NMR analyses of the interactions of human annexin I with ATP, Ca^{2+}, and Mg^{2+}

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Abstract Human annexin I is a member of the annexin family of calcium-dependent phospholipid binding proteins. The structure of an N-terminally truncated human annexin I (Δ-annexin I) and its interactions with Ca^{2+}, Mg^{2+}, and ATP were studied at the atomic level using nuclear magnetic resonance (NMR) spectroscopy. Since Δ-annexin I is a large protein, with a molecular weight of 35 kDa, a site-specific (carbonyl-^{13}C, amide-^{15}N) labeling technique was used to determine the interaction sites of Δ-annexin I with Ca^{2+}, Mg^{2+}, and ATP. The ^{13}C NMR study focused on the carbonyl carbon resonances of the histidine residues of Δ-annexin I. We found that ATP binds to Δ-annexin I, and that the ATP binding site is located in the 1-domain of annexin I. We also found that histidine-52 is involved in that site, and that the binding ratio of ATP to Δ-annexin I is 1:1.

Key words: Annexin I; Ca^{2+}; Mg^{2+}; Adenosine triphosphate; Nuclear magnetic resonance

1. Introduction

Annexins are a widely distributed family of homologous amphiphilic cytosolic proteins that bind phospholipids and membranes in a Ca^{2+}-dependent manner. They have been implicated in multiple aspects of cell biology, including the regulation of membrane trafficking and transmembrane channel activities, the inhibition of phospholipase A_2, the inhibition of coagulation, the transduction of mitogenic signals, and the mediation of cell-matrix interactions [1–3]. The similar properties of the annexins with regard to Ca^{2+} and phospholipids are due to a common primary structure. Each annexin is composed of two different regions, the unique N-terminal domain, also called the 'tail', and the C-terminal domain, named 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 for Ca^{2+} and phospholipid binding [4]. With one exception, it is always composed of four repeats of a $\sim$70 amino acid sequence containing an increased homology region [5]. The N-termini of the annexins show great diversity, in both composition and length. The annexin N-terminal domain is considered to be the regulatory region of the protein, since it contains the major sites for phosphorylation, proteolysis, and even interactions with other proteins [5].

Recently, the crystal structure of the calcium-bound form of an N-terminally truncated human annexin I was solved [6]. The X-ray crystal structure revealed that the four tandem repeats of the protein are folded into four domains with similar structures. Each domain consists of five α-helices, which are wound into a right-handed superhelix. The four domains are arranged in a planar array, with the Ca^{2+} binding sites located in a helix-loop-helix region. Annexin I binds Ca^{2+} in repeats 2, 3 and 4 through three sites: the (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 site as type II, with type I being the well-known EF-hand motif. Three additional Ca^{2+} binding sites, with a different structure (type III), have been identified in the annexin I crystal [6].

Many members of the annexin gene family, including annexin VII, annexin V and annexin I, are known to form ion channels in lipid bilayers [7,8]. Recently, it was reported that while calcium activates annexin I-driven chromaffin granule membrane aggregation and liposome fusion, these membrane activities are specifically and cooperatively regulated by cAMP, and that the calcium channels formed by annexin I in planar lipid bilayers can be profoundly altered by cAMP [9]. ATP and cAMP selectively alter ion channel activity only when the nucleotides are added to the *trans* chamber of a planar lipid bilayer system [9]. It would thus appear that a specific annexin I domain, which apparently interacts with cAMP and ATP, penetrates the bilayer and is exposed to the bulk phase on the *trans* side [9].

In the present work, the N-terminally truncated human annexin I protein (Δ-annexin I) was specifically labeled with stable isotopes ([^{13}C]carbonyl and/or [^{15}N]amide amino acids), and the carbonyl carbon resonances of Δ-annexin I labeled with [1-^{13}C]His were completely assigned by a double labeling technique [10] and a domain-specific resonance assignment method [11]. The effect of Ca^{2+} binding on the structure of annexin I was studied and was compared with that of Mg^{2+} binding. We have also investigated the interaction of ATP with Δ-annexin I using ^{1}H, ^{13}C nuclear magnetic resonance (NMR) spectroscopic methods.

