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Ca^{2+}-Dependent Annexin Self-Association on Membrane Surfaces†

William J. Zaks* and Carl E. Creutz*
Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908
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ABSTRACT: Annexin self-association was studied with 90° light scattering and resonance energy transfer between fluorescein (donor) and eosin (acceptor) labeled proteins. Synexin (annexin VII), p32 (annexin IV), and p67 (annexin VI) self-associated in a Ca^{2+}-dependent manner in solution. However, this activity was quite labile and, especially for p32 and p67, was not consistently observed. When bound to chromaffin granule membranes, the three proteins consistently self-associated and did so at Ca^{2+} levels (pCa 5.0-4.5) approximately 10-fold lower than required when in solution. Phospholipid vesicles containing phosphatidylserine and phosphatidylethanolamine (1:1 or 1:3) were less effective at supporting annexin polymerization than were those containing phosphatidylserine and phosphatidylcholine (1:0, 1:1, or 1:3). The annexins bound chromaffin granule membranes in a positively cooperative manner under conditions where annexin self-association was observed, and both phenomena were inhibited by trifluoperazine. Ca^{2+}-dependent chromaffin granule membrane aggregation, induced by p32 or synexin, was associated with intermembrane annexin polymerization at Ca^{2+} levels less than pCa 4, but not at higher Ca^{2+} concentrations, suggesting that annexin self-association may be necessary for membrane contact at low Ca^{2+} levels but not at higher Ca^{2+} levels where the protein may bind two membranes as a monomer.

The annexins are a newly described group of homologous proteins that bind phospholipid membranes in a Ca^{2+}-dependent manner [for reviews see Klee (1988), and Burgoyne and Geisow (1989)]. Some members of this group are also commonly known as lipocortins (Huang et al., 1986), calpactins (Glenney, 1986), chromobindins (Creutz et al., 1983, 1987), calelectrins (Sudhof et al., 1984), or placental anticoagulant proteins (Tait et al., 1988). Currently, 10 distinct members of this gene family, termed annexins I-X, have been identified (Pepinsky et al., 1988; Burns et al., 1989; Hauptmann et al., 1989; Johnston et al., 1990). Comparison of their amino acid sequences reveals a common structural theme: Each protein has two regions, a variable-length amino-terminal region lacking homology with other members of the family and a core region of four or eight repeating 70 amino acid domains, which share 40-60% homology between family members.

The biological function of the annexin proteins is unknown, but they have been hypothesized to play a role in signal transduction (Hollenberg et al., 1988), in exocytosis (Creutz et al., 1978, 1983), in the organization of membrane phospholipid domains (Geisow et al., 1987), as structural/regulatory elements of the cytoskeleton (Glenney, 1986), and as regulators of phospholipase A_2 (Huang et al., 1986). These theories are based on the preferential cellular localization of some of these proteins to the plasma membrane/cortical cytoskeleton and their ability to bind in a Ca^{2+}-dependent manner to phospholipid membranes and in some cases to cytoskeletal elements (Geisow et al., 1987). However, the mode of interaction of the annexins with phospholipids and proteins at the membrane surface is unclear. In addition to interactions with other proteins, some annexins have been reported to self-associate. This phenemonon was first reported for isolated synexin in solution, which formed 50 × 150 Å rods, bundles of rods, and paracrystalline arrays in a Ca^{2+}-dependent manner (Creutz et al., 1979). A similar self-association event was seen with isolated Torpedo calelectrin, which formed morphologically different structures: circular forms composed of 50-Å globular subunits in the absence of calcium and amorphous aggregates of polygonal structures each composed of the 50-Å globular subunit in the presence of calcium (Walker et al., 1983). Other reports, however, have claimed that other members of the annexin family such as p32 (annexin IV) or calpactin (annexin II) do not self-associate (Shadle et al., 1985). Similarly, it has been debated whether annexins self-associate on membrane surfaces. Some investigators have

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* Correspondence may be addressed to either author.

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reported cooperative annexin–membrane binding interactions (Zaks & Creutz, 1990) or visualization of membrane-bound annexin aggregates (Newman et al., 1989, 1991; Mosser et al., 1991), while others report only noncooperative membrane binding phenomena (Tait et al., 1989; Meers, 1990).

In this paper we show that a resonance energy transfer assay may be used to monitor the self-association of three annexin proteins: synexin (annexin VII; Creutz et al., 1978), p32 (annexin IV; Shadle et al., 1985; Geisow et al., 1986), and p67 (annexin VI; Owens & Crumpton, 1984; Shadle et al., 1985; Sudhof et al., 1984). Each of the three proteins was shown to be capable of Ca^{2+}-dependent self-association in solution and on some membrane surfaces. Furthermore, the self-association event enabled annexins to bind membranes in a positively cooperative manner and appeared to play a role in the ability of the annexins to aggregate membranes at Ca^{2+} levels of less than 100 μM.

Experimental Procedures

Materials. Fluorescein 5-isothiocyanate and eosin 5-isothiocyanate were purchased from Molecular Probes, Inc. Disuccinimidylsuberate was obtained from Pierce. Trifluoroperazine (TFP),[1] obtained from Smith, Kline and French, was a gift from Dr. M. J. Peach (University of Virginia). Phospholipids were from Sigma. Sephadex G-25 (PD-10) columns were obtained from Pharmacia Fine Chemicals.

Preparation of Chromaffin Granule Membranes and Phospholipid Vesicles. Chromaffin granule membranes were prepared by the method of Bartlett and Smith (1974). Multilamellar liposomes were prepared as follows. Chloroform solutions of phospholipids were mixed in various proportions, dried under nitrogen, and resuspended at 2 mg/mL by vortex mixing in buffer containing 0.24 M sucrose, 30 mM KCl, and 40 mM Hepes, pH 7.0.

