Annexin XII Forms Calcium-Dependent Multimers in Solution and on Phospholipid Bilayers: A Chemical Cross-Linking Study†

William S. Mailliard,‡ Hartmut Luecke,‡,§ and Harry T. Haigler*,‡

Department of Physiology and Biophysics and Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697

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ABSTRACT: The annexins are a family of proteins that bind in a Ca^{2+}-dependent manner to phospholipids that are preferentially located on the intracellular face of plasma membranes. Recent X-ray studies of hydra annexin XII showed that it crystallized as a homohexamer with an intermolecular Ca^{2+} binding site separate from the type II Ca^{2+}-dependent phospholipid binding site. On the basis of this hexamer structure, a novel mechanism was proposed to explain how annexins interact with membranes. The first step toward evaluating this proposal is to determine whether the annexin XII hexamer exists when the protein is not in a crystalline form. We now report that annexin XII in solution can be cross-linked with dimethyl suberimidate into multimers with apparent M_r's corresponding to trimers and hexamers as determined by SDS−polyacrylamide gel electrophoresis—the trimer band may correspond to incompletely cross-linked hexamers. Multimer formation was dependent on Ca^{2+} and was enhanced when the protein first was bound to phospholipid vesicles. To evaluate the role of the intermolecular Ca^{2+} site in annexin XII hexamer formation, one of the residues used to coordinate Ca^{2+}, glutamate 105, was replaced with lysine (E105K). In solution, the E105K mutation inhibited hexamer formation in the presence of moderate (3 mM) but not high (25 mM) Ca^{2+}. No inhibition of E105K annexin XII hexamer formation was observed in the presence of phospholipid, thereby suggesting that either (i) other interactions are capable of stabilizing the hexamer when bound to bilayers or (ii) only trimers form on bilayers and the observed hexamer bands were due to cross-linking of closely packed trimers. In summary, this study shows for the first time that annexin XII can form hexamers in solution and implicates the intermolecular Ca^{2+} site in hexamer formation. This study also shows that multimers form on bilayers but does not clearly establish whether the multimers are trimers or hexamers.

The annexins are a multigene family of proteins that share approximately 40−60% amino acid sequence identity and the property of high-affinity binding to certain negatively charged phospholipids that are enriched on the intracellular face of plasma membranes. Although annexins have been implicated in a number of biological processes involving membranes, including vesicular trafficking (*1, 2*) and ion conductance across membranes (*3, 4, 5*), their biological functions have not been clearly defined [see Moss (1992) for a review]. Because of the inherent complexities of studying annexin biology in mammals, we have used *H. vulgaris* as a simple animal model system in which to investigate annexin function. We have focused on hydra annexin XII which has approximately 50% amino acid sequence identity with vertebrate annexins (*6*), is a substrate for protein kinase C (*6*), and is selectively expressed in epithelial battery cells in tentacles (*7*).

Though little is known of function, a wealth of detailed information is emerging concerning the 3-D structures of annexins. X-ray crystallographic studies of annexin V provided the initial blueprint of annexin structure (*8*) and identified the "Type II" Ca^{2+} site that mediates phospholipid binding (*9, 10*). Later studies showed that annexins I (*11*), II (*12*), III (*13*), VI (*14*), and XII (*15*) fold in a manner highly homologous to annexin V.

Although the tertiary structures of all annexins studied by crystallography to date were very similar, only annexin XII crystallized as a hexamer (*15*). The annexin XII hexamer formed a biconcave disk with a prominent central hydrophilic pore. Six intermolecular Ca^{2+} sites were observed at the trimer−trimer interface of the hexamer. On the basis of the hexamer structure, we proposed that the annexin hexamer inserts symmetrically into the bilayer (*15*). The model offers plausible explanations for annexin-induced vesicle aggregation and ion channel formation (*15, 16*). Since several of the key residues apparently involved in annexin XII hexamer formation are highly conserved in other annexins, it is reasonable to speculate that other annexins may also form hexamers under the appropriate conditions.

