Calcium-induced Shape Change of Calmodulin with Mastoparan Studied by Solution X-ray Scattering*

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Solution x-ray scattering using synchrotron radiation as an x-ray source was used to analyze the Ca²⁺-dependent shape change of pig brain calmodulin in detail. The radius of gyration of calmodulin at 10 mg/ml was increased by 0.9 Å. The increase was nearly completed when 2.5 mol of Ca²⁺/mol of calmodulin was added, whereas the radius of gyration of calmodulin with mastoparan decreased by about 3 Å with an increasing Ca²⁺ concentration up to 4 mol of Ca²⁺/mol of calmodulin. At a moderate angle of region, both scattering profiles from calmodulin with or without Ca²⁺ displayed clear humps at \( s = 0.03 \) Å⁻¹ which are characteristic of a dumbbell structure. However, in the presence of mastoparan, the hump in the scattering profile became obscure and later disappeared with the third and fourth Ca²⁺ binding to calmodulin.

These findings are attributable to the Ca²⁺-induced shape change of calmodulin with mastoparan from a dumbbell structure to a non-dumbbell structure in which the distance between the two lobes of calmodulin become closer by a bend in the central helix.

A high-energy electron storage ring emits synchrotron radiation (SR) of a high brilliant x-ray which provides ideal conditions for solution x-ray scattering (SOXS) to investigate the shape-related parameters of the protein molecule in the solution. The scattering profile is free from smearing and its high signal/noise ratio enabled us to directly compare the experimental scattering profile with the theoretical calculation. The SR method makes it possible to investigate domain topology of the protein in solution directly because of its high quality of data at a moderate angle region of the scattering. We applied this technique to study the shape change of calmodulin induced by Ca²⁺ binding.

Since calmodulin is a ubiquitous protein in eukaryotic cells and regulates many cellular processes in a Ca²⁺-dependent manner (1). Ca²⁺-induced conformational changes of the protein have been studied extensively by conventional methods including CD, fluorescence, and NMR (2). In those studies it was noted that the changes of secondary structure of the calmodulin induced by calcium were almost completed by the binding of the first two Ca²⁺, although the third and fourth Ca²⁺ binding were needed to activate the target enzymes, such as phosphodiesterase (3) and myosin light chain kinase (4, 5). Recently, an NMR study showed that the local conformational change of calmodulin with mastoparan, one of the model peptides as a target protein of calmodulin (6), occurred by the third and fourth Ca²⁺ binding. However, this was not the case with mastoparan (5), suggesting that the Ca²⁺-induced conformational change of calmodulin bound on a target protein might be different from that of target protein-free calmodulin. Mastoparan consists of 14 amino acid residues of INLKALAALKKIL in the sequence, and the binding of 1 mol of mastoparan/mol of calmodulin with a high affinity \( K_D \approx 0.3 \) μM should be essential for a physiological function of calmodulin (6). Furthermore, the molecular weight of mastoparan is so small that it would not perturb the x-ray scattering profile from the calmodulin molecule. Therefore, mastoparan is the most suitable model peptide as a target protein of calmodulin on the SOXS study.

The molecular shape of calmodulin-calcium complex in its crystalline state takes on a dumbbell shape in which the N-terminal half (N-lobe) is connected with the C-terminal half (C-lobe) by a central α-helix (7). Using x-ray scattering measurements, two studies have shown that the radius of gyration \( R_g \) of Ca²⁺-saturated calmodulin was about 1 Å larger than that of Ca²⁺-free calmodulin. However, there is disagreement in the results whether the calmodulin in solution is still a dumbbell structure or not (8, 9).

In this paper, the calcium-dependent change of the \( R_g \) of calmodulin with or without mastoparan was investigated in detail. The high quality of the SOXS data at a moderate angle provided us directly with information about the correlations between the two lobes of calmodulin. We propose that the shape of calmodulin differs greatly from that of a dumbbell structure with the increasing of calcium binding in the presence of mastoparan.