Preparation of Annexins and Fluorescent Derivatives. Synexin was prepared from bovine liver as previously described (Zaks & Creutz, 1990). The protein was routinely reprecipitated in 20% saturated ammonium sulfate after the final FPLC chromatography step, to give a preparation greater than 85% pure as determined from Coomassie-stained SDS gels. The mammalian calelectrins (p32 and p67) were isolated from bovine liver as described (Creutz et al., 1987), and each protein was estimated to be greater than 95% homogeneous. For fluorescent labeling, 250 μg of synexin, p32, or p67 in buffer containing 0.24 M sucrose, 30 mM KCl, and 40 mM Hepes, pH 8.0, was allowed to react with a 10-fold molar excess of fluorescein 5-isothiocyanate (FITC) or eosin 5-isothiocyanate (EITC) (added as a concentrated solution in dimethylformamide) for 20 min at 4 °C. The unreacted free FITC or EITC was removed from the protein by Sephadex G-25 chromatography in the presence of a buffer containing 0.24 M sucrose, 30 mM KCl, and 40 mM Hepes, pH 7.0. The concentration of bound dye was determined by the absorbance with a molar extinction coefficient of 42 500 M^{-1} cm^{-1} at 495 nm for FITC and 84 000 M^{-1} cm^{-1} at 524 nm for EITC. In the various experiments the concentration of bound dye ranged from 0.2–0.4 mol of dye/mole of annexin. The labeled proteins were found to retain full chromaffin granule aggregating activity.

Self-Association Assays. The 90° light scattering from solutions of synexin, p32 or p67 was monitored at 350 nm in a 0.5 cm × 0.5 cm quartz cuvette as described (Sterner et al., 1985). Fluorescence experiments were performed on a SPEX Fluorolog 2 model 111C spectrofluorometer in a 0.5 cm × 0.5 cm quartz cuvette at 22 °C. Fluorescein- and eosin-labeled annexin proteins were mixed in a 1:1 molar ratio in 0.24 M sucrose, 30 mM KCl, and 40 mM Hepes, pH 7.0, buffer in the absence or presence of chromaffin granule membranes. The samples were excited at 470 nm (slit width 1.25 or 2.5 mm) and fluorescence emission was recorded either at 510 nm (slit width 2.5 mm) as a function of time or a 519 and 534 nm (slit width 2.5 mm) after 3 min of annexin self-association when polymerization reach a plateau. Annexin self-association was initiated by adding Ca^{2+} in the form of Ca^{2+}–HEDTA buffers prepared and standardized with a calcium electrode as previously described (Zaks & Creutz, 1990). Reported pCa values represent Ca^{2+} activity and not Ca^{2+} concentration. To compare these values with pCa values reported in concentration units, all values of pCa given in this paper should be decreased by approximately 0.26 units [i.e., pCa 6.0 (activity) becomes pCa 5.74 (concentration)].

Membrane-Binding Assays. Binding of FITC-labeled or iodinated annexin proteins to chromaffin granule membranes was measured by a centrifugation assay as previously described (Zaks & Creutz, 1990). Binding was quantitated either as the amount of radioactivity in the twice-washed pellet or as the decrease in fluorescence (λ_{ex} = 470, λ_{em} = 519) intensity of the supernatant relative to an identical sample in the presence of 2.5 mM HEDTA. Both assays gave essentially identical results.

Cross-Linking Experiments. p32 (50 μg/mL) was incubated for 10 min at 4 °C with a 5-, 10-, 50-, and 100-fold molar excess of disuccinimidylsuberate (added as a 100-fold concentrated solution in dimethylformamide) in 100 μL of buffer containing 0.24 M sucrose, 30 mM KCl, 40 mM Hepes, pH 8.0, 100 μg/mL phospholipid (PS/PC 1:2, w/w), and 2.5 mM HEDTA with 0 or 3.5 mM $CaCl_2$. The reactions were quenched by adding 5 μL of a saturated solution of Tris, and after 5 min the pH was neutralized with HCl. The mixture of cross-linked proteins was separated on a 10% SDS–polyacrylamide gel (Laemmli, 1970) and visualized by staining with silver (Morrissey, 1981).

Miscellaneous Procedures. Annexin-induced aggregation of chromaffin granule membranes was measured by a turbidity (at 540 nm) assay as previously described (Creutz et al., 1978). Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as the standard for p32, p67, and chromaffin granule membranes and bovine gamma globulin as the standard for synexin. With these standards, this assay gives protein concentrations that agree well with those determined by the Lowry assay (Pollard et al., 1978).

Results

Energy Transfer as a Measure of Annexin Self-Association. The Ca^{2+}-dependent self-association of synexin in solution has been detected by 90° light-scattering measurements (Sterner et al., 1985; Creutz et al., 1979) and by electron microscopic visualization of negatively stained synexin polymers (Creutz et al., 1979). In order to determine whether this process could be measured by a resonance energy transfer assay, the Ca^{2+}-dependent polymerization of mixtures of fluorescein (fluorescence donor) and eosin (fluorescence acceptor) labeled synexin was measured by both 90° light scattering and fluorescence donor (fluorescein) quenching at 510 nm. At this wavelength, representing the shoulder of the fluorescein emission spectrum, eosin makes only a negligible (5%) con-

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FITC, fluorescein 5-isothiocyanate; EITC, eosin 5-isothiocyanate; HEDTA, *N*-(hydroxyethyl)ethylenediaminetriacetic acid; TFP, trifluoperazine.

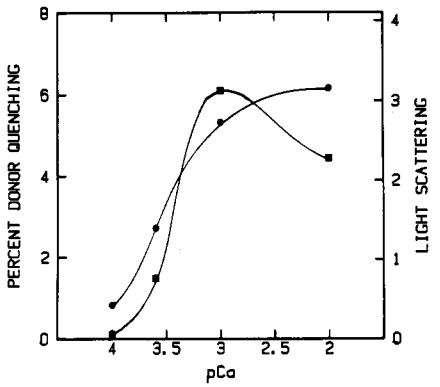

FIGURE 1: Ca^{2+} dependence of synexin self-association in solution. The self-association of a 1:1 mixture of fluorescein- and eosin-labeled synexin (1.76 μg/mL) was measured by donor (fluorescein) quenching at 510 nm (●) or light scattering (■) as described under Experimental Procedures.

tribution to the fluorescence signal, and the efficiency of energy transfer can be determined directly from the reduction in fluorescein quantum yield. As shown in Figure 1, measurement of donor quenching detects an event with a similar Ca^{2+} dependence to that of synexin polymerization determined by light scattering. To determine whether this donor quenching was due to energy transfer, the magnitude of fluorescein quenching in a 1:1 mixture of fluorescein-labeled annexin and unlabeled annexin was compared with that seen with a 1:1 mixture of fluorescein- and eosin-labeled annexin. It was observed that fluorescein quenching was enhanced 2.1-fold in the presence of eosin-labeled protein. Therefore, the quenching of the donor fluorescence was apparently primarily due to the interaction between the fluorescein- and eosin-labeled species. In studies conducted in the presence of membranes (see below) donor quenching was found to be similarly dependent upon the presence of the acceptor. Since fluorescein quenching was found to be insensitive to Ca^{2+}/phospholipid binding (Figure 3B), the donor quenching seen in the absence of eosin-labeled protein appears to represent fluorescein self-quenching. As an alternate measure of energy transfer, we also examined the ratio of acceptor (eosin) to donor (fluorescein) fluorescence, measured at 543 and 519 nm, respectively. Coincident with the decrease in the fluorescence at 510 nm was an increase in the 543/519 ratio, consistent with transfer of energy from the fluorescein to the eosin. However, the change in this ratio was due primarily to the reduction in donor fluorescence since a Ca^{2+}-dependent increase in eosin (acceptor) fluorescence was not observed in this study, possibly due to self-quenching of eosin at high concentrations in the annexin aggregates.

