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Conformational Change of Human Annexin I by the Binding of Ca^{2+}, ATP and cAMP

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Abstract : Human annexin I is a member of annexin family of calcium dependent phospholipid binding proteins, which have been implicated in various physiological roles including phospholipase A_2 (PLA_2) inhibition, membrane fusion and calcium channel activity. In this work, the structure of N-terminally truncated human annexin I (⊿-annexin I) and its interactions with Ca^{2+}, ATP and cAMP were studied at atomic level by using nuclear magnetic resonance (NMR) spectroscopy. The effect of Ca^{2+} binding on the structure of ⊿-annexin I was investigated. The addition of Ca^{2+} to ⊿-annexin I caused some changes in ^{13}C NMR spectra. Carbonyl carbon resonances of some histidines were significantly broadened by Ca^{2+} binding. However, in the case of methionine, phenylalanine, and tyrosin, small changes could be observed. We found that ATP and cAMP bind ⊿-annexin I, and the binding ratio of ATP to ⊿-annexin I is 1. These results are well consistent with the report that cAMP and ATP interact with annexin I, and affect the calcium channels formed by annexin I. Because ⊿-annexin I is a large protein with 35 kDa molecular weight, site-specific (carbonyl-^{13}C) labeling technique was used to study the interaction sites of ⊿-annexin I with Ca^{2+}. NMR study was focused on the carbonyl carbon resonances of tyrosine, phenylalanine, methionine and histidine residues of ⊿-annexin I because the number of these amino acids is small in the amino acid sequence of ⊿-annexin I.

INTRODUCTION

Annexins are a widely distributed family of homologous amphipathic cytosolic proteins that bind phospholipids and membranes in a Ca^{2+} dependent manner. They have been implicated in multiple aspects of cell biology including regulation of membrane

trafficking, transmembrane channel activity, inhibition of phospholipase A_2, inhibition of coagulation, transduction of mitogenic signal and settlement of cell-matrix interactions.[1,2,3] Most annexins are abundant intracellular proteins and annexin I, itself is approximately, 4% of the total soluble protein in human polymorphonuclear leucocytes.[4] Up to now at least ten different annexin members have been identified.

That all annexins display similar properties regarding Ca^{2+} and phospholipid is due to a common primary structure. Each annexin is constituted of two different regions, the unique N-terminal domain, also called the 'tail' and the C-terminal domain, called the core. The 34 kDa C-terminal domain is the conserved part of the molecule and strictly defines the annexin family, and direct evidence indicates that the C-terminal domain is sufficient to Ca^{2+} and phospholipid binding.[5] With one exception, it is always composed of 4 repeats of a 70 amino acid sequence containing an increased homology region.[6]

The N-termini of annexins show great diversity, both in composition and in length. The annexin N-terminal domain is considered as the regulatory region of the protein, since it contains the major sites for phosphorylation, proteolysis or even interactions with other proteins.[6] In intact cells, annexin I is substrate for the EGF,[7,8] insulin receptor kinases[9] and for $pp60^{v\text{-}src}$,[10] and has been shown to translocate from cytoplasm to the membrane upon Ca^{2+} treatment.[11] In assays *in vitro*, phosphorylation decreases their ability to bind membranes, and neutralizes the PLA_2 inhibitory activity.[12,13]

Corticosteroid hormons (glucocorticoids) such as cortisone are thought to act as anti-inflammatory drugs by inducing white blood cells to synthesize and/or secrete annexin I as a local chemical mediator. Annexin I inhibits the activity of phospholipase A_2 in the first step of the eicosanoid synthesis pathway, in which membrane lipids are degraded through arachidonic acid into inflammation mediating eicosanoids.[14,15]