**MATERIALS AND METHODS**

Calmodulin was prepared from frozen pig brain. The supernant of the pig brain homogenate in 20 mM phosphate buffer, pH 7.6, 126 mM NaCl, 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride was fractionated with ammonium sulfate. The protein precipitate of 35–70% ammonium sulfate was adjusted to 10 mM phosphate buffer, pH 6.0, 1 mM EDTA and applied to a phosphocellulose column equilibrated under the same conditions. Calmodulin was obtained from the flow-through of the column according to the methods of Yazawa et al. (10), and it was purified by phenyl-Sepharose (Pharmacia LKB Biotechnology Inc.) column chromatography (11), followed by extensive dialysis against EDTA to remove Ca²⁺. Finally, the medium conditions were adjusted to 50 mM Tris-HCl, pH 7.6, and 16 μM EDTA. The first step of phosphocellulose column was effective in excluding the contamination of low molecular weight proteins in calmodulin preparation. The purity of calmodulin was checked by
sodium dodecyl sulfate and/or 8 M urea-polyacrylamide gel electrophoresis (12, 13) as a single protein band, and the concentration was determined by the method of Lowry et al. (14). Mastoparan was purchased from the Peptide Institute Co. (Osaka, Japan) and used after an adjustment of pH of the solution.

The profile of SOXS using SR was obtained by the instrument BL23C which was installed at the Photon Factory in the National Laboratory for High Energy Physics at Tsukuba, Japan. Since the optics of SOXS is the point focusing type, the intensity data were used without slit correction. The details of the optics and instruments are given elsewhere (15). The data treatment was handled by the HITAC M680H computer of the Computer Center, Hokkaido University, Japan. SOXS profiles were obtained with 10–30 mg/ml calmodulin exposure for 20–30 min, depending on the protein concentrations, using a quartz cell of 70 µl in volume. The temperature of the scattering experiment was kept at 25 °C by circulating water through the cell holder. The medium conditions of scattering measurements were 50 mM Tris-HCl, pH 7.6, 120 mM NaCl, and the molar ratios of Ca2+ denoted in this paper were added molar ratios of Ca2+/mol of calmodulin, and Ca2+-free calmodulin was in 1 mM EDTA.

The scattering data at a very small angle region were analyzed by the Guinier method of approximation to calculate \( R_s \), in which \( R_s \) is written as a function of SOXS intensity, \( I(0) \), as \( \ln I(0) = -4\pi/3 R_s^2 \). In this paper, \( s \) is the reciprocal coordinate, \( 2\sin\theta/\lambda \) (scattering angle, \( \lambda \), wavelength of x-ray used, 1.448 Å).

Using the SR radiation as an x-ray source, we could obtain the scattering profiles of calmodulin in high quality at a moderate angle region which enables us to give direct information about domain topology. In fact, the scattering profiles from troponin C showed a hump at about \( s = 0.027 \) Å⁻¹ which relates principally to the distance between the two lobes (17). Thus, the measurement of the hump can be used as a criterion for dumbbell-shaped calmodulin. The theoretical analysis predicts that scattering profiles from a molecule consisted of two well separate domains display two minima but no zeros at a moderate angle. \( R_s \) of the domain could be calculated by the equation: \( R_s(\text{lobe}) = 0.56/s_{\text{min}} = 0.96/s_{\text{min}} \), where \( s_{\text{min}} \) are the position of the two minima and 0.56 and 0.96 are theoretical constants (18). Since calmodulin consists of two quite separate lobes, it is reasonable to analyze the size of the lobe by the theory described above on the assumption that the two lobes of calmodulin were not too much different from that of a sphere.