Annexin Self-Association in the Presence of Membranes. Although synexin self-associates in solution, there have been no direct studies to determine whether this self-association event occurs when the protein is membrane bound. However, we have previously shown that synexin binding to membranes shows positive cooperativity (Zaks & Creutz, 1990), which is suggestive of such a process. In order to provide more direct evidence for self-association on the membrane surface, we measured the Ca^{2+}-dependent energy transfer in a mixture of fluorescein- and eosin-labeled synexin in the presence of chromaffin granule membranes. As shown in Figure 2B, the extent of energy transfer was greatly enhanced in the presence of the membranes compared to that seen in the absence of membranes, as shown in Figure 1. These data suggest that synexin polymerizes on the membrane surface, possibly to a greater extent than in solution. However, we cannot eliminate the possibility that the greater magnitude of energy transfer

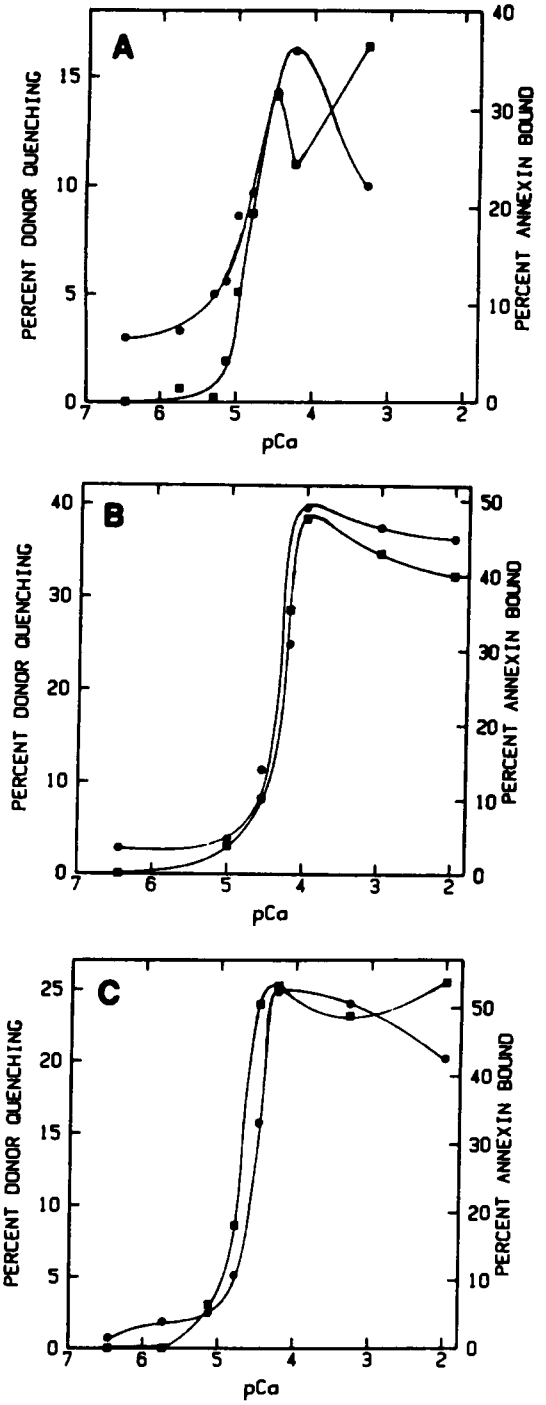

FIGURE 2: Ca^{2+} dependence of annexin self-association and membrane binding. A 1:1 mixture of fluorescein- and eosin-labeled p32 (8.4 μg/mL, A), synexin (4.0 μg/mL, B), or p67 (9.5 μg/mL, C) was incubated with chromaffin granule membranes (30 μg/mL, A and B; 10 μg/mL, C) at varying Ca^{2+} concentrations. The extent of annexin binding to the membranes (■) and energy transfer efficiency (donor quenching at 510 nm) (●) were determined as described under Experimental Procedures.

reflects a change in the geometry of the self-associating molecules as a result of membrane binding. Comparing the Ca^{2+} dependence for energy transfer seen in Figures 2 and 1, it also appears that synexin self-association occurs at lower Ca^{2+} levels when membrane bound. Such a phenomenon is similar to the ability of free fatty acids to decrease the Ca^{2+} levels required to enhance light scattering from solutions of synexin (Sterner et al., 1985). Both phenomena may result from the ability of lipids to modulate the Ca^{2+} sensitivities of the protein self-association event.

Table I: Effect of Surface Density on the Energy Transfer Efficiency of Membrane-Bound p67[a]

p67 bound (μg/mL)	rel surface density of membrane-bound p67	efficiency of energy transfer (%)
0.028	1	32
0.089	3	60
1.07	38	44
2.20	78	48
4.37	154	40
5.33	188	47

[a] Increasing amounts of fluorescein- and eosin-labeled p67 (0.23–10 μg/mL) were bound to chromaffin granule membranes (30 μg/mL) at pCa 4. Binding of the fluorescently labeled protein was quantitated as described under Experimental Procedures. The efficiency of energy transfer between membrane-bound p67 was calculated as the magnitude of Ca^{2+}-induced fluorescein quenching (at 510 nm), expressed relative to the fluorescence of bound fluorescein-labeled p67. The data is from a single experiment. The uncertainty in the calculated efficiency of energy transfer is estimated to be 50% of the given value at the two lowest p67 concentrations and 10% of value at the four higher concentrations.