Recently, the crystal structure of the calcium-bound form of N-terminally truncated human annexin I has been solved.[16] The X-ray crystal structure has shown that the four tandem repeats of the protein are folded into four domains of similar structures. Each domain consists of five α-helices which were wound into a right-handed superhelix(Fig. 1). The four domains are arranged in a planar array, with the Ca^{2+}-binding sites located in a region helix-loop-helix. Each domain has two helix-loop-helix regions, AB and DE loops. Annexin I binds Ca^{2+} in repeats 2, 3 and 4 through three sites: (E,M)-(K,R)-(G,R)-X-G-T(38 residues)-(D,E) motif. Huber and coworkers have defined this new type of Ca^{2+} binding sites as typeII, typeI being the well-known EF-hand motif. Three additional Ca^{2+} binding sites of different structure (typeIII) have been identified in the annexin I crystal.[16]

The overall sequence of the annexin core domain is fairly hydrophilic, due to abundance of polar and charged amino acids. Moreover, there is no significant strech of hydrophobic amino acids.[6] An important conformational modification has been reported in crystals having a Ca^{2+} ion bound in the tryptophan loop connecting helices 1 and 2 of the third repeat of annexin V. In crystals where Ca^{2+} is not bound to this site, the tryptophan loop is buried in the hydrophobic core of the protein.[17] On the other hand, when Ca^{2+} is

bound, the loop extends away from the molecule, exposing the tryptophan to the solvent.[18, 19] Thus, a significant but subtle rearrangement exposing the hydrophobic surfaces of the protein can allow annexin to span membrane in a conventional manner involving hydrophobic interactions.[6]

Many members of the annexin gene family including annexin VII, annnexin V and annexin I, are known to form ion channels in lipid bilayers.[20, 21]

Recently, it was reported that while calcium activates annexin I driven chromaffin granule membrane aggregation and liposome fusion, these membrane active properties are specifically and cooperatively regulated by cAMP and ATP, and that the calcium channels formed by annexin I in planar lipid bilayers can be profoundly altered by cAMP and ATP.[22] cAMP and ATP selectively alter ion channel activity only when the nucleotides are added to the trans chamber of the planar lipid bilayer system.[22] It would thus appear that specific Annexin I domain, able to interact with cAMP and ATP apparently, penetrates the bilayer and is exposed to the bulk phase on the trans side.[22]

In the present work, the N-terminally truncated human annexin I(Δ-annexin I) was specifically labeled with stable isotopes ([^{13}C]carbonyl - His, Met, Tyr, and Phe).[23, 24] The effect of Ca^{2+} binding on the struture of annexin I was studied. We have also investigated the interaction of ATP and cAMP with Δ-annexin I using ^{1}H NMR spectroscopic methods.

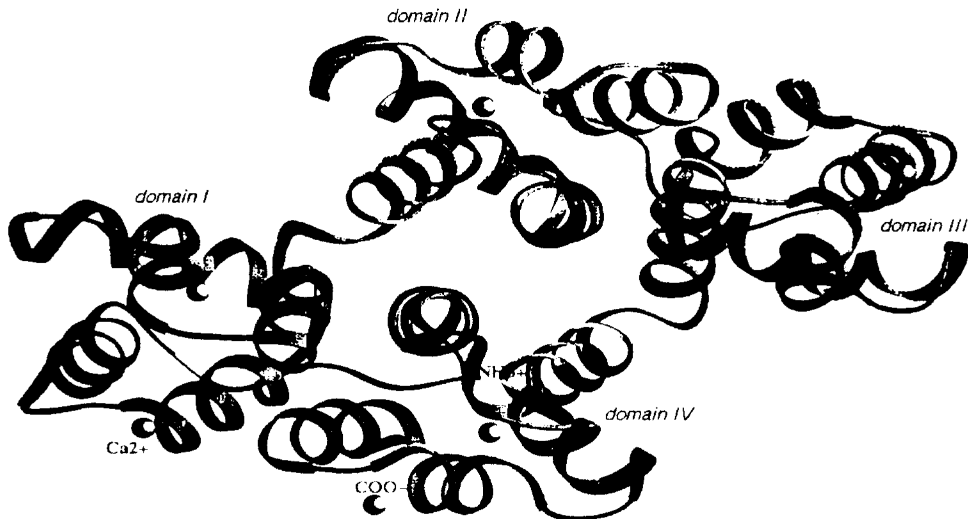

Fig. 1. Crystal structure of human annexin I generated by Ribbon 2.5.[16]

