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

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Abstract The denaturation of calmodulin (CaM) induced by urea has been studied by small-angle X-ray scattering, which is a direct way to evaluate the shape changes in a protein molecule. In the absence of Ca^{2+}, the radii of gyration (R_g) of CaM are 20.8 ± 0.3 Å in the native state and about 34 ± 1.0 Å in the unfolded state. The transition curve derived from Kratky plots indicates a bimodal transition via a stable unfolding intermediate around 2.5 M urea. In the presence of Ca^{2+} and in the presence of both Ca^{2+} and a target peptide, the R_g values are 21.5 ± 0.3 and 18.1 ± 0.3 Å in the native state and 26.7 ± 0.4 and 24.9 ± 0.4 Å at 9 M urea, respectively. The results indicate that a stable unfolding intermediate still persists in 9 M urea. The present results suggest that the shape of unfolding intermediates is an asymmetric dumbbell-like structure, one in the folded and one in the unfolded state.
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Key words: Calmodulin; Calcium binding; Peptide binding; Urea denaturation; Small-angle X-ray scattering; Unfolding intermediate

1. Introduction

The changes in the secondary and tertiary structure of proteins during folding and unfolding can be monitored by various methods such as circular dichroism (CD), intrinsic fluorescence, Fourier transform infrared spectroscopy and nuclear magnetic resonance (NMR). Direct measurement of changes in the compactness during protein folding (or unfolding) is, however, very difficult. Small-angle X-ray scattering (SAXS) is a powerful technique for such measurements [1–5]. It is well known that the SAXS profile is sensitive to the size and shape of a scattering molecule [1,2]. The size of a molecule can be estimated using the radius of gyration (R_g), obtained by analysis of the SAXS profile using a Guinier plot, while the shape and compactness of proteins can be described in terms of a Kratky plot and the distance distribution function. SAXS has been used for monitoring the shape changes of globular proteins [3–5]. The properties of the folding (or unfolding) intermediates characterized by the method are of importance for understanding protein folding (or unfolding).

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 μM EDTA (pH 7) after extensive dialysis against EDTA–water to remove free Ca^{2+} [12]. A mutant 18-residue peptide (RNKIR-A*K*GKMARVFSVLR) 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], $I(s) = I(0)\exp(-4\pi^2 R_g^2 s^2/3)$, where s is the reciprocal parameter given by $s = (2\sin\theta)/\lambda$ (2θ is the scattering angle and λ is the wavelength of

the X-ray) and $I(0)$ is the scattering intensity at zero angle. $I(0)$ is given by $I(0) = K(\Delta z)^2 \, cM$, where K is a constant, Δz 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
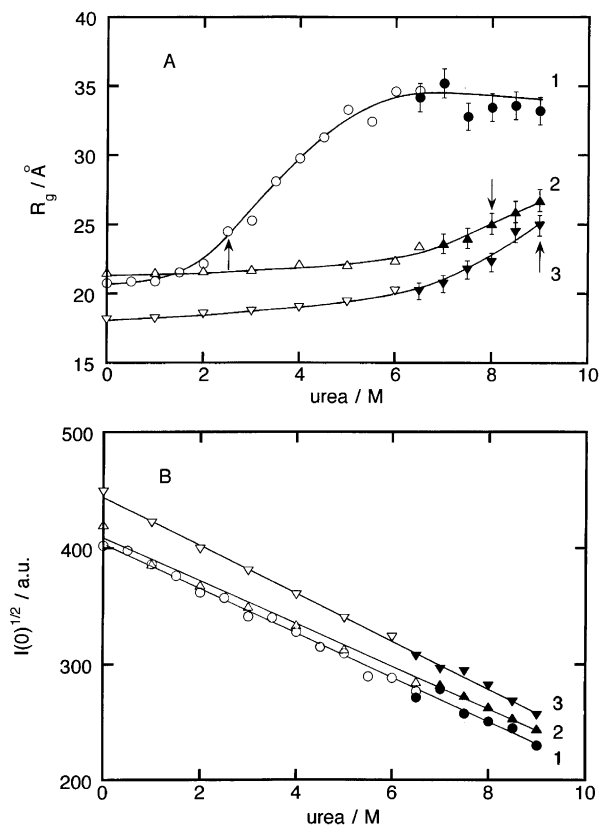

Fig. 1. Urea denaturation curves of CaM monitored by the apparent value of R_g (panel A) and by the square root of the apparent zero-angle intensity (panel B) for the apoCaM (curve 1), Ca^{2+}/CaM (curve 2), and Ca^{2+}/CaM/CN18p (curve 3), respectively. The protein concentration was 10 mg/ml. The SAXS 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.

