Effects of Mutagenesis of W343 in Human Annexin A6 Isoform 1 on Its Interaction with GTP: Nucleotide-Induced Oligomer Formation and Ion Channel Activity†

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Received August 16, 2005; Revised Manuscript Received February 21, 2006

ABSTRACT: Accumulated experimental evidence suggests that annexin A6 (AnxA6) is involved in ion transport in various tissues. Such a biological function is related either to the modulation of ion transport systems by AnxA6 or to the ion channel activity of the protein. While AnxA6 channel activity at low pH seems to be associated with a large conformational transition in the protein, the mechanism of GTP-induced ion channel formation remains obscure. This activity is not accompanied by changes in protein structure. The existence of a domain binding the phosphate groups of GTP in AnxA6 [Bandorowicz-Pikula, J., Kirilenko, A., van Deursen, R., Golczak, M., Kuhnel, M., Lancelin, J. M., Pikula, S., and Buchet, R. (2003) *Biochemistry 42*, 9137−9146] may provide some clues about the molecular mechanisms of GTP-induced ion channel formation. In addition, we observed that one of the AnxA6 tryptophan residues, W192 or W343, may be involved in GTP binding. Therefore, we created several site-directed mutants of AnxA6 in which selected amino acid residues within a consensus sequence of a putative nucleotide-binding domain of AnxA6 were replaced with other amino acid residues without affecting the overall structure of protein as examined by circular dichroism and infrared spectroscopies. Their properties were analyzed and compared to those of the native protein. In contrast to mutant W192S and wild-type annexin, mutant W343S neither bound GTP nor exhibited GTP-induced ion channel activity. In addition, we detected the likely formation of AnxA6 trimers in the presence of GTP. The ability of mutant W343S to form trimers was significantly impaired. Our findings suggest that W343 participates in the formation of AnxA6 trimers. We hypothesize that such trimers could lead to a functional unit of the GTP-induced ion channels formed by the annexin molecules.

Among all known mammalian annexins, there are two unique members, namely, vertebrate annexin A6 (AnxA6)[1] isoforms, AnxA6 isoform 1 and AnxA6 isoform 2 (*1*). They comprise two four-tandem repeats joined by a short linkage sequence. Phylogenic data suggest that AnxA6 evolved from two classical, four-repeat annexins, AnxA5 and AnxA10, early in chordate evolution (*2*).

In addition to its nucleotide binding properties explored under in vitro conditions, AnxA6 isoform 1 is a calcium-dependent membrane-binding protein that interacts with signaling proteins, including the GTPase-activating protein $p120^{GAP}$ (*3*), one of the most important mediators of Ras activity (*4*). It has been recently demonstrated that AnxA6 promotes membrane binding of $p120^{GAP}$ and plasma membrane targeting of $p120^{GAP}$ in living cells, each in a Ca^{2+}-dependent manner, which is consistent with AnxA6 promoting the Ca^{2+}-dependent assembly of $p120^{GAP}$-Ras at the plasma membrane (*5*). Moreover, AnxA6 was found to be involved in the regulation of GTP-dependent secretory processes, as it has been shown to interact with the 28 kDa protein, human tumor protein D52 (TPD52), in normal and in neoplastic epithelia (*6*).

Considering biological processes in which AnxA6 is likely to be engaged, the protein has been implicated in endosome aggregation and vesicle fusion in secreting epithelia during exocytosis, possibly acting as a negative regulator of exocytosis. In relation to exocytosis, the interaction or colocalization of AnxA6 with GTP-binding proteins, e.g., a GTPase, dynamin (*7*), and proteins mediating vesicle docking and/or fusion (e.g., v-SNARE, VAMP-2, and NSF) (*8*) or with GTPase-activating proteins (e.g., $p120^{GAP}$) (*3*) has been observed. Furthermore, in bovine lung, heart, and brain subfractions, it has been found that, following Ca^{2+}-dependent association of AnxA6 with membranes, a substantial fraction of this protein remains bound to membranes in a Ca^{2+}-independent way and behaves like an integral membrane protein (*9*). When associated with membranes, AnxA6 can act as an ion channel and/or ion channel regulator (*10*−*12*). For example, AnxA6 could play an important role in ion transport in placenta by regulating the maxi-chloride channel permeability. The maxi-chloride channel from the apical membrane of the syncytiotrophoblast (separating the maternal and fetal blood in placenta) is involved in chloride

† This work was supported by Grant 3 P04A 007 22 from the Polish State Committee for Scientific Research.

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[1] Abbreviations: AnxA, vertebrate annexin (protein); [α-^{32}P]GTP, [α-^{32}P]guanosine 5′-triphosphate; IPTG, isopropyl β-D-thiogalactopyranoside; TNP-GTP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)guanosine 5′-triphosphate.

10.1021/bi051629n CCC: $33.50
Published on Web 03/25/2006

Table 1: Primary Sequence Alignment of Guanine Binding Motifs of AnxA6 Isoforms and Other Calcium Binding Proteins

protein	NKXD consensus motif	EXSAX consensus motif
Sar1p	NKID	EELAI
calexcitin	NIID	FELAI
human AnxA1	NKVL	not present
human AnxA2	NKPL	not present
human AnxA4	NKSA	not present
human AnxA6	597-NKPL	344-ELSAV
chicken AnxA6	NKPA	ELSAV
bovine AnxA6	NKAY	ELSAV
rat AnxA6	NKPL	ELSAV
mouse AnxA6	NKPL	ELSAV
human AnxA8	NKSI	not present
human AnxA10	NKTV	not present
human AnxA11	NKAM	not present

conductance. AnxA6 is bound to apical placenta membranes in a calcium-dependent and calcium-independent manner (*12*). Anti-AnxA6 antibodies decreased the total current by at least one-third of control as well as the single-channel conductance and its amplitude by 50%. The open probability of the channel was also affected (*12*).