Although the self-association properties of the Torpedo calelectrin have been demonstrated (Walker et al., 1983; Sudhof et al., 1982), there have been no reports documenting self-association in solution of other members of the annexin family isolated from mammalian tissues. Using the sensitive light-scattering assay, we have been able to detect self-association of both p32 and p67 in solution at 1 mM Ca^{2+} at pH 7.0. However, this property appeared to be quite labile, as some preparations of these proteins failed to self-associate even though they were prepared in an identical manner and were of similar purity. In one instance, we were able to detect apparent Ca^{2+}-dependent self-association of p67 in solution using the fluorescence assay but were unable to repeat this observations after the sample of labeled protein sat for 4 h at 4 °C. However, the proteins that had lost their ability to self-associate in solution still showed the Ca^{2+}-dependent membrane-binding and membrane-aggregating activities of the native proteins. When chromaffin granule membranes were added to samples of fluorescently labeled p32 or p67 that were incapable of self-associating in solution, the proteins displayed Ca^{2+}-dependent energy transfer suggestive of self-association on the membrane surface (Figure 2A,C).

Two lines of evidence indicate that this phenomenon reflects true annexin self-association rather than energy transfer due to close proximity and/or diffusional contact of randomly distributed labeled proteins on the membrane surface. First of all, although the Ca^{2+} dependence for energy transfer is similar to that for annexin binding to the membrane, there is not an exact correlation between these two events at all pCa levels (Figure 2). Secondly, the magnitude of energy transfer between membrane-bound annexin molecules is independent of their apparent surface density on the membranes (Table I). Under these conditions, the maximum surface density of p67 may be calculated from the size and composition of isolated chromaffin granules [diameter = 2800 Å, 2.2 μmol of phospholipid/mg of membrane protein, 5.9×10^5 phospholipids/chromaffin granule (Winkler & Westhead, 1980)], the maximum amount of p67 bound (5.33 μg per 30 μg of chromaffin granule membrane protein), and the known efficiency of p67 labeling (~30%), to be 8.67×10^{-16} p67/Å^2, or, expressed per R_0^2, where R_0 = 46 Å for this donor–acceptor pair, 0.018 fluorescence acceptors/R_0^2. On the basis of a model for resonance energy transfer between noninteracting randomly distributed fluorophores in two dimensions (Wolber & Hudson, 1979), the efficiency of energy transfer at this surface density would be ~5% and linearly dependent on fluorophore concentration. The observed 40–50% quenching efficiency and its insensitivity to reduction in p67 surface density is thus incompatible with this model of randomly distributed fluorophores and implies the existence of tightly associated annexin polymers.

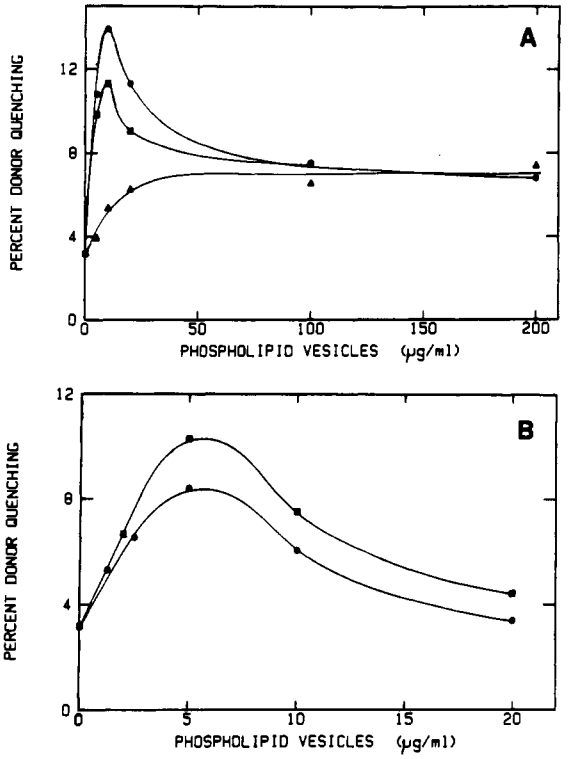

FIGURE 3: Effect of membrane composition of p32 self-association. Fluorescein- and eosin-labeled p32 (4.91 μg/mL) were bound to varying concentrations of phospholipid vesicles at pCa 4, and the efficiency of energy transfer was calculated from the quenching at 510 nm of total donor fluorescence. Membrane vesicle compositions were (A) PS (●), PS/PC (1:1 w/w) (■), PS/PC (1:3 w/w) (▲); (B) PS/PE (1:1 w/w) (■), PS/PE (1:3, w/w) (●).

Effects of Membrane Lipid Composition. Although chromaffin granule membranes are capable of promoting annexin self-association, it does not appear that all membranes are equally efficacious in this regard. As shown in Figure 3, energy transfer was not abolished by dilution of p32 on the membrane surface of PS or PS/PC vesicles but was eliminated by similar dilution on vesicles composed of PS/PE. For most lipid compositions, a maximum of energy transfer was seen, which was then reduced by further increasing the lipid concentration. This peak response appears to reflect a high surface density of p32 on certain vesicles, which produces a component of energy transfer due to close proximity rather than actual self-association. At high lipid concentrations, this proximity effect, or any weak annexin–annexin interaction, is abolished and only energy transfer from tightly associated polymers remains. In Figure 3a the surface density of annexin fluorophores in the presence of 200 μg/mL PS/PC (1:1) vesicles may be calculated to be $0.014/R_0^2$, given that 25% of p32 is bound (data not shown), R_0 = 46 Å, and the labeling efficiency is 30%, and assuming that exposed phospholipid has a molecular weight of 700 and a surface area of 60 Å^2 and represents 10% of total lipid in these multilamellar vesicles. At this surface density it can be calculated (Wolber & Hudson, 1979) that only 5% donor quenching, rather than the observed 28% (7% total quenching corrected for 25% annexin binding), can be attributed to proximity of randomly distributed monomers. A similar conclusion is reached if it is assumed that near-maximal p32 binding occurs at 10 μg/mL lipid (based

