, C., Frey, A. Z., & Wallner, B. P. (1988) *J. Biol. Chem. 263*, 10799–10811.

Ravanat, C., Torbet, J., & Freyssinet, J.-M. (1992) *J. Mol. Biol. 226*, 1271–1278.

Reutelingsperger, C. P. M., Hornstra, G., & Hemker, H. C. (1985) *Eur. J. Biochem. 151*, 625–629.

Schlaepfer, D. D., Mehlman, T., Burgess, W. H., & Haigler, H. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6078–6082.

Schlaepfer, D. D., Jones, J., & Haigler, H. T. (1992) *Biochemistry 31*, 1886–1891.

Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., & Sigler, P. B. (1990) *Science 250*, 1541–1546.

Tait, J. F., Gibson, D., & Fujikawa, K. (1989) *J. Biol. Chem. 264*, 7944–7949.

Tanford, C., Nozaki, Y., & Rhode, M. F. (1977) *J. Phys. Chem. 81*, 1555–1560.

Zaks, W., & Creutz, C. E. (1991) *Biochemistry 30*, 9607–9615.