A conformational equilibrium in a protein fragment caused by two consecutive capping boxes: ^1H -, ^{13}C -NMR, and mutational analysis

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Abstract

The conformational properties of an 18 residues peptide spanning the entire sequence, L₁KTPA₅QFDAD₁₀ELRAA₁₅MKG, of the first helix (A-helix) of domain 2 of annexin I, were thoroughly investigated. This fragment exhibits several singular features, and in particular, two successive potential capping boxes, T₃xxQ₆ and D₈xxE₁₁. The former corresponds to the native hydrogen bond network stabilizing the α helix N-terminus in the protein; the latter is a non-native capping box able to break the helix at residue D₈, and is observed in the domain 2 partially folded state. Using 2D-NMR techniques, we showed that two main populations of conformers coexist in aqueous solution. The first corresponds to a single helix extending from T₃ to K₁₇. The second corresponds to a broken helix at residue D₈. Four mutants, T3A, F7A, D8A, and E11A, were designed to further analyze the role of key amino acids in the equilibrium between the two ensembles of conformers. The sensitivity of NMR parameters to account for the variations in the populations of conformers was evaluated for each peptide. Our data show the $\delta^{13}\text{C}_\alpha$ chemical shift to be the most relevant parameter. We used it to estimate the population ratio in the various peptides between the two main ensembles of conformers, the full helix and the broken helix. For the WT, E11A, and F7A peptides, these ratios are respectively 35/65, 60/40, 60/40. Our results were compared to the data obtained from helix/coil transition algorithms.

Keywords: α -helix; annexins; capping box; non-native conformations; NMR; peptide; protein folding

The conformational properties of the partially folded states of proteins are related to major aspects of their folding and stability. These states can be considered as possible starting elements in the protein folding pathways, able to play an important role in the kinetics and thermodynamics of the folding process (Arcus et al., 1995; Muñoz et al., 1996; Shortle, 1996; Roder & Colon, 1997; Viguera et al., 1997). To precisely describe the partially folded

states at the residue level, several approaches were devoted to characterizing the nature of the reference "random coil" state (Serrano, 1995; Bolin et al., 1996; Fiebig et al., 1996; Smith et al., 1996b), analyzing the populations of conformations (Waltho et al., 1993; Jimenez et al., 1994a; Pintar et al., 1994; Yao et al., 1994), and investigating dynamic aspects associated with the fluctuating conformations (Farrow et al., 1997; Schwalbe et al., 1997). In this context, important insights have been gained from the study of small fragment peptides since their conformations reflect the local intrinsic propensity of specific sequences without the use of denaturing agents and in the absence of any cooperative long-range interactions (Dyson et al., 1992a, 1992b; Kemmink & Creighton, 1993; de Prat Gay et al., 1995; Muñoz et al., 1995b; Yang et al., 1995; Reymond et al., 1997b). Studies of these partially folded polypeptide chains, however, are difficult since they concern states with a high degree of flexibility and have to deal with an ensemble of conformers (Merutka et al., 1993). To tackle these problems, we are currently studying the folding and stability of protein fragments of the annexin family (Macquaire et al., 1992; Macquaire

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Abbreviations: CD, circular dichroism; DQF COSY, double quantum filtered correlated spectroscopy; DSS, 2,2-dimethyl-2-sylapentane-5-sulfonic acid; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; TFE, trifluoroethanol; TOCSY, total correlated spectroscopy; WT peptide, wild-type peptide; $\Delta\delta^{13}\text{C}_\alpha$, $^{13}\text{C}_\alpha$ conformational index; $\Delta\delta\text{H}_\alpha$, $\text{C}\alpha\text{H}$ conformational index; $\Delta^3J_{\text{NH}\alpha}$, $^3J_{\text{NH}\alpha}$ conformational index.

et al., 1993; Odaert et al., 1995; Cordier-Ochsenbein et al., 1996). Annexins (Moss, 1992; Raynal & Pollard, 1994) are composed of four homologous domains, each made of five helices, named A to E, folded into a characteristic superhelix motif.

We previously showed (Odaert et al., 1995) the formation of a DxxE non-native capping box in a fragment peptide partly spanning the α helix of the D2 domain of annexin. This first interesting and unexpected result led us to extensively investigate the whole sequence of the α helix. The sequence of the peptide studied in this paper, L₁KTPA₅QFDAD₁₀ELRAA₁₅MKG, now includes not only the full potential native capping box T₃xxQ₆ (Harper & Rose, 1993) but also the residue K₂, which, in the protein, is engaged in a stabilizing salt bridge with D₁₀. Our aim is now to investigate the interaction of the two potential capping boxes present in the protein fragment, T₃xxQ₆ and D₈xxE₁₁, and their influence on the helix stability. Interestingly, we find that this interaction gives rise to an equilibrium between two ensembles of conformations, one in which the helix extends from T₃ to practically the end of the sequence, and one in which the helix has a break due to the formation of the non-native D₈xxE₁₁ capping structure strengthened by the F₇-L₁₂ hydrophobic staple motif (Seale et al., 1994; Muñoz et al., 1995a). The interest of such a model is that both helix and capping conformations adopted at the D₈ residue can be clearly identified, not only through characteristic NOE signals, but also from chemical shift and $^3J_{\text{HN}\alpha}$ coupling constant indexes.