It is now important to determine whether the hexamer is a functional form of annexin XII in intact cells or simply the product of crystal packing forces. The first step toward this goal is to determine whether annexins can form hexamers in solution and on phospholipid bilayers. Although current models of annexin structure−function are dominated by the assumption that the proteins are monomeric, some degree of self-aggregation in solution has been reported for annexins

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* To whom correspondence should be addressed at the Department of Physiology and Biophysics, University of California, Irvine, CA 92697. Telephone: 714-824-6304. Fax: 714-824-8540. E-mail: hhaigler@uci.edu.

‡ Department of Physiology and Biophysics.

§ Department of Molecular Biology and Biochemistry.

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I (*17*), II (*17*), IV (*17, 18*), V (*17, 19, 20*), VI (*17, 18*), and VII (*18, 21*). In addition, electron microscopy identified an annexin V trimer in two-dimensional crystals on the surface of phospholipid monolayers (*22*). The annexin V trimer was similar to individual trimers in the annexin XII hexamer, and it is possible that annexin V hexamers were not detected by electron microscopy because monolayers were used instead of bilayers. In fact, previous chemical cross-linking studies of annexin V bound to phospholipid vesicles did detect the formation of oligomers that appeared to correspond to trimers and hexamers (*19*). In this report, we used the chemical cross-linking methods developed by Concha *et al.* (1992) to investigate Ca^{2+}-dependent multimer formation by annexin XII in solution and bound to phospholipid bilayers.

MATERIALS AND METHODS

Subcloning and Mutagenesis of the Annexin XII Gene. Polymerase chain reaction with pfxblue-p33H (*6*) as a template was used to recreate the annexin XII start methionine, resulting in an Nco 1 restriction site. The resulting annexin XII DNA was digested with *Nco*I and *Hin*dIII and subcloned into pSE420 to create pSE420-mrp33H (start methionine recombinant protein 33 kDa from hydra). The intermolecular Ca^{2+} site of annexin XII was disrupted by converting glutamate 105 to lysine using the single-site mutagenesis kit from ClonTech (Palo Alto, CA). All sequences were confirmed by dideoxy nucleotide sequencing.

Protein Purification. Recombinant annexin XII and the E105K[1] mutant were purified by a modification of a published procedure (*6*). DH5αf′IQ bacteria were transformed with pSE420-mrp33H or mutant expression vectors. An ampicillin-resistant colony was used to inoculate 500 mL of TB containing ampicillin (100 μg/mL) at 37 °C. When the cells reached an $OD_{600} \sim 1.0$, protein expression was induced by the addition of IPTG (0.5 mM). The cells were incubated overnight at 37 °C and then harvested by centrifugation (5000*g*, 10 min). The cells were frozen, thawed, suspended in Hepes buffer (20 mM, pH 7.4, containing 100 mM NaCl, 2 mM $MgCl_2$, 10 mg/mL leupeptin, 10 mg/mL aprotonin, 1 mM PMSF, and 1 mM EGTA), and then lysed with a French press. The insoluble matter was removed by centrifugation (100000*g*, 30 min), and the recombinant protein was isolated by reversible Ca^{2+}-dependent binding to phospholipid vesicles (*6*). The protein was further purified by size-exclusion chromatography on a G-100 column equilibrated in EPPS (25 mM, pH 8.0). Fractions were analyzed for purity by SDS−PAGE and Coomassie blue staining. Fractions containing the protein of interest were pooled, concentrated, and stored at −70 °C. This procedure typically produced 30−40 mg of purified protein per liter of recombinant bacteria culture.

Chemical Cross-Linking. Cross-linking of wild-type and mutant annexin XII was performed by modifications of methods published by Concha *et al.* (1992). Cross-linking in solution was performed in EPPS buffer (25 mM, pH 8.0) containing the indicated concentrations of Ca^{2+} and dimethyl suberimidate (DMS), 5% (v/v) 2-mercaptoethanol, and 40 μg of annexin in a final volume of 20 μL. Cross-linking in the presence of phospholipid vesicles [9 μg/μL; dioleoylphosphatidylglycerol/phosphatidylcholine, 2:1; prepared by the method of Reeves and Dowben (*23*)] was performed in EPPS buffer (20 mM, pH 8.0) containing NaCl (100 mM), $MgCl_2$ (2 mM), and 10 μg of annexin in a final volume of 30 μL. For both types of reactions, a 200 mM stock solution of DMS was freshly prepared in EPPS buffer (25 mM, pH 8.0). The reactions were allowed to proceed for 3 h at room temperature, and the products were analyzed by 7.5% SDS−PAGE and Coomassie blue staining.

