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Review

Annexin VI: An intracellular target for ATP*°

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Annexin VI (AnxVI), an Ca^{2+}- and phospholipid-binding protein, interacts *in vitro* with ATP in a calcium-dependent manner. Experimental evidence indicates that its nucleotide-binding domain which is localized in the C-terminal half of the protein differs structurally from ATP/GTP-binding motifs found in other nucleotide-binding proteins. The amino-acid residues of AnxVI directly involved in ATP binding have not been yet defined. Binding of ATP to AnxVI induces changes in the secondary and tertiary structures of protein, affecting the affinity of AnxVI for Ca^{2+} and, in consequence, influencing the Ca^{2+}-dependent activities of AnxVI: binding to F-actin and to membranous phospholipids, and self-association of the annexin molecules. These observations suggest that ATP is a functional ligand for AnxVI *in vivo*, and ATP-sensitive AnxVI may play the role of a factor coupling vesicular transport and calcium homeostasis to cellular metabolism.

Annexin VI (AnxVI), a multifunctional Ca^{2+}-dependent and membrane-binding protein, is expressed at the highest level in endothelial cells. In these cells, it exhibits various

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Abbreviations: Anx, annexin; CD, circular dichroism; K_D, dissociation constant; $K_{1/2}$, free ligand concentration for half-maximal response; RIDS, reaction-induced difference spectroscopy; TNP-ATP, 2′-(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; TNS, 2-*p*-toluidinylnapthalene-6-sulphonate.

activities, such as inhibition of phospholipase A_2 activity affecting blood coagulation, interaction with cytoskeletal proteins and proteases which possibly participate in membrane traffic during endo- and exocytosis, and interaction with Ca^{2+} transport proteins, as well as formation of transmembrane calcium channels playing a role in intracellular calcium homeostasis. Annexin VI is also phosphorylated by various protein kinases, including protein kinase C. Therefore, it is considered to be an element of the intracellular signaling cascade (for reviews see [1, 2]). The complete range of physiological functions of AnxVI has not been established. Recently, it has been reported that AnxVI can bind *in vitro* adenine nucleotides [3, 4]; this observation, however, has not been substantiated *in vivo*, and so far no related function has been discovered [5, 6].

The aim of this article is to provide a concise overview of recent progress in the field of nucleotide binding properties of AnxVI, as examined at various levels of structural organization of the annexin molecule, and to describe possible functional implications of AnxVI-nucleotide interaction.

NUCLEOTIDE-BINDING PROPERTIES OF ANNEXIN VI

AnxVI interacts with many structural and functional analogs of nucleotides (Fig. 1). In this regard, AnxVI fulfills the requirements established for ATP/GTP-binding proteins, e.g. AnxVI is photoaffinity labeled with 8-azido-[γ-^{32}P]ATP in a concentration-dependent manner (Fig. 2A) [3, 4]. The external fluorescence of 3′(or 2′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) becomes increase 2–3-fold in the presence of this protein, pointing to binding of TNP-ATP

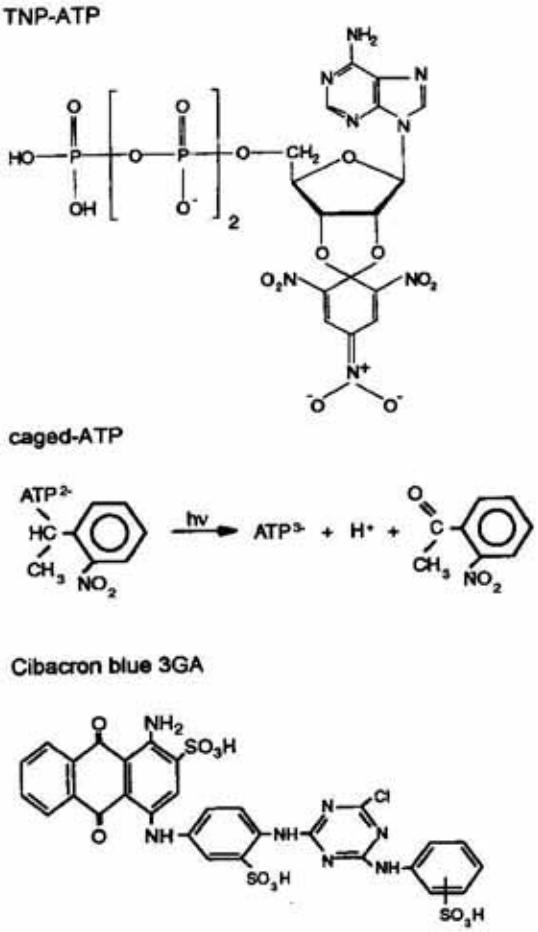

Figure 1. Chemical formulas of structural and functional analogs of nucleotides used in the present study.