MATERIALS AND METHODS

Materials

L-[1-^{13}C]Tyr, DL-[1-^{13}C]Met, DL-[1-^{13}C]His and L-[1-^{13}C]Phe were purchased from Isotec (Miamisburg, OH, USA). The isotope enrichment is 95% or higher for each of these amino acids. cAMP, Na_2ATP and IPTG were purchased from Sigma (St. Louis, MO, USA). All other materials were analytical or biotechnological grade. Selective deuteration of the H8 proton of the purine ring of the cAMP and ATP was respectively carried out by heating solutions of the cAMP and ATP in D_2O at 80℃ for 7h.[25]

Overexpression of isotopically labeled Δ-annexin I

The cDNA encoding Δ-annexin I was cloned in the expression vector pET28a controlled by T7 promoter by Professor D. S. Na. *E. coli* BL21 (DE3) strain carrying the expression constructs were grown in 5 mℓ of LB broth at 35℃ overnight. 10 μℓ of the culture was then transferred and grown in 5 mℓ of M9 minimal medium containing glucose as carbon source, NH_4Cl as nitrogen source and amino acids except the isotope-labeled amino acids. 1 mℓ of the culture was then transferred and grown in 100 mℓ of the same M9 minimal medium. When the culture was reached to 0.5 OD_{550nm}, 1 mℓ of the seed culture was transferred and grown respectively in eighteen 100 mℓ of M9 minimal media. After induction with 0.5 mM IPTG and addition of the isotope-labeled amino acids (30 mg/ℓ) at 0.5 OD_{550nm}, the cultures were then incubated with vigorous shaking. The cell cultures were harvested (8,000 g, 30 min, 4℃ centrifuge) over 3～4 hours post-induction.

Purification of Δ-annexin I

The harvested cells were suspended in 100 mℓ of buffer A (25 mM Tris-HCl, 1 mM β-mercaptoethanol, 1 mM EDTA and 5% glycerol, pH 7.7), and then lysed by using tip sonifier (Branson, CT, USA). The lysate was then centrifuged, and the supernatant was diluted to 200 mℓ of buffer B (25 mM Tris-HCl, 1 mM EDTA, pH 7.7). The lysate was then applied to DEAE-sepharose (anion exchange resin; Sigma, MO, USA) column previously equilibrated with buffer B at 4℃. Δ-annexin I protein eluted in pass-through was diluted to 300 mℓ of buffer C (50 mM potassium phosphate, pH 6.5). The sample was then applied to Bio-rex 70 (cation exchange resin; Bio-Rad, CA, USA) column previously equilibrated with buffer C at 4℃, and the column was washed extensively with the same buffer. Bound proteins were then eluted with 300 mℓ of linear gradients of 0 to 1 M NaCl in buffer C. Fractions containing Δ-annexin I protein were pooled and dialyzed against buffer D (50 mM potassium phosphate, 100 mM NaCl, 1 mM NaN_3, pH 6.5). The sample was then concentrated with Centriprep-10 and Centricon-10 concentrators (Ultrafiltration

membrane; Amicon, MA, USA) to 280 μl of NMR sample volume. The concentration was measured by Bradford method.[26] In the case of NMR measurement, the solvent water was exchanged with D_2O after lyophilization.