CD Spectra. Circular dichroism spectra were obtained using a Jasco J-720 spectropolarimeter with a 0.05 cm path length cell and a band-pass of 2 nm. Fifteen scans were collected at ambient temperature and averaged. Protein (10 μM) was suspended in Tris-HCl buffer (10 mM, pH 7.8).

Measurement of Phospholipid Binding. Annexin binding to phospholipid was analyzed by measuring the ability of the protein to copellet with vesicles [phosphatidylserine/phosphatidylcholine, 2:1; prepared by the method of Reeves and Dowben (*23*)] during centrifugation as previously described (*6*). Using this method, no detectable binding to vesicles of either wild-type annexin XII or the E105K mutant was observed in the absence of Ca^{2+}, and greater than 95% of either protein was bound in the presence of 500 μM Ca^{2+} (data not shown).

RESULTS

Previous X-ray crystallography studies detected a homohexamer of annexin XII in the asymmetric unit (*15*) but could not rule out that the hexamer was a result of crystal packing forces. The experiments described below used chemical cross-linking to determine whether annexin XII formed multimers either in solution or when bound to phospholipid bilayers.

Chemical Cross-Linking of Annexin XII in Solution. Annexin XII was incubated with increasing concentrations of the homobifunctional cross-linking reagent DMS in the presence and absence of Ca^{2+}. Cross-linking was evaluated by SDS−PAGE followed by Coomassie blue staining. In the absence of Ca^{2+}, only monomeric annexin XII (apparent $M_r = 33\,000$) and traces of other bands were detected (Figure 1, lanes f−h). In the presence of Ca^{2+} (25 mM), major bands were detected that had apparent M_r's of ∼95 000 and ∼155 000, corresponding to the approximate size of annexin XII trimers and hexamers, respectively (Figure 1, lanes a−e). We assume that the apparent M_r's of the putative trimer and hexamer bands were smaller than expected based on their calculated M_r's because the cross-linked protein was more compact and therefore migrated faster during electroporesis.

Experiments using the optimal concentration of DMS showed that the half-maximal formation of putative trimer and hexamer bands in solution occurred at approximately 1 mM Ca^{2+} and was maximal at 3 mM Ca^{2+} and above (Figure 2). Although only a small percentage of the protein was present in the putative trimer/hexamer bands, the limitations of this method do not allow us to determine the actual percentage of multimers in solution because the efficiency of cross-linking is not known. Likewise, it is possible that the putative trimer band corresponds to an incompletely cross-linked hexamer. Other studies using small-angle X-ray diffraction indicate that a high percent of annexin XII forms hexamers at high Ca^{2+} concentrations in solution (H. T.

[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; DMS, dimethyl suberimidate; E105K, annexin XII lysine 105 to glutamate mutation.

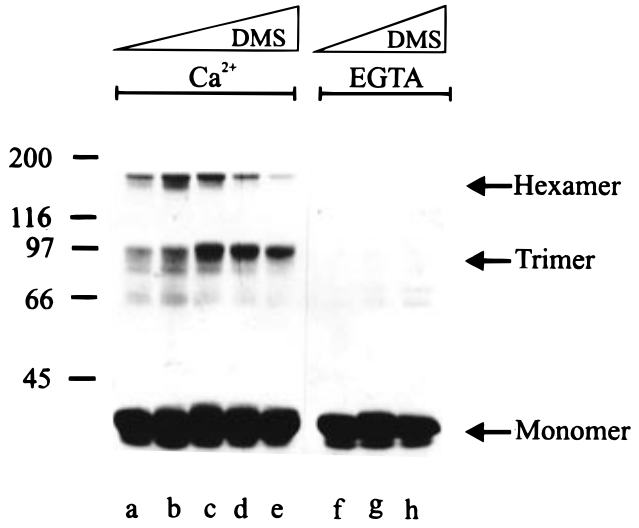

FIGURE 1: Chemical cross-linking of annexin XII in solution. Annexin XII was incubated with increasing amounts of the chemical cross-linker in the presence of 25 mM Ca^{2+} (lanes a−e) or 0.05 mM EGTA (lanes f−h) as described under Materials and Methods. The following concentrations of DMS were used: lanes a and f, 1.8 mM; lane b, 3.8 mM; lanes c and g, 15 mM; lanes d and h, 22 mM; lane e, 33 mM. The chemically cross-linked proteins were separated on a 7.5% SDS−PAGE, and the proteins were visualized by Coomassie blue staining. The migration of standard proteins of the indicated molecular mass (kDa) is noted to the left of the figure.