RESULTS

Guinier plots (\( \ln I(s) \) versus \( s^2 \)) of the data obtained with calmodulin at various Ca2+ concentrations are shown in Fig. 1. The Guinier plots show high quality data, and there is no evidence of any upward curvature at low \( s^2 \) value of calmodulin, indicating the monodispersity of the calmodulin molecule. The straight lines in the figure were obtained with data points between \( s^2 = 1.90 \times 10^{-3} \) and \( s^2 = 1.53 \times 10^{-4} \) using the least squares method. The \( R_s \) value of Ca2+-free calmodulin calculated from the slope of the Guinier plot was 19.9 Å. When Ca2+ was added, as shown in Fig. 2, it increased to 20.8 Å and was almost complete upon the addition of 2.5 mol of Ca2+/mol of calmodulin. The Ca2+-induced change in the \( R_s \) was reversible, since the \( R_s \) value went back to 19.9 Å when excess EDTA was added to the sample of Ca2+-saturated calmodulin after the scattering measurement (data not shown). As we recently reported in detail (19), both \( R_s \) values of Ca2+-free and Ca2+-saturated calmodulin were dependent on calmodulin concentrations. In our results, the infinite dilution values obtained by the extrapolation method were 20.9 ± 0.3 Å for Ca2+-free and 21.5 ± 0.3 Å for Ca2+-saturated calmodulin, respectively.

The slopes of \( R_s \) depending on calmodulin concentrations, were consistent with the results reported previously by other researchers (8, 9).

In the presence of equimolar mastoparan, however, the Ca2+-induced \( R_s \) changes of calmodulin were quite different from those without mastoparan. It is clear from Fig. 2 that the \( R_s \) value of calmodulin decreased drastically with the increase of Ca2+ concentration, and the change continues on to the fourth Ca2+ binding. The \( R_s \) value of Ca2+-free and Ca2+-saturated calmodulin are 20.5 ± 0.3 and 17.8 ± 0.3 Å, respectively. It is noted that the \( R_s \) value of calmodulin with mastoparan in the absence of Ca2+ is slightly but significantly

![Fig. 1. The Guinier plots of SOXS profiles from calmodulin at various Ca2+ concentrations. The x-ray scattering profiles were obtained by 20-min exposure of 10 mg/ml calmodulin in 50 mM Tris-HCl, pH 7.6, 120 mM NaCl at 25 °C. The molar ratios of Ca2+ added per mol of calmodulin were shown in the figure, and the ratio 0 is in 1 mM EDTA. The straight lines were obtained with data points between the arrows in the figure by the least squares method. The ordinate for each curve is shifted so as to demonstrate the Guinier plot clearly.](image1)

![Fig. 2. Ca2+-induced changes in \( R_s \) of calmodulin with or without mastoparan. The \( R_s \) values were calculated from the Guinier plots (shown in Fig. 1 for example) which obtained 10 mg/ml calmodulin in 50 mM Tris-HCl, pH 7.6, 120 mM NaCl at 25 °C. Circles are the results without mastoparan for two different preparations of calmodulin (open and closed). Triangles are the results with the equimolar mastoparan to calmodulin. The molar ratio of Ca2+ to calmodulin is based on added Ca2+ concentration, and 0 is in 1 mM EDTA.](image2)
**Ca^{2+}-induced Shape Change of Calmodulin**

Scattering profiles from calmodulin solution with different amounts of Ca^{2+} at moderate angles are shown in Fig. 3. As shown in panel A of Fig. 3, the characteristic features of all the scattering profiles without mastoparan are humps at around \( s = 0.056 \text{ Å}^{-1} \) and broad peaks which are characterized by two minima at \( s = 0.045 \) and \( s = 0.079 \text{ Å}^{-1} \) and peaks at \( s = 0.056 \text{ Å}^{-1} \). The peak height at \( s = 0.056 \text{ Å}^{-1} \) increases depending on the Ca^{2+} concentration. These changes are almost complete upon the addition of 2 mol of Ca^{2+}/mol of calmodulin and are reversible because trace 5 can be superimposed on trace 1. In the presence of equimolar mastoparan as shown in panel B of Fig. 3, however, the humps at around \( 0.03 \text{ Å}^{-1} \) decreased and disappeared in traces 3 and 4, although the humps could still be seen in traces 1 and 2. Panel B of Fig. 3 also shows that the broad peak at about \( s = 0.056 \text{ Å}^{-1} \) becomes obscure in traces 3 and 4, whereas the peak still can be perceived in traces 1 and 2.