The ion channel activities of AnxA6 found under in vitro conditions are induced by mildly acidic environments (*10*) or by the addition of GTP at pH 7 (*11*). While the ion channel activity induced in a mildly acidic environment is accompanied by a large conformational transition within the AnxA6 molecule leading to the insertion of the protein into a hydrophobic region of lipid membranes (*13*, *14*), the mechanism of GTP-induced ion channel formation by the annexin remains enigmatic. Neither significant changes of overall structure of the protein nor changes in hydrophobicity were observed (*11*). In fact, the protein in the presence of GTP does not behave as a real channel, e.g., membrane integral protein as observed for AnxA6 at pH <6.2 (*13*).

Our previous modeling, biochemical, and spectroscopic investigations enabled us to propose that a GTP-binding domain is present within the AnxA6 molecule (*15*) that is almost symmetrically distributed between the two N- and C-terminal halves of the protein. This domain comprises residues K296-Y297...Q341-M342-W343-XX-S346 and K598-XXX-L602...K644-Y645 (*15*), corresponding to a F-X-X-K-Y-D/E-K-S-L consensus sequence. A more general sequence (F/Y-X-X-X-F/Y-X-K-S-L) was found also in other annexins, suggesting that this motif is conserved in the annexin family of proteins. The domain responsible for binding nucleotides partially overlaps with a N-K-X-D motif found in some GTP-binding proteins (*17*). However, in the AnxA6 sequence, the conserved D residue is replaced with L, e.g., N597-K598-P599-L600. By comparing the AnxA6 sequence with that of calexcitin and Srp1, in which GTP-binding consensus motifs have been found (*18*), we identified the N-K-X-D motif in human AnxA6 and isoforms from other species (Table 1), suggesting its conservation during evolution. The N-K-X-D motif represents the so-called guanine recognition motif (*17*). The second identified motif in the GTP-binding proteins, namely, E-X-S-A, was suggested to play a role in the stabilization of the interactions with the phosphate groups of a nucleotide (*17*). In AnxA6, one of the tryptophan residues, W343 that is conserved in evolution, precedes the E-X-S-A motif, i.e., E344-L345-S346-A347. It is worth stressing that the E-L-S-A motif was not identified in other annexins but only in AnxA6 (Table 1), where it forms part of an exclusive linker region between the two symmetric lobes of the protein consisting of the N- and C-terminal halves of the annexin.

Table 2: Primers Used in the Construction of Mutants with a Single-Amino Acid Substitution

mutant	sense primer (5′ → 3′)
W192S	GGGAACTGAAATCAGGAACAGATGAAGCC
K296A	GAGATCTTCCGGACCGCGGTATGAGAAGTC
W343S	GCCTATCAGATGTCGGAACTTAGTGCAGTGG
W343F	GCCTATCAGATGTTTGAACTTAGTGCAGTGG
R560A	CGCACCTCCGGGCAGTCTTCCAGG
Q595A	GTGGCCATTGTTGCAAGTGTCAAGAAC
K598A	GTTCAAAGTGTCGCGAACAAGCCTCTCTTC
K644A	ATTCATTGAGGCATATGACAAGTCTCTCCACC

To investigate the mechanism of GTP-induced ion channel formation by the annexin in which the GTP-binding domain and residue W343 are involved, we created several site-directed mutants of AnxA6 by changing both tryptophan residues of the protein or selected amino acid residues within a consensus sequence of the nucleotide-binding domain of the protein. We found that in contrast to mutant W192S, which behaves like the wild-type AnxA6, mutant W343S neither binds GTP nor exhibits GTP-induced ion channel activity. In addition, using electrophoresis under nondenaturing conditions and cross-linking reagents, we observed stabilization of formation of AnxA6 trimers in the presence of GTP, while in mutant W343S, this ability is significantly impaired in comparison to that of the native protein. Our findings support a model in which W343 is involved not only in the guiding of the guanosine moiety of GTP to a binding domain of AnxA6 but also in the formation of AnxA6 trimers that serve as functional units of the GTP-induced ion channels observed in the physiological pH range. However, the molecular mechanism standing behind the stabilization of the AnxA6 trimers in the presence of GTP and formation of nucleotide-induced ion channels by AnxA6 remains to be determined.

MATERIALS AND METHODS

Chemicals. [α-^{32}P]Guanosine 5′-triphosphate ([α-^{32}P]GTP) was delivered by ICN Biomedicals, Inc. (Rzeszow, Poland). 2′-(3′)-*O*-(2,4,6-Trinitrophenyl)guanosine 5′-triphosphate (TNP-GTP) was obtained from Molecular Probes Inc. (Eugene, OR). GTP, asolectin (soybean lipids), and phosphatidylserine were purchased from Sigma-Aldrich (Poznan, Poland). Phosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL). The protein markers were obtained from Fermentas. All other chemicals were of the highest available purity. Data were analyzed and plotted using Origin.

Site-Directed Mutagenesis. Plasmid DNA was isolated using Mini-Prep Kits (QBiogene). Site-directed mutants were generated by the polymerase chain reaction (PCR) using a procedure developed by Stratagene (QuikChange kit, Stratagene, La Jolla, CA). The primers used for site-directed mutagenesis are listed in Table 2. Mutagenesis was carried out using 10 ng of template DNA and 125 ng of each primer in a total volume of 25 μL. A PCR program of 5 min at 95 °C was used, followed 16 cycles of 30 s at 95 °C, 1 min at 55 °C, and 5 min at 68 °C. The template (methylated) plasmid was digested with *Dpn*I. Then, 1 μL of reaction

mixture containing amplified plasmid was used for transfection of XL1-Blue competent cells. Plasmid DNA isolated from clones resistant to ampicilin were sequenced to verify the presence of the required mutation and then transformed into Bl21(DE3) cells.