Taken from Molecular Probes Inc., and Aldrich Chemical Co. catalogs.

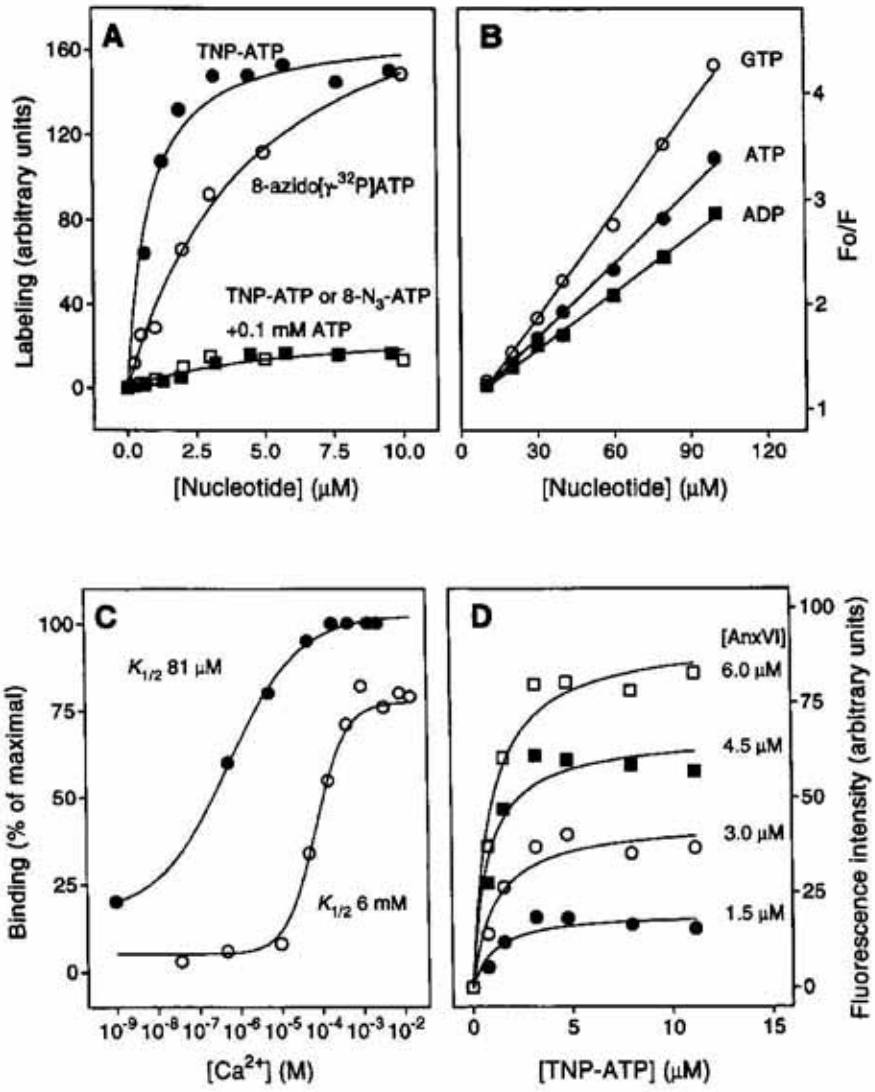

Figure 2. Interaction of annexin VI with nucleotides.