To elucidate the role of key amino acids in the equilibrium between conformers of the protein fragment, these were successively substituted by alanines. This well-known strategy is expected to uncouple their contribution to the formation of local interactions. The influence of each of the T₃, F₇, D₈, and E₁₁ residues on the distribution of both peptide conformations was precisely analyzed using several NMR parameters, NOE, H _{α} and $^{13}\text{C}_{\alpha}$ chemical shifts, and $^3J_{\text{HN}\alpha}$ coupling constants. Their relative sensitivity to variations in the populations of conformers is investigated. $^{13}\text{C}_{\alpha}$ chemical shifts indexes are found as the most reliable parameters to account for the equilibrium between several conformations and from their relative variations, we quantitatively estimate the contribution of the key residues to that particular equilibrium. Our results are also compared to the data provided by helix/coil transition algorithms. Comparison between the measured shifts in the helix population and those predicted by these algorithms can indeed be of interest to tune more accurately the theoretical parameters.

Results

Conformational properties of the WT peptide solubilized in aqueous solution

Figures 1A and 2, respectively, show the NOE, $\Delta\delta\text{H}_{\alpha}$, and $\Delta\delta^{13}\text{C}_{\alpha}$ data obtained for the WT peptide solubilized in aqueous solution. In the A₅-K₁₇ segment, we can observe a quasi complete set of $\alpha\beta(i, i+3)$ and $\alpha\text{N}(i, i+3)$ NOEs consistent with a helix conformation. Characteristic N β (T₃,Q₆) and N γ (T₃,Q₆) NOE contacts indicate that the helix is capped at its N-terminus by the native T₃xxQ₆ capping box structure (Fig. 3A) (Zhou et al., 1994). However, despite the presence of $\alpha\beta$ (D₈,E₁₁) and αN (D₈,E₁₁) NOEs, a N β (D₈,E₁₁) correlation attesting the formation of the second potential D₈xxE₁₁ capping box is observed. In addition, NOE contacts observed between F₇ and L₁₂ side chains reveal the existence of a hydrophobic staple motif associated with the capping box

Fig. 1. NOE data for the WT peptide solubilized (A) in pure aqueous solution and (B) in 20% TFE aqueous solution at pH 4.5 and 278 K. Line thickness corresponds qualitatively to the relative intensities of the NOEs. Asterisks indicate connectivities that could not be resolved due to overlap.

(Seale et al., 1994; Muñoz et al., 1995a). Simultaneous helix and capping conformations at the D₈ position are obviously not compatible. Therefore, it can be concluded that two families of conformers coexist and rapidly exchange with each other at the chemical shift NMR time scale. The first corresponds to a native-like WT helix, whereas the second includes the D₈xxE₁₁ capping box acting as a helix breaking structure (Odaert et al., 1995). In the following, we will discuss the conformational properties of the peptides using a two-state approximation in which one state represents the subset corresponding to the native helix extending from T₃ to K₁₇ and the other state represents the subset in which the helix is broken by the formation of the non-native capping box.

Fig. 2. A: H_α chemical shift index for the WT peptide in pure aqueous solution (open circle) and in 20% TFE aqueous solution (filled circle) at pH 4.5 and 278 K. B: $^{13}C_\alpha$ chemical shift index for the WT peptide in pure aqueous solution (open circle) and in 20% TFE aqueous solution (filled circle) at pH 4.5 and 278 K.

As regards the $\Delta\delta H_\alpha$ index, except for F₇ and D₈, all the residues within the A₅–M₁₆ segment exhibit negative values close to or greater than -0.1 ppm in agreement with a helix conformation. The singularly low F₇ and D₈ $\Delta\delta H_\alpha$ values most probably reflect the coexistence of two conformers revealed by the NOE data. This coexistence is more accurately depicted by the $\Delta\delta^{13}C_\alpha$ profile. Although all the residues within the P₄–K₁₇ segment exhibit positive $\Delta\delta^{13}C_\alpha$ values characteristic of the helix conformation, the indexes of residues P₄ to F₇ are twice as small as those of residues A₉ to R₁₃. Such a feature can readily be attributed to the influence of the D₈xxE₁₁ capping structure, which prevents, for one family of conformers, the stabilization of the helix turn in the T₃–F₇ segment. In agreement with a capping conformation, D₈ $\delta^{13}C_\alpha$ exhibits a significant upfield shift (Gronenborn & Clore, 1994). Lastly, in the C-terminal A₁₄–K₁₇ helix turn, the decrease in the $\Delta\delta^{13}C_\alpha$ indexes reflects a helix-end fraying.

