Calcium-dependent Changes in Structure of Calmodulin with Substance P*

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Solution x-ray scattering using synchrotron radiation as an x-ray source has been used to study the solution structure of calmodulin complexed with substance P, a undecapeptide neurotransmitter. The x-ray data indicate that the complex has a compact globular structure, the formation of which is dependent upon the binding of Ca²⁺ to calmodulin. In the Ca²⁺-saturated condition, the radius of gyration of complexed calmodulin was 4.2 Å smaller than that of uncomplexed calmodulin. The Ca²⁺-dependent change in radius of gyration of calmodulin with substance P is complete by the third and fourth Ca²⁺ binding. The behavior of the Guinier plot at small-to-moderate angles for uncomplexed calmodulin corresponds to a dumbbell shape. The Guinier plot for complexed calmodulin, however, corresponds to a non-dumbbell structure.

These biochemical and x-ray data suggest that the binding of substance P to calmodulin is completed when the C-terminal half of calmodulin is occupied by Ca²⁺, while a significant structural change of calmodulin in the complex is still induced by successive Ca²⁺ occupancy on the N-terminal half of this molecule.

Calmodulin is a key protein in many signal-transduction processes mediated by Ca²⁺ in living cells (1, 2). In last two decades, numerous target proteins of calmodulin have been identified in various tissues, indicating that the calmodulin is a multi-functional protein. Therefore, many studies have focused on the structure-function relationships of calmodulin.

In the crystalline state, Ca²⁺-saturated calmodulin is a dumbbell shape in which the N-terminal half (N-lobe) is connected to a C-terminal half (C-lobe) by a long central linker (3, 4). Recently, a series of solution x-ray scattering (SOXS) data suggested that the solution structure of calmodulin with or without Ca²⁺ is basically a dumbbell-like shape (5-11). In contrast, in the presence of mastoparan, a calmodulin-binding peptide, the molecular shape of calmodulin changed sequentially from a dumbbell to a globular structure depending on Ca²⁺ binding (7, 8). Similar shape changes occurred with other calmodulin-binding peptides such as melittin (10), M13 (a synthetic peptide corresponding to the calmodulin-binding domain of myosin light chain kinase) (9), Pfk5 (a synthetic peptide corresponding to the calmodulin-binding domain of phosphorylase kinase) (11), and C24W (a synthetic peptide corresponding to the calmodulin-binding domain of the Ca²⁺ pump of human erythrocytes) (12). The idea of a bend in the middle of the central linker of calmodulin has been proposed to explain the globular structure of the Ca²⁺-calmodulin-peptide complexes. In this model, peptides were localized to a cavity between the N-lobe and C-lobe by hydrophobic interaction (13, 14).

A bend model may help interpret the function of calmodulin. In the presence of target proteins, a small change of bending angle in the linker region of calmodulin induced by the Ca²⁺ binding may result in a large change of the relative positions of two lobes affecting the structure of the target proteins. Furthermore, calmodulin could adapt itself to various kinds of calmodulin-binding protein, since the size of the cavity formed by the two lobes would be also regulated by a small change of the bending angle in the linker.

The current consensus for the structure of peptides complexed with calmodulin is a basic-amphiphilic α-helix (Baa-helix) (15, 16). Indeed, synthetic simple peptides designed to form a Baa-helix bound to calmodulin with high affinity. Crystallographic, NMR, and CD data proved the Baa-helical structure of melittin (17-19) and mastoparan (20) in a crystalline state or under hydrophobic circumstances. However, it is still uncertain that the predicted structure of all calmodulin-binding peptides, or the domains of the target proteins, satisfy the criterion of the Baa-helix.

Extensive studies on the binding of hormones and neurotransmitters including substance P by calmodulin have been reported (21-24). Substance P is one of a group of related peptides called tachykinins that are distributed widely in the mammalian nervous system (25). Because the sequence of substance P is RPKPQQFFGLM, the predicted conformation of substance P in the complex with calmodulin is unlikely to form a typical Baa-helical structure. Based on an NMR study, the conformation of substance P in methanol is composed of a flexible basic portion (Arg-1-Lys-3) and a hydrophobic α-helical portion (Pro-4-Phe-8) (20). Furthermore, substance P has the smallest number of residues in a peptide which could bind to calmodulin with micromolar $K_d$.

In this paper we characterize the complex of Ca²⁺-calmod-
ulin-substance P using SOXS and report the susceptibility of calmodulin to proteolytic attack with trypsin.

