Annexin 24 from Capsicum annuum  
X-RAY STRUCTURE AND BIOCHEMICAL CHARACTERIZATION*

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This work provides the first three-dimensional structure of a member of the plant annexin family and correlates these findings with biochemical properties of this protein. Annexin 24(Ca32) from Capsicum annuum was purified as a native protein from bell pepper and was also prepared by recombinant techniques. To overcome the problem of precipitation of the recombinant wild-type protein in crystallization trials, two mutants were designed. Whereas an N-terminal truncation mutant turned out to be an unstable protein, the N-terminal His-tagged annexin 24(Ca32) was crystallized, and the three-dimensional structure was determined by x-ray diffraction at 2.8 Å resolution. The structure refined to an R-factor of 0.216 adopts the typical annexin fold; the detailed structure, however, is different from non-plant annexins, especially in domains I and III and in the membrane binding loops on the convex side. Within the unit cell there are two molecules per asymmetric unit, which differ in conformation of the IAB-loop. Both conformers show Trp-35 on the surface. The loop-out formation is stabilized by tight interactions of this tryptophan with residue side chains of a symmetry-related molecule and enforced by a bound sulfate. Characterization of this plant annexin using biophysical methods revealed calcium-dependent binding to phospholipid vesicles with preference for phosphatidylcholine over phosphatidylserine and magnesium-dependent phosphodiesterase activity in vitro as shown with adenosine triphosphate as the substrate. A comparative unfolding study of recombinant annexin 24(Ca32) wild type and of the His-tag fusion protein indicates higher stability of the latter. The effect of this N-terminal modification is also visible from CD spectra. Both proteins were subjected to a FURA-2-based calcium influx assay, which gave high influx rates for the wild-type but greatly reduced influx rates for the fusion protein. We therefore conclude that the N-terminal domain is indeed a major regulatory element modulating different annexin properties by allosteric mechanisms.

Annexins have been a focus of research for nearly 20 years, and a large amount of data has accumulated especially for mammalian members of this protein family although annexin proteins are abundant throughout all species but yeast. In 1989, the first annexin-like proteins in plants were identified (1), and it is now well established that annexins are just as ubiquitous in the plant kingdom as elsewhere. The amount of annexin in plant cells makes up to 0.1% of the total protein content (2). As deduced from amino acid sequences, plant-type and vertebrate annexins show similarities up to 40%, whereas the plant-type members share up to 97% similarity with each other (see Table I). A more detailed analysis of the phylogenetic aspects led to the conclusion that within the overall annexin family a unique subset is made up by the plant-type members. In contrast to mammalian and vertebrate tissues, where varying amounts of different annexin proteins are expressed, plants possess only two different annexins, often described as p32/p35/p38 for bell pepper (p34/p35 for tomato, p33/p35 for maize, etc.). Following a recent classification, we are denoting annexin 24(Ca32) as the name for the annexin p32 from Capsicum annuum. Due to their high sequence similarity, it was supposed recently that three annexins from tobacco, tomato, and bell pepper, namely Anx(Nt32), Anx(Le34), and Anx24(Ca32), might constitute a distinct type of annexins, which were called Sp32 annexins (3) (see Table I).

Structurally, the highly conserved annexin core is made up by four similar domains with about 70 amino acids each. From comparison of primary structures between vertebrate and plant-type annexins, the most prominent differences are observed in the loop regions between the second and third domain and within the third domain (Fig. 1). The putative calcium binding sites of plant annexins are of particular interest, as in most cases the typical endonexin fold (4) is only conserved within the first domain. The interaction of these annexins with calcium and calcium/phospholipids is therefore expected to be different from that of the known vertebrate annexins.

In recent publications (5, 6) annexins from Medicago sativa and Lycopersicon esculentum were reported to have the conserved endonexin fold present in two domains. The calcium dependence of phospholipid binding of the tomato annexin was investigated, including the effects of mutations within the (putative) membrane binding loops (6). This study also confirmed the ability of tomato annexin to interact with membranes in a calcium-dependent manner; however, much higher calcium concentrations were required than in the case of vertebrate annexins.

The present study was aimed at elucidation of structure-function relationships of bell pepper annexin 24(Ca32) using x-ray crystallography and various biophysical methods. The first three-dimensional structure of a plant annexin shows clearly the high structural conservation of the annexin fold for members of this protein family throughout all kingdoms. How-
ever, the membrane binding mechanism is significantly different from other annexin proteins. The structure presented here particularly demonstrates the unique feature of a membrane binding loop in the first domain of annexin 24(Ca32) being able to adopt different conformations similar to the IIIAB-loop of annexin V. Moreover, the attachment of the N-terminal region to the protein core, as observed in the present structure, has not been observed in any other annexin crystal structure to date.

