Unfolding intermediate of a multidomain protein, calmodulin, in urea as revealed by small-angle X-ray scattering

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Calmodulin (CaM) is a highly conserved, eukaryotic calcium binding protein, which mediates a variety of physiological processes by interacting with high affinity to activate a number of different enzymes. The structures of CaM and some target peptide complexes have been obtained (6) and references therein. CaM itself consists of two structurally similar globular domains, connected by a flexible linker, which allows the two domains to adopt different positions with respect to each other and to bind targets. Previous studies on the stability of CaM have been performed with optical spectroscopy and/or calorimetric methods and showed that CaM in the absence of Ca\(^{2+}\) (apoCaM) is much less stable than Ca\(^{2+}\)-saturated CaM (Ca\(^{2+}\)/CaM) [7,8]. Several studies showed that for an intact apoCaM, the N-domain is more stable than the C-domain [9], while for Ca\(^{2+}\)/CaM, the N-domain is less stable than the C-domain and required the general postulate of an unfolding intermediate, one in the folded and one in the unfolded state [10]. In the present study, the changes in the size and shape of CaM during unfolding in urea were first revealed.

2. Materials and methods

2.1. Materials

Rat CaM was expressed in Escherichia coli and purified as described previously [11]. The purified CaM fraction was kept in water with 10 mM EDTA (pH 7) after extensive dialysis against EDTA-water to remove free Ca\(^{2+}\) [12]. A mutant 18-residue peptide (RNKIR-AVGKMARVFSLVR) from the sequence corresponding to the CaM binding domain (residues 397–414) of calcineurin (human) (referred to as CN-18p) was synthesized and purified as described previously [13]. Ultra-pure urea (ICN821519) was obtained from Wako Pure Chemical Industries, Ltd., Japan. The SAXS data were collected within 3–4 h after addition of series of urea to the CaM solutions.

2.2. SAXS measurements

The measurements were performed using synchrotron orbital radiation with an instrument for SAXS installed at BL-10C of the Photon Factory, Tsukuba, Japan. The details of the optics and instruments are given elsewhere [14]. For the SAXS measurements, apoCaM, Ca\(^{2+}\)/CaM, and a mixture of the target peptide and Ca\(^{2+}\)/CaM at 1:1 molar ratio (Ca\(^{2+}\)/CaM/CN18p) were prepared, all containing 50 mM Tris-HCl and 120 mM NaCl at pH 7.6. To get Ca\(^{2+}\)/CaM and Ca\(^{2+}\)/CaM/CN18p, 0.1 M CaCl\(_2\) solution was added, while to get apoCaM, 0.1 M EDTA was added. The temperature of the SAXS experiment was kept at 25.0 ± 0.1°C by circulating water through the cell holder. The protein concentration was 10.0 mg/ml. The concentration range of urea was 0–9 M.

The \(R_g\) value was evaluated by the Guinier approximation [1,2].

\[
R_g(S) = \frac{g(0)}{g(2\theta)}(2\theta) = \frac{s^2}{2\theta} \int_0^\infty \frac{I(\theta)}{2\theta} d\theta,
\]

where \(s = \frac{2\sin \theta \lambda}{\lambda}\) is the scattering angle and \(\lambda\) is the wavelength of


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the X-ray) and \( I(0) \) is the scattering intensity at zero angle. \( I(0) \) is given by \( I(0) = K(\Delta \rho)^2 cM \), where \( K \) is a constant, \( \Delta \rho \) is a contrast factor, \( c \) is the concentration of the protein, and \( M \) is the molecular weight. The compactness of the protein molecule was examined with a Kratky plot, i.e. \( s^2 I(s) \) versus \( s \).