(A) Concentration-dependent binding of 8-azido-[γ-^{32}P]ATP (○, in the absence of Ca^{2+}) and TNP-ATP (●, in the presence of 0.5 mM Ca^{2+}) to AnxVI. The competitive effect of 0.1 mM ATP on the two processes is shown (the respective symbols are □,■). (B) Quenching of intrinsic fluorescence of AnxVI by nucleotides (in the presence of 20 mM Ca^{2+}). (C) Calcium-dependent binding of AnxVI to ATP-agarose (●) or Cibacron blue 3GA-agarose (○). (D) Protein concentration-dependent enhancement of TNP-ATP fluorescence accompanied by a blue shift of emission maximum of TNP-ATP.

Fluorescence titrations were determined at room temperature at λ_{ex} = 415 nm and λ_{em} = 530 nm, in the presence of 1.5–6.0 μM AnxVI + 1 mM $CaCl_2$, at increasing TNP-ATP concentrations, using a Double Spectrometer, model 1680B, equipped with DM 3000 software (Spex Fluorolog Industries Inc., U.S.A.). Titration curves were fitted by hyperbolic regression. Mean values of two experiments are shown. Fluorescence due to unspecific binding was measured in the presence of 2 mM ATP. Concentration of TNP-ATP bound to AnxVI, fluorescence yield (ϕ) and the binding stoichiometry were calculated essentially, as described by Huang *et al.* [12]. The results presented in panel A are taken from [3], in panel B from [4], in panel C from [3] and [9], and in panel D from J. Bandorowicz-Pikuła, A. Wrzosek, M. Danieluk, S. Pikuła & R. Buchet, Biochem. Biophys. Res. Commun., in press.

to AnxVI. In addition, a blue shift of emission maximum of the TNP-ATP fluorescence spectrum is observed; from 550 nm to 530–535 nm. The binding of TNP-ATP to AnxVI is a nu-

Figure 3. Primary structure of human AnxVI [19, 20] revealing a repeat-domain nature of the protein.

Partial amino-acid sequences of the porcine liver AnxVI isoform are also shown, indicating a high level of homology between the two proteins. Asterisks denote identical amino-acid residues. Proteolytic fragments of AnxVI after digestion with V8 protease from *Staphylococcus aureus* were purified as described previously [8, 9]. Direct sequencing of N-terminal portions of these fragments, after their separation by ATP-agarose affinity chromatography, SDS/PAGE, and electroblotting to Immobilon-P transfer membrane, was performed in the Protein Chemistry Laboratory (University of Texas Medical Branch at Galveston, TX, U.S.A.).

cleotide concentration-dependent (in the presence of Ca^{2+} $K_{D\ TNP\text{-}ATP} \approx 0.6 \pm 0.1\ \mu M$, n = 3) and it is by more than 80% inhibited by 0.1 mM ATP (Fig. 2A) [3]. Furthermore, the intrinsic fluorescence of AnxVI is quenched by adenine and guanine nucleotides in the following order of effectiveness: GTP > ATP > ADP > cAMP (Fig. 2B) [4]. In the case of TNP-ATP, the quenching of intrinsic fluorescence of AnxVI by a nucleotide is accompanied by a fluorescence energy transfer from Trp343 in the AnxVI molecule to TNP-ATP [4]. The rate of quenching depends on AnxVI conformation [3, 7]. AnxVI binds also specifically and in a Ca^{2+}-dependent manner to ATP-agarose (Fig. 2C). The binding is reversed by ATP and GTP [3, 8]. Annexin VI binds also to Cibacron blue 3GA-agarose, the binding depending on calcium concentration (Fig. 2C) [9, 10]. Cibacron blue 3GA is not a structural but rather a functional analog of ATP, e.g. it has been found to evoke P2 purinergic receptor antagonism and to affect the activity of many ATP-binding proteins [11]. Thus, also this observation favors the view of AnxVI being a nucleotide-binding protein. From experiments in which various concentrations of AnxVI were used to titrate the external fluorescence of TNP-ATP (Fig. 2D), the TNP- ATP:AnxVI binding stoichiometry was calculated (using a standard method described by Huang *et al.* [12]; the experimental details are described in the legend to Fig. 2), to be amounted to 1:1 (TNP-ATP/AnxVI, mol/mol). Binding revealed no cooperativity (Hill coefficient = 1.0 ± 0.1, n = 3).