2. Materials and methods

2.1. Materials

DL-[1-^{13}C]His, L-[^{15}N]Leu, L-[^{15}N]Gln and L-[^{15}N]Lys were purchased from Isotec (Miamisburg, OH, USA) and Cambridge Isotope (Andover, MA, USA). The isotope enrichment was 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 either analytical or biotechnological grade. Selective deuteration of the H8 proton of the ATP purine ring was achieved by heating solutions of ATP in D_2O at 80°C for 7 h [12].

2.2. Overexpression and purification of native and isotopically labeled Δ-annexin I

The cDNA encoding Δ-annexin I was cloned in the expression vector pET28a, which is controlled by the T7 promoter. The *Esche-*

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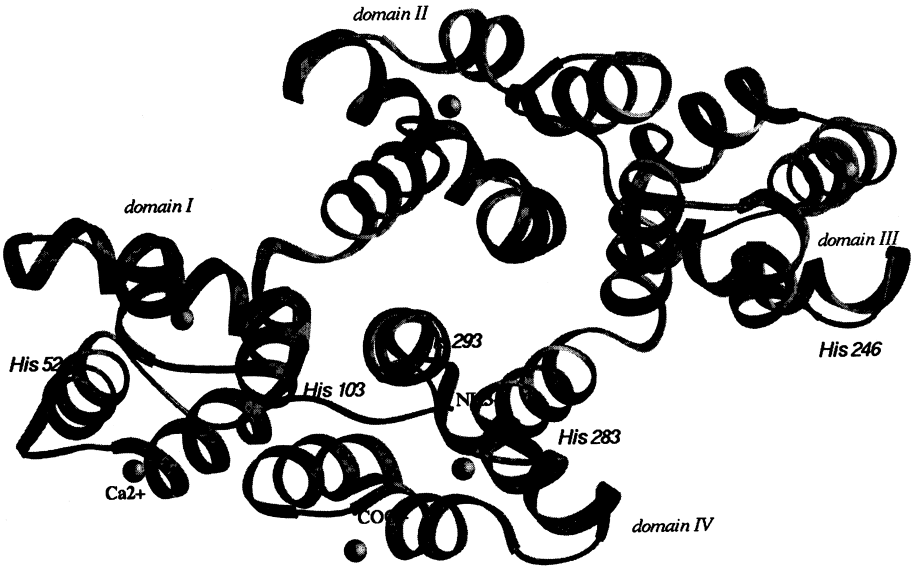

Fig. 1. Crystal structure of human annexin I, which was generated by the Ribbon 2.5 program [6].

richia coli strain BL21 (DE3) was used as a host for the expression constructs [13].

A DEAE-Sepharose (anion exchange resin; Sigma) column and a Bio-rex 70 (cation exchange resin; Bio-Rad, Hercules, CA, USA) column were used for purification. The sample was concentrated with Centriprep-10 and Centricon-10 concentrators (Amicon, MA, USA) for NMR analyses. The concentration was measured by the Bradford method [14].

2.3. Preparation of the [1-^{13}C]histidine-labeled 1,2 domain of annexin I

The cDNA encoding the 1,2 domain of annexin I (amino acids 44–185) was cloned and expressed in *E. coli* strain BL21 (DE3). The overexpression and purification procedures for the 1,2 domain of annexin I were the same as those for the Δ-annexin I protein.

2.4. NMR measurements

The buffer for the NMR measurements was 50 mM potassium phosphate buffer, pH 6.5, containing 100 mM NaCl and 1 mM NaN_3. 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. Chemical shifts are given in ppm from the methyl group resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). For the ^{1}H NMR measurement, the probe temperature was 30°C. Spectra in H_2O were recorded by means of the 1-1 echo water suppression pulse.