NMR measurements

For NMR measurement, the samples were prepared to a final volume of 280μl in 50 mM potassium phosphate buffer, pH 6.5, containing 100 mM NaCl and 1 mM NaN_3 in D_2O. Shigemi micro-NMR tube is used. The final concentration of Δ-annexin I was 0.3 ~ 1.2 mM. NMR measurements were made on a Bruker AMX-500 FT NMR spectrometer in Seoul National University. ^1H NMR spectra was recorded with a spectral width of 7042.25 Hz and 8192 real data points. Prior to Fourier transformation, the data obtained were multiplied by exponential window function with broadening factor of 0.2 in D_2O and zero-filled to 16384 real data points. Chemical shifts were given in ppm from the methyl group resonance of 2,2-dimethyl-2- silapentane-5-sulfonate. In ^1H NMR measurement, the probe temperature was 30℃. Spectra in H_2O were recorded by means of the 1-1 echo water suppression pulse.

^{13}C NMR spectra were recorded at 125 MHz by using WALTZ-16 composite decoupling pulse sequence. The free induction decay was recorded with 32 K data points, a spectral width of 25,000 Hz. All FIDs were multiplied by an exponential window function with broadening factor of 5 Hz prior to Fourier transformation. Chemical shifts were given in ppm from external dioxane. In ^{13}C NMR measurement, the probe temperature was 27℃.

RESULTS AND DISCUSSION

Production of isotopically labeled Δ-annexin I

^1H NMR spectroscopy has had some problems with studies of protein structure as its molecular weight increase. In order to study the structure of large protein over 30 kDa by NMR, it is obviously necessary to obtain isotopically labeled protein. We prepared Δ-annexin I samples labeled with various carbonyl ^{13}C amino acids including tyrosine, phenylalanine, methionine and histidine.

Observation of carbonyl carbon resonance in the ^{13}C NMR spectra of Δ-annexin I

Being a large protein with 35 kDa molecular weight, Δ-annexin I was labeled with specific amino acid isotopes. Δ-annexin I contains nine phenylalanine , ten tyrosine , seven methionine, and five histidine residues. In the ^{13}C NMR spectrum of Δ-annexin I labeled with [1-^{13}C]Phe or [1-^{13}C]His, nine phenylalanine and five histidine carbonyl resonances were well resolved (Fig. 2A, 3A). Also, in the case of tyrosine, almost all the individual carbonyl resonances were resolved (Fig. 4A). However, three methionine carbonyl

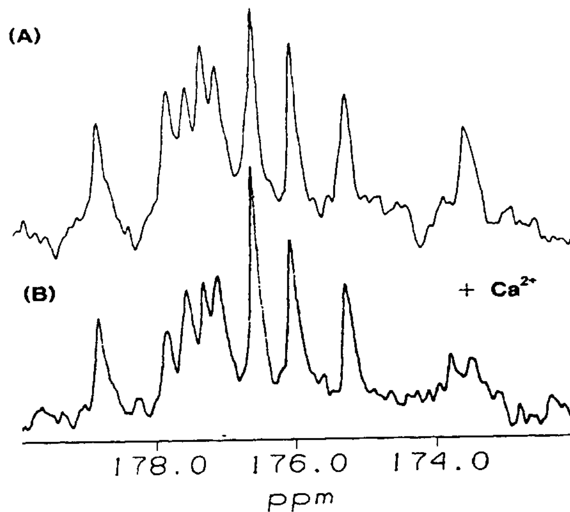

Fig. 2. 125-MHz ^{13}C NMR spectra of (A) [1-^{13}C]Phe labeled □-annexin I, and (B) □-annexin I* Ca^{2+} complex. □-annexin I in D_2O containing 50mM potassium phosphate, pH 6.5, 100mM NaCl, and 1mM NaN_3. The concentrations of □-annexin I and Ca^{2+} were 0.6mM and 6mM respectively. The probe temperature was 27 ℃