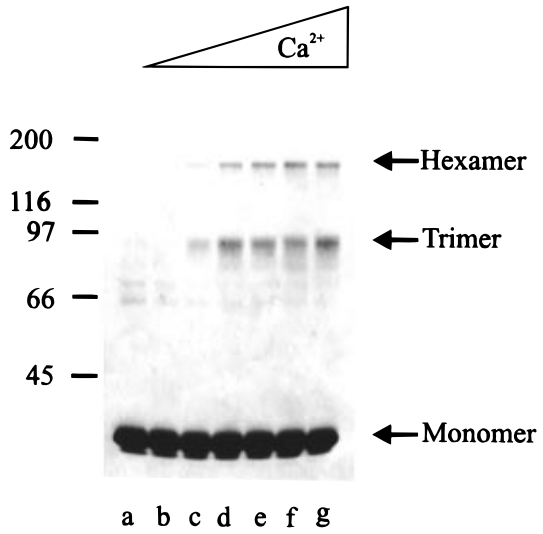

FIGURE 2: Ca^{2+}-dependent cross-linking of annexin XII. Annexin XII was incubated in the presence of 15 mM DMS and increasing concentrations of Ca^{2+} as described under Materials and Methods. Lane a, 0.05 mM EGTA; lane b, 0.5 mM Ca^{2+}; lane c, 1 mM Ca^{2+}; lane d, 3 mM Ca^{2+}; lane e, 6 mM Ca^{2+}; lane f, 12 mM Ca^{2+}; lane g, 25 mM Ca^{2+}. The chemically cross-linked proteins were separated on a 7.5% SDS−PAGE and the proteins visualized by Coomassie blue staining. The migration of standard proteins of the indicated molecular mass (kDa) is noted to the left of the figure.

Tsurata, W. S. Mailliard, H. T. Haigler, and H. Luecke, unpublished results).

Chemical Cross-Linking of Annexin XII Bound to Phospholipid Vesicles. To determine whether annexin XII formed multimers when bound to phospholipid bilayers, cross-linking was analyzed in the presence of vesicles containing phosphatidylglycerol/phosphatidylcholine (2:1). The experiments were performed at concentrations of Ca^{2+} and annexin XII that were too low to promote cross-linking in solution in the absence of vesicles (data not shown). As expected, no

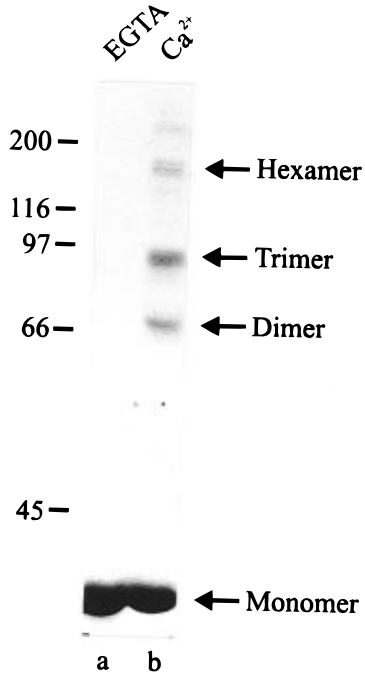

FIGURE 3: Chemical cross-linking of annexin XII bound to phospholipid vesicles. Annexin XII was incubated with phospholipid vesicles in the presence of 0.05 mM EGTA (lane a) or 1 mM Ca^{2+} (lane b) and reacted with DMS as described under Materials and Methods. The chemically cross-linked proteins were separated on a 7.5% SDS−PAGE and the proteins visualized by Coomassie blue staining. The migration of standard proteins of the indicated molecular mass (kDa) is noted to the left of the figure.