**EXPERIMENTAL PROCEDURES**

**Purification of Native Protein**—Bell pepper leaves and fruits of mature plants were washed, crushed manually, and ground in a Waring Blender by adding glass pearls (0.25–0.5 mm). Per 1 kg of raw material, 1 liter of buffer A (0.15 M NaCl, 5 mM benzamidinium hydrochloride, 10 mM EDTA, 2 mM β-mercaptoethanol, 0.25 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml DNase, 0.1 mg/ml RNase, 10 mM HEPES, pH 7.4) was added. To prevent heating, liquid nitrogen was added to the blender. Glass pearls and solid components were separated by filtration over a Perlon net, and the resulting suspension was centrifuged for 1 h at 100,000 g. The supernatant was dialyzed against PEG 40,000 to concentrate it, again dialyzed against buffer B (5 mM benzamidinium hydrochloride, 2 mM β-mercaptoethanol, 0.25 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml DNase, 0.1 mg/ml RNase, 10 mM HEPES, pH 7.4) and D5 buffer (10 mg/ml Chelex (Sigma) in D4 buffer).

**Purification of Recombinant Protein**—C. annuum annexin 24(Ca32) was subcloned from the clone E511 (7) by polymerase chain reaction using primers C7 (5'-TCTAACCGTTCCAGC-3') and C2 (5'-d(CCGGAATTCAGTCCTCCTC- TTGTCACCAGC)-3') and C2 (5'-GCGGAATTCAGTCCTCCTC- TTGTCACCAGC-3') and C2 yielded the His-tag fusion protein. Best results were obtained using Pfu polymerase and 10% Me₂SO in the polymerase chain reaction mixture. The amplified DNA was ligated into pRSET 5d (8) via NcoI and EcoRI restriction sites. Positive clones were obtained by transformation into Escherichia coli DH5α, selection via ampicillin resistance and DNA sequencing. Expression was carried out in E. coli BL21(DE3). A 1-liter culture of transformed BL21(DE3) was grown overnight at 37 °C in LB medium containing 50 mg/liter ampicillin. The overnight culture was diluted into 10 liters of LB medium (50 mg/liter ampicillin) and grown at 37 °C until the absorbance at 600 nm reached values above 1.0. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 0.5 mM, and the concentration of ampicillin was increased 4-fold. Cell growth was continued for 4 h.

The protein purification is based on reversible calcium-dependent binding of annexins to phospholipid membranes and follows well documented protocols (9). After harvesting the cells and preparing spheroplasts by resuspending the cell pellet in diluted D2 buffer (750 mM sucrose, 0.5 mM EDTA, 200 mM Tris, pH 8.0) (1:1), the spheroplasts were lysed by osmotic shock in low salt D1 buffer (3 mM MgCl₂, 100 mM NaCl, 5 mM benzamidinium chloride, 1 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml pepstatin A, 0.1% Triton X-100, 20 mM Tris, pH 8.0). Insoluble components were separated by overnight Ultracentrifugation at 100,000 g. The supernatant was applied to liposomes made from bovine brain extract (Sigma) and resuspended in liposome buffer (100 mM NaCl, 3 mM MgCl₂, 20 mM Tris, pH 8.0) containing 20 mM calcium. The protein was detached from the vesicles using D4 buffer (15 mM EDTA, 100 mM NaCl, 10 mM MgCl₂, 20 mM Tris, pH 8.0) and D5 buffer (10 mg/ml Chelex (Sigma) in D4 buffer).

**Crystallization**—Protein from the native purification grew in different crystal forms, small square boxes or cubes, large plates, and paralelepipedic boxes. Crystallization was achieved with recombinant Anx24(Ca32) and a broad variety of different screenings did not succeed, as precipitation and spherolytic deposition occurred. 150 mg of protein were used for crystallization trials with 40 µg of recombinant annexin 24(Ca32)/drop. From the recombinant fusion protein His-Anx24(Ca32), hexagonal prisms and cubes grew within 6 to 8 weeks from 1.7M (NH₄)₂SO₄, 0.1 M sodium acetate, pH 5.0, 30% glycerin). Aimed for higher resolution, a data set at beamline BW6 (DESY, Hamburg) was collected at a wavelength of λ = 1.07 Å with a MAR CCD detection system. Data analysis was performed with the MOSFLM program (10) and data reduction was performed with SCALa from the CCP4 program suite (11). Statistics for different data sets are summarized in Table II.