MATERIALS AND METHODS

Calmodulin was prepared from frozen bovine brain. From the supernatant of the bovine brain, homogenized in 50 mM phosphate buffer (pH 5.7), 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, the calmodulin fraction was obtained according to the method of Yazawa et al. (27). The calmodulin was purified by phenyl-Sepharose (Pharmacia LKB Biotechnology Inc.) column chromatography using an EDTA gradient elution (0-0.5 mM). Calmodulin was eluted at high EDTA concentration, whereas contaminant Ca\(^{2+}\)-binding proteins eluted at lower EDTA concentration. SDS- or urea-polyacrylamide gel electrophoresis (PAGE) showed a single protein band of calmodulin (see lanes 1 of Figs. 5 and 6). The purified calmodulin fraction was kept in water (pH 7) after extensive dialysis against EDTA to remove free Ca\(^{2+}\).

The trypsin digestion of calmodulin was performed in 50 mM Tris-HCl (pH 7.6), 120 mM NaCl at 25 °C. The reaction was started by addition of 1/100 (w/w) trypsin in 0.1 mM HCl to 5 mg/ml calmodulin solution. At appropriate times, 10 μl of the reaction mixture was drawn out and added to 90 μl of 9 M urea, 20 mM Tris-glycine buffer (pH 8.5), 1 mM EDTA with bromphenol blue to terminate the digestion. The protein composition of the mixture was analyzed by polyacrylamide gel electrophoresis (PAGE) (28). The gel was stained with Coomassie Blue, and the densitometric pattern of the gel was obtained by a dual-wavelength scanner (Shimazu CS-910).

The concentration of proteins were determined by the method of Lowry et al. (29).

L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was purchased from Sigma. Substance P and mastoparan (synthesized products) were purchased from the Peptide Institute Co. (Osaka, Japan) and used after adjustment of pH of the solution.

The SOXS data were obtained with the BL-1OC instrument, which was installed at the Photon Factory in the National Laboratory for High Energy Physics at Tsukuba, Japan. The radius of gyration of calmodulin was calculated from the scattering data in the very small angle region by the Guinier method (28). The gel was stained with Coomassie Blue, and the denstometric pattern of the gel was obtained by a dual-wavelength scanner (Shimazu CS-910).

The concentration of proteins were determined by the method of Lowry et al. (29).

RESULTS

All the SOXS data for the calmodulin-substance P complex were obtained at pH 6.0, because the mixture of calmodulin with substance P at neutral pH became turbid irreversibly. Therefore, we evaluated the effect of pH on the Rg for calmodulin. With a decrease in pH from 7.6 to 6.0, the Rg values of Ca\(^{2+}\)-saturated calmodulin at fixed protein concentration (~11 mg/ml) changed from 20.6 to 21.1 Å, and that of Ca\(^{2+}\)-free calmodulin changed from 19.8 to 19.0 Å, respectively (Fig. 1). The changes are small, but they go in the opposite direction depending on pH. The Rg values of calmodulin-Ca\(^{2+}\)-mastoparan complex, however, are almost constant irrespective of pH.

The protein concentration dependence of the Rg values of calmodulin at pH 6.0 are shown in Fig. 2. It is noted that the slope of Ca\(^{2+}\)-saturated calmodulin is quite different from that of Ca\(^{2+}\)-free calmodulin. Table I summarizes the intrinsic Rg values obtained by extrapolation to infinite dilution in Fig. 2. This table also shows the intrinsic Rg values of calmodulin at pH 7.6 presented in our previous paper (7). The intrinsic Rg value of Ca\(^{2+}\)-saturated calmodulin is 0.3 Å larger than that of Ca\(^{2+}\)-free calmodulin at pH 6.0. In contrast, Ca\(^{2+}\)-saturated calmodulin with substance P is 4.2 Å smaller than that without substance P.

The Ca\(^{2+}\)-dependent Rg change of calmodulin was measured at a fixed calmodulin concentration of 11 mg/ml. The Rg value of calmodulin with substance P decreased from 19.0 to 16.8 Å with increasing Ca\(^{2+}\) concentration, and the change continued

![Fig. 1. Effect of pH on the Rg for calmodulin. The Rg values of calmodulin were obtained at a fixed protein concentration of 11 mg/ml in 50 mM PIPES (pH 6 - 7) or 50 mM Tris-HCl (pH 7.6 ~ 9) buffer and 117 mM NaCl. Other conditions to the media were as follows: O, 3.3 mM Ca\(^{2+}\); ▲, 1 mM EDTA; ◆, 3.3 mM Ca\(^{2+}\) and 0.66 mM mastoparan.](image)