Expression and Purification of the Wild Type and Mutants of AnxA6. Human recombinant AnxA6 and its mutants (W192S, K296A, W343S, W343F, R560A, Q595A, K598A, and K644A) were expressed in *Escherichia coli* strain B121-(DE3) after induction with isopropyl β-D-thiogalactopyranoside and purified to homogeneity as described previously (*11*). The efficiencies of mutant expression were comparable to that of the wild-type protein (7 mg of protein/L of bacterial culture).

Circular Dichroism Measurements. The far-UV CD spectra of AnxA6 were collected at 25 °C using an AVIV CD spectrophotometer (AVIV Associates Inc.) in a 2 mm optical path length quartz cuvette, as described previously (*10*). The assay medium contained 25 mM Tris-HCl (pH 7.4) buffer, 0.2 mg/mL AnxA6 or its mutants, and 0.25 mM EGTA. Each spectrum was recorded as an average of three scans between 195 and 260 nm. The participation of α-helix and β-sheet structures in protein secondary structure was calculated with the aid of Selcon 3.

FTIR Spectroscopic Measurements. The Fourier transform infrared spectra of AnxA6 and its mutants were determined in ^2H_2O buffer containing 100 μM protein, 100 mM Tris-HCl buffer (p^{2}H 7.5), 2 mM $CaCl_2$, or 1 mM EGTA. Infrared data were acquired with a Nicolet 510M FTIR spectrometer equipped with a DTGS detector, using a temperature-controlled flow-through cell (model TFC-M25, Harrick Scientific Corp., Ossining, NY) with 50 μm spacers and CaF_2 windows, as previously described (*15*).

Steady-State Fluorescence Measurements of AnxA6 and Its Mutants. All fluorescence measurements were monitored at 25 °C with a Fluorolog 3 spectrophotometer (Jobin Yvon Spex, Edison, NJ). The fluorescence titrations with various TNP-GTP concentrations (0−15 μM) were monitored at a λ_{em} of 545 nm (λ_{ex} of 415 nm) at 1 nm resolution of slits for excitation and emission. The 5 mm × 5 mm cuvettes with an internal volume of 0.6 mL contained 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM $CaCl_2$, asolectin liposomes (0.25 mg/mL), and 2 μM AnxA6 mutants. Dilution of samples did not exceed 10%, and the fluorescence intensity was corrected for the dilution factor. To minimize inner filter effects, the total absorbance of the protein/ligand mixtures did not exceed 0.2 AU. To compensate for the decrease in fluorescence due to the increased absorption of TNP-GTP, the measured fluorescence was corrected by a factor $f(\Delta A_1,\Delta A_2)$ equal to $10^{(\Delta A_1+\Delta A_2)(1/2)}$, where ΔA_1 and ΔA_2 are the increases in absorption at the excitation and emission wavelengths, respectively, upon addition of the ligand (*16*). The dissociation constants (K_D) were calculated as previously described (*15*). For each individual and independent measurement, the error was calculated using Origin on the basis of 16 experimental points per each individual and independent measurement by fitting them to the hyperbolic regression curve. The K_D values from three independent determinations per mutant were then compared, and the standard error was calculated.

The intrinsic fluorescence of AnxA6 mutants W192S and W343S (each at 1 μM) was monitored by excitation at 295 nm (emission at 305−400 nm). Measurements were performed in a buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM $CaCl_2$, and liposomes (0.033 mg/mL) made of asolectin or phosphatidylserine and phosphatidylcholine (at a 4:1 weight ratio) as indicated in the text. The width of excitation and emission slits was set at 3 nm.

Photoaffinity Labeling of the Wild Type and Mutants of AnxA6 with [α-^{32}P]GTP. The incubation mixture, in total volume of 25 μL, contained 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.5 mM EGTA, 5 μCi of [α-^{32}P]GTP (25 Ci/mmol) (e.g., final GTP concentration of 40 μM), and 20 μg of protein. After a 10 min incubation on ice, the samples were exposed to broad-spectrum UV light for 20 min at a distance of 10 cm and at 4 °C. Then, the samples were resuspended in sample buffer containing SDS and loaded on 12% polyacrylamide gels. The developed gel was exposed to medical X-ray film (Foton, Warsaw, Poland).

Single-Ion Channel Recordings. The single-channel activities of AnxA6 mutants were measured by the black lipid membrane technique. Lipid membranes were formed in a Derlin cell filled with an appropriate electrolyte solution (2 mL in the cis compartment and 3 mL in the trans compartment). The composition of the solution was 10 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, and 200 mM KCl. The membrane-forming solution contained 25 mg/mL asolectin resolved in *n*-decane. The formation of stable bilayers was controlled by measurement of membrane capacitance and resistance. Membranes, characterized by the final capacitance of bilayer ranging from 130 to 200 pF and a resistance of 10−30 GΩ, were found to be suitable for measurements. The final concentration of protein added to the cis side of the bilayer was 20 nM with 4 mM GTP. The current through membranes was measured using a bilayer membrane admittance meter (model ID 562, IDB, Gwynedd, U.K.).

Chemical Cross-Linking of AnxA6 and Mutant W343S. AnxA6 (10 μg) (or the W343S mutant) was incubated at room temperature for 0.5 h in a buffer (25 μL) containing 50 mM Hepes-KOH (pH 7.4), 50 mM NaCl, 2 mM dithiothreitol (DTT), 0.5% glutaraldehyde, and 2 mM $CaCl_2$, in the presence or absence of 20 mM GTP. After incubation, sample buffer containing SDS was added and the probes were incubated at 100 °C for 5 min. Then, the probes were subjected to SDS−PAGE (10% gels) and proteins stained with Coomassie Brilliant Blue.

