Role of the N-terminus in the structure and stability of chicken annexin V

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Abstract The role of the short N-terminal region of chicken annexin V in the maintenance of the protein structure and its influence in the conformation of the calcium binding regions was analyzed. The N-terminal domain is not essential for protein folding, wild-type and dnt-annexin V showing almost identical secondary structures. However, the partial truncation of the N-terminus significantly decreases the melting temperature of the protein and induces the partial exposure of Trp^{187} which is normally located in a hydrophobic pocket of the calcium binding region of domain 3 of annexin V in the Ca^{2+}-free form.

Key words: Annexin V; Calcium binding; Circular dichroism spectroscopy; Fluorescence emission spectroscopy

1. Introduction

Annexin V is a member of the ubiquitous family of proteins that bind to acidic phospholipids in the presence of calcium [1]. Several functions have been proposed for annexin V in vitro. It is involved in the control of blood coagulation, in the regulation of the inflammatory response by inhibition of phospholipase A_2 activity, and it is a high-affinity inhibitor of protein kinase C [1,2]. Moreover, it interacts with collagens type II and X being a potential collagen receptor in chondrocytes [3–5]. Its activity as a voltage-gated calcium channel has also been described [6,7] and it is responsible for the calcium uptake by cartilage matrix vesicles in vivo [8].

The annexin core structure is composed of four (or eight, in annexin VI) homologous domains of 70 amino acid residues. Unlike these conserved repeats, the N-terminal region is highly variable in both length and sequence. It is formed by 10–40 residues except in annexins VII and XI that possess larger N-termini [9]. In spite of its small size, this region is supposed to mediate the specific biological functions of individual annexins. In fact, the N-terminus is involved in the interaction with annexin-associated proteins, it can be phosphorylated by protein kinase C or tyrosine kinases, it has been found to be sensitive to proteolysis, it may be expressed as splice variants with different properties regarding phosphorylation or the interaction with other proteins, and modifications or mutations in this region dramatically alter some of the protein functions [1,2,10–15].

The crystal structure of several annexins from different sources has been solved so far. The overall shape of the molecule is a slightly bent ring surrounding a hydrophilic channel, with a concave surface with the N-terminus, and a convex one where the calcium and phospholipid binding sites are located. The homologous domains consist of five amphipathic α-helices (A–E) of about 7–15 residues in length. Helices A, B, D and E form a right-handed superhelix with helix C situated perpendicular to the others [16–18]. Every domain presents a highly conserved region of 17 amino acids with a consensus sequence, named endonexin fold, that contains the high-affinity calcium binding site. The calcium binding site sequence in domain 3 of chicken annexin V is slightly different from the standard endonexin fold, and contains the unique tryptophan residue (W^{187}) whose side chain is buried in the structure in the absence of calcium [17–19].

It has been described that Ca^{2+} binding may alter the conformation of the N-terminal region of annexin I, and vice versa, alterations in the N-terminal region modify the calcium binding properties [13,15,20]. This bidirectional influence between the two domains has also been described for other annexins, but not for annexin V. For this reason, we have studied by circular dichroism and fluorescence emission spectroscopy the role of the N-terminal domain of chicken annexin V in the overall structure and thermal stability of the protein, as well as the alterations induced by a partial truncation of the N-terminus in the conformation of the calcium binding sites.

2. Materials and methods

2.1. Protein preparation

Recombinant chicken annexin V and its mutant dnt-annexin V lacking amino acid residues 2–9 have been produced and purified essentially as previously reported [5] using the ability of both proteins to interact reversibly with PS-enriched liposomes in the presence of calcium. Vesicles were prepared as described in [5] but using phospholipids from Brain Extract (Folch fraction III from bovine brain; Sigma, St. Louis, MO). The final EGTA extracts were dialysed versus 50 mM Tris, pH 7.4, 1 mM EGTA, loaded onto a DEAE-cellulose column equilibrated in the same buffer, and eluted using a 0–0.5 M NaCl linear gradient. Fractions containing pure annexin V or dnt-mutant were pooled and dialysed against 20 mM HEPES, pH 7.4, 0.1 M NaCl (buffer A), filtered through 0.22 μm membranes and stored at 4°C. Protein concentration was determined by amino acid analysis (Beckman System 6300 analyzer) or by UV spectroscopy using molar extinction coefficients at 280 nm of 22155 and 20720 M^{-1} cm^{-1} for annexin V and the dnt-mutant, respectively.