**Spacegroup Determination and Patterson Search Methods**—Indexing of diffraction patterns was successful with a trigonal space group.
p-Chloromercury benzoic acid.

| Derivative | A24_1 | A24_2 | A24_3 | A24_4 | A24_5 | A24_6 | A24_7 |
|------------|-------|-------|-------|-------|-------|-------|-------|
| Crystals   | Native | Native | Native | Native | Native | Native | Native |
| X-ray source | In-house | In-house | In-house | In-house | In-house | In-house | In-house |
| Cryo       | No     | No     | No     | No     | No     | No     | No     |
| Cell constants (in Å) | 98.15, 98.15 | 103.16, 103.16 | 101.12, 101.12 | 99.05, 99.05 | 101.13, 101.13 | 100.71, 100.71 |
| Max. resolution | 3.9 Å | 3.7 Å | 3.7-5.0 Å | 4.0 Å | 3.7 Å | 3.5 Å |
| No. of measurements (I > 2σ) | 55,561 | 28,972 | 40,123 (3.7 Å) | 33,003 | 24,500 | 42,344 |
| No. of independent reflections | 7,630 | 8,230 | 4,262 | 7,812 | 5,532 |
| Completeness | 82.3% | 55.8% | 86.5% | 87.1% | 45.4% |
| Redundancy | 2.4 | 1.6 | 2.1 | 2.0 | 2.5 |
| Rmerge (%) | 12.6% | 15.5% | 14.2% | 32.5% | 31.3% |

\[ R_{merge} = \frac{\sum I(k) - \langle I \rangle}{\sum I(k)} \]

Starting with P3, self-rotation functions were calculated with GLRF (12), indicating the presence of crystallographic 2-fold axes (\( \kappa = 180^\circ \)) at \( \phi = 30^\circ \) and \( 90^\circ \). Additionally, the intensities of reflections along the direction [00l] show high values only for \( l = 2n+1 \), indicating the presence of crystallographic 2-fold axes (12). Therefore the space group was determined to be either P3\(_2\)1 or P3\(_2\)2\(_1\). The Matthews coefficient of molecules; therefore, a first 2\(_d\) direction [00l] show high values only for \( l \) even, indicating the presence of crystallographic 2-fold axes (12), suggesting the presence of two monomers.

For the Patterson search, a library of 81 annexin models was constructed consisting of annexins I, III, IV, V, VIa, VIb, and XII as full models, poly(Ala) models either with or without the N-terminal domain, and single modules (domains I/IV and II/III). The calculations were carried out with the programs AMoRe (13) and BLANC (14); however, only the former gave suitable results with poly(Ala)-annexin VIa (without the N-terminal tail) as the search model. Also at this stage, it became evident that the final space group had to be P3\(_2\)2\(_1\) (highest correlation coefficient: 0.634), since there was no equivalent Patterson solution available, model building and refinement were carried out with these data. Several rounds of refinement with inspection and manual adjustment of the model were performed at a resolution range of 12.0 to 3.0 Å.

For further refinement, only the CNS program was used. Iteratively, the model was subjected to simulated annealing slow cool protocols in torsion space, positional refinement, and B-factor refinement using the parameter set derived from Engh and Huber (20). NCS restraints were released slowly, and the resolution range was increased to 50, 2.8 Å. The observed structure factors were scaled anisotropically (tensor: \( B_{11} = -9.624; B_{22} = -9.624; B_{33} = 19.247; B_{12} = -7.677; B_{13} = 0.000; B_{23} = 0.000 \)), and a bulk solvent correction was applied (limiting resolution 6.0 Å). \( 2F_o - F_c \) and \( F_o - F_c \) difference maps were calculated for inspection and model building on an Evans and Sutherland graphics terminal with the program FRODO (16) and also O (19) running on an SGI indigo2. Model geometry was verified with PROCHECK (21). Refinement results are summarized in Table III. Coordinates and structure factors have been submitted to the Protein Data Bank with access code 1dk5.

**Mass Spectrometry**—Mass spectra of diluted acidified samples (~1 mg/ml) were obtained from a Perkin-Elmer API 165 mass spectrometer equipped with an electrospray application device. The native purified annexin 24(Ca32) yielded a molecular mass of 35,845 g/mol (theoretical: 35,850 g/mol). For the recombinant proteins, values of 35,759 g/mol were obtained for annexin 24(Ca32) wild type and 36,790 g/mol for the His-tagged fusion protein. This indicates that in both cases the starting methionine is processed, and a calcium (Anx24 wild-type) or two sodium ions (His-Anx24) are associated with the proteins.

**Fluorescence Spectroscopy**—Fluorescence spectra of recombinant annexin 24(Ca32) wild type and fusion protein were recorded on a Perkin-Elmer LS 50B using a 500-μl sample with 0.3 mM to 0.5 mM protein in 5 mM Tris, pH 8.0. For elucidation of calcium-dependent annexin interaction with liposomes, the samples additionally contained PS/PE (3:1) liposomes with a total lipid content of 0.5 nmol and the appropriate concentration of CaCl\(_2\). Circular Dichroism—To elucidate the secondary structure of recombinant annexin 24(Ca32) and the fusion protein His-Anx24, their circular dichroic spectra were recorded with a Jobin-Yvon Auto-Dichrograph Mark IV. The samples had a concentration of 0.15 mg/ml in 5 mM Tris, pH 8.0.
vesicles was elucidated by a centrifugation assay. A sample with the dependent binding of recombinant annexin 24(Ca32) to phospholipid was incubated for 15 min and then centrifuged for 30 min at 130,000 r.p.m., root mean square. Calcium influx is characterized by the slopes 

$$
\gamma = \sqrt{\left(\frac{\alpha}{1 \text{ min}}\right)^2 + \left(\frac{\beta}{1 \text{ min}}\right)^2}
$$

(Eq. 1)

The activity parameter \( \gamma \) of each measurement was normalized with respect to a control measurement without protein (\( \gamma_0 \)). We expressively note that the assay is validated by the above-mentioned stability assay. Experimental procedures follow the protocols described (23). All experiments were carried out three times independently.