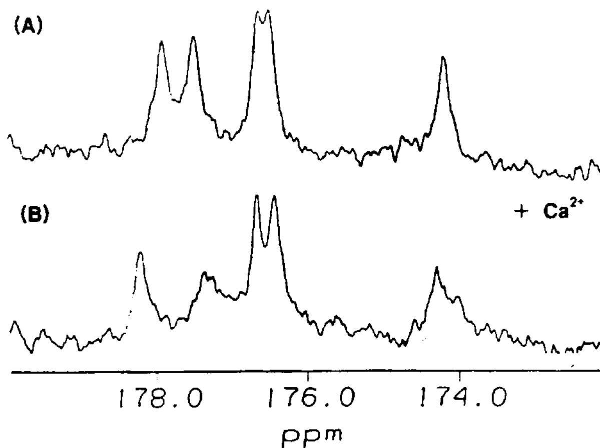

Fig. 3. 125-MHz ^{13}C NMR spectra of (A) [1-^{13}C]His labeled □-annexin I, and (B) □-annexin I* Ca^{2+} complex. The concentrations of □-annexin I and Ca^{2+} were 0.8mM and 8mM respectively. The probe temperature was 27 ℃

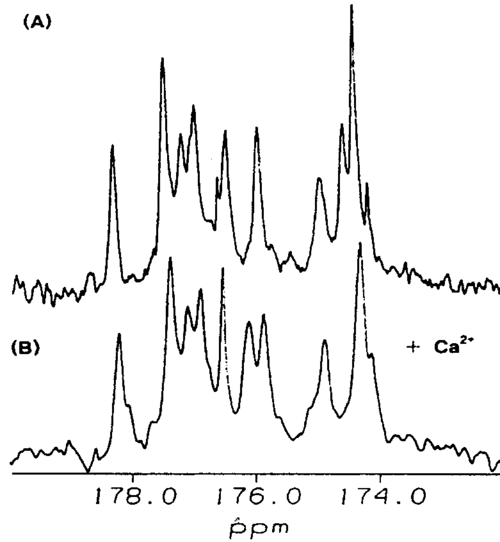

Fig. 4. 125-MHz ^{13}C NMR spectra of (A) [1-^{13}C]Tyr labeled Ɑ-annexin I, and (B) Ɑ-annexin I* Ca^{2+} complex. Experimental conditions were identical to those of the measurement of Phe labeled Ɑ-annexin I. The concentrations of Ɑ-annexin I and Ca^{2+} were 0.8mM and 8mM respectively. The probe temperature was 27 ℃

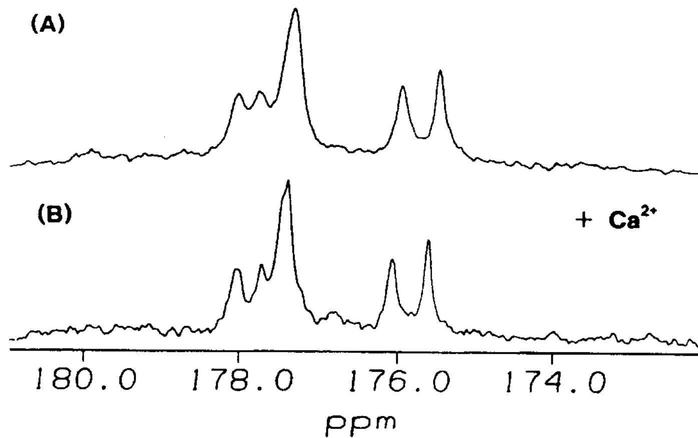

Fig. 5. 125-MHz ^{13}C NMR spectra of (A) [1-^{13}C]Met labeled Ɑ-annexin I, and (B) Ɑ-annexin I* Ca^{2+} complex. Experimental conditions were identical to those of the measurement of Phe labeled Ɑ-annexin I. The concentrations of Ɑ-annexin I and Ca^{2+} were 1mM and 10mM respectively. The probe temperature was 27 ℃

resonances were overlapped and four resonanaces were resolved in the ^{13}C NMR spectrum of Δ-annexin I labeled with [1-^{13}C]Met (Fig. 5A).

These carbonyl ^{13}C NMR data provide information on the structure of the polypeptide backbone.

