Nereis sarcoplasmic Ca^{2+}-binding protein has a highly unstructured apo state which is switched to the native state upon binding of the first Ca^{2+} ion

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Abstract NSCP, a sarcoplasmic Ca^{2+}/Mg^{2+}-binding protein from *Nereis diversicolor*, shows an allosteric change during Ca^{2+} binding and a high positive cooperativity for Mg^{2+} binding. Here we report the results of CD and NMR experiments aiming to characterize the apo state and the Ca^{2+}-induced conformational changes in this protein. Circular dichroism spectra of the apo form are indicative of a reduced helical structure. In contrast, NMR spectra show no element of regular secondary or tertiary structure. Addition of one Ca^{2+} determines large spectral changes bringing the molecule in a conformation which is very close to the native three Ca^{2+} state. Addition of the second and third Ca^{2+} shifts this equilibrium progressively towards the liganded conformation but affects only minimally the spectrum of the liganded species.

Key words: Protein folding; Sarcoplasmic calcium-binding protein; Nuclear magnetic resonance; Apo-protein

1. Introduction

The cellular calcium concentration and its function as second messenger are usually mediated and controlled by cytoplasmic Ca^{2+}-binding proteins. Most of them contain a common helix-loop-helix ion-binding motif (EF-hand). The calcium-binding proteins (CaBP) found in the sarcoplasm of muscle cells were called sarcoplasmic Ca^{2+}-binding proteins (SCP). Large amounts of this type of protein, with a wide structural and functional heterogeneity, were found in invertebrate muscle tissues. They have no capacity to control enzyme activity and are thought to play a role in the regulation of intracellular calcium concentration and fluxes. A member of this class, NSCP (174 residues), was isolated from the annelid *Nereis diversicolor* [1] and extensively characterized [2–4]. X-ray analysis revealed the presence of 4 EF-hand motifs but, due to critical side-chain changes and deletions, only 3 of them (I, III and IV) bind the ligand. In contrast with the CaM or TnC, which have a dumbbell shape, NSCP has a highly compact structure with a hydrophobic core created by aliphatic and aromatic residues [2]. However, by controlled trypsinolysis it is possible to cut apo NSCP into two halves which have specific properties, different from the intact molecule [5].

The basic functional event of many CaBPs is binding of Ca^{2+} ions which switches the protein to an active and more stable conformation. Understanding of the equilibrium and kinetic aspects of the ion-binding process requires detailed structural information on the apo, partially liganded and saturated forms of the proteins. Crystallographic and NMR structures were resolved for a large number of CaBPs in the Ca^{2+}-loaded state [6]. In contrast, the crystallographic analysis of calcium-free proteins is more difficult due to the difficulty of crystallizing the samples in the apo form or in the presence of well-defined calcium concentrations. A number of CaBPs have a moderate size (9–18 kDa) and could be studied by NMR spectroscopy in solution. In this case one can easily control the medium conditions (such as the ion concentration), and the structural flexibility is not incompatible with the spectrum recording. Recent NMR studies of metal-free CaM and TnC showed that metal removal maintains the secondary structure elements but induces specific tertiary structural changes [7–9] and increases the conformational flexibility [10].

NSCP, by its particular structural features (three binding sites, lack of a central helix) and its specific ligand binding properties, represents a particularly attractive system for NMR investigation. We initiated a long-term project aiming to characterize its structure and dynamics in various Ca^{2+}-liganded forms in solution. Here we report the results of CD and NMR experiments dedicated to a global characterization of the apo and partially liganded forms. Surprisingly, in contrast with the previously studied CaBPs, the apo form is mainly unstructured and highly flexible. Addition of the first Ca^{2+} ion induces large spectral changes, bringing the protein in a physicochemical state close to the native liganded state.

2. Materials and methods

2.1. Sample preparation

NSCP was purified according to the method of Cox and Stein [1], modified as described in [3]. For removal of contaminating metals and for equilibration in the assay buffer, NSCP was precipitated with 3% trichloroacetic acid in the presence of 1 mM EDTA and then passed trough a column of Sephadex G-25, equilibrated in the assay buffer. As was previously shown [3], this procedure does not lead to irreversible protein changes. Ca^{2+} contamination was less than 0.05 mol/mol protein.