Test of ATPase Activity—To test ATPase activity of recombinant annexin 24(Ca32), 10 \( \mu \)g of protein were incubated in ATPase buffer (3 mM MgCl\(_2\), 50 mM KCl, 5 mM MES, pH 6.0) for 5 min at room temperature. To start the reaction 3 mM Na\(_2\)ATP were added to yield a final sample volume of 200 \( \mu \)l. After incubation for either 0.5, 1, and 2 h at room temperature, the enzyme reaction was stopped by the addition of Ammonium sulfate (6 ml of 10 mM (NH\(_4\))\(_2\)O\(_4\), 0.5 M H\(_2\)SO\(_4\), 1 ml of 500 mM ascorbic acid) (28). The coloring reaction was allowed to proceed for 20 min. Then the samples were centrifuged (15,000 \( g \), 1 min), and the absorption at 820 nm was determined. The method is based on determination of free phosphate produced by the enzyme reaction. With Ammonium solution a blue molybdenum phosphate complex was formed.

The reaction was carried out at pH 6.0, since Calvert and co-workers (29) localized the optimal pH for the phosphodiesterase activity of tomato annexin at this value. If additives like lipids, CaCl\(_2\) or EDTA were used, the amount of H\(_2\)O was adjusted to obtain a sample volume of 200 \( \mu \)l. The specific ATPase activity \( \nu \) is calculated as “amount of released phosphate per mass of enzyme and time,” with \( |d| = 1 \mu \text{mol} \times \text{mg}^{-1} \times \text{h}^{-1} \). To obtain the amount of released phosphate from the absorption at 820 nm, a calibration curve was measured first using KH\(_2\)PO\(_4\) in the range from 0 to 100 \( \mu \text{g} \). A linear regression curve could be determined according to the following equation.

$$
\text{[phosphate]} = (3.0905 \times 10^{-3} \, \mu \text{mol}^{-1}) \times (A_{820\text{nm}} - 7.048 \times 10^{-2})
$$

(Eq. 2)

All experiments were carried out as triplicate.

Urea-induced Unfolding—Determination of the folding stability of wild-type and mutant protein was carried out by monitoring intrinsic protein fluorescence at varying concentrations of urea. If the denaturation is a two-state process, the data can be analyzed according to the method of Pace (30), finally yielding the free stability energy of the hypothetical denaturation in aqueous solution, \( \Delta G(H_2O) \), and the degree of interaction of the denaturing reagent with the protein, \( m \).

Fluorescence emission spectra were recorded for every sample with excitation wavelengths set at \( \lambda_{ex} = 280 \text{ nm} \) and \( \lambda_{em} = 295 \text{ nm} \). The data were analyzed with respect to the wavelength at the maximum of the emission spectrum and the emission intensity at that maximum, respectively. All fluorescence spectra were corrected against buffer-only samples, and three independent unfolding series were performed.
RESULTS

Native Annexins from C. annuum—Annexin 24(Ca32) like all annexins comprises a tetrad repeat of approximately 70 amino acids and has all of the highly conserved residues generally found in other annexins involved in salt bridge interactions. The endonexin fold, typical for non-plant annexins where it is present in the AB-loops of domains I, II, and IV, is only restored in the IAB loop of annexin 24(Ca32) (see Fig. 1). A prominent feature of plant-type annexins was the existence of (at least) two annexin proteins in every plant species investigated so far (29, 31–33); thus, a second annexin, Anx(Ca38), is found in C. annuum. The two proteins Anx24(Ca32) and Anx(Ca38) differ not only in their primary structure but also in their migration behavior on SDS-PAGE, despite having nearly the same molecular weights. Fig. 2A shows a SDS-PAGE of annexin (Ca) purified from red bell pepper fruits, clearly indicating the presence of two different proteins, p32 and p38. Additionally, proteolytic digestion with V8 protease reveals distinct differences between both proteins.

In a second experiment, the behavior of natively purified annexin (Ca) was compared with recombinant annexin 24(Ca32) wild type. Anx(Ca) was purified from green and red bell pepper fruits and subjected to SDS-PAGE. As can be seen from Fig. 2B, the native material appeared at molecular masses of 32 and 38 kDa, whereas recombinant wild-type Anx24(Ca32) appeared at approximately 38 kDa despite its molecular mass of 32 kDa. Cross-reaction with anti-annexin(Zm) antibody is only positive with natively purified material and not with the recombinant protein, strongly suggesting the presence of post-translational modifications in the native protein.