Ca^{2+} binding to Δ-annexin I

The effect of Ca^{2+} binding on the structure of Δ-annexin I was studied. In the ^{13}C NMR spectra, it could be observed that some phenylalanine and tyrosine resonances were small affected by Ca^{2+} binding (Fig. 2, 4).

In the case of methionine, almost no change could be observed by Ca^{2+} binding (Fig. 5).

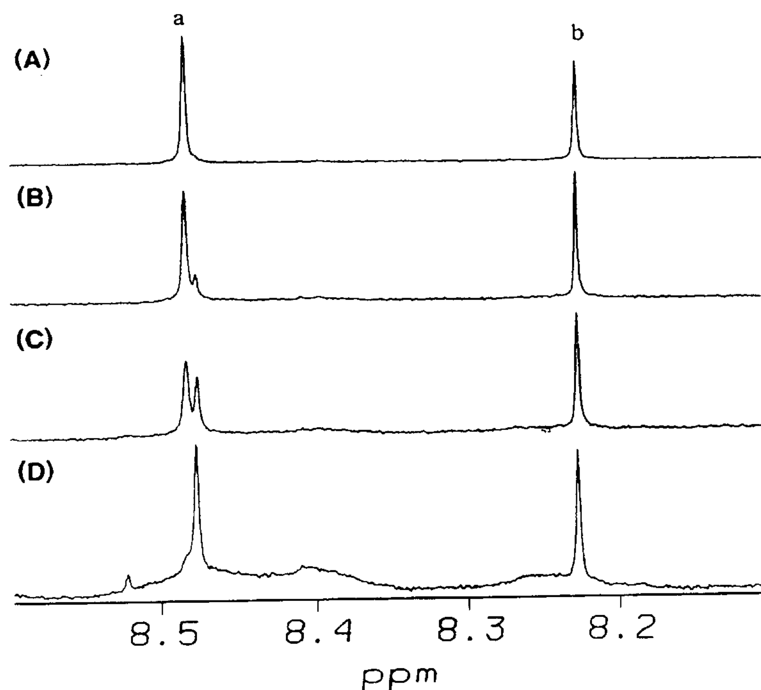

Fig. 6. ^1H NMR spectra of ATP in the absence and presence of Δ-annexin I. (A) In the absence of Δ-annexin I; [ATP] = 0.3mM. In the spectra of (B), (C) and (D), the molar ratio of Δ-annexin I to ATP is 0.1, 0.3 and 1, respectively.

In Fig. 3, the ^{13}C NMR spectra of Δ-annexin I labeled with [1-^{13}C]His alone (Fig. 3A) and in its complex with Ca^{2+} (Fig. 3B) are shown. When Ca^{2+} was added, some histidine resonances were significantly perturbed and broadened. On the basis of these data, we concluded that Ca^{2+} selectively binds Δ-annexin I, and causes the conformational changes around histidine residues in the structure of Δ-annexin I.

It has been suggested that the molecular structure of annexins undergo modest conformational change in the presence of Ca^{2+} and the orientation of the amphiphilic α-helices could be reoriented to expose their hydrophobic surfaces to the exterior, with only slight changes in the specific α-helical character of the domains.[5]

Thus, a significant but subtle rearrangement exposing the hydrophobic surfaces of the protein can allow annexin to span membrane in a conventional manner involving hydrophobic interactions.[5] We also suggest that these changes in the aromatic residues of Δ-annexin I by Ca^{2+} binding is an important factor on the phospholipid binding properties of annexin I.