The ^{13}C NMR spectra were recorded at 125 MHz using a WALTZ-16 composite pulse decoupling sequence. Chemical shifts are given in ppm from the methyl group resonance of DSS. In the case of the ^{13}C NMR measurement, the solvent water was changed to D_2O after lyophilization, and the probe temperature was 27°C.

3. Results and discussion

3.1. Observation and assignment of the carbonyl carbon resonances in the ^{13}C NMR spectra of Δ-annexin I

To assign the carbonyl peaks of the histidine residues, the ^{13}C-^{15}N double labeling method [10] and a specific subdomain of annexin I were used in this study. Five histidine residues exist in Δ-annexin I (Fig. 1). Two histidine residues (His^{52} and His^{293}) are followed by lysine, and the others (His^{103}, His^{283}, and His^{246}) are followed by leucine, glutamine, and aspartic acid, respectively, in the amino acid sequence of Δ-annexin I [15]. The Δ-annexin I was doubly labeled with either [1-^{13}C]His and [α-^{15}N]Lys, [1-^{13}C]His and [^{15}N]Leu, or [1-^{13}C]His and [α-^{15}N]Gln. In addition, the 1,2 domain of annexin I, containing His^{52} and His^{103}, was labeled with [1-^{13}C]His.

In the ^{13}C NMR spectrum of Δ-annexin I labeled with [1-^{13}C]His, the five histidine carbonyl resonances were well resolved. Fig. 2 shows a ^{13}C NMR spectrum of Δ-annexin I labelled with [1-^{13}C]His and [^{15}N]Leu, where the intensity of one histidine peak was significantly reduced due to $^1J_{CN}$ spin

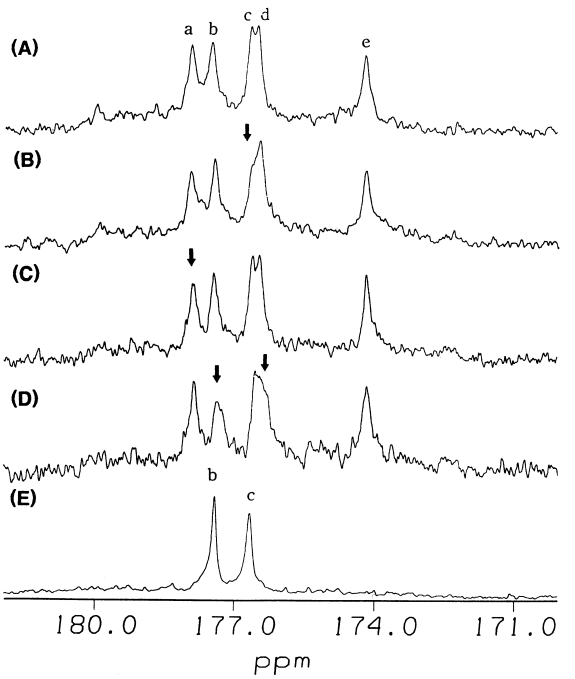

Fig. 2. 125-MHz ^{13}C NMR spectra of (A) [1-^{13}C]His-labeled Δ-annexin I, (B) [1-^{13}C]His- and [^{15}N]Leu-labeled Δ-annexin I, (C) [1-^{13}C]His- and [α-^{15}N]Gln-labeled Δ-annexin I, (D) [1-^{13}C]His- and [α-^{15}N]Lys-labeled Δ-annexin I, and (E) the [1-^{13}C]His-labeled 1,2 domain of annexin I. Experimental conditions: 0.8–1.2 mM Δ-annexin I in D_2O containing 50 mM potassium phosphate, pH 6.5, 100 mM NaCl, and 1 mM NaN_3. The probe temperature was 27°C.