General Characterization of Recombinant Proteins—Purified proteins were characterized by mass spectrometry and amino acid sequencing. N-terminal sequencing of recombinant proteins purified via calcium-triggered binding to liposomes (Fig. 3A) indicated the lack of the first methionine, in agreement with results from mass spectrometry. In SDS-PAGE, both recombinant proteins migrate as though they have a molecular mass of 38–40 kDa. A significant cross-reaction with an anti-annexin V antibody is observed with both recombinant proteins (data not shown). Results from gel filtration experiments or native PAGE (data not shown) indicate that the plant protein has a tendency to aggregate. Fluorescence spectra of both recombinant annexin 24(Ca32) wild type and the fusion protein indicate a hydrophilic environment of the fluorophore residues, as there is a bathochromic effect (347 nm for the wild type and 345 nm for the fusion protein). Circular dichroism revealed typical annexin spectra for the recombinant wild-type and the
fusion protein. As annexins are all-helical proteins, there are two minima in the CD spectra visible, as expected. However, there is a considerable difference in the circular dichroic spectra of the wild-type and the fusion protein. The peak wavelengths for annexin 24(Ca32) wild type are 209 nm and 219 nm, whereas the peak wavelengths for the fusion protein are 208 nm and 221 nm. Moreover, the fusion protein displays a hypochromic effect. The molar ellipticity is reduced to 66% (blue) and 71% (red) (Fig. 3B).

Structure Determination—Similar crystals from the fusion protein were obtained by either hanging or sitting drop with the vapor diffusion method at 22 °C. Crystallization conditions are summarized in Table IV. The crystals belong to the space group P3221 with approximate cell dimensions of \( a \approx 5 \) Å, \( b \approx 5 \) Å, and \( c \approx 172 \) Å (for data collection statistics, see Table II) and contain two molecules per asymmetric unit. Molecular replacement was successful with a truncated poly(Ala) model derived from annexin VIa.

The three-dimensional crystal structure of His-tagged annexin 24(Ca32) adopts the typical annexin fold known from other members of this protein family (Fig. 4). The protein core is built by four domains, each comprising five \( \alpha \)-helices, four of them arranged parallel and anti-parallel to each other (helices A, B, D, and E) and the fifth (helix C) lying almost perpendicular to the four-helix bundle. However, the extension of individual helices is slightly different compared with annexin V, resulting in some variations regarding particular sites within the annexin 24 structure. The two molecules in the asymmetric unit adopt similar conformations. While the N-terminal tail is constituted by a 20-amino acid sequence, it is only visible beyond position 8 in the second molecule and beyond position 6 in the first molecule. On the C-terminal side, molecule #1 can be traced up to residue 320 and molecule #2 up to position 321. However, the poor density in the very N- and C-terminal regions indicates high flexibility. Additionally, the high solvent content accounts for rather high temperature factors in both molecules. The structure has been refined to an R-factor of 0.216 at 2.8 Å resolution; refinement statistics are summarized in Table III.

Despite similar overall conformations of both molecules of the present structure, the arrangement of the IAB loops differs significantly. Trp-35 of the second molecule is located at the very surface on the convex side, and together with residues Glu-76 and His-79, it participates in coordination of a sulfate...
ion. The situation is completely different in the first molecule, where the sulfate ion is located in the region of the IAB and IDE loops. Trp-35 is forced to relocate to the outside of the first domain, sitting in a pocket that is created by a symmetry-related molecule and lined by Tyr-192, Phe-234, and Ser-193.

**Calcium-dependent Binding to Phospholipid Vesicles**—One of the most interesting features of annexin 24 is the calcium-dependent binding of this protein to liposomes, since the calcium binding sites of plant-type annexins are altered relative to non-plant annexins. As can be seen from Fig. 5A, recombinant wild-type annexin 24(Ca32) binds only moderately to PS/PE (3:1) liposomes. A much higher degree of binding is achieved by using liposomes made from PC/PS (1:1) liposomes. The half-maximal calcium concentration needed for binding is in the latter case $c_{1/2} \approx 1.1$ mM.
measurements. Normalization was carried out against a sample with (3:1) liposomes. Data shown are the average of three independent and recombinant annexin 24(Ca32) (\(m\)) in the presence of 500 \(\mu M\) dithiothreitol (\(A24/DTT\)), Anx24(Ca32) with 1 \(mM\) dithiothreitol (\(A24/DDT\)), Anx24(Ca32) with 1 \(mM\) \(\beta\)-mercaptoethanol (\(A24/ME\)), and His-Anx24(Ca32) (\(HA24\)). The data are averages from three independent measurements and are normalized with respect to a sample without protein. The control activity is therefore \(\gamma_\lambda = 1\).