2.2. Circular dichroism

Spectra were recorded at room temperature on a Jasco-710 spectropolarimeter in a cell of 0.1 cm, with a protein concentration of either 2.0 μM in 1 mM phosphate buffer, pH 6.5 containing 10 μM $CaCl_2$

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Abbreviations: CaBP, Ca^{2+}-binding protein; CaM, calmodulin; CD, circular dichroism; DQF-COSY, double-quantum filtered correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; NSCP, *Nereis diversicolor* Ca^{2+}-binding protein; SCP, sarcoplasmic Ca^{2+}-binding protein; TnC, troponin C

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(ligand-saturated NSCP), or 4 μM in the same buffer containing 500 μM EDTA (free NSCP).

2.3. NMR spectroscopy

NMR samples were 1.1 mM protein in sodium phosphate buffer (50 mM), pH 6.5 containing 5% ^2H_2O for the spectrometer lock system. The spectra were obtained on a Varian Unity 500 NMR spectrometer. Standard methods [11] were used to obtain pure absorption DQF-COSY and NOESY spectra. Usually 512 experiments were performed with 64–128 scans and 2K complex points each.

3. Results and discussion

3.1. Circular dichroism

Far-UV CD spectra of NSCP were recorded in the presence and absence of Ca^{2+} ions (data not shown). The spectrum recorded on the ion-saturated form shows two minima at 208 and 222 nm and a maximum at 192 nm, characteristic for α-helical secondary structure [12]. Removal of Ca^{2+} by EDTA reduces the molar ellipticity at 222 nm from −12000 to −10000 deg cm^2 dmol^{-1} as was already observed at a higher pH [1]. From the ellipticity at 222 nm, which is a good measure of the helix content in a protein [13], the holo NSCP contains about 37% α-helix, less (by about 20%) than observed in the X-ray structure [2]. The CD spectrum of the ion-free sample suggests a small decrease (about 16%) of the α-helix content relative to the holoprotein. The quantitative interpretation of the CD changes in α-helical proteins was recently questioned [14]. In order to explain some inconsistencies between NMR and CD results on TnC the authors suggested that variation of far-UV ellipticity may also depend on tertiary changes, like rearrangement of the helices or alteration of the environment of clustered Phe side chains. In any case, beyond the quantitative aspects, the CD spectrum of apo NSCP may be considered qualitative evidence for the presence of α-helix elements.

3.2. Nuclear magnetic resonance of the apo form

The 1D NMR spectra of NSCP were recorded as a function of Ca^{2+} content at 288 K. In absence of Ca^{2+} the 1D spectrum (Fig. 1) has the main characteristics of a denatured soluble protein [11]. A lower buffer concentration (25 mM) or a high ionic strength (300 mM KCl) have no significant effect on the spectrum of the apo form. The linewidth is compatible with a monomeric, non-aggregated state for a molecule of this size. The aromatic and amide resonances are concentrated in a small range (2 ppm) of the lowfield region. The hydrogen bonds between amide protons of the residues in position 6 with side chain carboxylates in position 1 of the Ca^{2+}-binding loops are crucial for the stability of the Ca^{2+}-binding sites. The amide protons involved in such intramolecular hydrogen bonds (Gly^{21}, Asn^{109}, Gly^{143}) undergo large downfield chemical shifts in the liganded form of NSCP (10.28, 10.27 and 10.47 ppm, respectively) (Prêcheur, B. and Craescu, C.T., unpublished results). Inspection of the 1D spectrum shows that these resonances are upfield shifted in the apo form by at least 2.0 ppm, suggesting a considerable alteration of the EF-hand motifs. The protons corresponding to Val, Leu and Ile methyl groups resonate up to 0.60 ppm which is only 0.30 ppm upfield shifted from the random coil values. Spectral assignment in the Ca^{2+}-loaded state of two regions, one situated between 4.8 and 5.6 ppm and the other between 9.0 and 9.84 ppm, were shown to correspond to the β-strand $C_\alpha H$ and NH,