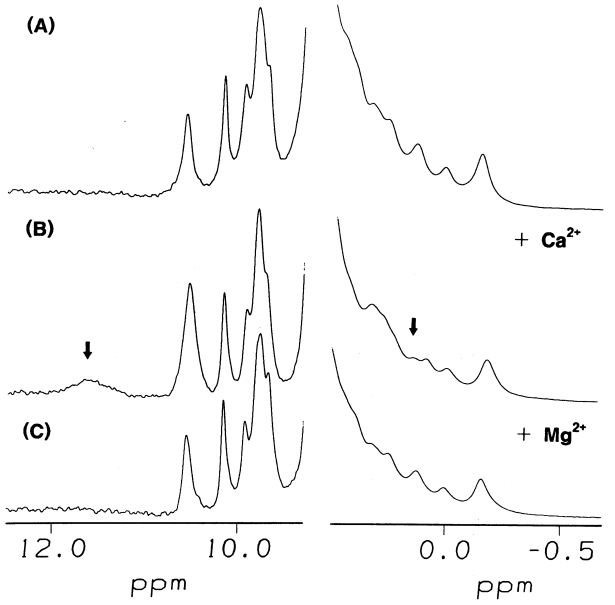

Fig. 3. The high field and low field regions of the ^{1}H NMR spectra of (A) Δ-annexin I, (B) the Δ-annexin I-Ca^{2+} complex, and (C) the Δ-annexin I-Mg^{2+} complex. Experimental conditions: 0.3 mM Δ-annexin I in H_2O containing 50 mM potassium phosphate, pH 6.5, 100 mM NaCl, and 1 mM NaN_3. The concentration of Ca^{2+} in (B) and Mg^{2+} in (C) was 3 mM. The probe temperature was 30°C.

coupling. Among the five histidine residues existing in Δ-annexin I, only His^{103} is directly followed by leucine. Therefore, the reduced resonance of peak c was unambiguously assigned to His^{103} of Δ-annexin I. With this method, resonance a was also assigned to His^{283} of Δ-annexin I. The histidine resonances observed for the 1,2 domain of annexin I, containing His^{52} and His^{103}, were compared with those of Δ-annexin I (Fig. 2A,E). As Fig. 2 shows, all of the resonances observed for the 1,2 domain of annexin I were conserved in the spectrum of Δ-annexin I. Resonance c had already been assigned to His^{103} of Δ-annexin I. Therefore, resonance b could be assigned to His^{52} of Δ-annexin I. Fig. 2D shows a ^{13}C NMR spectrum of Δ-annexin I labeled with [1-^{13}C]His and [α-^{15}N]Lys, where the intensities of the two histidine peaks were reduced due to $^1J_{CN}$ spin coupling. Two histidine residues, His^{52} and His^{293}, are directly followed by lysine. Resonance d could be assigned to His^{293} of Δ-annexin I, since resonance b had been previously assigned to His^{52} of Δ-annexin I. Four of the five histidine resonances were assigned by the aforementioned analyses. Therefore, the last resonance, labeled e in Fig. 2A, could be assigned to His^{246} of Δ-annexin I. These carbonyl ^{13}C NMR data provide information about the structure of the polypeptide backbone, and the assignments of the histidinyl carbonyl carbon resonances will help to identify the active sites of annexin I, which consists of four structurally homologous domains.

3.2. Ca^{2+} binding to Δ-annexin I

The Δ-annexin I protein is composed of four domains, which are repeats of a ~70 amino acid sequence containing an increased homology region (Fig. 1). Each domain has two helix-loop-helix regions, the AB and DE loops. The Δ-annexin I molecule binds Ca^{2+} in repeats 2, 3, and 4 through three sites: the (E,M)-(K,R)-X-G-T-(38 residues)-(D,E) motif. Huber and coworkers defined this new type of Ca^{2+} binding site as type II, with type I being the well known EF-hand motif. Three additional Ca^{2+} binding sites, with a different structure (type III), have been identified in the annexin I crystal [6].