The above characterized behavior of AnxVI in the presence of purine nucleotides justifies, in our opinion, further structural studies aimed at identification and characterization of the nucleotide-binding domain of AnxVI.

NUCLEOTIDE-BINDING DOMAIN OF ANNEXIN VI: PRIMARY STRUCTURE

Analysis of primary structure of AnxVI did not reveal the presence of Walker consensus motifs A and B involved in ATP/GTP binding [13] and other motifs found in ATP/GTP-binding proteins characterized to date [14]. Some similarity to cAMP-binding proteins has

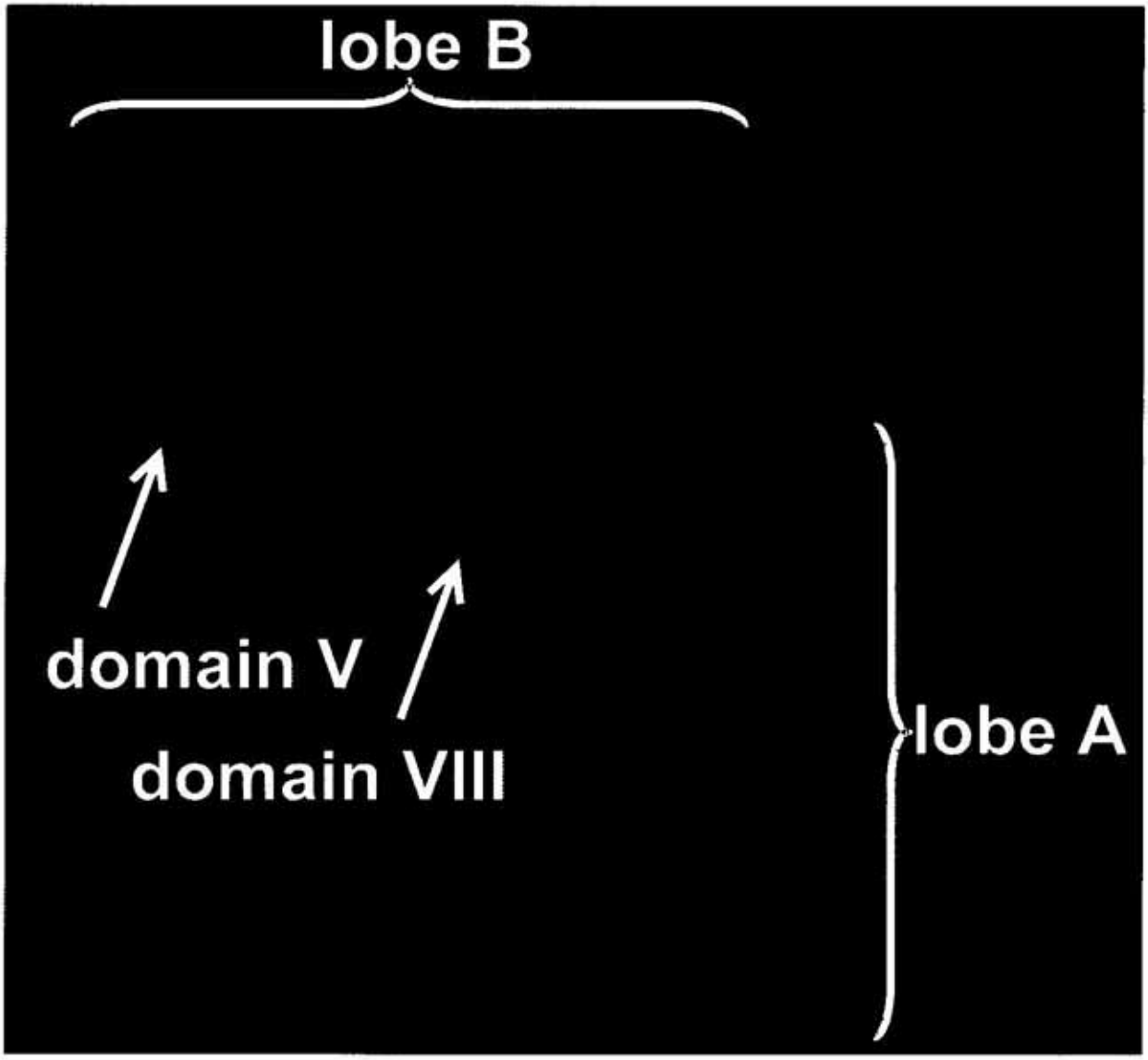

Figure 4. Ribbon representation of the annexin VI molecule, showing a tight association of domains V and VIII within the C-terminal portion of the protein.