bands corresponding to multimers were observed following incubation with DMS in the presence of vesicles but the absence of Ca^{2+} (Figure 3; lane a). However, when annexin XII was incubated with vesicles in the presence of Ca^{2+} (1 mM) under conditions where greater than 95% of the protein was bound to the vesicles, the membrane-permeant cross-linker DMS induced the formation of putative trimer and hexamer bands (Figure 3; lane b). The banding pattern was similar to that observed when annexin XII was cross-linked in solution except the following subtle differences were noted: the yield of multimeric bands was higher; the multimeric bands were broader and sometimes could be resolved into multiple closely spaced bands; and a prominent band with the mobility of a dimer was observed. It is not known whether the dimer and trimer bands were derived from an incompletely cross-linked annexin XII hexamer. It is also possible that the observed hexamer bands resulted from cross-linking of adjacent trimers on the surface for the bilayer—we think this is of concern on vesicles but not in solution because the protein is sequestered at high density on the vesicles.

Evaluation of a Site-Directed Mutant of the Annexin XII Intermolecular Ca^{2+} Site. These chemical cross-linking experiments show that annexin XII formed a Ca^{2+}-dependent hexamer in solution and that multimer formation was promoted by phospholipid vesicles. Thus, the hexamer seen in the crystal structure might be a physiologically relevant form of the protein. To determine whether the intermolecular Ca^{2+} site observed by crystallography was involved in hexamer formation in solution and on vesicles, one of the residues involved in formation of the site, glutamate 105, was changed to lysine (E105K). The E105K mutant annexin XII retained the ability to bind in a Ca^{2+}-dependent manner

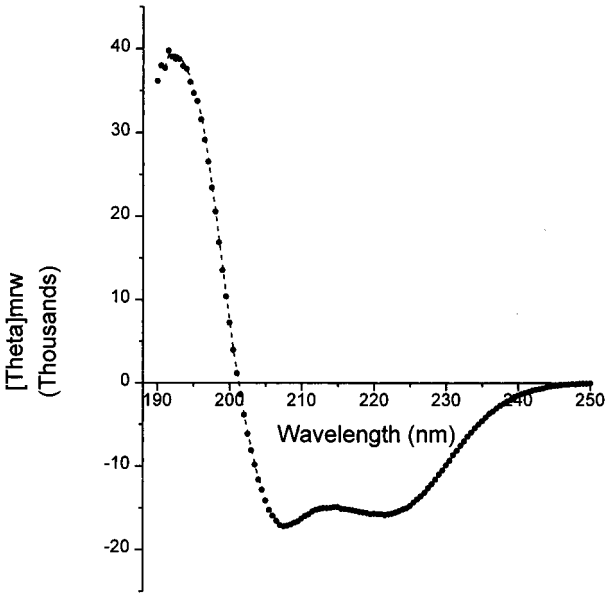

FIGURE 4: Circular dichroism spectra of wild-type and E105 K annexin XII. Circular dichroism spectra were obtained for annexin XII (−) and the E105K mutant (●) in the absence of Ca^{2+} as described under Materials and Methods.

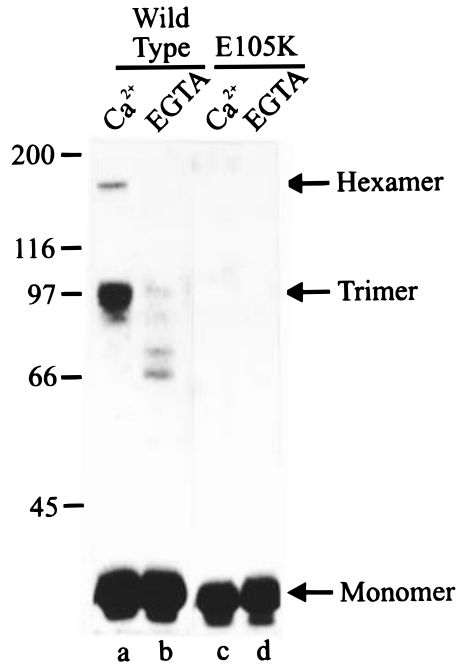

FIGURE 5: Chemical cross-linking of wild-type and E105K annexin XII in solution. Wild-type annexin XII (lanes a and b) and the E105K mutant (lanes c and d) were reacted with DMS (15 mM) in the presence of 3 mM Ca^{2+} (lanes a and c) or 0.05 mM EGTA (lanes b and d) as described under Materials and Methods. The chemically cross-linked proteins were separated on a 7.5% SDS−PAGE and the proteins visualized by Coomassie blue staining. The migration of standard proteins of the indicated molecular mass (kDa) is noted to the left of the figure.

to phospholipid vesicles (see Materials and Methods)—the retention of this activity is not unexpected because the type II Ca^{2+} binding sites that mediate vesicle binding (*10*) were not disrupted. The E105K mutant had a CD spectrum that was indistinguishable from wild-type annexin XII (Figure 4), thereby showing that the mutation did not grossly disrupt the secondary structure of the protein.