Gel Electrophoresis under Nondenaturing Conditions. AnxA6 (10 μg) was incubated in a buffer (25 μL) containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM $CaCl_2$, and 0, 1, 5, or 20 mM GTP at 4 °C. Then, samples were resuspended in sample buffer. The polyacrylamide gels (10%) were loaded at 4 °C according to the same procedure that was used for SDS−PAGE, but sodium dodecyl sulfate and β-mercaptoethanol were omitted from samples and buffers. Proteins from the developed gel were electrotransferred onto nitrocellulose membranes (Bio-Rad). Western blot analysis was performed with a monoclonal antibody to AnxA6 (Transduction Laboratories) as the primary antibody (diluted 1:5000) and anti-mouse IgG−horseradish peroxidase conjugates (Amersham Bioscience) as a secondary antibody (diluted 1:10000).

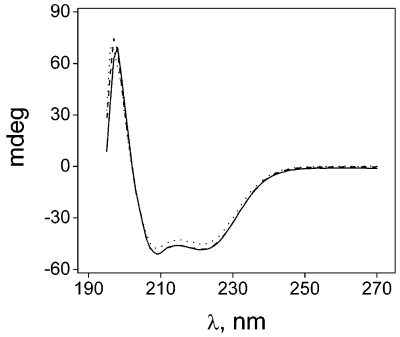

FIGURE 1: CD spectra of AnxA6 and its single-tryptophan mutants, W192S and W343S. Far-UV CD spectra of AnxA6 (—), W192S (- - -), or W343S (•••) were recorded at 25 °C in the assay medium (of a total volume of 0.6 mL) containing 25 mM Tris-HCl (pH 7.4), 0.25 mM EGTA, and 2 mg/mL protein. Unsmoothed and buffer-corrected spectra were averaged from three independent determinations. The secondary structure content of AnxA6 and its mutants was calculated from the spectra with the aid of Selcon 3. The region between 205 and 230 nm with two minima is characteristic for the mostly α-helix secondary structure of AnxA6 and its single-tryptophan mutants.

Table 3: Secondary Structure of AnxA6 Mutants with Single-Amino Acid Substitutions[a]

	α-helix structure (%)	β-structure (%)	unordered structure (%)
wild type	81.4 ± 0.3	5.6 ± 0.3	14.9 ± 1.7
W192S	81.5 ± 1.9	5.2 ± 0.3	13.3 ± 0.2
W343S	80.9 ± 4.9	5.8 ± 0.4	15.2 ± 2.5
R560A	56.2 ± 7.1	22.7 ± 1.9	22.8 ± 4.9
K598A	80.9 ± 5.4	5.3 ± 1.1	14.8 ± 1.8

[a] Calculations are based on the CD spectra measured under the conditions detailed in Materials and Methods. The mean values ± the standard deviation from at least three determinations are shown and were calculated with the aid of Selcon.

RESULTS

Secondary Structure of Single-Tryptophan Mutants of AnxA6. To study a possible role of tryptophan residues in GTP binding by AnxA6, we prepared two tryptophan mutants, W192S and W343S, e.g., the AnxA6 molecules with a single tryptophan residue, in positions 192 and 343, respectively. The site-directed mutagenesis method must be, however, used with caution as it may elicit a significant impact on the protein structure. To rule out this possibility, we have examined the secondary structure of the single-tryptophan mutants of AnxA6 using circular dichroism and Fourier transform infrared spectroscopy. The far-UV circular dichroism spectra (Figure 1 and Table 3) revealed that the overall secondary structures of the single-tryptophan mutants are mainly α-helical as in wild-type AnxA6 (*15*), suggestive of rather local changes in the secondary structure of AnxA6 evoked by a single replacement of one of the tryptophan residues of the protein with serine. This was confirmed by analysis of Fourier transform infrared spectra of AnxA6. The 1652 cm^{-1} band in Figure 2 was assigned to the amide 1 region highly characteristic of α-helix structures, and the infrared spectra of human recombinant AnxA6 and its mutants were identical to the infrared spectrum of AnxA6 already published (*11*).

Two tryptophan residues present in human AnxA6, W192 and W343, are located in different regions of the protein (Figure 3), as evidenced by the crystal structures of human

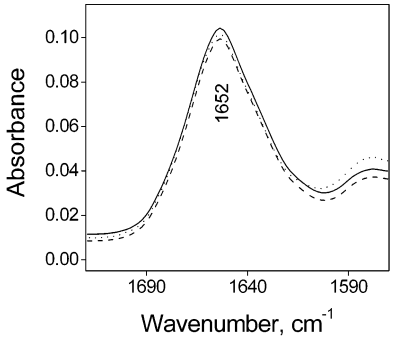

FIGURE 2: Fourier transform infrared spectra of AnxA6 and its single-tryptophan mutants. Infrared spectra of AnxA6 (—) or its single-tryptophan mutants W192S (- - -) and W343S (•••) in ^2H_2O buffer containing 100 mM Tris-HCl (p^2H 7.4), 2 mM $CaCl_2$, and 6−8 mg/mL protein, after subtraction of the infrared absorption of the buffer without protein. Each spectrum represents nine spectra that were averaged. Other details are given in Materials and Methods.

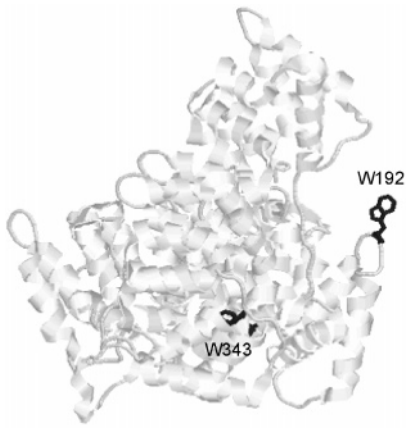

FIGURE 3: Cartoon representation of the crystal structure of phosphorylation-mimicking mutant T356D of human AnxA6, isoform 1 (PDB entry 1M9I) (*19*). The localization of amino acid side chains of W192 and W343 within the protein molecule (sticks) is shown. The figure was created using RasMol version 2.6. The orientation of the molecule in comparison to the orientation of AnxA6 from the PDB file was changed as follows to better visualize the W192 and W343 residues: *x*, 20°; *y*, 180°; *z*, 0°.