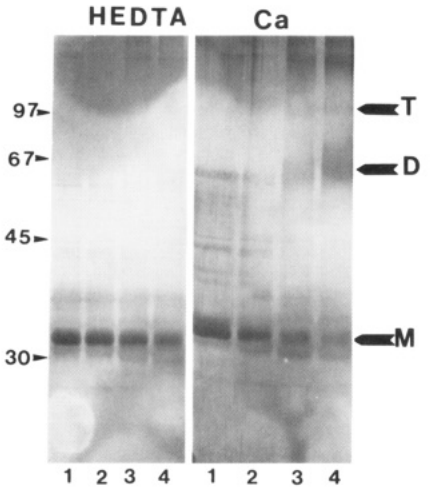

FIGURE 4: Cross-linking of p32. Crosslinking was performed with disuccinimidylsuberate as described under Experimental Procedures. p32 (50 μg/mL) was incubated in the presence of a (1) 5-, (2) 10-, (3) 50-, and (4) 100-fold molar excess of cross-linker in the presence of 100 μg/mL PS/PC (1:2) lipid vesicles and HEDTA or 1 mM free Ca^{2+}. The numbers on the left mark the positions of migration of molecular weight standards: 30, carbonic anhydrase, 30 kDa; 45, ovalbumin, 45 kDa; 67, serum albumin, 67 kDa; 97, phosphorylase, 97 kDa. Cross-linked forms of p32 are apparently generated in the presence of calcium (Ca) and the higher levels (3, 4) of cross-linker: M = p32 monomer, D = p32 dimer, T = p32 tetramer. This gel was stained first with Coomassie blue, then destained and stained with silver. The broad scalloped pattern at the top of the gel is due to residual Coomassie stain. In the original gel the protein bands representing the p32 monomers, dimers, and trimers can be distinguished by their gray color due to the silver stain.

on maximum energy transfer at this lipid concentration). Under these conditions p32 surface density (and energy transfer efficiency from a random distribution) would be only 5% as great at 200 μg/mL phospholipid and thus contribute only 10% of the observed energy transfer seen at this lipid concentration. For PS/PC (1:3) vesicles, energy transfer was a monotonic function of lipid concentration. It appears these vesicles may not bind enough protein for this proximity effect to be realized. Liposomes prepared from chromaffin granule lipid extracts also showed a monotonic response similar to that seen with PS/PC (1:3) vesicles although with a higher maximum efficiency of energy transfer (data not shown).

Chemical Cross-Linking of p32 Bound to Lipid Vesicles. Chemical cross-linking studies corroborate the formation of annexin polymers on membranes as detected by resonance energy transfer. As shown in Figure 4, p32 dimers and higher order aggregates, possibly tetramers, are detected by cross-linking when p32 is bound to 100 μg/mL PS/PC (1:2) vesicles in the presence of 1 mM free calcium but not when the protein is free in solution in the presence of HEDTA. The dimer and tetramer bands are fairly broad, perhaps reflecting multiple modes of cross-linking that lead to a distribution of mobilities in each case. The formation of the higher order aggregates is correlated with the loss of the monomeric form of p32. The polymeric forms were less prominent when PS/PE (1:3) vesicles were substituted or no vesicles were present (not shown).

Inhibition of Annexin Self-Association by Trifluoperazine. Since many annexin functions, such as their ability to bind membranes (Shadle & Weber, 1987) and induce membrane aggregation (Pollard et al., 1983, Meers et al., 1987) have been shown to be inhibited by drugs in the phenothiazine class such as trifluoperazine (TFP), we examined whether self-association was also sensitive to these drugs. We observed that TFP inhibited self-association of p32, p67, and synexin on the

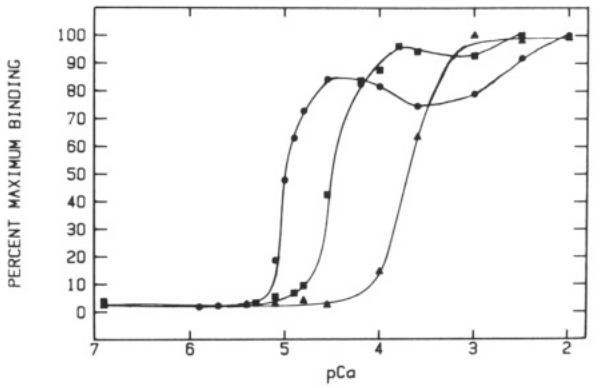

FIGURE 5: Effect of trifluoperazine on the Ca^{2+} dependence of p32 binding to membranes. The binding of ^{125}I-labeled p32 (20 nM) to chromaffin granule membranes (52 μg/mL) at varying Ca^{2+} levels was measured in the presence of zero (●), 30 μM TFP (■), or 100 μM TFP (▲) as described under Experimental Procedures. Results are expressed as a percent of maximal activity bound.

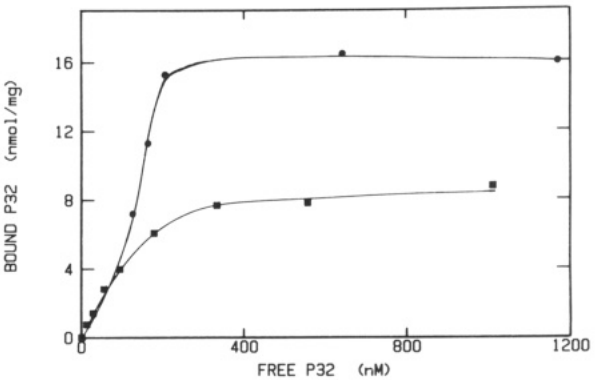

FIGURE 6: P32 Binding to chromaffin granule membranes in the presence and absence of trifluoperazine. Varying concentrations of ^{125}I-labeled p32 were bound to chromaffin granule membranes (52 μg/mL) at pCa 3.0 in the absence (●) or presence (■) of 100 μM TFP as described under Experimental Procedures.

chromaffin granule membrane at pCa 4 with a $K_{1/2}$ of 10 μM. An investigation into the mechanism of action of TFP revealed that the drug appeared to act as a competitive Ca^{2+} antagonist, as illustrated by its effects on the membrane-binding properties of p32 shown in Figures 5 and 6. The data in Figure 5 show that increasing concentrations of TFP progressively lower the protein's apparent affinity for Ca^{2+}, while the data in Figure 6 show that the extent of binding and the degree of cooperativity is reduced in the presence of TFP in a manner similar to that we have reported to occur when the Ca^{2+} concentration is reduced (Zaks & Creutz, 1990). It is unclear, however, whether the TFP is binding to the annexin or to the membrane to produce these effects.