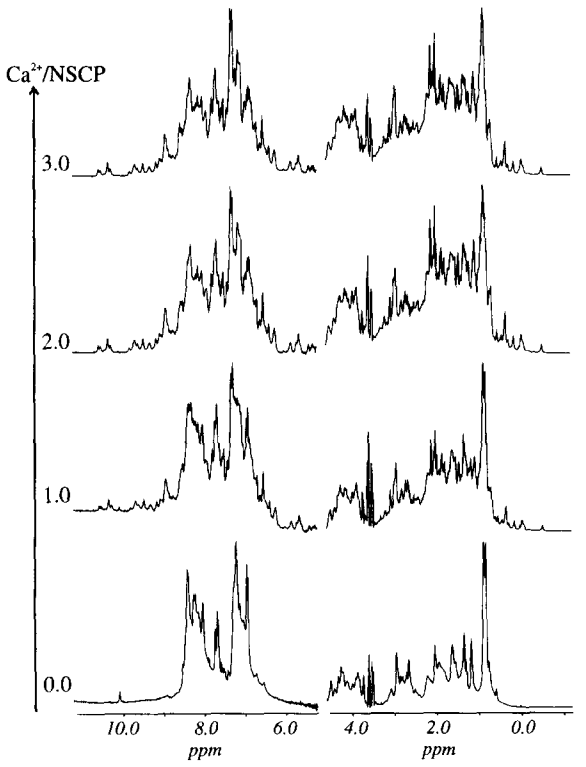

Fig. 1. 500 MHz ^{1}H-NMR spectra of NSCP at 288 K as a function of added Ca^{2+} equivalents. NSCP is 1.1 mM in 50 mM potassium phosphate buffer, pH 6.5.

respectively. Upfield shift of these resonances in the apo state strongly suggests weakening or disappearance of the β-type secondary structure. All these observations may be explained by a complete absence of any regular secondary and tertiary structure and/or by a highly fluctuating structural state in which one or more ordered structures are in rapid equilibrium with irregular structures.

2D NMR spectroscopy was used to characterize further the apo state of NSCP. The COSY spectrum (Fig. 2) is similar to that which would be expected for an ideal random coil polypeptide. The cross-peaks have a very low dispersion and are clustered around the values characteristic for each amino acid type. Nevertheless, the dispersion of these resonances is somewhat larger than in random coil. Resonances from $C_{\delta 1}$ and $C_{\zeta 2}$ protons in the three Trp residues are upfield shifted by about 0.08 ppm while some methyl signals undergo even larger (up to 0.30 ppm) upfield shift from the random coil values. Generally, a tendency towards higher field values relative to the random coil values was noted. The presence of these residual chemical shifts may be the result of an averaged ring current effect over different non-native conformations driven by hydrophobic interactions between aromatic and long-chain alkyl residues clustered in a relatively compact space. This kind of organization was proposed for several non-native protein states [15–17]. It is important to note that NSCP is particularly rich in apolar aromatic residues (3 Trp and 15 Phe).

Analysis of proton dipolar interactions observed in NOESY spectra provides additional information on the three-dimensional architecture of the apo form. The lowfield region of the NOESY spectrum in the Ca^{2+}-loaded form presents a large number of sequential amide/amide cross-peaks, characteristic