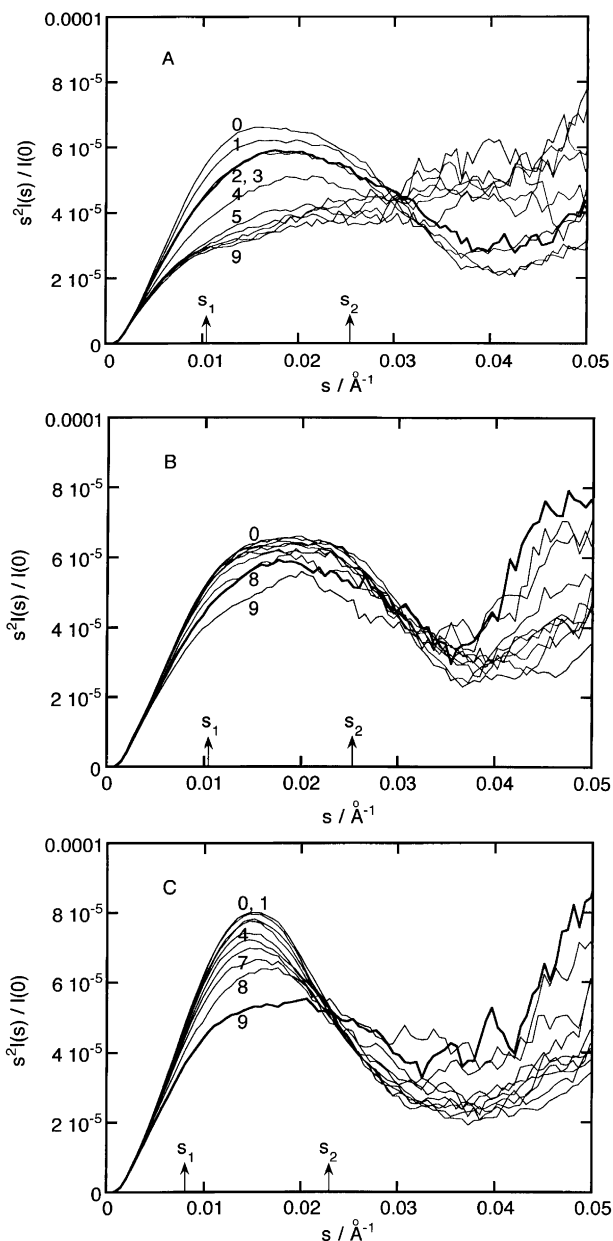Fig. 2. Kratky plots for the apoCaM (panel A), Ca^{2+}/CaM (panel B), and Ca^{2+}/CaM/CN18p (panel C) at various concentrations of urea. The numbers on each curve denote urea molar concentrations. The symbols denoted by arrows, s_1 and s_2, correspond to the lower and upper limits of the integrated intensity shown in Fig. 3, respectively.

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
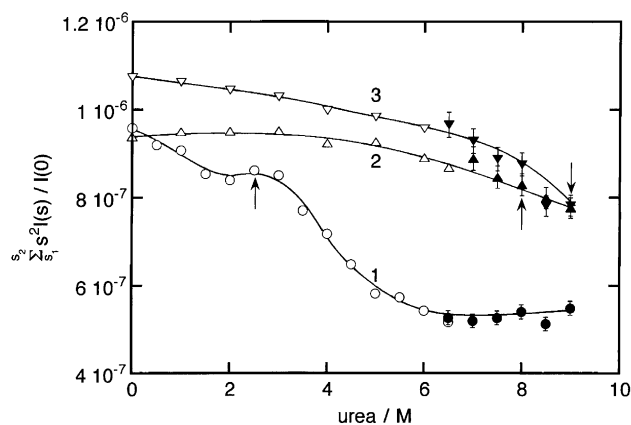
Fig. 3. Urea denaturation curve of CaM monitored by the integrated intensity for the apoCaM (curve 1), Ca^{2+}/CaM (curve 2), and Ca^{2+}/CaM/CN18p (curve 3), respectively. The data points are evaluated by the integrated intensity between two arrows shown in Fig. 2. The curves are drawn for easy viewing. The arrows for the apoCaM, Ca^{2+}/CaM, and Ca^{2+}/CaM/CN18p indicate the integrated values corresponding to the R_g value of about 25 Å.

native state. The decreasing behaviors indicate that the values of $(I(0))^{1/2}$ are proportional to each contrast factor, Δz, indicating that the molecular weight does not change during the denaturation. It is noted that the difference in the values of $I(0)$ 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, Ca^{2+}/CaM, and Ca^{2+}/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 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 Ca^{2+}/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 Ca^{2+}. The peak slightly decreases and broadens below 6 M urea, suggesting that the shape change is induced by the dissociation of Ca^{2+} from one domain of CaM. The peak decreases gradually below 9 M urea with a further increase of the urea concentration but it does not disappear even at 9 M urea, indicating that a native-like structure persists even in 9 M urea, suggesting that the structure is a more expanded dumbbell-like structure than the native state. In Fig. 2C, that of Ca^{2+}/CaM/CN18p in the native state (curve 0) shows a sharp peak around an s value of 0.015 Å^{-1}, typical of the compact globular structure. The peak decreases gradually and broadens below 6 M urea. With a further increase of urea concentration, the peak shifts to a higher s value and broadens, but it does not disappear even at 9 M urea as in the case of Fig. 2B. Thus, the result in Fig. 2C indicates that the shape changes

from the compact globular structure to a dumbbell-like structure similar to that of Ca^{2+}/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 $(I(0))^{1/2}$, as shown in Fig. 1B, does not change during the denaturation, irrespective of the presence of 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 Ca^{2+}/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 Ca^{2+}/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 Ca^{2+}/CaM with a further increase of the urea concentration, suggesting that the shape is similar to that of Ca^{2+}/CaM at 9 M urea. The previous result on the stability of CaM based on the far-UV CD measurements showed that Ca^{2+}/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 Ca^{2+}/CaM and Ca^{2+}/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 Ca^{2+}/CaM, and 9 M urea for Ca^{2+}/CaM/CN18p, respectively. The bold lines in Fig. 2 indicate the corresponding Kratky plots for apoCaM, Ca^{2+}/CaM, and Ca^{2+}/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, Ca^{2+}/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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