2.2. Circular dichroism measurements

CD spectra were recorded in a Jasco J-715 dichrograph at 25°C (Neslab RTE-111 thermostat). The far-UV CD spectra were monitored between 200 and 250 nm and near-UV CD spectra were measured between 250 and 320 nm. Melting curves were determined following CD at 208 nm between 25 and 70°C and increasing

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Abbreviations: CD, circular dichroism; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; UV, ultraviolet

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temperature at 30°C/h. Spectra in the absence of calcium were recorded in buffer A containing 1 mM EGTA.

2.3. Steady-state fluorescence spectroscopy

Tryptophan fluorescence emission spectra were recorded at 25°C between 300 and 420 nm on a SLM Aminco 8000C spectrofluorimeter (Urbana, IL) with an excitation wavelength of 295 nm and 4-nm excitation and emission bandwidths. Scattering was minimized by crossed Glan-Thompson polarizers. Stern-Volmer quenching constants were calculated from plots of F_0/F versus acrylamide concentration. Static quenching was non-significant up to 150 mM acrylamide.

3. Results and discussion

The N-terminal extension of annexins is supposed to play a key role in their biological functions. However, very few studies have been performed to evaluate the importance of this short region that holds together domains 1 and 4 of annexin V. It has been reported that the truncation of 14 amino acid residues from the N-terminus of human annexin V eliminates the annexin V-mediated calcium influx into Fura-2-loaded liposomes [21]. On the other hand, in a previous report we showed that dnt-annexin V, which lacks eight residues of the N-terminus, presents the same collagen binding activity as the wild-type chicken annexin V [5]. Here we report on the spectroscopic differences between wild-type and dnt-annexin V, mainly regarding the conformational changes affecting the Ca^{2+} binding domains.

Recombinant chicken annexin V and the dnt-mutant were purified from JA221 *Escherichia coli* cultures transformed with constructions pACII.E and pdntACII.E [5] after induction with 1 mM IPTG. The final protein preparations were free of any detectable contaminations after analysis by SDS-PAGE followed by silver staining.

CD spectra of chicken annexin V and the dnt-mutant are almost identical in the presence of 1 mM EGTA (Fig. 1A,B). The overall shape of the CD spectra in the n-π* and π-π* transition regions of the amide chromophores indicates that the truncation of the N-terminus of annexin V does not induce major conformational changes in the secondary structure of the protein. Analysis of the spectra using the CCA algorithm [22] predicts an α-helical content of around 70–80% and almost no β-pleated structures, which is in good agreement with the crystal structure described for chicken annexin V [17]. Thus, it can be concluded that the forces involved in the interaction between the N-terminus and domain 4 of annexin V are not required for the proper folding of the molecule.

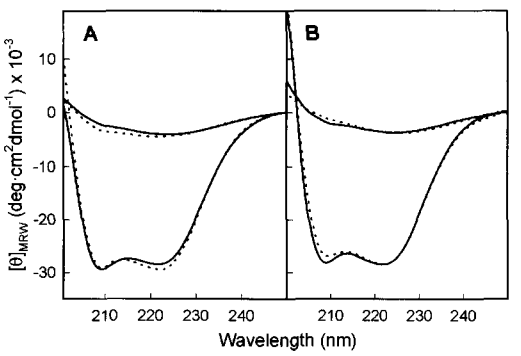

Fig. 1. Circular dichroism spectra of annexin V (A) and dnt-annexin V (B). Spectra were recorded in the far-UV in the presence of 1 mM EGTA (solid line) or 10 mM free Ca^{2+} (dotted line) in buffer A at 25 and 70°C, using a 0.1-cm optical path cuvette and a protein concentration of 0.25 mg/ml. Spectra with intermediate free Ca^{2+} concentrations are not represented, but were almost identical to the ones recorded in 10 mM Ca^{2+}. The different free calcium concentrations were obtained by addition of the required volumes of a 0.5 M $CaCl_2$ stock solution (no changes in the pH were detected). CD is expressed as molar ellipticity per residue ($[\theta]_{MRW}$).

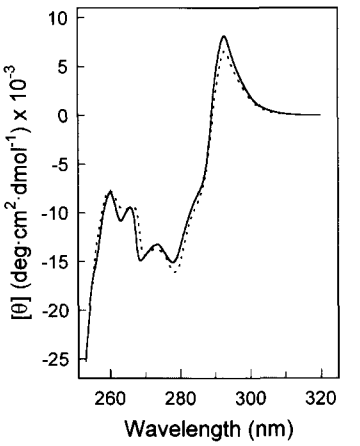

Fig. 2. Circular dichroism spectra in the near-UV of annexin V (solid line) and dnt-annexin V (dotted line) were recorded in buffer A containing 1 mM EGTA, using a 1.0-cm optical path cuvette and a protein concentration of 0.8 mg/ml. Molar ellipticity at 292 nm is 20% lower in the mutant. CD is expressed as molar ellipticity ([θ]).