3. Results

3.1. Changes in the \( R_g \) value and \( I(0) \) of CaM during denaturation by urea

Fig. 1A shows that the \( R_g \) value of apoCaM (curve 1) in the native state is 20.8 ± 0.3 Å, a value typical of the dumbbell-like structure without Ca\(^{2+}\) [15], and increases gradually to a value of 22.2 ± 0.3 Å below 2 M urea. The \( R_g \) value increases sharply at urea concentrations between 2 and 6 M, reaching a value of about 34 Å, a value typical of the chain-like structure [16]. Further increases of the urea concentration result in no further changes in the \( R_g \) value. On the other hand, the \( R_g \) value of Ca\(^{2+}\)/CaM (curve 2) in the native state is 21.5 ± 0.3 Å, a value typical of the dumbbell-like structure with Ca\(^{2+}\) [15], and increases gradually to a value of 22.4 ± 0.3 Å below 6 M urea. It reaches a value of about 26.7 ± 0.4 Å with a further increase of the urea. The \( R_g \) value of Ca\(^{2+}\)/CaM/CN18p (curve 3) in the native state is 18.1 ± 0.3 Å, a value typical of the globular structure [12,15], and increases gradually to a value of 20.2 ± 0.3 Å below 6 M urea. It reaches a value of about 24.9 ± 0.4 Å with a further increase of the urea. It is noted that these \( R_g \) values at 9 M urea are clearly larger than those in each native state and smaller than that in the chain-like structure.

Fig. 1B shows that the values of \( (I(0))^{1/2} \) for apoCaM, Ca\(^{2+}\)/CaM, and Ca\(^{2+}\)/CaM/CN18p decrease linearly with urea concentrations and to a value of about 60% in each

![Fig. 1. Urea denaturation curves of CaM monitored by the apparent value of \( R_g \) (panel A) and by the square root of the apparent zero-angle intensity (panel B) for the apoCaM (curve 1), Ca\(^{2+}\)/CaM (curve 2), and Ca\(^{2+}\)/CaM/CN18p (curve 3), respectively. The protein concentration was 10 mg/ml. The SAXS data were collected within 3-4 h after addition of series of urea to the CaM solutions. Open symbols indicate that 9 M urea solution was added to the CaM samples to get the desired concentrations of urea below 6.5 M. Filled symbols indicate that the solid urea was directly added to the CaM samples to get the desired concentrations of urea above 6.5 M. The curves are drawn for easy viewing. The arrows correspond to the \( R_g \) value at about 25 Å for the apoCaM, Ca\(^{2+}\)/CaM, and Ca\(^{2+}\)/CaM/CN18p, respectively.](image)

![Fig. 2. Kratky plots for the apoCaM (panel A), Ca\(^{2+}\)/CaM (panel B), and Ca\(^{2+}\)/CaM/CN18p (panel C) at various concentrations of urea. The numbers on each curve denote urea molar concentrations. The symbols denoted by arrows, \( s_1 \) and \( s_2 \), correspond to the lower and upper limits of the integrated intensity shown in Fig. 3, respectively.](image)
natural state. The decreasing behaviors indicate that the values of \((l(0))^{1/2}\) are proportional to each contrast factor, \(\Delta z\), indicating that the molecular weight does not change during the denaturation. It is noted that the difference in the values of \(l(0)\) with or without peptide corresponds to the increment by the binding of peptide.

### 3.2. Shape changes of CaM during denaturation by urea

Fig. 2 shows the Kratky plots of apoCaM, \(\text{Ca}^{2+}/\text{CaM}\), and \(\text{Ca}^{2+}/\text{CaM/CN18p}\) at different concentrations of urea. In Fig. 2A, the Kratky plot of apoCaM in the native state (curve 0) shows a broad peak around an \(s\) value of 0.016 Å\(^{-1}\), typical of the dumbbell-like structure without \(\text{Ca}^{2+}\) [13]. The peak broadens and decreases about 10% at 2 M urea but it almost does not change between 2 and 3 M, suggesting the appearance of a stable unfolding intermediate around the urea concentrations. Furthermore, the peak broadens and decreases rapidly at urea concentrations between 3 and 6 M. The peak completely disappears at urea concentrations above 6 M, indicating a chain-like structure in the unfolded state [16].

In Fig. 2B, the Kratky plot of \(\text{Ca}^{2+}/\text{CaM}\) in the native state (curve 0) shows a broad peak around an \(s\) value of 0.018 Å\(^{-1}\), typical of the dumbbell-like structure of CaM with \(\text{Ca}^{2+}\). The peak slightly decreases and broadens below 6 M urea, suggesting that the shape change is induced by the dissociation of \(\text{Ca}^{2+}\) from one domain of CaM. The peak decreases gradually below 9 M urea, suggesting that the shape change is induced by the dissociation of \(\text{Ca}^{2+}\) from the compact globular structure to a dumbbell-like structure similar to that of \(\text{Ca}^{2+}/\text{CaM}\) at 9 M urea.