Annexin Self-Association during Membrane Aggregation. It has previously been proposed that self-association may take place between annexin molecules bound to separate membranes (Creutz & Sterner, 1983) thus providing a mechanism for the ability of these proteins to promote membrane aggregation. Alternatively, it has been suggested that each protein may contain several membrane-binding sites and cross-link membranes as a monomer (Hong et al., 1982). In order to test whether intermembrane annexin self-association occurs when chromaffin granules aggregate, we bound fluorescein- and eosin-labeled annexins to separate membrane populations in the presence of varying Ca^{2+} concentrations and measured the extent of energy transfer between the fluorescein on one membrane surface and the eosin on another membrane surface as Ca^{2+}-dependent membrane aggregation occurred. In order

for this experiment to work, the exchange of bound proteins between membranes must be slow relative to the kinetics of membrane aggregation. To determine if this was the case, we measured the rate of dissociation of fluorescein- and eosin-labeled p32 from chromaffin granule membranes in the presence of Ca^{2+}. This was done by first measuring the energy transfer associated with the binding of fluorescein- and eosin-labeled p32 to the chromaffin granule membrane at pCa 4 and then measuring the change in energy transfer following the introduction of an excess of PS vesicles. Since the extent of energy transfer had previously been found to be less when the protein bound to the mixed population of chromaffin granule membranes and PS vesicles than when it bound to chromaffin granule membranes alone (data not shown), any dissociation of annexin from the granule membranes and rebinding to PS vesicles should have resulted in a decreased energy transfer signal. However, no change in energy transfer was observed for over 30 min following addition of PS vesicles, indicating an extremely slow rate of dissociation of chromaffin granule membrane-bound annexin. This result is consistent with the observation of others (Hong et al., 1982) who have seen a similar slow (nonmeasurable over minutes) dissociation rate of synexin from membranes composed of pure PS.

When eosin- and fluorescein-labeled p32, each prebound to a separate chromaffin granule population in the presence of varying Ca^{2+} concentrations, were mixed, energy transfer was observed (Figure 7). The kinetics of this intermembrane energy transfer were similar to those of membrane aggregation measured by turbidity changes (data not shown). This intermembrane energy transfer differs in two major ways from the energy transfer phenomenon shown in Figure 2, where the fluorophores were added simultaneously to a single chromaffin granule population before binding and aggregation were induced by Ca^{2+}. First, intermembrane energy transfer for p32 and synexin was completely inhibited at the higher Ca^{2+} levels, whereas energy transfer shown in Figure 2 was much less affected. Second, the magnitude of intermembrane energy transfer is much smaller than the energy transfer shown in Figure 2. It may be that this smaller magnitude is the result of limiting the donor–acceptor interactions to sites of intermembrane contact in Figure 7 but not in Figure 2 where these interactions would occur on membranes as well as between them.

The decline in energy transfer seen at high Ca^{2+} levels (Figure 7) does not appear to be due to a lack of interaction between extensively preaggregated membranes. First, the decline is not seen with p67 even though this protein produces a degree of membrane aggregation similar to that seen with p32 (Figure 7). Second, data similar to those seen in Figure 7 may be obtained under conditions where no membrane preaggregation occurs by analyzing the slower of the two components of the energy transfer response seen in Figure 2, which represents the sum of intra- and intermembrane energy transfer. Although we cannot entirely eliminate the possibility that the energy transfer seen in Figure 7 represents close proximity rather than actual self-association of the annexin molecules between membranes, a proximity effect appears unlikely since it does not readily explain the absence of intermembrane energy transfer seen with p32 and synexin at the higher Ca^{2+} levels where extensive membrane aggregation occurs (Figure 7a,b).

At Ca^{2+} levels greater than pCa 4, it thus appears that synexin or p32 molecules are capable of promoting intermembrane contacts by binding two membrane surfaces in a bivalent fashion. However, intermembrane annexin self-as-