The Figure is based on the crystal structure of bovine AnxVI (resolution 2.9 Å, space group P43, unit cell dimensions: *a* 67.40 Å, *b* 67.40 Å *c* 200.08 Å, unit cell angles: $\alpha = \beta = \gamma$ 90°), crystallized in the presence of Ca^{2+} [21] (calcium ions bound are shown as dark grey balls). The picture prepared with the use of MOLSCRIPT was obtained from Protein Data Bank (code 1AVC). The position of domains V and VIII, tightly connected to each other, is depicted. A linker between domains V and IV, containing residue Trp343, is not well visible from this perspective. We suggest that amino-acid residues located within the domains V and VIII, as well as in a linker region between domains IV and V, form a nucleotide-binding domain of AnxVI. Other explanations are in the text.

been reported for AnxI [15], but no structure-function relationship has been described.

In most species, as in rat [16], mouse [17], *Bos taurus* [18] and man [19, 20] AnxVI is composed of 673 amino-acid residues. The molecule is organized into two symmetrical lobes A and B connected by a linker, as established from crystal structure of bovine [21] and human [22] AnxVI isoforms. These lobes consist of four domains, each containing conservative consensus motifs for Ca^{2+}- and phospholipid-binding, so-called "annexin folds". Annexin folds represent Ca^{2+}-dependent phospholipid-binding sites, alternative to C2

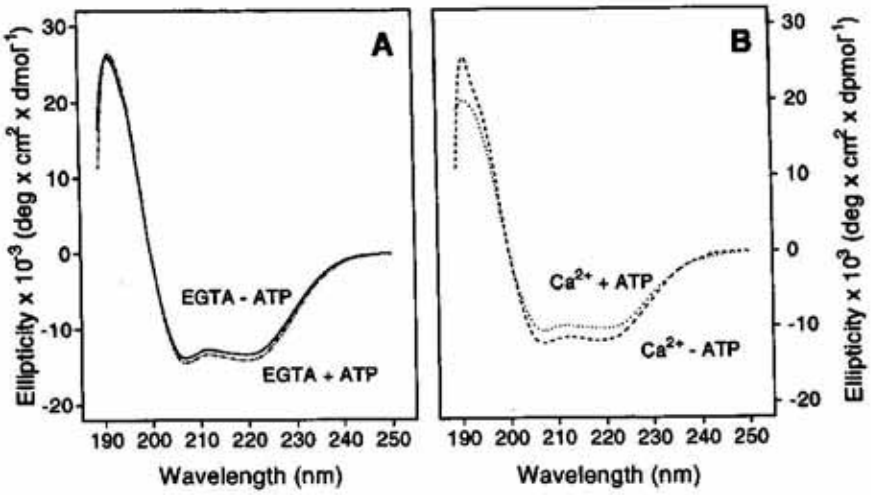

Figure 5. The effect of ATP on secondary structure of annexin VI.

Far-UV CD spectra of AnxVI were recorded in the presence of EGTA (A) or $CaCl_2$ (B) at room temperature on an AVIV CD spectrophotometer (AVIV Associates Inc., U.S.A.), essentially as described previously [43]. The assay medium contained AnxVI (0.25 mg protein/ml) in 5 mM Tris/HCl, pH 7.4, 20 mM NaCl, and 1 mM EGTA (solid line), 2 mM Ca^{2+} (dashed line), 1 mM EGTA and 0.2 mM ATP (dashed-dotted line) or 2 mM Ca^{2+} and 0.2 mM ATP (dotted line). On the Figure the representative smoothed spectra chosen from 3–8 experiments are shown.

domains, which allow protein binding to lipid membranes. Annexin folds are present exclusively in annexins while C2 domains can be found in various protein families, such as protein kinase C isoforms, phospholipase A_2 and proteins involved in membrane traffic [23]. Annexin folds differ from C2 domains in having, unlike the C2 domains, a high α-helix content [24–27].