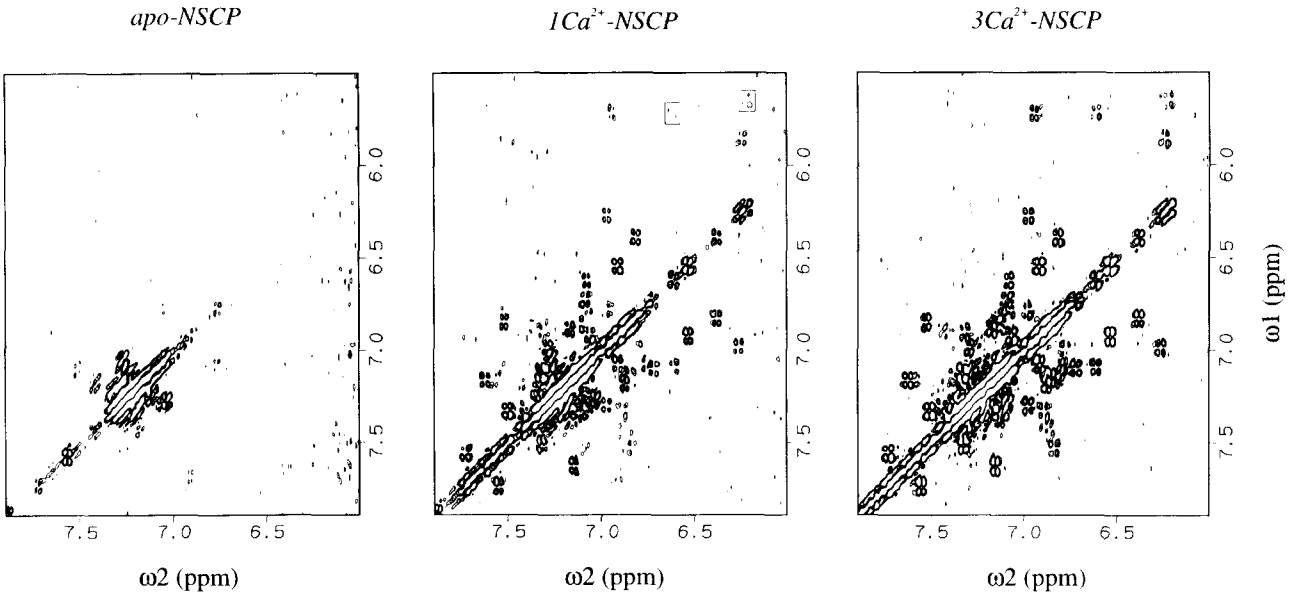

Fig. 2. Aromatic region of 2D DQF-COSY spectra of apo, one Ca^{2+}, and three Ca^{2+} NSCP. The same physicochemical conditions as in Fig. 1. The boxes indicate low intensity cross-peaks which are visible at a lower plot level.

for a highly helical structure (Fig. 3). In contrast, only few cross-peaks of this type are visible in the apo form, indicating the absence of significantly stable secondary structures of α-helix type. In order to avoid the saturation transfer (through hydrogen exchange) from water to amide protons, we performed a NOESY experiment with a jump-and-return read pulse [18] and no water presaturation. The obtained spectrum, which is practically unchanged, shows no additional amide-amide cross-peaks.

3.3. Effect of Ca^{2+} binding

Addition of the first Ca^{2+} ion induces substantial spectral changes (Fig. 1), yielding an NMR spectrum typical for a well folded protein. Further addition of the second and third Ca^{2+} equivalent appears to induce only minimal spectral changes and the final spectrum is practically identical to that of the purified Ca^{2+}-loaded protein. This strongly suggests that the main conformational transition from the apo to the liganded states takes place upon addition of the first ion. The structural changes induced by the ion binding were further investigated by 2D NMR experiments. As shown in the COSY spectra in Fig. 2, the first Ca^{2+} ion is sufficient to completely restore the aromatic cross-peak pattern of the holo form. The same trend was observed in other spectral regions (data not shown). This means that the secondary and tertiary native structure, defined by stable spatial relationships between amino acid side chains, in particular between the aromatic rings, is mainly established in the first step of ion loading.

The first Ca^{2+} binding studies [1] suggested that at pH 7.5 NSCP displays 3 identical intrinsic binding constants of 2×10^8 M^{-1}. However, conformational and microcalorimetric studies on the intact protein [4] or on the isolated N- and C-terminal halves [5] led to the proposal of a new model of sequential binding, with induction of high-affinity sites. In apo form only one site is functional and this may be the site I, since the N-terminal half appears to be the more stable structural nucleus in NSCP. When Ca^{2+} binds to this site, a concerted conformational change occurs that strongly increases the affinity of sites III and IV. The present equilibrium data provide additional support for this model.

A similar Ca^{2+} binding mechanism, in which the chelation of the first ion induces the main structural changes (much smaller than in our case), was recently described for the one-domain, two Ca^{2+}-binding sites, calbindin D_{9K} [19]. But generally, the CaBPs show a sequential ion binding reflected in spectroscopic and conformational progressive changes of similar amplitude [20].

The resonance at 10.08 ppm in the spectrum of apo NSCP could be easily assigned to the Nε protons of the three Trp residues. In particular, their dipolar interactions with the $C_{\delta1}H$ and $C_{\zeta2}H$ are clearly visible in the NOESY spectrum (Fig. 3). The intensity of the 10.08 ppm peak decreases progressively in the one and two Ca^{2+} species and vanishes in the three Ca^{2+} species. This suggests that in the intermediate liganded states a small population (less than 10% in presence of 1 mol of Ca^{2+}) of NSCP is still in a apo-like conformation which is in equilibrium with liganded states. Absence of line broadening and comparison of the chemical shift values of the $N_\varepsilon H$ in the apo and three Ca^{2+} states (Prêcheur, B. and Craescu, C.T., unpublished results) enable us to evaluate that the rate of the conformational change is within the slow limit on the NMR chemical shift scale (less than 100 s^{-1}). Absence of any exchange cross-peak at 10.08 ppm in the NOESY spectrum recorded on the one Ca^{2+} species is indicative of an even lower exchange rate (less than 10 s^{-1}). The slow interconversion rate, observed here, is in qualitative agreement with previous results obtained by fluorescence spectroscopy on NSCP [1,3] showing that the rate constant of conformational changes induced by Ca^{2+} dissociation (holo to apo transition) is about 0.02–0.06 s^{-1}. It is interesting to note that coexistence of several (two or three) conformations with slow interconversion rates were observed in other Ca^{2+}-binding proteins in apo or partially liganded forms [10,21,22].