When CD spectra of wild-type or dnt-annexin V are recorded in the presence of increasing concentrations of free calcium, up to 10 mM, no significant changes in the spectra are detected (Fig. 1A,B). Only a minor increase in the negative molar ellipticity at 222 nm and a decrease at 209 nm, both around 3% of the original values, are observed in the far-UV CD spectra corresponding to wild-type and dnt-annexin V, respectively.

It has been reported that the binding of calcium triggers small conformational changes that affect mainly the loops between helices A and B in the four domains [18,23,24]. These structural alterations are small when the whole structure is considered and do not significantly affect the overall secondary structure of the protein core, not even in the mutant lacking the additional stabilizing forces of interaction between the N-terminus and domain 4. We have analyzed the conformational changes in the AB loops by near-UV CD and fluorescence emission spectroscopy taking advantage of the presence of W^{187} in the endonexin fold of the third domain of annexin.

Near-UV CD spectra of annexin V and the dnt-mutant present a characteristic positive peak at 292 nm, negative ones at 262, 269, and 277 nm, and a shoulder at 287 nm (Fig. 2). The positive peak probably corresponds to the tryptophanyl 0-0 ^1L_b band [19,25]. When wild-type and dnt-annexin V spectra are compared, several differences can be observed. The maximum at 292 nm corresponding to W^{187} presents a lower molar ellipticity in the mutant (6500 versus 8112 deg cm^2 dmol^{-1} in the wild-type), showing that the microenvironment of W^{187} is less structured in the mutant than in the wild-type protein. Possible artifacts due to errors in the protein concentration can be discounted since it is determined by UV spectroscopy and amino acid analysis, the two values being always in agreement.

Fluorescence emission spectra of annexin V and the dnt-

mutant are shown in Fig. 3A,B, respectively. When the excitation wavelength is set at 295 nm, the fluorescence emission of W^{187} is maximal at 324 nm in the absence of calcium, which is characteristic of a tryptophan residue in a hydrophobic environment inaccessible to the solvent [23,24]. The fluorescence emission spectrum of the tryptophan residue present in dnt-annexin V is red-shifted by 4 nm, indicating that it is more exposed to the solvent than that present in the wild-type protein. The increased exposure of W^{187} is confirmed by the higher Stern-Volmer constant obtained in the dnt-mutant by acrylamide quenching, 5.96 ± 0.20 versus 1.94 ± 0.14 M^{-1} for annexin V (Fig. 3C). Moreover, normalized spectra (Fig. 3A,B) show an increase in the quantum yield of W^{187} in the dnt-mutant compared with annexin V.

Calcium binding alters the microenvironment of W^{187} in annexin V and the truncated mutant. In both proteins, a progressive red shift of the fluorescence emission maximum parallel to an increase in the quantum yield is observed. At 10 mM $CaCl_2$ and 0.05 mg/ml protein (7200 $CaCl_2$/protein molar ratio), the emission maxima are 334 and 332 nm for wild-type and dnt-annexin V, respectively. These results confirm the conformational change described in the loop between helices A and B in domain 3 of chicken annexin V and agree with the previously reported data for human annexin V by X-ray crystallography [16,26]. The tryptophan residue can, therefore, be present in two possible conformations, either buried in a hydrophobic pocket or exposed to the solvent in the presence of calcium. Sopkova et al. [19] have suggested that, in human annexin V, the ε-OH and α-carbonyl groups of T^{224} are close enough to the indole ring of the buried W^{187} to establish hydrogen bonds that induce quenching of the tryptophan residue. The conformational change after calcium binding increases the distance between W^{187} and these groups and, therefore, quenching decreases. The T^{224} residue is also present in chicken annexin V and could be also responsible for this effect. The effect of calcium binding is less significant in dnt-annexin V probably because the tryptophan residue in the mutant is already more exposed to the solvent. This is also in agreement with the data obtained by CD in the near-UV, where this residue appears to be in a less organized environment in the mutant when compared to the wild-type protein (Fig. 2). The indole ring is probably not so close to T^{224}, and thus fluorescence quenching by the polar groups in the hydrophobic pocket is lower and the quantum yield of W^{187} higher.