Comparison of the intrinsic protein fluorescence in the absence and the presence of PS/PE (3:1) liposomes reveals a considerable blue-shift of the wavelength at maximal emission intensity (~9 nm). Calcium dependence of this hypochromic effect is not significant, as seen from Fig. 5B. Similarly, the intensity at peak emission decreases in the presence of the lipid vesicles (hypochromic effect). There is no effect of calcium on fluorescence intensity in the presence of liposomes; however, in their absence, Anx24(Ca32) fluorescence intensity is decreased by high calcium concentrations (Fig. 5C).

Carboxyfluorescein Leakage and Membrane Permeabilization Assay—The CF leakage assay provides data about the ability of proteins to stabilize or destabilize lipid vesicles. With annexins, stabilization of liposomes in this assay validate results from the FURA-2-based calcium influx assay (23). Numbers of the stability parameter \(\delta_\lambda\) lower than 1.0 account for more stable liposomes relative to the control experiment. The results for human recombinant annexin V and recombinant wild-type annexin 24(Ca32) clearly indicate that both proteins stabilize liposomes in the presence of calcium (Fig. 6A).

The ability of recombinant wild-type annexin 24(Ca32) to induce calcium influx into PS/PE (3:1) liposomes was elucidated with a calcium influx assay. The lipid composition was chosen to maintain compatibility with previous permeabilization measurements in our laboratory. Annexin 24(Ca32) was shown to be the most active wild-type annexin in a comparative study with more than seven different proteins from this family (34). It displays a 1.4-fold higher normalized activity than annexin II, the most active mammalian annexin in our hands (Fig. 6B). Surprisingly, the activity induced by the His-tag fusion protein is substantially decreased; the activity parameter \(\gamma_\lambda = 0.07\) is only 4% that of the activity displayed by recombinant annexin 24(Ca32) wild type.

**ATPase Activity—**Although annexins from maize and tomato have already been reported to display phosphodiesterase activity (29, 31), nothing was known so far about the behavior of annexin 24 from *C. annuum* or mammalian annexins. Using the protocol described in the experimental section, the hydrolase activity of recombinant wild-type annexin 24(Ca32) from *C. annuum* was tested with Na\(_2\)ATP as substrate. The activity was determined by analyzing the amount of monophosphate generated from ATP\(^-\) by the enzyme. A sample of recombinant wild-type annexin 24 yielded a specific activity of \(\nu = 0.7 \mu\)mol/l/(mg × h), which is slightly but insignificantly increased by the addition of 10 mM CaCl\(_2\). However, in the presence of 10 mM EDTA, the specific activity nearly vanished (Fig. 7). This leads to the conclusion that in the pure protein sample there is either some divalent metal ion present, or the hydrolase activity is catalyzed by Mg\(^2+\) present in the ATPase buffer.

As a control, we also tested recombinant human annexin V under the same conditions, which did not display hydrolase activity. A sample of recombinant (Na\(^+\)-K\(^-\))-ATPase from pig kidney was tested in this assay yielding a specific activity of \(\nu = 14.3 \mu\)mol/l/(mg × h) with 3 mM MgCl\(_2\) present.

**Folding Stability—**Folding stability of both recombinant proteins, annexin 24(Ca32) wild-type and His-Anx24, was investigated by urea-induced unfolding monitored by fluorescence spectroscopy. The comparison of both proteins is particularly interesting, since the fusion protein crystallizes while the wild-type protein precipitates. For the wild-type protein, 4.65 \(M\) urea is required for half-maximal unfolding, whereas the unfolding curve of the fusion protein yields \(c_{\text{U}}(\text{urea}) = 3.82 \ M\). Measurements of the free stability energy show a significantly higher value for the fusion protein than for the wild type. \(\Delta G(H_2O)_{\text{ext}} = 4.69 \text{kJ/mol} (\gamma_\lambda = 0.96 \text{kJ/mol})\), and \(\Delta G(H_2O)_{\text{His}} = 9.32 \text{kJ/mol} (\gamma_\lambda = 0.07 \text{kJ/mol})\).

**DISCUSSION**

**General Features and Comparison with Annexin V—**Several biochemical properties of annexin 24(Ca32), like electrophoretic migration or aggregation behavior, clearly distinguish this plant annexin from its typical non-plant relatives, for example, annexin V. Topologically, the most prominent difference between this plant annexin and other well known mammalian annexins is certainly the lack of the endonexin sequence in domains II, III, and IV. Despite these differences,
cross-reaction with an anti-annexin V antibody emphasizes the overall similarity of different members of the annexin protein family even throughout different organisms; however, post-translational modification of Anx24(Ca32) in vivo seems likely, since anti-maize antibodies are able to recognize the native protein only (Fig. 2B).