3.4. Comparison with other CaBPs

The Ca^{2+}-dependent conformational changes described for

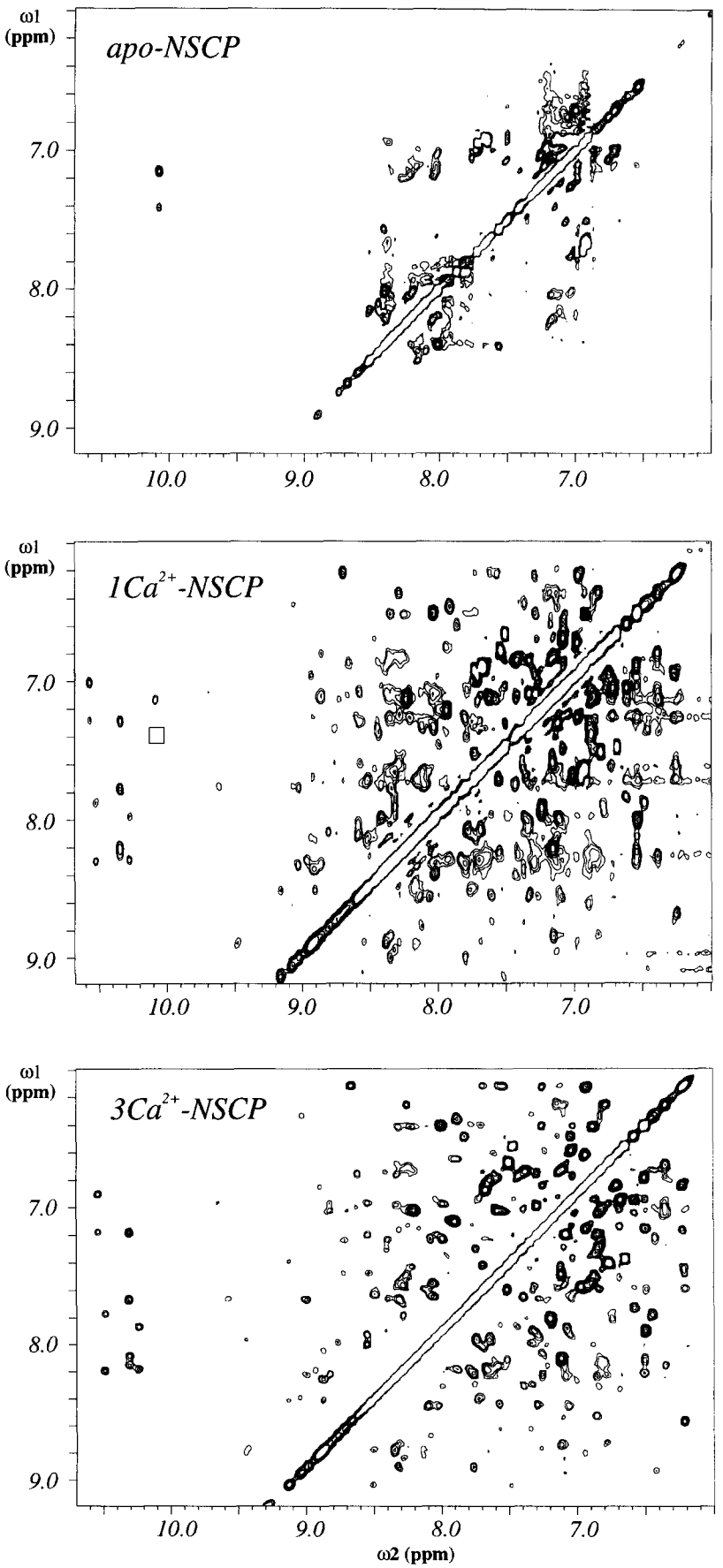

Fig. 3. Aromatic and amino region of the NOESY spectra recorded on apo, one Ca^{2+}, and three Ca^{2+} NSCP in the same conditions as in Fig. 1. The mixing time was 150 ms. The box indicates a cross-peak visible only at lower contour plot.