The following regions can be recognized in the AnxVI molecule: the N-terminal tail (numbering of residues in parentheses) (1–21), domain I (22–91), domain II (92–163), coil II–III (164–172), domain III (173–250), domain IV (251–325), a linker (326–354) preceding lobe B coil (355–365), domain V (366–434), domain VI (435–506), coil VI–VII (507–515), domain VII (516–599), and domain VIII (600–673) [21]. The actin-binding site, found in lung AnxII [28], is present in AnxVI (TLIRIMVSR) starting, however, from Tre273, and not from valine, as in AnxII. Annexin VI is also able to bind heparin [29], although it does not contain typical Cardin–Weintraub recognition sequences for glycosaminoglycans localized in other annexins, as for example in AnxII [30, 31].

On the basis of the following experimental data it can be hypothesized that the nucleotide-binding domain of AnxVI is located within the C-terminal half of the protein molecule. The fluorescence energy transfer from Trp343 to TNP-ATP occurs only in the case of a large, eight-domain AnxVI [4], but not in homologous four-domain AnxIV [32]. Partial digestion of AnxVI with protease V8 followed by affinity chromatography on ATP-agarose gives three proteolytic fragments of protein with molecular masses of 33.2 kDa, 18.8 kDa and 14.3 kDa. Their N-terminal sequences reveal that these fragments originate from the C-terminal half of the AnxVI molecule (Fig. 3). In addition, these fragments, especially those of 33.2 kDa and 14.3 kDa, retain covalently attached 8-azido-[γ-^{32}P]ATP [9]. It can be speculated, on the basis of the crystal structure of human [22] and bovine [21] AnxVI, that the putative high affinity nucleotide-binding site is located within the calcium-binding domains V and VIII of AnxVI (Fig. 4), since both do-

mains were found to interact tightly with each other, and are spatially close to Trp343, allowing fluorescence energy transfer from this residue to TNP-ATP. These speculations are substantiated by observations made by other investigators suggesting that a nucleotide-binding domain is present in human AnxI [15, 33], in bovine and recombinant human AnxVII [34], and in the four-domain GTP-binding plant annexin [35].

CHANGES IN SECONDARY AND TERTIARY STRUCTURE OF ANNEXIN VI UPON ATP BINDING

By using circular dichroism (CD) spectroscopy, we investigated the mechanism of the interaction of AnxVI with ATP and found a 5% decrease in α-helix content upon ATP binding in the presence of Ca^{2+} but not of EGTA (Fig. 5). Since we have used only a self-consistent method to calculate a change in the secondary structure of AnxVI upon ATP binding, the observed decrease in α-helix content has to be taken qualitatively. However, this interpretation of CD spectra is corroborated by infrared spectroscopy in conjunction with photoactivated release of ATP (J. Bandorowicz-Pikuła, A. Wrzosek, M. Danieluk, S. Pikuła & R. Buchet, in press) from an inactive photolabile substrate, caged-ATP (at Fig. 1). This method used was based on measurements before and after illumination of two infrared spectra of protein, reflecting the two states of the protein: nucleotide free and nucleotide bound. This is the so-called reaction-induced difference spectroscopy (RIDS), used earlier in the case of Ca^{2+}-ATPase [36, 37], creatine kinase [38] and arginine kinase [39]. Secondary structural changes were also observed by infrared spectroscopy upon interaction of AnxV with lipids [40, 41]. However, so far the putative effects of nucleotides on the secondary structure of any annexin isoform, except AnxVI, have not been described.

The results of CD determinations suggest a moderate rearrangement of AnxVI secondary structure upon ATP binding. Therefore, we investigated the possible changes in the tertiary structure of AnxVI. In fact, various adenine and guanine nucleotides quenched the intrinsic fluorescence of AnxVI, which implies that some tyrosine and tryptophan residues located in the vicinity of ATP-binding site of AnxVI undergo relocation resulting in changed exposure of these residues to the surrounding milieu [4, 7, 10]. The findings are in agreement with the results of experiments with the use of a hydrophobic fluorescent probe, 2-*p*-toluidinylnaphthalene-6-sulphate (TNS). Mani & Kay [42] have earlier shown that this compound occupies a larger hydrophobic space when bound to AnxVI in the presence of Ca^{2+} than in the presence of EGTA, as it was evident from the enhancement of TNS fluorescence. We have found that addition of ATP abolishes the Ca^{2+}-dependent enhancement of TNS fluorescence, suggesting that the structure of the calcium-binding sites within an AnxVI molecule is affected upon nucleotide binding [43].