Conformational properties of the WT peptide solubilized in H₂O/TFE (80/20)

Since TFE is known to enhance the helix proportion in peptides, it was of interest to investigate its effect on the equilibrium between the two conformers of the WT peptide. NOE data (Fig. 1B) indicate that the helix proportion in the P₄–K₁₇ segment is globally increased in the presence of TFE. In addition, the N β (D₈,E₁₁) and

Fig. 3. Contour plots of 500 MHz 1H -NMR NOESY spectra of selected NH- β - γ H region of (A) the WT peptide in pure aqueous solution which shows both capping box signals; (B) the D8A peptide in the mixed H₂O/TFE (80/20) solution which highlights the correlations between NH₇ and K₂ side-chain protons (spectrum obtained in H₂O/TFE solution is shown but similar, though less intense NOE contacts are found in aqueous solution).

N γ (D₈,E₁₁) NOEs are also strengthened. These results show that both helix turns and capping structures still coexist in the presence of TFE.

The $\Delta\delta^{13}C_\alpha$ index (Fig. 2B) shows that the increase of helix population in the P₄–F₇ segment is approximately half that found in the A₉–K₁₇ segment. Besides, no significant change of the D₈ $\Delta\delta H_\alpha$ and $\Delta\delta^{13}C_\alpha$ indexes is observed upon addition of TFE (Fig. 2A,B). TFE effect on the WT peptide can be interpreted considering three families of conformers in exchange with each other: the “full helix” state, in which the peptide is helical from T₃ to K₁₇, the “broken helix” state, in which the helix begins only at the D₈ residue, and lastly, a common random coil state. Using this description, our data show that TFE shifts, as expected, the equilibrium in favor of the helical states (“full” and “broken” helix) at the expense of the random coil state. However, TFE has almost no effect on the equilibrium between the “broken helix” and the “full helix” states. Therefore, D₈ $\Delta\delta H_\alpha$ and $\Delta\delta^{13}C_\alpha$ indexes are unchanged upon addition of TFE since the contributions of both “full helix” and “broken helix” to the D₈ δH_α and $\delta^{13}C_\alpha$ chemical shifts compensate each other. Furthermore, in the P₄–F₇ segment, only the “full helix” family of conformers contribute to the increase of $\Delta\delta^{13}C_\alpha$ whereas, in the A₉–K₁₇, both “full helix” and “broken helix” contributions are added.

T3A substitution: Suppression of the first capping box

NOE data obtained for the T3A peptide solubilized in aqueous solution are shown in Figure 4A. As expected, no $N\beta(A_3, Q_6)$ correlation is observed in the NOESY spectra. In contrast, the $N\beta(D_8, E_{11})$ NOE intensity increases when passing from the WT to the T3A NOESY spectrum. Downstream from the $D_8 \times E_{11}$ capping box, the $(i, i + 3)$ NOE network is quite similar to that observed for the WT peptide. On the other hand, in the A_5 - D_8 region, the $\alpha\beta(i, i + 3)$ (P_4/F_7 , A_5/D_8 , F_7/D_{10}) NOE intensities are found significantly lower than those corresponding to the WT peptide. In the absence of the $T_3 \times Q_6$ capping box, the first helix turn is, therefore, marginally formed (Fig. 8A).

Fig. 4. NOE data for the (A) T3A peptide, (B) D8A peptide, solubilized in aqueous solution at pH 4.5 and 278 K. Line thickness corresponds qualitatively to the relative intensities of the NOEs. Asterisks indicate connectivities that could not be resolved due to overlap.

Fig. 5. H_α chemical shift index for the WT peptide in pure aqueous solution (open circle) and for the T3A peptide in pure aqueous solution (filled square) at pH 4.5 and 278 K.

The $\Delta\delta H_\alpha$ and $\Delta\delta^{13}C_\alpha$ indexes for both WT and T3A peptides obtained in pure aqueous solution are reported in Figures 5 and 6, respectively. In the A_9 - K_{17} region, no significant variation is observed between WT and T3A peptides, whereas in the P_4 - D_8 segment, most of the residues of the T3A mutant exhibit a δH_α downfield shift and a $\delta^{13}C_\alpha$ upfield shift. Both variations show that the conformational equilibrium is nearly completely shifted toward the “broken helix” state, in which the second $D_8 \times E_{11}$ capping box is formed. The $\Delta^3J_{HN\alpha}$ coupling constant and $\Delta\delta^{13}C_\alpha$ chemical shift indexes, are superimposed in Figure 7A. In the A_9 - G_{18} segment forming a well-defined helix conformation, both parameters are well correlated along the whole sequence. In contrast, although the A_5 and F_7 residues exhibit $^3J_{HN\alpha}$ values characteristic of helix conformation, their $\Delta\delta^{13}C_\alpha$ indexes reflect a random coil conformation. Since $\delta^{13}C_\alpha$ mainly depends on both φ and ψ torsion angles while $^3J_{HN\alpha}$ is sensitive only to φ angle variations, this discrepancy can be simply related to specific ψ

Fig. 6. $^{13}C_\alpha$ chemical shift index for the WT (black bold line), the T3A (red square), the D8A (green triangle), the E11A (purple circle), the F7A (cyan lozenge) peptides solubilized in pure aqueous solution at pH 4.5 and 278 K.