(*19*) and bovine (*20*) isoforms. Their different accessibilities to the surrounding milieu were observed by using various quenchers of their fluorescence (*21*, *22*). In this report, we investigated their molecular characteristics using steady-state fluorescence measurements. The obtained mutants allowed us to measure the emission spectra of W192 or W343 within the AnxA6 molecule in various environments, without interference from the other tryptophan residue. The emission spectra of W192 within the W343S mutant in the absence or presence of phosphatidylserine/phosphatidylcholine liposomes are shown in Figure 4A. The maximum of emission in the absence of liposomes (Figure 4A, dotted line) occurred at 347 nm, indicating that W192 is in a rather aqueous environment. When mutant W343S of AnxA6 is bound to the membrane (in the presence of calcium and liposomes), the fluorescence intensity of W192 increased (Figure 4A, solid line) probably due to the fact that under these conditions W192 is less exposed to the aqueous medium (water molecules could act as a quencher of W192 fluorescence). Alternatively, the mobility of the W192 residue is restricted due to the interaction of W192 or its surrounding amino acid

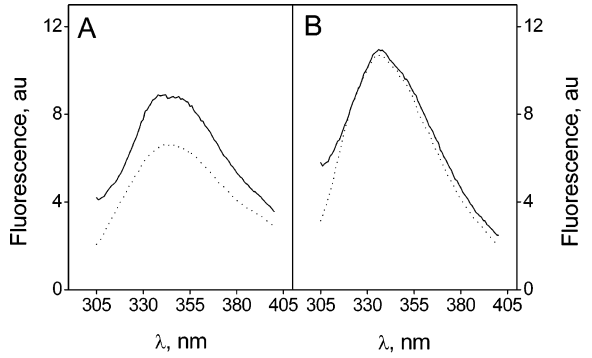

FIGURE 4: Fluorescence emission spectra of single-tryptophan AnxA6 mutants W343S (A) and W192S (B). The measurements were performed in the presence of 1 mM $CaCl_2$ (···) or in the presence of 1 mM $CaCl_2$ and phosphatidylserine/phosphatidylcholine liposomes (at a weight ratio 4:1, 0.033 mg/mL) (—). The samples were excited at 295 nm.

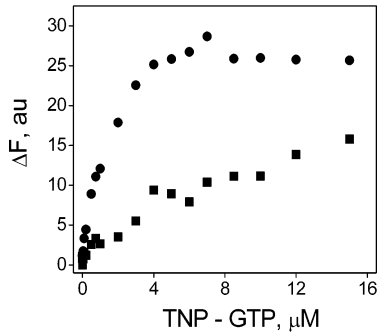

FIGURE 5: Binding of TNP-GTP to AnxA6 mutants W192S (●) and W343S (■). Upon excitation at 415 nm, the binding of TNP-GTP to mutants was specifically monitored by the increase in the TNP group fluorescence at 545 nm. The buffer consisted of 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM $CaCl_2$, and asolectin liposomes (0.25 mg/mL). The specific fluorescence enhancement ΔF was calculated as the difference between the fluorescence intensities in the absence and presence of 5 mM GTP.

residues with lipids, thereby inducing an increase in the fluorescence of this tryptophan residue. The emission maximum of W343 within the W192S mutant in the presence of calcium (Figure 4B, dotted line) is located around 336 nm, suggesting that W343 is less accessible to aqueous medium. The addition of phosphatidylserine/phosphatidylcholine liposomes neither increased significantly its fluorescence nor changed its emission maximum (Figure 3B, solid line). Similar results were obtained with asolectin liposomes (not shown). The fluorescence intensity of W343 was higher and less sensitive to changes in the solvent than that of W192.

Binding of TNP-GTP to Tryptophan Mutants of AnxA6. We observed earlier fluorescence resonance energy transfer (FRET) from tryptophan residue(s) of AnxA6 to the phenyl ring of TNP-GTP (*11*), which might suggest that the nucleotide-binding site within the AnxA6 molecule is localized close to one of the tryptophan residues. To identify which tryptophan residue may participate in nucleotide binding, we assessed binding of TNP-GTP to W192S and W343S mutants of AnxA6 (Figure 5). Fitting curves to the experimental points revealed that the W192S mutant bound TNP-GTP with a K_D value similar to that of the wild-type protein (1 μM) (*11*), while the K_D value for mutant W343S increased significantly (at least 100 times), suggesting that W343 but not W192 is involved in GTP binding (Table 4).

Table 4: K_D Values of Binding of TNP-GTP to AnxA6 and Its Mutants in the Presence of 2 mM Ca^{2+} and Asolectin Liposomes

mutant	K_D (μM)[a]	mutant	K_D (μM)[a]
wild type	1.01 ± 0.13	R560A	1.30 ± 0.19
W192S	1.13 ± 0.13	Q595A	1.46 ± 0.14
K296A	1.44 ± 0.11	K598A	2.60 ± 0.30
W343S	—[b]	K644A	1.09 ± 0.12
W343F	1.04 ± 0.25		

[a] From three independent experiments. [b] The K_D value cannot be determined in the used range of TNP-GTP concentrations (see the text).

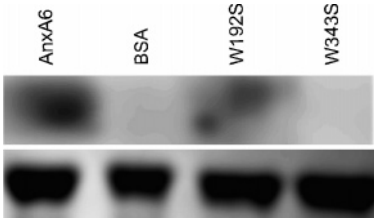

FIGURE 6: GTP binding assay of AnxA6 and its single-tryptophan mutants. Proteins (20 μg) were incubated with 5 μCi of [α-^{32}P]-GTP in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 0.5 mM EGTA, photolabeled by UV irradiation, incubated with SDS sample buffer, and analyzed by SDS−PAGE. The gel was autoradiographed (top panel) and visualized by Coomassie Brilliant Blue staining (bottom panel).