In the present work, the effect of Ca^{2+} binding on the structure of Δ-annexin I was studied and compared with that of Mg^{2+} binding. Fig. 3 shows the high field and low field regions of the ^{1}H NMR spectra of Δ-annexin I. When Ca^{2+} was added in a 10-fold excess to Δ-annexin I, a new broad peak appeared in the low field region, and some peaks in the high field regions were changed. The broad peak in the low field region (at 11.7 ppm) may be the result of hydrogen bonding of the histidine imidazole proton by Ca^{2+} binding, since the backbone NH resonance usually does not appear in this region. Upon the addition of Mg^{2+} to Δ-annexin I, only slight changes were observed. Fig. 4 shows the aromatic region of the ^{1}H NMR spectra of Δ-annexin I. The striking features are the extremely sharp lines in the histidine region 7.6–8 ppm), and these histidine peaks were significantly affected by Ca^{2+} binding. The changes due to Ca^{2+} binding were larger than those caused by Mg^{2+} binding (Fig. 4). In Fig. 5, the ^{13}C NMR spectra of Δ-annexin I labeled with [1-^{13}C]His alone (Fig. 5A) and in its complex with Ca^{2+} (Fig. 5B) and Mg^{2+} (Fig. 5C) are shown. When Ca^{2+} was added, the His^{52} and His^{246} resonances were significantly perturbed, and the His^{283} resonance was shifted slightly downfield by Ca^{2+} binding (Fig. 5B). In contrast, upon addition of Mg^{2+} to Δ-annexin I, the histidine resonances were not affected (Fig. 5C). On the basis of these data, we concluded that Ca^{2+} selectively binds Δ-annexin I, and causes conformational changes in its structure.

It has been suggested that the molecular structures of the annexins undergo modest conformational modifications in the presence of Ca^{2+}, and that 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

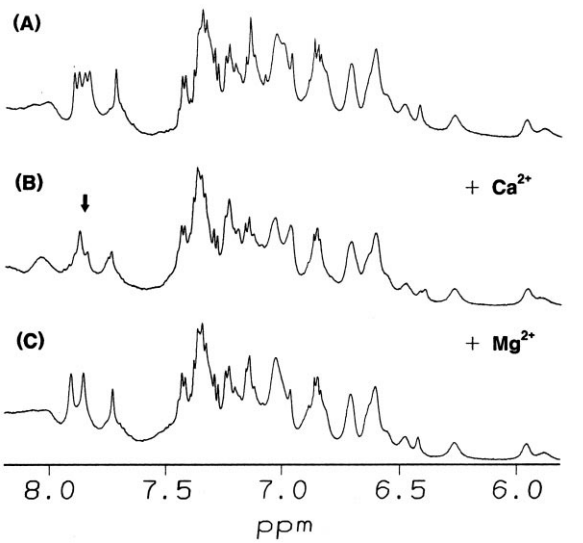

Fig. 4. Aromatic region of the ^{1}H NMR spectra of (A) Δ-annexin I, (B) the Δ-annexin I-Ca^{2+} complex, and (C) the Δ-annexin I-Mg^{2+} complex. Experimental conditions: 0.3 mM Δ-annexin I in D_2O containing 50 mM potassium phosphate, pH 6.5, 100 mM NaCl, and 1 mM NaN_3. The concentration of Ca^{2+} in (B) and Mg^{2+} in (C) was 3 mM. The probe temperature was 30°C.