Fig. 7. $^{13}\text{C}_\alpha$ chemical shift index (filled square) and $3J_{\text{HN}\alpha}$ coupling constant index (open lozenge), (A) for the T3A peptide and (B) for the D8A peptide solubilized in pure aqueous solution at pH 4.5 and 278 K. $^3J_{\text{HN}\alpha}$ coupling constants were extracted from DQF COSY and measured from double lorentzian curve fitting procedure.

angles in average significantly different from canonical α helix values. Lastly, the D₈ residue has a $\Delta^3 J_{\text{HN}\alpha}$ close to zero, in agreement with a φ torsion angle around -90° characteristic of the N-cap residue in capping box structures.

D8A substitution: Suppression of the second capping box

As with the T3A peptide, the D8A substitution induces significant variations on the peptide conformation. As expected, no characteristic $\text{N}\beta(\text{A}_8, \text{E}_{11})$ capping correlation is observed in Figure 4B. Moreover, NOEs between F₇ βH , and L₁₂ δH are less intense than those observed for the WT peptide, which simply results from the destabilization of the F₇-L₁₂ hydrophobic cluster associated with the D_{8xx}E₁₁ capping box in the WT peptide. As regards the native T_{3xx}Q₆ capping sequence, the $\text{N}\beta(\text{T}_3, \text{Q}_6)$ and $\text{N}\gamma(\text{T}_3, \text{Q}_6)$ NOEs are more intense than those observed in the WT peptide spectra. In addition, the increase in the magnetic nonequivalence of Q₆ βH protons (0.23 ppm) also confirms an increase in the population of conformers, in which the T_{3xx}Q₆ capping box is formed. Furthermore, the complete set of $(i, i+3)$ NOEs associated with numerous $\alpha\text{N}(i, i+4)$ NOEs observed for the D8A peptide definitely shows that the A₅-K₁₇ segment adopts a native-like helix conformation. Figure 7B shows that, in general, chemical shift and coupling constant indexes are markedly correlated along the whole sequence and are consistent with an extended helix structure. However, for Q₆ and A₉ residues, significant discrepancies are found

between both indexes. This particular point is discussed at the end of the following paragraph.

Interestingly, NOE contacts between F₇ backbone amide proton and K₂ side-chain protons as well as between F₇ and K₂ side-chain protons are observed (Fig. 3B). It has to be pointed out that these correlations may exist in the spectra of other mutants but were not detected because of signal overlaps. They suggest that K₂ and F₇ side chains constitute a hydrophobic staple motif described as a capping box stabilizing structure (Seale et al., 1994; Muñoz et al., 1995a). Besides, NOE contacts are found between F₇ aromatic ring protons and E₁₁, as well as D₁₀ side-chain protons. Therefore, though no K₂-E₁₁ and K₂-D₁₀ NOE is observed, the combination of F₇-K₂ NOEs with F₇-E₁₁/D₁₀ side-chain NOEs supports the existence of transient ionic interactions between the K₂ amino group and E₁₁/D₁₀ carboxylate groups. Chemical shift data support such interactions. First, the K₂ ^1H protons are magnetically nonequivalent. Second, the K₂ side-chain proton signals are sensitive to pH variation around the pK_a value of acidic residues ($\Delta\delta > 0.05$ ppm), whereas the K₂ H $_\alpha$ chemical shift is not affected. To ensure the specificity of the K₂ chemical shift pH dependence, we checked that no variation of K₁₇ side-chain chemical shifts occurred upon pH titration. The large shifts observed for the K₂ side-chain signals with pH variations are undoubtedly due to a decrease of F₇ ring current contributions, and thus, reflect a change in the relative orientations of K₂, F₇, and E₁₁/D₁₀ side chains. We may reasonably assume that this change occurs because the protonation of the E₁₁/D₁₀ carboxyl groups prevents the formation of ionic interactions between K₂ and E₁₁/D₁₀ side chains (Fig. 8B). Therefore, our data suggest that the K₂ side chain, flanked by the F₇ aromatic ring, lies on average along the helix axis allowing ionic interactions between K₂ and D₁₀/E₁₁. Furthermore, such a specific side-chain to side-chain interaction provides an explanation for the small $\Delta^3 J_{\text{HN}\alpha}$ index found for Q₆ and A₉ mentioned above. Indeed, interactions between K₂, F₇, and E₁₁/D₁₀ residues can constitute a strain that would distort the peptidic planes at Q₆ and A₉ residues and induce singular correlated $^3J_{\text{HN}\alpha}$ values. This hypothesis is also supported by the large amplitude of both F₇ $\Delta\delta^{13}\text{C}_\alpha$ and $\Delta^3 J_{\text{HN}\alpha}$ values, which may also be associated with a distortion of both F₇ φ and ψ torsion angles.