IMPACT OF ATP/GTP BINDING FOR ANNEXIN VI FUNCTIONING

The effect of binding of nucleotides to AnxVI on protein function was extensively studied in the case of ATP. It has been shown that ATP shifts the affinity of AnxVI for Ca^{2+} while binding to erythrocyte ghosts (shifting $K_{1/2}$ value for Ca^{2+} from 1.5 μM to 21.1 μM in the absence or presence of 4 mM ATP, respectively) [3]. A similar effect of ATP was observed in the case of calcium-dependent interaction of AnxVI with F-actin (10-fold increase of $K_{1/2}$ for Ca^{2+} in the presence of nucleotide) [3]. These results strongly suggest that ATP may influence *in vivo* the binding of AnxVI to membranes and cytoskeleton [44]. This was further substantiated by the results of experiments, revealing changes in affinity of AnxVI

for Ca^{2+} [7], in which AnxVI in complex with ATP aggregated phosphatidylserine liposomes. In our opinion, these observations are in agreement with the results of structural studies, suggesting rearrangement of calcium-binding domains within an AnxVI molecule upon nucleotide binding. This hypothesis should be verified in experiments *in vivo*. Some results, as those of Tagoe *et al.* [45] indicating that binding of AnxVI to hepatocyte plasma membrane is enhanced by ATP at relatively high calcium concentrations, point in that direction. The hypothesis is also supported by the results of Hoyal *et al.* [46], showing that, under oxidative stress accompanied by the depletion of intracellular ATP, AnxVI detaches from the plasma membrane of alveolar macrophages releasing a substantial amount of Ca^{2+}, leading thus to an overall elevation of intracellular Ca^{2+} concentration.

Further experiments are required to confirm the importance of AnxVI/nucleotide interaction for cell functioning. We are currently considering two possibilities. First, AnxVI is activated by ATP/GTP binding. In that case the nucleotide signal should be stopped by slow rate hydrolysis of nucleotide, perhaps regulated by Ca^{2+}, as shown for plant annexins [35, 47, 48] and AnxVII exhibiting GTPase activity [34, 49]. As a second possibility, we suggest that AnxVI interacts with other nucleotide-binding proteins, e.g. with synapsins [50, 51]. AnxVI may also play a role of a guanine exchange factor (GEF) or of a GTPase activating protein (GAP) or may interact with other GAPs. In favor of the latter possibility is the observation that AnxVI binds to $p120^{GAP}$, an activator of GTPase $p21^{ras}$ [52, 53]. The suggestion that AnxVI is a GAP is in agreement with the observation that AnxVI interacts with dynamin, a GTPase involved in endocytosis [54]. Thus, it appears that the AnxVI/nucleotide interaction might play a role in membrane traffic processes (for review see [44]).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The hypothesis of ATP/GTP being functional ligands for AnxVI requires, in our opinion, the following verification. First, the crystal structure of AnxVI/nucleotide complex has to be solved and amino-acid residues involved in binding of nucleotides identified. Second, the nucleotide binding properties of the annexin family has to be provided. It has been earlier shown that AnxI [15, 33], AnxIV [32], AnxVII [34, 49], and probably AnxV [55, 56] are able to bind cAMP, ATP or GTP/GDP, although neither structural data nor convincing results of *in vivo* experiments have been given. Third, one of the most important tasks we foresee in the near future is an elaboration of experimental conditions which will elucidate the significance of the AnxVI/nucleotide interaction *in vivo*. We believe that such efforts will allow to define a new function for some members of the annexin family. We suggest that ATP-sensitive AnxVI may play the role of a factor coupling the intracellular calcium homeostasis to cellular metabolism.

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