**DISCUSSION**

All the scattering profiles of calmodulin without mastoparan clearly display the humps at around \( s = 0.03 \text{ Å}^{-1} \) (panel A of Fig. 3). Since the \( s \) value of 0.03 Å\(^{-1}\) is equivalent to 33 Å of Bragg spacing (\( s = 1/d, d, \) Bragg spacing), the hump would be a characteristic profile of a dumbbell structure in which the N-lobe correlates with the C-lobe at a distance of 33 Å. The value of 33 Å seems to be reasonable with the value estimated from the crystal structure of calmodulin (7). In the two-domain model, the scattering profiles in Fig. 3 give us information of each lobe size as described under “Materials and Methods.” The \( R_1(\text{lobe}) \) value estimated by the equation (see “Materials and Methods”) is about 12 Å, which is in good agreement with the value estimated from the crystal structure of calmodulin (7). It is noted that the apparent differences among the height of the peaks between the two minima shown in Fig. 3 correspond to the local conformational change since the ratio of \( s_n/s_{\text{so}} \) is close to the theoretical value of 1.74, irrespective of the Ca^{2+} concentrations (18). Presumably, rearrangements of the secondary or tertiary structures in the lobes occur by the binding of Ca^{2+}.

It has been well established that the high affinity binding sites for Ca^{2+} are located in the C-lobe (5, 20). Therefore, the Ca^{2+}-induced small \( R_1 \) change of calmodulin without mastoparan shown in Fig. 2 indicates that Ca^{2+} binding to only the C-lobe can be attributed to the change in \( R_1 \). The C-lobe might partially wrap around the central helix because of its rather slack structure in the absence of Ca^{2+}. The conformation of the C-lobe, however, would become so tight by the binding of Ca^{2+} (3) that it might slightly move away from the central helix and increase the \( R_1 \). However, the size of C-lobe did not change significantly as described above; therefore, the conformational changes of the C-lobe would be very local, and the shape of the whole calmodulin may still be a dumbbell-like structure irrespective of the Ca^{2+} binding.

Consequently, in the absence of mastoparan the shape of the calmodulin molecule and the structure of each lobe in the solution would be similar with those in the crystal.

On the other hand, the Ca^{2+}-induced \( R_1 \) change of calmodulin with mastoparan is significant enough to consider that the whole molecular shape of calmodulin would be changing sequentially by the binding of 4 mol of Ca^{2+}. It is noted that position at the hump and two minima described in the text, respectively. Dotted lines on traces 2–5 are sketches of trace 1. In panel B, dotted lines on traces 1–4 are sketches of corresponding traces 1–4 in panel A.
the distinctive shape change of calmodulin was induced by the Ca\(^{2+}\) binding to the N-lobe. As we showed elsewhere (19), the infinite dilution value of \(R_r\) with Ca\(^{2+}\)-saturated calmodulin in the presence of mastoparan was 17.8 ± 0.3 Å; therefore, the decrease of \(R_r\) dependent on Ca\(^{2+}\) was not caused by the concentration effect of the calmodulin-mastoparan complex. This result has led to the question as to whether the calmodulin molecule still maintains a dumbbell structure in the presence of mastoparan-Ca\(^{2+}\) or not.