Comparison of the present structure with the crystal structure of annexin V (Fig. 4C) shows distinct local differences in various regions of the proteins; for example, a shortening of the IIDE loop by four amino acids, which renders the convex side of the intermolecular space more accessible. Several sulfate ions are found within this area, providing evidence that the higher accessibility of this region can be a distinctive feature of annexin 24(Ca32). The presence of several polar residue side chains and the proposed conformational flexibility in this region provides an excellent environment for the phosphodiesterase activity of this protein. Additionally, the molecular surfaces of both proteins are strikingly different in shape and also in surface potential. The overall structure of annexin 24(Ca32) appears to be much more fissured, presenting several clefts and pockets (see Fig. 9) not observed with annexin V. This is also supported by the calculated geometrical extensions of annexin 24 and annexin V, which reveal a larger molecular surface area for annexin 24(Ca32), whereas the volumes of both proteins are very similar (see Table IV).

Of remarkable interest is the association of the N-terminal domain with the protein core in the present structure, since this has not been observed in other annexin structures so far. The N-terminal tail in both molecules of the annexin 24(Ca32) structure is attached to the core via H-bonding between His-48 and backbone carbonyl CO-13 (Fig. 8). Furthermore, stability measurements indicate that the fusion protein is more stable than the wild-type (ΔΔG = 4.6 kJ/mol). According to results from CD spectroscopy, the artificial elongation of the N-terminal tail also affects the overall conformation of the protein. This further emphasizes the modulatory role of the N-terminal region on the protein core, as already proposed from a mutation study with N-terminal annexin V truncations.²

Membrane Binding Loops—Of major interest are, of course, the membrane binding loops located on the convex side of the protein. The AB loops of domains I, II, and III each contain an aromatic residue capable of effective interaction with a membranous lipid layer. Analysis of the canonical calcium binding sites, as defined by annexin V, shows a complete disruption within annexin 24(Ca32). None of the four calcium ions from annexin V could be bound because of structural divergence of the loops, believed to contribute to calcium coordination. It is known from the primary structure that the endonexin fold is only conserved within the first domain of annexin 24(Ca32). Still, canonical calcium binding within this site is doubtful, as Lys-40 with its positive charge prevents coordination of a cation.

The strict conservation of Trp-35 in the IAB loop within the plant-type annexins (see Fig. 1) indicates a major role of this residue for membrane binding or annexin oligomerization (see the next paragraph). The present structure anticipates that the switching mechanism from the loop-in to the loop-out conformation of the IAB loop might be a conserved feature of all plant-type annexins. A possible scenario transforming both conformers would involve a relocation of the sulfate ion along the protein surface to the position in molecule #1, where it prevents the loop-in conformation of Trp-35. The creation of a binding pocket by a second annexin molecule further contributes to energetically stabilizing the loop-out conformation of the IAB loop in molecule #1 in the crystal structure.

Interaction with Calcium and Membranes—Binding of annexin 24(Ca32) to PS/PE (3:1) liposomes as monitored by intrinsic protein fluorescence results in a blue-shift of the wavelength at the emission maximum. It seems likely that this is due to the residues Trp-35, Trp-107, and Tyr-192, all located on the convex side of the protein within the AB loops. This agrees well with the structural data and strongly suggests that these loops serve as membrane binding regions, with the aromatic residues penetrating the lipid layer. The annexin-membrane interaction observed here is not strongly dependent on calcium, emphasizing differences in the protein-membrane interaction mechanisms between annexin 24(Ca32) and annexin V. However, calcium-induced structural rearrangements are suggested by the observed changes in the intrinsic protein fluorescence in the absence of lipids. With increasing calcium concentration we observe a decrease in emission intensity, indicating enhanced access of quencher molecules (like e.g. solvent) to the fluorophores. This effect is nearly absent in the presence of lipids, suggesting a protection of fluorophores by surrounding lipid layers. One possible explanation is a calcium-induced interaction of two annexin proteins either in the membrane-bound or soluble form. To compensate for the unfavorable situation of exposure of aromatic residues, these might interact with a second annexin molecule, thereby accounting for the observed spectroscopic behavior. In the membrane-bound form, this would result in a membrane-supported two-dimensional protein layer (triggered by calcium). Interestingly, a similar mechanism has been suggested for Trp-187 in annexin V recently (35).

Differences between annexin V and annexin 24(Ca32) are also observed regarding the affinity for certain constituents of a phospholipid bilayer. The degree of binding observed with PS/PE (3:1) liposomes is only 30% in the case of the plant annexin. Nearly complete binding, however, is observed with PS/PC (1:1) liposomes. The affinity for PS is therefore much lower for annexin 24(Ca32) than for annexin V. Neglecting any calcium effect on membrane binding, an interesting view is obtained from a closer look at the protein surface potential on the convex side (Fig. 9). In addition to aromatic residues being able to interact with a membrane bilayer after conformational rearrangements, there are positively charged residues present on the convex side that might well be able to interact with the glycerol backbone/ester region of the membrane bilayer. The very surface of the protein on the convex side, however, is highly negatively charged. This agrees well with the preference of positively charged lipid head-groups, as observed in the liposome binding assay.