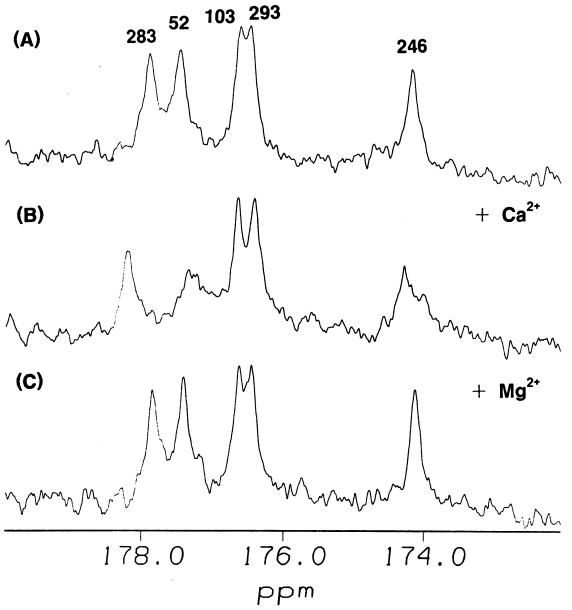

Fig. 5. 125-MHz ^{13}C NMR spectra of (A) [1-^{13}C]His-labeled Δ-annexin I, (B) the Δ-annexin I-Ca^{2+} complex, and (C) the Δ-annexin I-Mg^{2+} complex. Experimental conditions are as in the legend to Fig. 1, except that the concentrations of Δ-annexin I, Ca^{2+}, and Mg^{2+} were 0.8 mM, 8 mM, and 8 mM, respectively.

of the domains [4]. Thus, a significant, but subtle, rearrangement that exposes the hydrophobic surfaces of the protein could allow annexin to span the membrane in a conventional manner involving hydrophobic interactions [4]. We also suggest that these changes in the aromatic residues of Δ-annexin I due to Ca^{2+} binding could also be an important factor in the phospholipid binding properties of annexin I.

3.3. Interaction of ATP with Δ-annexin I

Many members of the annexin gene family, including annexin VII, annexin V, and annexin I, are known to form ion channels in lipid bilayers [7,8]. Recently, it was reported that while calcium activates annexin I-driven chromaffin granule membrane aggregation and liposome fusion, these membrane activities are specifically and cooperatively regulated by ATP and cAMP, and that the calcium channels formed by annexin I in planar lipid bilayers can be profoundly altered by ATP and cAMP [9].

In this study, we investigated the interactions of ATP with Δ-annexin I using ^1H, ^{13}C NMR spectroscopic methods. To analyze the binding of ATP to Δ-annexin I, we recorded the ^1H NMR spectra of ATP in the absence and presence of Δ-annexin I. Fig. 6 shows the ^1H NMR spectra of ATP in D_2O. We already knew that peaks a at 8.482 ppm and b at 8.226 ppm in Fig. 6A are due to the H8 and H2 protons, respectively, in the purine ring of ATP, from the selective deuteration experiment (data not shown). As Δ-annexin I was added to the ATP, at ratios of 0.1, 0.3, and 1, the H8 proton resonance of ATP was gradually shifted 0.01 ppm upfield, and this change was completed when the ratio of ATP to Δ-annexin I was 1:1. These results show that ATP binds Δ-annexin I, and that the binding ratio of ATP to Δ-annexin I is 1:1.

In order to determine whether there is a conformational change in the structure of Δ-annexin I caused by ATP binding, or not, the ^1H NMR spectra of Δ-annexin I were recorded in the absence and presence of ATP in H_2O (data not shown). The ^1H NMR spectra of Δ-annexin I in the presence of ATP did not exhibit any difference in the high field and low field regions from that of free Δ-annexin I. These results suggest that ATP binding causes only minor conformational changes in the structure of Δ-annexin I.