In fact, the exact K_D value for the W343S−TNP-GTP complex cannot be properly calculated by this method within the used TNP-GTP concentration range; the experimental data did not fit to the hyperbolic curve as for mutant W192S (Figure 5) and the wild-type protein (*11*), and the use of higher concentrations of TNP-GTP is not recommended. It is worth stressing that mutant W343F bound TNP-GTP with a K_D similar to that of the wild-type protein (Table 4), suggesting that the tryptophan residue can be replaced with another aromatic residue but not with a nonaromatic residue as in mutant W343S.

Covalent Labeling of Tryptophan Mutants of AnxA6 with [α-^{32}P]GTP. To confirm the results of TNP-GTP binding experiments, we employed photoaffinity labeling of the protein and its mutants with 25 Ci/mmol [α-^{32}P]GTP (Figure 6). This method has been recently used to investigate the GTP binding properties of various proteins, including G_h/transglutaminase (*23*) and the Ga_{i1} subunit of the heterotrimeric G-protein (*24*). In the absence of UV irradiation, there is no covalent labeling of AnxA6. After UV irradiation of AnxA6 and the single-tryptophan mutants, intense labeling of AnxA6 (Figure 6, lane 1) and mutant W192S (Figure 6, lane 3) was observed. The photoincorporation of the label did not occur in the case of bovine serum albumin (Figure 6, lane 2), which does not bind either GTP or TNP-GTP, pointing to the specificity of the method. As expected, the W343S mutant with an apparent affinity for GTP in the submillimolar concentration range (as revealed from experiments with TNP-GTP) was not photolabeled in the presence of 40 μM [α-^{32}P]GTP (Figure 6, lane 4), suggesting that residue W343 may be directly involved in GTP binding. These findings confirmed the observation obtained with TNP-GTP.

Ion Channel Activity of Tryptophan Mutants of AnxA6. As a next step, we addressed the question of whether the W343S mutant with impaired GTP binding properties possesses in vitro a GTP-induced ion channel activity, as described for the wild-type protein (*11*). For measurements

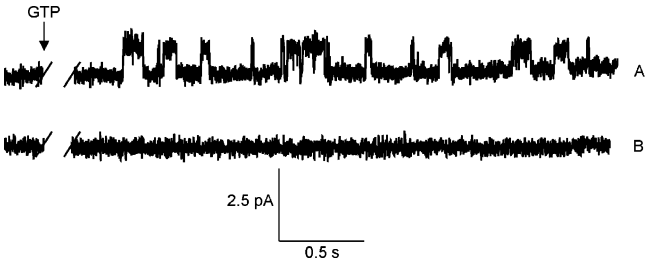

FIGURE 7: Ion channel activity of single-tryptophan mutants of AnxA6 in the presence of GTP. (A) Single-channel recordings of W192S incorporated into asolectin membranes upon addition of 4 mM GTP. Both chambers contained 10 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, and 200 mM KCl. The protein concentration was 20 nM in the cis chamber. (B) Trace for the W343S mutant. No current changes were observed.

of ion channel activity of AnxA6, W192S, or W343S, the proteins were placed in the cis chamber of a bilayer membrane admittance meter, separated from the trans chamber with a planar lipid bilayer. Both chambers contained 200 mM KCl (symmetric conditions). GTP was added to the same chamber as the protein to a final concentration of 4 mM, as described previously (*11*). The obtained recordings revealed that only mutant W192S (Figure 7A), which efficiently binds GTP, exhibited ion channel activity similar to that of wild-type annexin (*11*). The W343S mutant does not form ion channels in the lipid bilayer under any conditions tested in this report (Figure 7B). It can be, therefore, concluded that the mutation of the W343 residue to S343 impaired not only the GTP binding properties of AnxA6 but also its GTP-induced ion channel activity, implying that a nucleotide-binding domain of AnxA6 may form a molecular basis for the ion channel activity of the protein. To verify this hypothesis, we created other mutants of AnxA6 in which some amino acid residues within the nucleotide binding site (*15*) were replaced with alanine and tested their ability to bind GTP.

Binding of GTP to Single Mutants of AnxA6. A knowledge of a consensus sequence within a GTP binding site of AnxA6 (ref *15* and Table 1) allowed us to select amino acid residues other than W343 that are probably involved in the GTP binding. We replaced the chosen residues with alanine and examined the interaction of the obtained mutants with TNP-GTP (Table 4). We found that among the tested mutants, residue K598 may be involved in the binding, because the K_D value of the K598A−TNP-GTP complexes was by a factor of 2.6 higher than the K_D value determined for the wild-type protein (*11*). This may suggest that the K598-XXX-L602...K644-Y645 sequence within the C-terminal domain of the protein is involved in GTP binding. To confirm this observation, we labeled isolated mutants with [α-^{32}P]-GTP as in the case of the single-tryptophan mutants. In this assay, the mutations of residues located outside the consensus sequence, R560A and Q595A, did not change the GTP binding properties of the protein, as revealed with TNP-GTP (Table 4), while instead of mutant K598A, other mutants such as K644A from the C-terminal domain and K296A from the N-terminal domain revealed impaired binding properties (Figure 8). Therefore, both independent methods lead to similar findings for the W343 and K598 residues, suggesting that both these residues are involved in the GTP binding of AnxA6. Other mutants did not produce clear-cut conclusions,

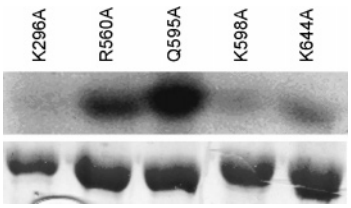

FIGURE 8: Photoaffinity labeling of AnxA6 mutants with [α-^{32}P]-GTP. Proteins were treated in the same way as single-tryptophan mutants. The autoradiographed gel is the top panel, and the bottom panel was visualized by Coomassie Brilliant Blue staining.

for example, K296A and K644A, which bind TNP-GTP with an affinity similar to that of the wild-type protein (Table 4), and are weakly photolabeled with [α-^{32}P]GTP. In our opinion, labeling of AnxA6 mutants with [α-^{32}P]GTP is more reliable than using TNP-GTP which is a chemically modified nucleotide with an additional chemical moiety that may change the binding properties of the ligand. It is worth stressing that for residues not located within the consensus sequence for GTP binding in AnxA6 (W192S, R560A, and Q595A mutants), both methods gave entirely the same results, showing an efficient binding of TNP-GTP and labeling with [α-^{32}P]GTP.