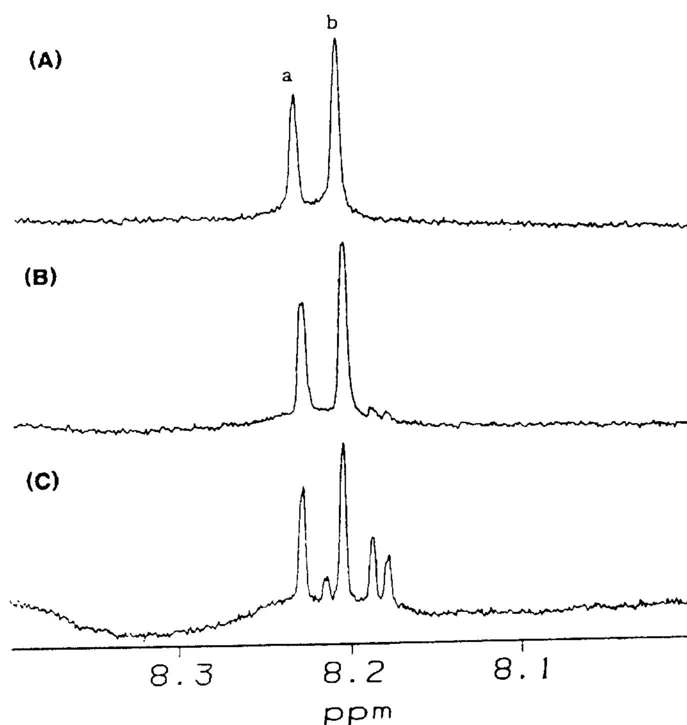

Fig. 7. ^1H NMR spectra of cAMP in the absence and presence of Δ-annexin I. (A) In the absence of Δ-annexin I; [cAMP] = 0.3mM. In the spectra of (B), (C), the molar ratio of Δ-annexin I to ATP is 0.1, 0.3 and 1, respectively.

Interaction of ATP and cAMP with Δ-annexin I

We investigated the interaction of ATP and cAMP with Δ-annexin I using ^{1}H NMR spectroscopic methods. In order to ascertain the binding of ATP to Δ-annexin I, we have recorded ^{1}H NMR spectra of ATP in the absence and presence of Δ-annexin I. Fig. 6 shows ^{1}H NMR spectra of ATP in D_2O. The peaks, a at 8.48 ppm and b at 8.22 ppm in Fig. 6A are due to the H8 and H2 protons in the purine ring of ATP, respectively. As Δ-annexin I was added to ATP in the ratio of 0.1, 0.3 and 1, H8 proton resonance of ATP was gradually appeared at 0.01 ppm upfield, and this change was completed when the ratio of ATP to Δ-annexin I was 1. These results show that ATP binds Δ-annexin I, and the binding ratio of ATP to Δ-annexin I is 1. Fig. 7 shows ^{1}H NMR spectra of cAMP in D_2O. The peaks, a and b in Fig. 7A are due to the H2 and H8 protons in the purine ring of cAMP, respectively. Upon addition of Δ-annexin I to cAMP, some peaks around 8.2 appeared newly (Fig. 7). This indicate that cAMP also binds Δ-annexin I.

In order to check whether there is a conformational change on the structure of Δ-annexin I by ATP and cAMP binding or not, the ^{1}H NMR spectra of Δ-annexin I was recorded in the absence and presence of ATP or cAMP in H_2O (data not shown). ^{1}H NMR spectra of Δ-annexin I in the presence of ATP or cAMP did not exhibit any difference in the high field and low field regions from that of free Δ-annexin I. These results may be interpreted that ATP and cAMP binding cause little conformational change on the structure of Δ-annexin I.

The classical Walker A consensus sequence (GXXXXGKT) for binding of ATP or GTP could not be found in the annexin I. However, we showed that ATP binds Δ-annexin I and the binding ratio of ATP to Δ-annexin I is 1 by the above studies.

Recently, Cohen and coworkers reported that the calcium channels formed by annexin I in planar lipid bilayers can be profoundly altered by cAMP and ATP, and cAMP and ATP selectively alter ion channel activity only when the nucleotides are added to the trans chamber of the planar lipid bilayer system. It would thus appear that specific annexin I domain, able to interact with cAMP and ATP apparently, penetrates the bilayer and is exposed to the bulk phase on the trans side.[22]

Acknowledgements

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