NSCP are significantly different from those observed for other intact CaBPs. Generally, the apo form preserves the secondary structure elements and have a conformation similar to the liganded form [9,23–25]. The ligand-induced changes are considerably smaller in calbindin D_{9K}, which is a single-domain two-calcium binding protein, relative to the two-domain, four-calcium-binding proteins like CaM and TnC [7–9]. This small differential conformational answer to the Ca^{2+} binding was invoked to explain the functional variety of CaBPs from Ca^{2+} buffering to signal transduction [7,23]. In contrast with the limited structural changes, the internal dynamics, studied by amide proton exchange or relaxation measurements, showed a substantial increase upon metal removal [10,26].

Which could be the specific structural properties which may explain the NSCP behavior on Ca^{2+} depletion? The X-ray analysis showed that the holo protein has a particular three-dimensional structure, devoid of the long central helix observed in CaM and TnC. The two EF-hand motifs are closely packed, creating a highly hydrophobic core containing 20 aromatic side chains [2]. Removal of all Ca^{2+} ions should expand the structure of individual EF-hands and relax the contacts between the neighboring ion sites. This more flexible structure can easily switch into an ensemble of non-native structures in which the hydrophobic side chains are loosely clustered to avoid unfavorable contacts with the solvent. Local perturbations induced by the first Ca^{2+} binding on one selected site create an ordered structural nucleus able to induce a global conformational change over the whole molecule.

3.5. Nature of the apo conformational state

The NMR features of apo NSCP, presented here, define a conformational state which is clearly distinct from the native, fully liganded state and lacks any persistent secondary and tertiary structure. Observation of several sequential amide-amide NOEs is compatible with a collapsed (rather than extended) structure with several turn-like local conformations, as was reported for the denatured SH3 domain [27] or the reduced unfolded bovine pancreatic trypsin inhibitor [28]. Thus, removal of Ca^{2+} ions switches the protein in a soluble, stable, and monomeric state which is closer to an unfolded state [29,30]. In addition, the apo NSCP is not in a 'dead end' or a trapped state on the folding pathway since addition of Ca^{2+} ions restores the native conformation. To our knowledge, this type of ligand-induced folding has been observed only for the isolated fragments or protein domains like the EF-hand motif [31], the isolated C-terminal domain89 of TnC [32] or the Zn-finger domain [33].

Recently, Ptitsyn and his colleagues [34] proposed the existence of a new equilibrium intermediate state in the folding process whose structural properties are intermediate between random coil and molten globule. This state, named 'pre-molten globule', is similar to a kinetic intermediate, called 'burst' state, which was found to present a significant far-UV CD ellipticity but lack any significant protection of NH protons in the NMR experiments. NSCP has many similarities with this early folding state. In this state the hydrophobic collapse has already taken place, as indicated by the NMR data and as can be inferred from previous Trp fluorescence [1] and difference spectrophotometric [4] studies on apo NSCP. Moreover, native apo NSCP is cleaved by trypsin at only two positions, 80–81 and 89–90 [5], indicating that only this short central segment is highly flexible and exposed. Such a restricted proteolysis pattern was reported to be characteristic of the denatured state of α-lactalbumin [35].

The present experiments indicate that apo NSCP conserves a large part of its helical-like CD signal. In contrast, NMR observations such as the paucity of NH/NH NOE cross-peaks and inefficiency of ring-current effects suggest that there is no preferred regular conformation with a significant lifetime. The elements of secondary structure in the highly fluctuating apo NSCP may be localized dynamically in various positions of the sequence, probably in regions having a significant propensity to form helices. The CD spectroscopy thus detects an average content of α-helix, independent of their localization. In contrast, NMR experiments observe average values for nuclear magnetic properties in every local region of the polypeptide chain. In this case, extensive structural fluctuations result in observed values which are characteristic of random coil states. Similar discrepancies between CD and NMR observations were previously reported for the non-native states of other proteins [36–38]. This situation should be more easily encountered in helical proteins in which small elements of secondary structure requires only short- and medium-size interactions along the peptide chain.

The apo and one Ca^{2+} states are sufficiently stable at normal physicochemical conditions to be used for an in-depth analysis of the folding and ion binding mechanisms. This project, requiring detailed multidimensional and multinuclear NMR methods, is currently under way in our laboratories.

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