F7A and E11A substitutions: Influence of F₇ and E₁₁ residues on the stability of the D_{8xx}E₁₁ capping box structure

F7A and E11A peptides were studied to probe the influence of F₇ and E₁₁ residues on the stability of the D_{8xx}E₁₁ capping box. In a well-defined capping box structure, the E₁₁ carboxylate group is hydrogen bonded to the D₈ amide proton while F₇ is associated with L₁₂ to form a hydrophobic staple motif. Therefore, F7A as well as E11A substitution should affect the peptide conformation. However, NOE data obtained for both F7A and E11A peptides in aqueous solution are quite similar to those obtained for the WT peptide (data not shown).

The parameter the most sensitive to the effects of F7A and E11A substitutions is the $\Delta\delta^{13}\text{C}_\alpha$ index (Fig. 6). As regards the F7A peptide, whereas the index profile in the P₄-Q₆ and A₉-G₁₈ segments is similar to that observed for the WT peptide, F₇ and D₈ residues exhibit a small but significant increase in their chemical shift index indicating that the helix conformation is more populated for these residues. This increase in local helix population can be related to the expected destabilization of the D_{8xx}E₁₁ capping

Fig. 8. Molscript stereoviews (Kraulis, 1991) of the major features of (A) the conformation in the T3A peptide in which the D₈xxE₁₁ capping box initiates the helix at A₉; (B) the helix conformations in the D8A peptide. Green balls represent the hydrogen bonding network linking T₃-F₇ and Q₆-D₁₀. Side chains drawn on B illustrate the interaction described between K₂, F₇, D₁₀-E₁₁.

box in the absence of the hydrophobic F₇-L₁₂ interaction. The F7A substitution introduces an alanine residue, which may also take part in the stabilization of helical conformations. In the E11A peptide, a similar increase in the $^{13}\text{C}_\alpha$ chemical shift index is observed for the F₇ and D₈ residues. In addition, the E11A profile exhibits a small but uniform increase in the $^{13}\text{C}_\alpha$ shift of the indexes in the P₄-Q₆ and A₉-R₁₃ regions, which corresponds to a slight but significant stabilization of the whole helix conformation. However, in both F7A and E11A peptides, D₈ still acts as a single capping residue and conserves its ability to break the helix.

Estimation of the conformer population in the WT fragment

The observed consistency between the NOE, chemical shift, and coupling constant data obtained for the various mutants of the WT peptide prompted us to use the $^{13}\text{C}_\alpha$ chemical shift for quantitatively estimating the populations of the two main ensembles of conformers in the WT peptide. Our main assumption is to consider the conformational equilibrium in the WT peptide as a two-state

equilibrium represented by both T3A and D8A conformations. The random coil constitutes a third state in equilibrium with both former states. Nevertheless, we may reasonably assume that the random coil contribution to the chemical shifts is equivalent for all the peptides. Thus, considering the D₈ residue as the pivotal residue in the equilibrium, we use the D₈ $\Delta\delta^{13}\text{C}_\alpha$ value as a measure of the population ratio for the WT peptide as well as for the F7A and E11A mutants. We find a ratio (full helix/broken helix) of $35 \pm 5/65 \pm 5$ for the WT peptide and of $60 \pm 5/40 \pm 5$ for both F7A and E11A peptides. This population ratio can be expressed as $P_{\text{WT}} = (\Delta_{\text{D8A}} - \Delta_{\text{WT}})/(\Delta_{\text{WT}} - \Delta_{\text{T3A}})$ where Δ 's represent the $\Delta\delta^{13}\text{C}_\alpha$ indexes of D₈ in each peptide. P_{WT} may be used to calculate an order of magnitude of the free energy between both states:

$$\Delta G_{\text{WT}}(\text{full helix-broken helix}) = -RT \ln(P_{\text{WT}}). \quad (1)$$

We found

$$\Delta G_{\text{WT}} = 0.3 \pm 0.1 \text{ kcal} \cdot \text{mol}^{-1} \quad (2)$$

for the WT peptide and similarly

$$\begin{aligned}\Delta G_{F7A}(\text{full helix-broken helix}) &\approx \Delta G_{E11A}(\text{full helix-broken helix}) \\ &= -0.2 \pm 0.1 \text{ kcal} \cdot \text{mol}^{-1}.\end{aligned}\quad (3)$$

From these values, we estimate that the F₇ or E₁₁ alanine substitutions destabilizes the DxxE capping box structure approximately by the same $\Delta\Delta G$ value:

$$\Delta G_{WT} - \Delta G_{F7A} = \Delta G_{WT} - \Delta G_{E11A} = 0.5 \pm 0.2 \text{ kcal} \cdot \text{mol}^{-1}.\quad (4)$$

This value is in agreement with that of $0.65 \pm 0.3 \text{ kcal} \cdot \text{mol}^{-1}$ (Muñoz & Serrano, 1995a) characterizing the contribution to α -helix stability of an F-L hydrophobic staple motif inside a capping box with respect to an A-L interaction.