AnxA6 Can Form Homooligomeric Complexes in the Presence of GTP. At this stage of our investigation, we addressed the molecular mechanism leading to the formation of ion channels by AnxA6 in the presence of GTP. So far, we described experimental evidence suggesting that the proposed GTP binding domain in AnxA6 is divided into two regions: a phosphate-binding region that is split into two symmetrical subdomains located in the N- and C-terminal halves of the annexin, as identified previously (*15*), and a hydrophobic domain in the linker region of the protein containing the crucial residue, W343. We suggest that the tryptophan residue (its aromatic ring), as in other GTP-binding proteins, transglutaminase 2 (*25*) and T4 DNA ligase (*26*), is probably involved in steering the guanosine moiety of GTP, being therefore responsible for the specificity of the binding. GTP binding does not evoke large conformational or hydrophobicity changes in annexins, as observed for AnxA6 (*13*, *14*, *27*), AnxA5 (*28*), or AnxB12 (*29*) at mildly acidic pH. Therefore, there must be some alternative to the mechanism of alteration of hydrophobicity of annexin introduced above, possibly involving oligomerization, as suggested for AnxB12 (*30*). To test this possibility, we followed protein oligomerization by performing gel electrophoresis of AnxA6 under nondenaturing conditions. In the presence of Ca^{2+}, AnxA6 migrates as a doublet in accordance with earlier observations that AnxA6 is in equilibrium between monomers and dimers in solution (*31*). By supplementing the incubation medium with increasing concentrations of GTP, we observed the formation of high-molecular mass oligomers of AnxA6 under nondenaturing conditions (Figure 9).

To verify the molecular mass of the observed oligomers, we used glutaraldehyde as a cross-linking agent. We found that AnxA6 incubated without GTP, but in the presence of millimolar Ca^{2+} concentrations and 0.5% glutaraldehyde, forms large aggregates (the monomer band is faint; see Figure 10, lane 2) that did not migrate into the gel. In the presence of 20 mM GTP, the protein is probably stabilized in such a conformation that two bands can be detected, one corre-

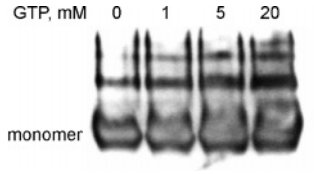

FIGURE 9: Effect of GTP on AnxA6 oligomerization. Western blot of a native electrophoresis of AnxA6. Samples were preincubated in the presence or absence of GTP in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM $CaCl_2$, and liposomes on ice for 30 min. Then, protein was applied to a 10% nondenaturing gel, which was electrotransferred onto a nitrocellulose membrane. Western blot analysis was carried out by using a monoclonal antibody to AnxA6.

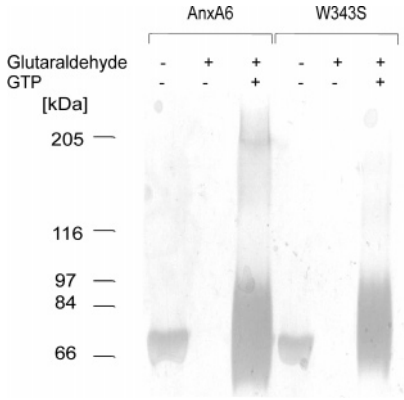

FIGURE 10: Glutaraldehyde cross-linking of AnxA6 (left) and the W343 mutant (right). GTP was used at a concentration of 20 mM with 0.5% glutaraldehyde. The samples after incubation were diluted with sample buffer, boiled, run on a 10% SDS−PAGE, and stained with Coomassie Brilliant Blue.

sponding to the monomer and a second as a protein oligomer with an apparent molecular mass of >200 kDa (most likely the trimer, Figure 10, lane 3).

The W343S AnxA6 Mutant Probably Does Not Form Oligomers in the Presence of GTP. We examined whether AnxA6 mutant W343S, which does not strongly bind GTP and does not exhibit GTP-induced channel activity, may form oligomers. We incubated W343S in the presence of GTP and added 0.5% glutaraldehyde (under the same conditions as for the wild-type protein). The mutant was separated via SDS−PAGE under reducing conditions. In the absence of GTP, the W343S mutant behaves like the wild-type annexin (Figure 10, lane 5). However, in the presence of GTP, it did not form oligomeric complexes (Figure 10, lane 6); one may notice that the monomer band is still clearly visible, as for nonmutated AnxA6.

DISCUSSION

W343 Is a Crucial Residue for Binding of GTP to AnxA6 and for Ion Channel Activity. Within the AnxA6 molecule, there are two tryptophan residues, W192 and W343, characterized by different levels of exposure to agents present in the surrounding milieu. Our previous data indicated that one of them might be involved in GTP binding. Using site-directed mutagenesis, we replaced the tryptophan residues with serine residues. W192S bound TNP-GTP with the same K_D value as the wild type of AnxA6 (1 μM), whereas the affinity of W343S for TNP-GTP significantly decreased in comparison with that of the wild-type protein. Interestingly, the W343F mutant fully retained the ability to bind TNP-GTP, suggesting that the aromatic ring of this residue is important for GTP binding and perhaps also for oligomer formation by AnxA6, as described for example for *E. coli* primary replicative helicase DnaB (*32*, *33*) and for hexameric helicase RepA (*34*). These data were confirmed with labeled GTP, [α-^{32}P]GTP. We conclude that W343 is a key residue involved in GTP binding. Besides AnxA6, there are also other proteins in which tryptophan residues play a crucial role in binding nucleotide. This is the case for transglutaminase 2 (TG2), in which the W241 residue is pivotal for protein activity (*25*) and T4 DNA ligase (*26*).