In striking contrast to wild-type annexin XII, the E105K mutant did not produce any observable DMS cross-linked multimers when incubated in solution with 3 mM Ca^{2+} in the absence of vesicles (Figure 5). However, wild-type and E105K annexin XII formed similar yields of cross-linked bands corresponding to trimers and hexamers in solution in the presence of very high concentrations of Ca^{2+} (25 mM, data not shown). In the presence of phospholipid vesicles, similar cross-linking patterns were observed for both wild-type and E105K annexin XII even at relatively low Ca^{2+} concentrations (Figure 6). The implications of these data with regard to the function of the intermolecular Ca^{2+} site are discussed below.

DISCUSSION

Previous X-ray crystallography studies showed that annexin XII formed a homohexamer that appeared to be stabilized by six Ca^{2+} ions bound to sites formed, in part, by the γ-carboxyl groups of Glu105 and Glu76 in opposing trimers (*15*). Extensive intermolecular interactions occur in the hexamer with 19% of the surface area per monomer being buried (*15*). The extent and apparent specificity of these interactions lead us to propose that the hexamer might be a biologically relevant form of the protein and not just a crystal-packing artifact. Studies reported herein provide preliminary support for this proposal. A multimer corresponding to the approximate size of the annexin XII hexamer was detected by chemical cross-linking both in solution (Figures 1 and 2) and when the protein was bound to phospholipid vesicles (Figure 3). Multimer formation was not observed in the absence of Ca^{2+} (Figures 1−3). Although we also detected Ca^{2+}-dependent cross-linked trimers of annexin XII in solution (Figures 1 and 2) and on bilayers (Figure 3), inherent limitations of the method prevent us from determining whether the trimers correspond to incompletely cross-linked hexamers. We think the data establish that annexin XII can form hexamers in solution, but more caution is required in interpretation of the cross-linking in the presence of vesicles. Previous studies of annexin V showed that it forms trimers on the surface of monolayers (*22*). If annexin XII were to form similar trimers on the surface of vesicles, it is possible that these densely packed trimers could cross-link to form the hexamer-sized bands observed (Figures 3 and 6).

Ca^{2+}-dependent annexin XII trimers and hexamers formed in relatively low yields in these assays, but limitations of the method prevent us from drawing quantitative conclusion regarding the extent of multimer formation. We suspect that the low yields were due to incomplete cross-linking because solution studies using small angle X-ray scattering indicate that annexin XII forms hexamers in high yields in the presence of high concentrations of protein and Ca^{2+} (H. T. Tsurata, W. S. Mailliard, H. T. Haigler, and H. Luecke, unpublished results).

As in the small-angle X-ray scattering studies, high concentrations of protein (~60 μM) and Ca^{2+} (~3 mM) were required in order to detect annexin XII hexamer formation in solution in the absence of vesicles in our chemical cross-linking assay (Figures 1 and 2). However, in the presence of phospholipid vesicles, cross-linked hexamer-sized bands could be detected (Figure 3) at much lower concentrations of protein (~10 μM) and Ca^{2+} (~1 mM). We speculate that in a biological context annexin XII hexamer formation would