Discussion

Investigation of conformer populations in partially folded polypeptide chains

Relationships between a given peptide sequence and the populations of its conformers are governed by a complex balance between intrinsic φ, ψ propensities of the various amino acids and their abilities to form local hydrophobic or electrostatic side chain to side chain or side chain to backbone interactions. Helpful information for understanding these relationships can be obtained by combining amino acid substitutions and NMR conformational analysis. We applied this strategy to a singular peptide spanning the A-helix of the D2 domain of annexin I, which exhibits two well-defined subsets of conformations exchanging with each other, which can be summarized as the native-like helix and the non-native broken helix. NMR studies of four single mutants allowed us to distinguish between the influence of the T₃, D₈, F₇, and E₁₁ key residues. Indeed, our annexin peptide constitutes an appropriate model for evaluating the relevance of NMR parameters (NOE, chemical shifts, and coupling constants) as probes of changes in conformer proportions induced by amino acid substitutions.

In this context, we first emphasize that information derived only from NOE data may support misleading interpretations. When found in NMR spectra, consecutive sets of $\alpha N(i, i+3)$ and $\alpha\beta(i, i+3)$ NOEs observed for a given segment are in general readily related to a relatively stable helix conformation. As in our case, coexistence of the helix with another stable conformation can be revealed by characteristic NOEs, such as $N\beta(i, i+3)$, incompatible with a single continuous helix. In other cases, these singular NOE correlations may not be detected either because of signal overlaps or because motifs such as simple capping are not systematically associated with characteristic NOEs. Combined with NOE data, $\Delta\delta H_\alpha$ indexes that account for the whole set of conformations adopted by the peptide can be used as a suitable probe to reveal the existence of singularities in secondary structure elements. For example, in the case of the helical peptide from the C terminus of myohemerythrin (Reymond et al., 1997a), it was proposed that despite the absence of strong NOE evidence for a capping box conformation, the $\Delta\delta H_\alpha$ profile could be used as evidence for an aspartate capping. Nevertheless, $\Delta\delta H_\alpha$ may significantly be affected by other factors such as charge field perturbations, aromatic ring current

effects, and variations due to the amphipathic character of the peptide, which may in some cases be ambiguous.

In contrast, our data showed that $^{13}\text{C}_\alpha$ chemical shifts appear to be a very suitable probe for quantifying the conformer distributions in peptides with a reasonable precision and with more confidence. Ab initio calculations (de Dios et al., 1993; de Dios & Oldfield, 1994, 1996; Le et al., 1995; de Dios, 1996) already outlined the sensitive dependence of $^{13}\text{C}_\alpha$ chemical shift upon both φ, ψ torsion angle variations. In addition, several previous studies of helical peptides showed that, in general, $\Delta\delta^{13}\text{C}_\alpha$ chemical shift indexes were less affected than $\Delta\delta H_\alpha$ by periodic variations along the sequence. From various publications (Lee et al., 1992; Zhang et al., 1993; Arvidsson et al., 1994; Thanabal et al., 1994; Maciejewski & Zehfus, 1995), we estimated that, given one helix turn, the $\Delta\delta^{13}\text{C}_\alpha$ index fluctuated less than 10% around its mean value along the corresponding sequence whereas the $\Delta\delta H_\alpha$ sequence usually varied by more than 25% around its mean value. Therefore, $\Delta\delta^{13}\text{C}_\alpha$ indexes constitute valuable and relevant parameters not only for estimating the relative stability of helix turns along the sequence but more importantly for detecting the existence of singularities related to conformational exchanges (Yao et al., 1997).

From the $\Delta^3 J_{\text{HN}\alpha}$ index, the distribution of torsion angles for each residue can be investigated to evaluate the helix propensity in each peptide segment. Nevertheless, in capping structures torsion angles are around -80° and thus, give rise to a $\Delta^3 J_{\text{HN}\alpha}$ index close to zero, as for a random conformation. As shown from our study, combined with the analysis of $^3 J_{\text{HN}\alpha}$ values, $\Delta\delta^{13}\text{C}_\alpha$ indexes enable us to distinguish between $\Delta\varphi$ and $\Delta\psi$ changes. The marked correlation ($R = 0.85$) (data not shown) that we calculated for each peptide between the variations of both $\Delta\delta^{13}\text{C}_\alpha$ and $\Delta^3 J_{\text{HN}\alpha}$ indexes suggests that both φ and ψ torsion angles vary in a concerted way upon helix stabilization. As a comparison, a much lower correlation ($R = 0.65$) was found between the variations of $\Delta\delta H_\alpha$ and $\Delta^3 J_{\text{HN}\alpha}$ indexes. Nevertheless, it has to be recalled that using chemical shifts and coupling constants, we are only measuring mean parameters. Coupled to NMR data sets, simulated molecular dynamics running in the nanosecond time range should allow characterizing the transitions between the various conformational sets that are in equilibrium. Moreover, the role of water molecules in these transitions can be investigated. In this view, the ensemble of NMR data constitutes a comprehensive database for the calibrations of molecular dynamics simulations. Extensive analysis of such simulations is presently in progress in our lab. Our NMR data can also be useful to tune more accurately the experiments and the prediction algorithms.