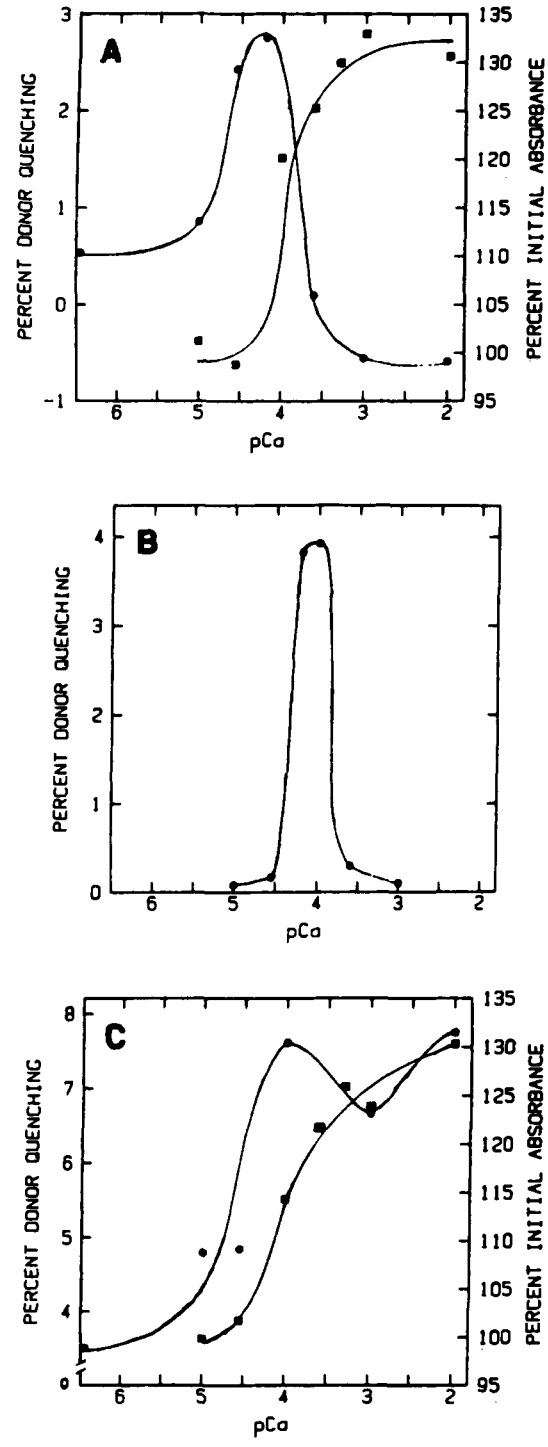

FIGURE 7: Ca^{2+} dependence of intermembrane annexin self-association and membrane aggregation. For determination of intermembrane energy transfer, two populations of chromaffin granule membranes (each 5 μg/mL) were incubated separately with equivalent amounts of fluorescein- or eosin-labeled p32 (2.33 μg/mL each, A), synexin (2.02 μg/mL each, B), or p67 (6.8 μg/mL each, C). After the fluorescence emission intensity at 510 nM was recorded from the fluorescein-containing sample (containing 15–20-fold greater volume than the eosin-containing sample), Ca^{2+} in varying concentrations was added to both samples. This produces a small decrease in fluorescein fluorescence as a result of self-quenching during self-association. When a stable fluorescence intensity was achieved (1–3 min) the eosin-labeled sample was introduced and the percent change in fluorescein quenching (●) expressed relative to the fluorescein fluorescence prior to Ca^{2+} addition was determined as a measure of resonance energy transfer efficiency. The Ca^{2+} dependence of chromaffin granule aggregation (■) was measured under identical total annexin and membrane concentrations as described under Experimental Procedures.

sociation may still play a role in promoting membrane aggregation induced by p67, as well as by synexin or p32 at Ca^{2+} levels less than pCa 4.

Discussion

A resonance energy transfer assay was developed to monitor the self-association of annexin proteins in solution and on membrane surfaces. However, this assay is subject to some limitations. Because the efficiency of energy transfer depends on the inverse sixth power of the distance between the fluorescence donor (fluorescein) and acceptor (eosin), energy transfer becomes appreciable only when these fluorophores are separated by less than the Förster critical distance, approximately 46 Å for this donor–acceptor pair. For proteins 40–60 Å in diameter, with less than 1 mol of dye/mol of protein, this implies that the efficiency of energy transfer is dependent on the location of the label with respect to the site of protein self-association. Because of the variable extent and unknown location of labeling, and the possibility of multiple sites of annexin contact, different self-association events, for example at different Ca^{2+} levels or when the proteins are free in solution versus bound to a membrane, may not yield equivalent transfer signals. Hence, the assay as used in this study provides only a qualitative or perhaps semiquantitative measure of annexin self-association.

It is also important to emphasize that the assay used in this study is specific for annexin self-association and does not detect membrane binding events as evidenced by the fact that the measured resonance energy transfer is independent of the surface density of membrane-bound annexin and is insensitive to annexin binding to PS/PE vesicles. In a related study, Tait et al. (1988) used a similar assay based on the quenching of fluorescein-labeled annexin V to measure the binding of the protein to PS/PC (1:4) vesicles. Our data suggest that Tait et al. may have been measuring annexin self-association occurring during membrane binding rather than the membrane-binding event itself. Their failure to detect cooperative binding interactions suggestive of such self-association is not unusual. In fact, we obtain data essentially identical with those of Tait et al. when we measure the fluorescein quenching associated with the interaction of p32 with PS/PC (1:3) vesicles, as shown in Figure 3a. We have also found that the binding of iodinated annexins to chromaffin granule membranes at pCa 5.0 appears noncooperative despite the presence of resonance energy transfer occurring under these conditions, suggestive of annexin self-association (Zaks & Creutz, 1990). It thus appears that modeling annexin–membrane interactions in terms of well-defined nonvariant "binding sites" may be inaccurate and fail to provide a complete picture of events occurring at the membrane surface.

The evidence presented here indicates that the annexins self-associate in solution and on some membrane surfaces. However, it is impossible to determine the size of the aggregates formed by this technique. Electron micrographs of the structures formed by synexin and Torpedo calelectrin in solution would suggest that these aggregates may be quite large (Creutz et al., 1979; Walker et al., 1983). Using chemical cross-linking, we were able to detect aggregates as large as tetramers on phospholipid vesicles. Larger structures, not detected by this technique, may also exist, as suggested by the formation of extensive two-dimensional crystals of annexins IV, V, and VI on lipid monolayers (Newman et al., 1989, 1991; Mosser et al., 1991). The enhancement of resonance energy transfer associated with membrane binding suggests that the latter event may actually promote annexin self-association. This phenomena cannot be due solely to the increased effective concentration of protein on the membrane surface, since it persists at high lipid concentrations and is sensitive to the type of lipid present. The efficiency of energy transfer was greatest (40–50%) when the proteins bound to chromaffin granule membranes or phospholipid vesicles derived from granule lipids. Less efficient (~28%) energy transfer was seen on vesicles composed of pure phosphatidylserine or phosphatidylserine/phosphatidylcholine (1:2) mixtures, while no energy transfer was seen on phosphatidylserine/phosphatidylethanolamine (1:3) vesicles. It thus appears that the extent and/or type of annexin polymerization may be regulated by membrane phospholipid composition. It remains to be seen how this occurs and whether it is physiologically important.

The ability to self-associate appears to be a general feature of molecules in the annexin class. It is likely that this property is a function of the "core" region common to all such proteins. The core of these proteins is composed of repeating 70 amino acid domains arranged in a planar array with domains I + IV and II + III, respectively, forming tightly associated modules (Huber et al., 1990a). Each domain is a cube-shaped structure formed from two helix–turn–helix substructures linked by an additional α-helix. The Ca^{2+}-binding site in each domain was interpreted to be located on the convex surface of the almost planar array in contact with the phospholipid bilayer (Huber et al., 1990b). The annexin monomers interact extensively to form two-dimensional crystals of varying geometry on lipid monolayers (Newman et al., 1989, 1991; Mosser et al., 1991). It is possible that these interactions are responsible for the intramembrane energy transfer seen in this study. However, additional modes of annexin interaction are likely to account for the observed intermembrane energy transfer. To explain this mode of self-association, we postulate that the concave surfaces of the two-domain modules also interact. At low Ca^{2+} concentrations, such interactions may occur between annexins bound to opposing membranes. At higher Ca^{2+} levels, however, each four-domain annexin may undergo a conformational change bringing its own two modules into a similar, but intramolecular, association. The formation of such a structure would prevent the formation of similar associations between different annexins on opposing membranes. The reorientation of the calcium- and phospholipid-binding sites would allow an annexin monomer to simultaneously bind two membrane surfaces. This model would explain the membrane-aggregating ability of the four-domain annexins seen at high Ca^{2+} concentrations under conditions where no intermembrane energy transfer was observed. The eight-domain annexin, p67, might be unable to undergo this intramolecular conformational change since its moveable modules are physically connected, thus perhaps accounting for the persistence of intermembrane complex formation seen at high Ca^{2+} concentration with this protein. Additional structural and dynamic information will be needed to determine if this model is correct.