### 4. Discussion

The changes in the size and shape of the CaM molecule during the urea-induced denaturation were directly monitored with the SAXS technique. SAXS gives unique and essential information for describing the unfolding process. The changes in the \(R_g\) values reflect those in the size of the protein molecule during unfolding, while the Kratky plot shows directly the shape of the molecule. Each slope of \((l(0))^{1/2}\), as shown in Fig. 1B, does not change during the denaturation, irrespective of the presence of \(\text{Ca}^{2+}\) or not, indicating that there is no intermolecular association and dissociation. The transition curve based on a bulk structure can also be obtained from the Kratky plot, in which a region between two arrows in Fig. 2 is selected. The selected region is considered to be a measure of compactness for the unfolding molecule.

Fig. 3 shows the transition curves obtained from Kratky plots at different concentrations of urea. The transition curve for apoCaM (curve 1) shows a bimodal transition via a stable unfolding intermediate around 2.5 M urea, while the corresponding \(R_g\) values do not show this noticeable tendency. Based on the dumbbell-like structure, the central linker as well as two domains of CaM contribute to the \(R_g\) value, while the central linker almost does not contribute to the transition curve derived from the Kratky plots. The present result is consistent with that obtained by the far-UV CD study [10].

The transition curve of \(\text{Ca}^{2+}/\text{CaM}\) (curve 2) compares well with the changes of the \(R_g\) values. The compactness decreases slightly below 6 M urea and decreases gradually with a further increase of the urea concentration. At 9 M urea, it reaches a value of about 80% in the native state. It is noted that the \(R_g\) value at 9 M urea is clearly smaller than that in the unfolded state and the corresponding Kratky plots show an expanded dumbbell-like structure. The transition curve of \(\text{Ca}^{2+}/\text{CaM/CN18p}\) (curve 3) also compares well with the changes of the \(R_g\) values. The compactness decreases gradually below 6 M urea and it reaches a value of \(\text{Ca}^{2+}/\text{CaM}\) with a further increase of the urea concentration, suggesting that the shape is similar to that of \(\text{Ca}^{2+}/\text{CaM}\) at 9 M urea. The previous result on the stability of CaM based on the far-UV CD measurements showed that \(\text{Ca}^{2+}/\text{CaM}\) unfolds completely at 9 M urea [10]. However, the present results are not consistent with the previous result. The disagreement between both results would be explained by the differences of protein concentrations employed and the ionic strength.

Finally, we compare the shape of the intermediate for apoCaM with those for \(\text{Ca}^{2+}/\text{CaM}\) and \(\text{Ca}^{2+}/\text{CaM/CN18p}\) at the \(R_g\) value of about 25 Å. The intermediates with the \(R_g\) value of about 25 Å are observed around 2.5 M urea for apoCaM, 8 M urea for \(\text{Ca}^{2+}/\text{CaM}\), and 9 M urea for \(\text{Ca}^{2+}/\text{CaM/CN18p}\), respectively. The bold lines in Fig. 2 indicate the corresponding Kratky plots for apoCaM, \(\text{Ca}^{2+}/\text{CaM}\), and \(\text{Ca}^{2+}/\text{CaM/CN18p}\), respectively. These Kratky plots are similar to each other and the shapes are typical of an expanded dumbbell-like structure. Fig. 3 shows that these unfolding intermediates have similar compactness, as seen from the position of the arrows. Previous NMR studies on the unfolding of CaM reported that, at 2 and 6 M urea, \(\text{Ca}^{2+}/\text{CaM}\) retains a dumbbell-like structure similar to the native state, while at the
same concentrations of urea, apoCaM reveals a typical random coil spectrum [17]. As the NMR spectra have been assigned to amino acid residues in the C-domain of CaM like Phe89, Phe92, Tyr138, etc., the result should be applicable only to the C-domain of CaM. In fact, recent far-UV CD measurements suggested the possibility of a stable unfolding intermediate, in which there is a specific interaction between the two domains of a given CaM molecule, one in the folded and one in the unfolded state [10]. Furthermore, previous results showed that for an intact apoCaM, the N-domain is more stable than the C-domain [9], and for Ca$^{2+}$/CaM, the N-domain is less stable than the C-domain [10]. Combined with these results, the present result could provide direct evidence on a stable unfolding intermediate which consists of one in the folded and one in the unfolded state and a direct interaction between two domains of a given CaM molecule.

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References