Comparison with data obtained from helix/coil transition algorithms

Helix/coil transition algorithms constitute an important tool to progress in the dissection of the complex combinations of local interactions that stabilize or destabilize a helix conformation. We, therefore, compared our $\Delta\delta^{13}\text{C}_\alpha$ index profiles to the data provided by two prediction programs, SCINT (Stapley et al., 1995) and the last version of AGADIR (Muñoz & Serrano, 1995b, 1995c, 1997). Regarding the two extreme cases, D8A and T3A peptides, in which only one staple motif can be formed, both algorithms correctly predict the stabilization of the helix conformers, respectively, downstream either the first T₃xxQ₆ or the second D₈xxE₁₁ capping box. Quantitatively, the AGADIR predictions are significantly closer to the $\Delta\delta^{13}\text{C}_\alpha$ profiles than those of SCINT. In the cases of higher

complexity, i.e., for the WT, F7A, and E11A peptides, the agreement between prediction and experimental data appears to fluctuate more. The AGADIR data well account for the conformational equilibrium present in the WT peptide but overestimate the stability of the helix conformers in both E11A and F7A peptides. Conversely, the SCINT data underestimate the helix stability in each of the WT, E11A, and F7A peptides. Assuming that the WT peptide conformations can be described as an equilibrium between the "full helix" state (D8A) and the "broken helix" state (T3A), the AGADIR data predict a (full helix–broken helix) ratio of (33/67), which is very close to the experimental ratio (35/65). In both E11A and F7A peptides, the predicted (full helix–broken helix) ratios are, respectively, (90/10) and (85/15), which are above the (60/40) ratio measured experimentally.

Aspartate residues and local conformations in partially folded states

Our study emphasizes the marked role of the D₈ residue as a helix breaking residue in the peptide spanning the A-helix of the D2 domain of annexin I. Similar features were observed for a model peptide (Huyghues Despointes et al., 1993) in which Asp amino acids located in the middle of a helix act either as helix breaking or helix stabilizing residues. Other studies of helical peptides (Jimenez et al., 1994a, 1994b) showed that when a short helical peptide, N-capped by an aspartate, was further elongated at the N terminus, this aspartate no longer acted as a helix breaking residue. In our case, D₈ conserves the property of breaking the helix, though the first capping box structure is formed and induces helix stabilization from the T₃ residue. In contrast, the D₁₀ residue does not break the helix. This is probably due to the helix stabilizing D₁₀–R₁₃ salt-bridge (Odaert et al., 1995) and to the possible K₂–D₁₀ salt-bridge mentioned above. The breaking and stabilizing properties of aspartate residues are undoubtedly defined by the sequence pattern formed by the neighboring residues.

Therefore, this study points out that aspartate residues can play, because of their propensity to form side chain to backbone interactions, a central role in local residual structures found in partially folded states. Local interactions, in which D₈ and D₁₀ residues play a major role, are non-native structures that have to be released during protein folding (Cordier-Ochsenbein et al., 1996). Numerous studies emphasized the existence of non-native local structures in partially folded states, but most of those described in the literature concern small hydrophobic clusters (Lumb & Kim, 1994; Smith et al., 1994; Ruiz Sanz et al., 1995; Wang & Shortle, 1995; Rothwarf & Scheraga, 1996; Zhang et al., 1997). These local non-native structures were shown to have significant effect in slowing down the global folding kinetics, and also in playing a role in the thermodynamic stability of proteins (Muñoz et al., 1994; Rothwarf & Scheraga, 1996; Prieto et al., 1997). Detailed investigation of non-native structures and quantitative estimation of their energetic contribution to partially folded states is, therefore, of high interest in the scope of protein folding and protein engineering.

Materials and methods

Peptide synthesis

Peptides were synthesized by the Merrifield solid-phase method on an Applied Biosystems 430 A synthesizer using Boc chemistry.

Stepwise elongation of the peptide chains was done using the standard coupling-capping protocols on p-methylbenzylidrylamine resins. To obtain the peptide in the N-terminal acetylated form, the peptide resins were treated with acetic anhydride using our own program. After the low-high HF cleavage, the crude peptides were purified by MPLC (Medium Pressure Liquid Chromatography) on a HD-SIL 15–25 mm C18 100 Å, using a 25–75% linear gradient of acetonitrile in 0.08% aqueous TFA (pH 2) over 60 min at a 25 mL/min flow rate. Pure fractions were pooled, lyophilized, and the final purity (>95%) of the peptides were checked on a Nucleosil 5 mm C18 300 Å analytical column. Amino acid analyses were performed, after 6 N HCl, 110°, 20-h peptide hydrolysis, on a Beckman 6300 analyzer and were all in agreement with the expected amino acid compositions.