Asp-297 and Glu-68, which have both been identified in a recent study as being important for membrane binding of

² J. Benz, personal communication.
Figure 9. Surface potential map of His-tagged annexin 24(Ca32). The molecular surfaces of both molecules #1 and #2 are colored according to the electrostatic potential (red, negative; blue, positive). The figure was created with GRASP (38).

Anx(Le35) (5), are also conserved in Anx24(Ca32) (residues Asp-304 and Glu-76). These amino acids align to the more distant residues of the endonexin sequence and are responsible for bidentate coordination of calcium in the case of vertebrate annexins. In the present structure Glu-76 in both molecules is involved in coordination of a sulfate ion, which might be substituted by a phosphate group in the membrane-bound state.

Functional Properties—Assays characterizing the membrane activity of annexin 24(Ca32) show a peculiar behavior of this protein. On one hand, the calcium concentration needed for half-maximal binding to PS/PE (3:1) liposomes is about 10-fold higher than for annexin V, the degree of binding is much lower, and the results from the CF leakage assay indicate no vesicle destabilization by annexin 24. On the other hand, membrane permeabilization activity as measured by the calcium influx assay is higher by a factor of 10 for recombinant wild-type annexin 24(Ca32) than for annexin V and about 1.5-fold higher than for annexin II, the most active non-plant type annexin protein. On one hand, the calcium concentration needed for half-maximal binding to PS/PE (3:1) liposomes is about 10-fold higher than for annexin V, the degree of binding is much lower, and the results from the CF leakage assay indicate no vesicle destabilization by annexin 24. On the other hand, membrane permeabilization activity as measured by the calcium influx assay is higher by a factor of 10 for recombinant wild-type annexin 24(Ca32) than for annexin V and about 1.5-fold higher than for annexin II, the most active non-plant type annexin measured in our laboratory. This high increase in membrane permeabilization ability can not be explained by the amino acid sequence. All pore residues conserved among the non-plant annexins are also conserved within annexin 24(Ca32); the only exception is E120 (AnxV), which is replaced in annexin 24 by a lysine, resulting in a change of charge at this position. In light of other results from calcium influx experiments with annexins, the most likely explanation is an allosteric regulation of this membrane activity (23). This conclusion is further supported by the extremely reduced activity of the His-tag fusion protein, where the elongation of the N-terminal domain may affect the structure and dynamics of the membrane active species. The differences in the CD spectra of wild-type and fusion protein agree well with this explanation.

Phosphodiesterase activity has been already reported for annexins from maize (31) and tomato (29). As the presence of MgCl₂/Na₂ATP or BDA452 has no effect on the membrane permeabilization assay, the ATPase activity or a possible complex formation between the benzodiazepine derivative and annexin 24 does not interfere with the permeabilization behavior. The former result agrees well with a recent study (5), presenting evidence for the independence of the phospholipid binding and phosphodiesterase activity of tomato annexin Anx(Le35). The present work is the first report of this enzymatic activity for recombinant wild-type annexin from C. annuum. Compared with recombinant (Na⁺⁻K⁺)-ATPase, the enzymatic activity of annexin 24 is about 5%. It is important to note that annexin V does not hydrolyze ATP²⁻ under these conditions.

Conclusion—The present work provides the first three-dimensional structure of a member of the plant annexin family and correlates these findings with the biochemical properties of this protein. Annexin 24(Ca32), as purified in its native form or recombinant from bacterial expression, shows distinct variations when compared with vertebrate annexins like annexin V. Post-translational modifications of annexin 24(Ca32) are very likely to occur since anti-maize antibody recognizes the native purified protein only. Though adopting the same overall fold, the detailed structure is very different from annexin V, resulting in a much less compact shape with high intrinsic flexibility. The N-terminal region has a considerable effect on the protein core, since the His-tag elongation stabilizes the protein in unfolding experiments and is required for successful crystallization. Concerning this modification, one might argue that some structural features of the recombinant wild-type protein remain unrevealed. However, we would rather suggest that the effect of the N-terminal elongation is mainly an overall stabilization of the otherwise flexible structure of annexin 24(Ca32). From the present structure, the observed membrane binding behavior can be explained, and even the prediction of the most important residues is possible. The absence of calcium within the structure prevents insights into the calcium binding mechanism. Binding of calcium certainly requires structural rearrangements in the membrane binding loops; these conformational changes are likely to occur as the two molecules in the present structure already adopt different conformations of the IAB loop, forced by different positions of a sulfate ion and crystal packing effects. Domain I is supposed to play an important role for protein-membrane and protein-protein interactions, since the switching tryptophan residue observed in the present structure is conserved throughout all plant annexins. Calcium influx activity as well as phosphodiesterase activity were demonstrated with recombinant wild-type annexin 24(Ca32). Future experiments aiming at structural location of the enzymatic activity of this annexin will contribute to clarification of the physiological role of these plant representatives of the annexin family of proteins.

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