Striking differences were observed in SOXS profiles of calmodulin with mastoparan at a moderate angle. The hump at 0.03 Å⁻¹, which reflects the correlation between two lobes as described previously, decreased and disappeared in traces 3 and 4, although the hump could still be seen in traces 1 and 2 in panel B of Fig. 3. Furthermore, the two minima discussed above in calculating the lobe size are also unclear in traces 3 and 4 of panel B in Fig. 3, although the minima are still perceptible in traces 1 and 2. The ratio of \(\beta_{01}/\beta_{00}\) equals 1.73 in both traces 1 and 2, indicating that the size of lobes did not change significantly.

These findings clearly indicate that the Ca\(^{2+}\) binding to the N-lobe made the shape of the calmodulin molecule far as from a dumbbell structure in which the distance between the two lobes might be so close that the correlation characterized as the hump disappeared. The two domains model could no longer be applied to the calmodulin. Therefore, the \(R_r\) value decreased drastically by the third and fourth Ca\(^{2+}\) binding to calmodulin as shown in Fig. 2. Presumably, the distance between the two lobes became close enough to transmit the information of the Ca\(^{2+}\) binding on the N-lobe to the C-lobe by an almost direct manner (5). We could not interpret the decreasing distance between the two lobes except for the idea of a bending in the central helix of the calmodulin (21, 22). As we reported recently (19), the calculation of pair distance distribution function, \(P(r)\), from the extended scattering profiles which involves information about interdomain distance (8, 9) supported the bending model.

Based on the Chou and Fasman structure prediction (23), the sequence of mastoparan is considered a high helix former. Helical wheel representation of mastoparan displays two hydrophobic regions which locate at opposite surfaces of the helix. It is, therefore, reasonable to consider that one molecule of mastoparan could interact with two lobes of Ca\(^{2+}\)-saturated calmodulin at the same time by the interaction between hydrophobic patches of two lobes (21) and hydrophobic surfaces of mastoparan. Thus, the Ca\(^{2+}\)-saturated calmodulin in the presence of mastoparan underwent a bending at the linker region of this molecule. A recent report on the crystal structure of calmodulin refined at a high resolution indicates that residues 79–81, which are at the middle region of the central helix, show significant deviations from ideal \(\alpha\)-helical geometry, and also these residues have high mean temperature factors (24). It is conceivable that a part of the linker region including residues 79–81 would be in disorder for the secondary structure in solution (25).

Since the target enzymes, such as myosin light chain kinase and phosphodiesterase, were activated by the third and fourth Ca\(^{2+}\) binding to calmodulin as already described, the whole molecule of calmodulin was needed for the activation (26, 27). It is therefore possible to consider that the key role of the molecular mechanism of the activation would be in the structural change of calmodulin from a dumbbell to a bending structure induced by the third and fourth Ca\(^{2+}\) binding. The protein structure of the target enzymes could be affected by the structural change of calmodulin induced by the Ca\(^{2+}\) binding. However, we do not as yet know the physiological significance of the Ca\(^{2+}\) binding to the C-lobe. In the presence of mastoparan, the Ca\(^{2+}\) binding to the C-lobe induced the decrease of \(R_r\) (Fig. 2), although the SOXS profiles at a moderate angle still displayed the characteristic features of a dumbbell structure as described previously. Presumably, mastoparan interacts strongly with the C-lobe but weakly with the N-lobe, because the hydrophobicity of each lobe was intensified with the Ca\(^{2+}\) binding. Therefore, at intermediate Ca\(^{2+}\) concentrations, the molecular shape of calmodulin-mastoparan complex is still a dumbbell-like shape, but the shape might have a diversity in which the distance between two lobes is variable depending on the degree of hydrophobic interaction between the lobes and mastoparan. The diversity of the shape of this molecule which is caused by a pliable structure of the central helix, therefore, might be essential to interpret that the calmodulin could bind to various kinds of target enzymes. The studies on \(R_r\) of calmodulin with mastoparan at various Ca\(^{2+}\) concentrations depending on the calmodulin concentration may give us vital information about the second virial coefficients of molecules.

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