NMR samples

Peptides in aqueous solution

For each sample, 3.9 mg (~4 mM) of lyophilized peptide was dissolved in 0.5 mL of 20 mM NaHPO₄ buffer, 0.1 mM EDTA, 0.1 mM DSS, pH 4.5, and 10% D₂O.

Peptides in trifluoroethanol aqueous mixture

For each sample, 3.9 mg (~4 mM) of lyophilized peptide was dissolved in 0.5 mL of 20 mM NaHPO₄ buffer, 0.1 mM EDTA, 0.1 mM DSS, 10% D₂O, and 20% perdeuterated trifluoroethanol (Eurisotop, France).

Absence of any aggregation effects for the WT, D8A, and T3A peptides was tested by acquiring two far-UV CD spectra (Jobin-Ivon CD6) at 500 μ M and 5 μ M peptide concentrations in 0.1 mm and 0.5 mm cuvettes, respectively. The shape of the CD curve showed no modification between both concentrations for the three peptides.

NMR experiments

NMR spectra were recorded using Bruker AMX and DRX 500 MHz spectrometers. Standard homonuclear two-dimensional phased experiments TOCSY, NOESY, and DQF-COSY (Rance et al., 1983; Wüthrich, 1986; Markley, 1989) were performed. To check the absence of spin diffusion, mixing times from 75 ms to 300 ms were used for the WT and D8A peptide NOESY spectra. For the other NOESY spectra, a mixing time of 180 ms was used. Water suppression was achieved using presaturation. Spectra in aqueous solution and in the trifluoroethanol aqueous mixture were recorded at 278 K. These were recorded in the phase-sensitive mode with quadrature detection in the w1 dimension achieved using TPPI (Marion & Wüthrich, 1983). In general, spectra were acquired with a recycling delay of 1 s. Two-dimensional homonuclear NOESY and TOCSY spectra were recorded with 400 increments of 2 K data points. For the DQF-COSY spectra, 2K points were recorded with t2 digital filtering (spectral width of 3 ppm) of an amide proton region resulting in a 0.73 Hz/data points resolution.

Natural abundance ¹³C heteronuclear HMQC spectra (Bax et al., 1983) were recorded at 278 K using a GARP ¹³C-decoupling sequence (Shaka et al., 1985). One hundred twenty-eight scans were acquired for each of 300 t1 values. The spectral width in w2

was 5,500 Hz and in w1 was 11,300 Hz. The ^{13}C transmitter frequency was placed at 125.76 ppm.

Shifted sine-bell functions were used for apodization of NOESY, TOCSY, and HMQC spectra. DQF-COSY spectra were apodized with a single Lorentzian function. ^1H and ^{13}C chemical shifts data were referenced from the DSS signal at 0 ppm. Two-dimensional data were processed using XWINMR software (Bruker).

Complete sequential assignment of all the backbone and >95% of the side-chain proton resonances was accomplished for the WT, D8A, T3A, F7A, E11A, D10S, peptides in both H_2O and $\text{H}_2\text{O}:\text{TFE}$ 80:20. It is noteworthy that the P_4 residue gives rise to two sets of resonances corresponding to the proline *cis/trans* equilibrium. From integration of the one-dimensional spectra, the population of *cis* conformers was estimated at 6% of the whole ensemble of conformations. Because the *cis/trans* exchange is slow compared to the chemical shift time scale, it does not influence the consideration of the two-state conformational equilibrium (full helix/broken helix) within the population of *trans* conformers. H^α chemical shift index were referenced using (Merutka et al., 1995) random coil values and $^{13}\text{C}^\alpha$ chemical shift index were referenced using random coil values (Wishart et al., 1995). The $^3J_{\text{NH}\alpha}$ coupling constants, measured in aqueous solution at 278 K, were carefully extracted from the anti-phase doublets in COSY cross-peaks within a fitting procedure using two Lorentzian functions in order to take the signal line width into account. In the case of our peptides, the line width is typically below 3 Hz so that the error on $^3J_{\text{NH}\alpha}$ was minimized to ± 0.2 Hz. $\Delta^3J_{\text{NH}\alpha}$ index data were calculated from the relation $\Delta^3J_{\text{NH}\alpha} = ^3J_{\text{NH}\alpha}(\text{exp.}) - ^3J_{\text{NH}\alpha}(\text{coil})$, using $^3J_{\text{NH}\alpha}(\text{coil})$ reference values calculated in Smith et al. (1996a). Similar study, done by Serrano (1995), gave the same reference values.

Electronic supplementary material

Tables T1–T5, respectively, list the chemical shifts values of WT, T3A, D8A, E11A, F7A peptides in aqueous solution. Tables T6–T10, respectively, list the chemical shifts values of WT, T3A, D8A, E11A, F7A peptides in mixed water/TFE (80/20) solvent.

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