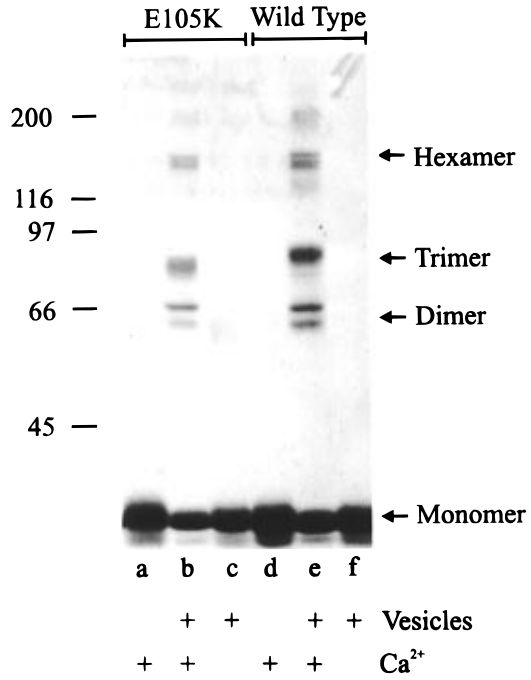

FIGURE 6: Chemical cross-linking of wild-type and E105K annexin XII bound to phospholipid vesicles. Wild-type annexin XII (lanes d, e, and f) and the E105K mutant (lanes a, b, and c) were reacted with DMS (4 mM) as described under Materials and Methods in the presence (lanes b, c, e, and f) or absence (lanes a and d) of phospholipid vesicles and either 1 mM Ca^{2+} (lanes a, b, d, and e) or 0.05 mM EGTA (lanes c and f). The chemically cross-linked proteins were separated on a 7.5% SDS−PAGE and the proteins visualized by Coomassie blue staining. The migration of standard proteins of the indicated molecular mass (kDa) is noted to the left of the figure.

not occur while the protein is free in the cytosol. If multimerization occurs in biological settings, it probably would occur following binding to the cytosolic face of the plasma membrane in response to a stimulation-dependent increase in Ca^{2+} concentration.

Previous crystal studies of annexin XII suggested that the hexamer was stabilized by a novel intermolecular Ca^{2+} site (*15*). To evaluate the role of this Ca^{2+} site in hexamer formation, we mutated one of the residues which coordinated Ca^{2+} from a glutamate to lysine (E105K). The E105K mutant did not form cross-linked trimers or hexamers in solution under conditions (3 mM Ca^{2+}) where multimerization of wild-type annexin XII was near-maximal (Figure 5). These data provide support for the involvement of this Ca^{2+} site in hexamer formation and strengthen the proposal that the cross-linked band with the apparent size of a hexamer corresponds to the hexamer observed by X-ray crystallography. The observation that the E105K mutant could be cross-linked into hexamer-sized multimers in the presence of phospholipid vesicles (Figure 6) is more difficult to interpret. It is possible that other interactions besides the intermolecular Ca^{2+} site are capable of stabilizing the hexamer in the presence of phospholipid. Alternatively, the putative hexamer band observed in the presence of vesicles might be the product of cross-linking of adjacent trimers on the surface of the vesicles. Additional experiments using more discriminating methods are required to evaluate mutimerization of annexins on bilayers.

In contrast to annexin XII, the other annexins studied to date by crystallography were not hexameric. Although trimers were not noted in the original publications, in four out of six crystal forms reported to date, trimers very similar to the trimer comprising the annexin XII hexamer were present (Brookhaven Protein Data Bank accession codes 1AVH, 1AVR, 1ANX, and 2RAN). In one of these four crystal forms, the trimer is generated by noncrystallographic symmetry (1ANX); in the other three cases, the trimers are generated by crystallographic symmetry elements. Although hexamers were not noted in the above annexin crystals, the residues that are involved in formation of the intermolecular Ca^{2+} site of the annexin XII hexamer are highly conserved in other annexins. We speculate that other annexins can also form hexamers under the appropriate conditions. In fact, a previous chemical cross-linking study detected Ca^{2+}-dependent trimers and hexamers of annexin V (*19*). Annexin V multimers were detected only in the presence of phospholipid; however, annexin V was not tested in solution at the very high concentrations of protein and Ca^{2+} that were required to induce hexamer formation of annexin XII in solution.

In summary, chemical cross-linking studies provide the first evidence that annexin XII can form hexamers outside crystals. These studies also provide preliminary evidence that the intermolecular Ca^{2+} site stabilizes hexamer formation. Although the chemical cross-linking method has severe limitations, these studies show that the hexamer forms under conditions which suggest it may be a physiologically relevant form of annexin XII. It should thus be worthwhile to investigate hexamer formation on bilayers with more discriminating methods.

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