In our previous paper, we showed that GTP induces the channel activity of AnxA6 (*11*). In this work, we checked whether the W343S mutant, which did not bind GTP, would retain its GTP-induced channel activity. The W343S mutant did not form ion channels in lipid bilayers (Figure 7B), while mutant W192S did (Figure 7A). The mutation of W343 to S impaired not only the GTP binding properties of AnxA6 but also its GTP-induced ion channel activity.

GTP-Binding Motif in AnxA6. AnxA6 is a GTP-binding protein. However, AnxA6 does not contain the most frequently identified GTP-binding motif, G-X-X-X-X-G-K-(S/T), which is conserved in GTPases (*35*), including Ras protein (*36*) and adenylate kinase (*37*). We identified a different GTP phosphate group binding motif within the annexins, F/Y-X-X-X-F/Y-X-K-S-L. In AnxA6, there are two such consensus motifs, located almost symmetrically within the AnxA6 molecule (*15*). The mutation of the K296 residue in the first phosphate-binding motif (N-terminal half of the protein) or the mutation of K644 in the second (the C-terminal half of the protein) impaired the labeling of AnxA6 with [α-^{32}P]GTP (Figure 8). Montaville et al. (*38*) reported that the R-X-X-X-K sequence is responsible for recognition of phosphatidylserine by AnxA5, involving its phosphate groups. The sequence was found in the middle of the F/Y-X-X-X-F/Y-X-K-S-L consensus motif for some annexins, consistent with its importance for phosphate binding (*15*).

The AnxA6 sequence is similar to the consensus motif of GTPases that is responsible for guanine recognition, N-K-X-D (*17*). In the AnxA6 sequence, we found the N-K-P-L sequence (residues 597−600). The D residue in the GTPase sequence here is replaced with a L residue. When we replaced the K598 residue with A, the affinity of the nucleotide for the protein decreased. Another motif characteristic for GTPases is the E-X-S-A sequence (*17*). Such a sequence was also found in AnxA6, in the vicinity of the W343 residue. It seems that AnxA6 may possess a motif similar to that of GTP-binding proteins, but with the main characteristic that it recognizes phosphate groups. We found other proteins, which also have atypical GTP-binding motifs, for example, calexcitin, a calcium-binding protein from the "EF-hand" protein family (*18*). The degree of sequence similarity of calexcitins and GTP-binding proteins is very low, as is the case for AnxA6. However, calexcitin possesses regions that are more homologous to GTP-binding proteins as in the case of the ADP-ribosylation factor family, particularly sar1p (*18*). Calexcitin not only binds GTP but also hydrolyzes the nucleotide. Danieluk et al. (*39*) did not observe any GTPase activity of AnxA6, but other annexins, especially from plants, are most probably GTPases (*40*−*43*). The atypical GTP-binding motifs were also found in

endonuclease McrBC, forming a new subfamily within the superfamily of GTP-binding proteins (*44*).

Oligomerization of AnxA6 May Be Important for Its Ion Channel Activity. Annexins, including mammalian AnxA5 (*45*), AnxA7 (*46*), and AnxB12 (*45*, *47*) as well plant annexins (*48*), are well-known to form oligomers both at the membrane surface and in solution, but it is, to our knowledge, the first description of such annexin oligomers being stabilized by GTP. The addition of GTP favored AnxA6 oligomerization, probably formation of trimers (Figure 10). The W343S mutant was able to weakly bind GTP but did not form oligomers. GTP is responsible for the stabilization of oligomeric complexes inducing ion channel activity of AnxA6. The activity of annexins as ion channels is still controversial because they do not contain well-defined transmembrane domains. The mechanism of a possible rearrangement in the AnxB12 molecule as a response to a drop in pH has been proposed (*45*, *47*), and there are examples of other cytosolic proteins that may interact with membranes such as Hsc70 protein, a member of the Hsp70 family of heat shock proteins (*49*). Although Hsc70 is predominately a cytosolic protein, the presence of Hsp70s in association with, or in the proximity of, cellular membranes has been reported (*49*). Hsc70 self-associates to form oligomers in an ATP-dependent manner (*49*). The mechanism of the process is still unknown, as in the case of AnxA6. AnxA6 may exist in at least two different states, soluble and attached to the surface of the membrane (*50*). Maybe one of these states is stabilized by GTP, allowing the formation of multimers that are functional units of ion channels formed by AnxA6 in the presence of GTP. However, the molecular mechanism linking formation of the AnxA6 trimers in the presence of GTP and ion channel activity of the protein still awaits its final elucidation.

ACKNOWLEDGMENT

We thank Professor R. Huber from the Max-Planck-Institut fur Biochemie (Martinsried, Germany) for providing cDNA for human annexin AnxA6 and Professor R. Donato from Universita di Perugia (Perugia, Italy) for sending *E. coli* transformed with AnxA6 cDNA. We acknowledge fruitful discussion with Professor Rene Buchet from Universite Claude Bernard Lyon 1 (Villeurbanne, France) and Dr. Patrick Groves from the University of Madrid (Madrid, Spain). The infrared spectra of AnxA6 and its mutants were determined and analyzed at Universite Claude Bernard Lyon 1. The participation of Dr. Marcin Golczak in the preparation of AnxA6 mutants is kindly acknowledged.

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BI051629N