3.4. ATP binding site of annexin I

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

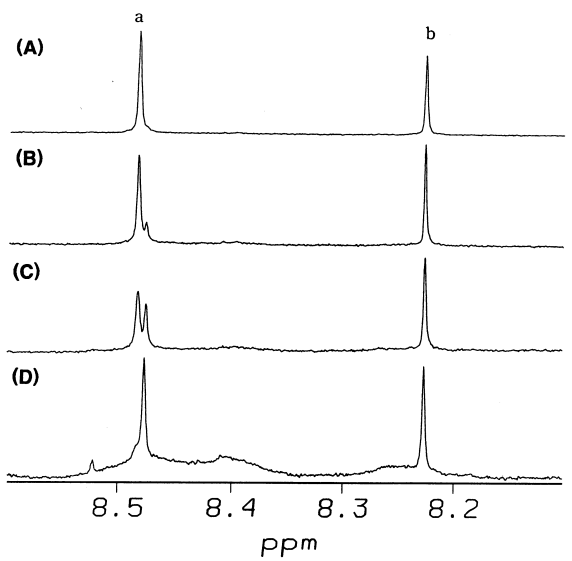

Fig. 6. ^1H NMR spectra of ATP in the absence and presence of Δ-annexin I. A: In the absence of Δ-annexin I: [ATP] = 0.3 mM. In the spectra of B, C, and D, the molar ratios of Δ-annexin I to ATP were 0.1, 0.3, and 1, respectively. Other experimental conditions were as in the legend to Fig. 4.

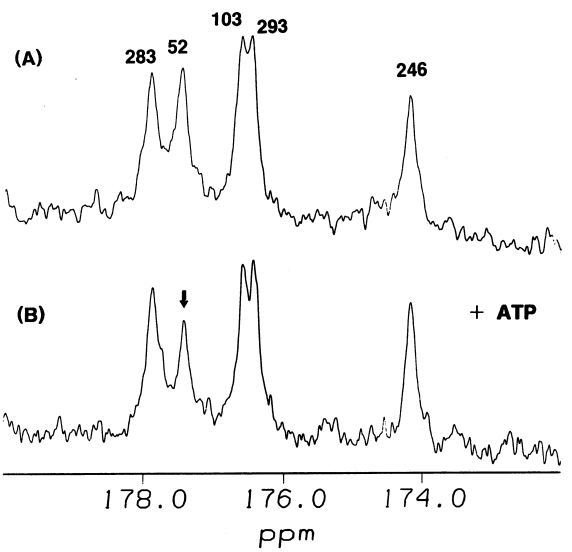

Fig. 7. 125-MHz ^{13}C NMR spectra of (A) [1-^{13}C]His-labeled Δ-annexin I, (B) the Δ-annexin I-ATP complex. Experimental conditions were as in the legend to Fig. 1, except that the concentration of Δ-annexin I was 1 mM. In the spectra of B, the molar ratio of ATP to Δ-annexin I was 1:1.

1:1 by the above studies. We have hypothesized that ATP might bind to a specific domain of annexin I.

The ATP binding site of Δ-annexin I was investigated by ^{13}C NMR. The ^{13}C NMR spectra of Δ-annexin I labeled with [1-^{13}C]His in the absence and presence of ATP are shown in Fig. 7A,B, respectively. The resonance originating from His^{52} was significantly reduced by ATP binding, whereas the other His resonances were not affected (Fig. 7B). Therefore, His^{52} in the 1 domain of annexin I is involved in the ATP binding site of Δ-annexin I.

Recently, Cohen and coworkers reported that the calcium channels formed by annexin I in planar lipid bilayers can be profoundly altered by ATP and cAMP. ATP and cAMP selectively alter ion channel activity only when the nucleotides are added to the *trans* chamber of a planar lipid bilayer system. It would thus appear that a specific annexin I domain, which apparently is able to interact with ATP or cAMP, penetrates the bilayer and is exposed to the bulk phase on the *trans* side [9]. It was also reported that the structural features of domains 1 of annexins I and V determine the overall phospholipid affinity, while domains 2, 3, and 4 determine the Ca^{2+} requirements for phospholipid binding [16].

On the basis of these results, we conclude that the ATP binding site is located in the 1 domain of annexin I, and that His^{52} of annexin I is involved in that site. Also, we think that the 1 domain of annexin I may be the major site that binds and penetrates phospholipids, and interacts with ATP.

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