Self-association appears to play an important role in annexin function in vitro. Although cooperative membrane-binding interactions between annexins may occur through several mechanisms, including indirect effects through the lipid bilayer, it is likely that direct protein self-association is a major contributor to this phenomenon since there is a good correlation between cooperative binding and self-association (e.g., Figure 6). Annexin self-association may also be important for the membrane-aggregating property of these proteins. Although an annexin monomer may bind two membranes, self-association may serve to aggregate annexins at the site of intermembrane contact and strengthen, through cooperative effects, the adhesive interaction between the bilayers. This theory is

consistent with the data of Meers et al. (1988), which suggests that synexin polymers are more active than monomers in promoting aggregation of phospholipid vesicles. At the Ca^{2+} concentrations used in the Meers et al. (1988) study (2 mM) only intramembrane polymers would be expected to form. Self-association may also be important for membrane aggregation at saturating annexin concentrations where divalent membrane-binding modes might be prohibited.

The physiological significance of annexin self-association remains to be determined. Although the Ca^{2+} levels required for this phenomenon are quite high, they are similar to those observed for the binding of these proteins to membranes. Since the latter's Ca^{2+} dependence is sensitive to membrane lipid composition (Zaks & Creutz, 1990; Schlaepfer et al., 1987) and perturbation of the protein's amino-terminal tail (Powell & Glenney, 1987; Glenney & Zokas, 1988), it is not inconceivable that the Ca^{2+} dependence of self-association may be similarly modulated so as to occur at more physiological Ca^{2+} levels. It is unknown whether annexin proteins self-associate in the cell in a manner similar to that seen with other membrane components such as spectrin. It is possible, however, that such self-association may account for the punctate distribution of some annexins seen by immunofluorescence (Zokas & Glenney, 1987) and immunoelectron microscopy (Nakata et al., 1990). It is also possible that annexin self-association is an in vitro manifestation of the exposure of a hydrophobic protein-binding site of broad specificity. Perhaps this site is the same one that enables some annexins to bind F-actin and spectrin as well as a variety of other proteins (Cheney & Willard, 1989). Consistent with this idea is the observation that the Ca^{2+} dependencies of both self-association (this study) and F-actin binding (Saris et al., 1986) are lowered in the presence of acidic phospholipids. An additional indication of the broad specificity of the self-association site is the apparent ability of the annexins to undergo "heterologous" association between different members of the annexin family leading to cooperative membrane binding phenomena (Zaks & Creutz, 1990). Perhaps the annexins are analogous to calmodulin in this respect and are capable of interacting in a semiselective manner with several macromolecules so as to amplify and distribute a Ca^{2+} signal at the cell membrane.

Registry No. Ca, 7440-70-2.

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The Carboxyl Modifier 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) Inhibits Half of the High-Affinity Mn-Binding Site in Photosystem II Membrane Fragments†

Christopher Preston‡ and Michael Seibert*
Photoconversion Research Branch, Solar Energy Research Institute, Golden, Colorado 80401
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ABSTRACT: The diphenylcarbazide(DPC)/Mn^{2+} assay [Hsu, B.-D., Lee, J.-Y., & Pan, R.-L. (1987) *Biochim. Biophys. Acta 890*, 89-96] was used to assess the amount of the high-affinity Mn-binding site in manganese-depleted photosystem II (PS II) membrane fragments from spinach and *Scenedesmus obliquus*. The assay mechanism at high DPC concentration was shown to involve noncompetitive inhibition of only half of the control level of DPC donation to PS II by micromolar concentrations of Mn at pH 6.5 (i.e., one of two DPC donation sites is inhibited). At low DPC concentration both DPC and Mn^{2+} donate to PS II additively. Treatment with the carboxyl amino acid modifier 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) inhibited half of the high-affinity Mn-binding site in spinach and *Scenedesmus* WT PS II membranes and all of the available site in *Scenedesmus* LF-1 mutant PS II membranes. A similar EDC concentration dependence was observed in all cases. Addition of 2 mM $MnCl_2$ to the 10 mM EDC modification buffer provided complete protection for the Mn-binding site from modification. This protection was specific for Mn^{2+}; six other divalent cations were ineffective. We conclude that EDC modifies that half of the high-affinity Mn-binding site that is insensitive to the histidine modifier diethyl pyrocarbonate (DEPC) [Seibert, M., Tamura, N., & Inoue, Y. (1989) *Biochim. Biophys. Acta 974*, 185-191] and directly affects ligands that bind Mn. The effects of EDC and DEPC that influence the high-affinity site are mutually exclusive and are specific to the lumenal side of the PS II membrane. Removal of the two more loosely bound of the four functional Mn from PS II membranes uncovers that part of the high-affinity site associated with carboxyl but not histidyl residues. We suggest that carboxyl residues on reaction center proteins are associated with half of the high-affinity Mn-binding site in PS II and are involved along with histidine residues in binding Mn functional in the O_2-evolving process.

The O_2-evolving system of photosystem II (PS II)[1] is a metalloenzyme containing four Mn per reaction center. These Mn atoms are believed to form a complex that stores the oxidizing equivalents required for the water oxidation process in a concerted fashion and serves as the catalytic site for O_2 evolution [see Amesz (1983), Dismukes (1986), Babcock (1987), and Hansson and Wydrzynski (1990) for reviews]. Considerable effort has been expended on elucidating the nature of this complex, and while significant progress has been

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*Address correspondence to this author.
‡Current address: Department of Crop Protection, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond S.A. 5064, Australia.

[1] Abbreviations: Chl, chlorophyll; D, redox-active tyrosine 160 on the D2 reaction center protein; DCIP, 2,6-dichlorophenolindophenol; DEPC, diethyl pyrocarbonate; DPC, 1,5-diphenylcarbazide; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; LF-1, low fluorescence mutant of *Scenedesmus obliquus*; MES, 4-morpholineethanesulfonic acid; PS II, photosystem II; Tris, tris(hydroxymethyl)-aminomethane; WT, wild type; Z, redox-active tyrosine 161 on the D1 reaction center protein.

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