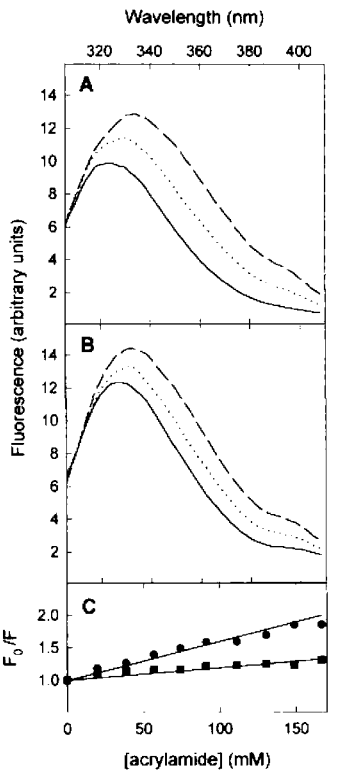

Fig. 3. Fluorescence emission spectra of W^{187} in annexin V (A) and dnt-annexin V (B), and acrylamide quenching of both proteins (C). Spectra were recorded at 25°C in buffer A containing 1 mM EGTA (solid lines) or in the presence of increasing concentrations of free Ca^{2+}, using a 0.4-cm optical excitation and 1.0-cm emission path cuvette. Buffer blanks were always subtracted in the same experimental conditions. The protein concentration was 0.05 mg/ml. Only spectra at 5 mM (dotted line) and 10 mM free Ca^{2+} (dashed line) are shown. All spectra were normalized versus the actual protein concentration to avoid artifacts. Fluorescence is expressed in arbitrary units.

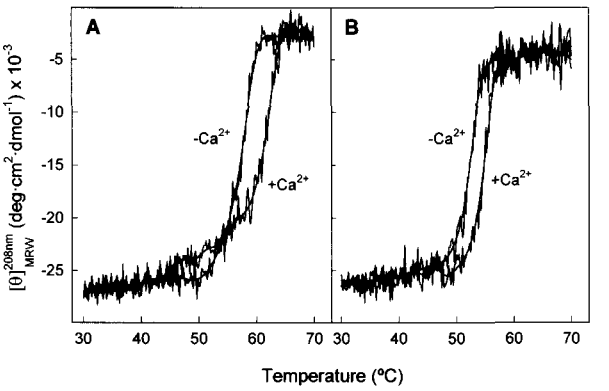

Fig. 4. Irreversible thermal unfolding curves of annexin V (A) and dnt-annexin V (B) obtained by monitoring molar ellipticity per residue at 208 nm in buffer A containing 1 mM EGTA ($-Ca^{2+}$) or 10 mM free Ca^{2+} ($+Ca^{2+}$). Temperature was increased by 30°C/h. CD spectra were recorded at the beginning (25°C) and at the end of the experiment (70°C); initial and final spectra of both proteins were almost identical (Fig. 1).

The N-terminus of annexin V is not essential for the correct folding of the protein, since the dnt-mutant has the same secondary structure as the wild-type protein. Moreover, the dnt-mutant is still able to interact with acidic phospholipids in the presence of calcium. The calcium requirement for phospholipid binding is slightly lower than that required by annexin V, and the mutant is also able to interact with type I and type II collagens following the same pattern as the wild-type protein [5]. However, the N-terminus may be involved in the stabilization of the protein core structure since it interacts with residues present in the C-terminal domain of the protein (domain 4). Thus, we have analyzed the melting curves of wild-type annexin V and the truncated mutant recording the variation of molar ellipticity per residue at 208 nm (Fig. 4A,B).

The partial truncation of the N-terminus of annexin V induces a significant decrease in the melting temperature (Tm) of the protein (Fig. 4). In both proteins, thermal unfolding is irreversible and highly cooperative. The Tm for the wild-type protein in the absence of Ca^{2+} is 58.1°C (Fig. 4A) whereas it is only 50.7°C for dnt-annexin V (Fig. 4B). Thus, it can be concluded that the N-terminus contributes greatly to the sta-

bilization of the annexin V structure by establishing interactions with the fourth domain and closing the overall ring-shaped structure. The effect of calcium binding on the thermal stability of the proteins was also studied. Both proteins are more stable in the Ca^{2+}-bound form even though no significant modifications in the secondary structure were detected (Fig. 1); at 10 mM $CaCl_2$, the Tm values increase by 2.9 and 3.4°C in wild-type and dnt-annexin V, respectively (Fig. 4A,B).

In summary, our results indicate that the N-terminal region of chicken annexin V is not essential for the proper folding of the protein, but contributes significantly to the stabilization of the overall structure. A relationship between the N-terminus and the conformation of the calcium binding loops, at least in domain 3, has been found since the partial truncation of this region induces a more relaxed structure where the unique tryptophan residue of annexin V is partially exposed to the solvent. This could explain the apparent lower calcium concentration required for membrane binding in the dnt-mutant compared to the wild-type protein [5].

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