![Fig. 2. Protein concentration dependence of Rg values for calmodulin at pH 6.0. The Rg values of calmodulin were obtained in 50 mM PIPES buffer (pH 6.0) and 117 mM NaCl. Other conditions are as follows: O, in the presence of a 5-fold molar excess of Ca\(^{2+}\) to calmodulin; ◆, in the presence of a 5-fold molar excess of Ca\(^{2+}\) to equimolar substance P to calmodulin; ▲, in the presence of 1 mM EDTA; ▲, in the presence of 1 mM EDTA and equimolar substance P to calmodulin.](image)

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<tr>
<td>Ca(^{2+})</td>
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<td>Mastoparan</td>
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*(The Rg values at pH 7.6 are from Matsushima et al. (7)).
on the third and fourth Ca\(^{2+}\) binding as shown in Fig. 3. The apparent decrease of 2.2 Å in R\(_g\) is consistent with the difference between R\(_g\) values at a calmodulin concentration at 11 mg/ml, as seen in Fig. 2. The apparent R\(_g\) value of calmodulin without substance P increased from 19.6 to 22.1 Å with increasing Ca\(^{2+}\) concentration, and the change saturated at about 2 mol of Ca\(^{2+}\)/mol of calmodulin. The apparent increase of 2.5 Å in R\(_g\) is also consistent with the difference seen in Fig. 2.

Since the slope of the plot in Fig. 2 reveals the interparticular interference between calmodulin molecules, the molecular state of Ca\(^{2+}\)-free calmodulin would be different from that of Ca\(^{2+}\)-saturated calmodulin. The apparent change in R\(_g\) values of calmodulin with or without substance P due to binding of Ca\(^{2+}\) (Fig. 3), therefore, might include both contributions of an intrinsic structural change and molecular state change of calmodulin. In the absence of substance P, both changes in calmodulin are completed by binding the first two Ca\(^{2+}\). These results suggest that there is not a mixture of Ca\(^{2+}\)-free and Ca\(^{2+}\)-saturated calmodulin at intermediate Ca\(^{2+}\) concentrations. In the presence of substance P, binding of the first two Ca\(^{2+}\) to calmodulin caused an increase in the R\(_g\) value, while Ca\(^{2+}\)-dependent substance P binding to calmodulin caused a decrease in the R\(_g\) value. The profile of the Ca\(^{2+}\)-dependent R\(_g\) change of calmodulin with substance P at low Ca\(^{2+}\) concentration shown in Fig. 3, might reflect a balance of the two effects.

The Guinier plot of calcium-saturated calmodulin at pH 6.0 displayed a quasi-straight line at higher scattering angles (s\(^2\) = 3 \times 10^{-4} \sim 8 \times 10^{-5}) in addition to the intrinsic straight line at very small scattering angles (s\(^2\) = 2 \times 10^{-5} \sim 1.5 \times 10^{-4}) (Fig. 4, trace 1). Similar behavior is seen in Ca\(^{2+}\)-saturated calmodulin and Ca\(^{2+}\)-free calmodulin at pH 7.6 (Fig. 4, traces 2 and 3). The appearance of two distinct linear regions on Guinier plots of the small-to-moderate scattering angle shown in Fig. 4 is consistent with the calculated profile of a dumbbell-shaped model particle (31). However, Guinier plots of calcium-saturated calmodulin with equimolar substance P or mastoparan (Fig. 4, traces 4 and 5) approximates a simple straight line between s\(^2\) = 1.5 \times 10^{-5} and 6 \times 10^{-4}.

The susceptibility of calmodulin to digestion with trypsin was used to examine the structural effects accompanying substance P and mastoparan binding. When Ca\(^{2+}\)-saturated calmodulin was digested for 10, 20, 30, 60, and 90 min under the conditions described under "Materials and Methods," the amounts of degradation of the calmodulin were 16, 34, 52, 77, and 95%, respectively. Therefore, a digestion time of more than 60 min should be appropriate to examine the effect. The rate of digestion of Ca\(^{2+}\)-saturated calmodulin decreased remarkably with increasing molar ratios of the added peptides as shown in Fig. 5. Since the changes level off at a molar ratio of about one in both the 60- and 90-min digestions, binding of 1 mol of the peptides is sufficient to induce a structural change in calmodulin. The binding of substance P or mastoparan produces a similar effect upon the structure of calmodulin.

With or without peptides, the degradation of calmodulin is sensitive to the added molar ratio of Ca\(^{2+}\) to calmodulin, as shown in Fig. 5. All curves display similar dependence to the molar ratio of Ca\(^{2+}\), although the susceptibility of calmodulin to degradation without the peptides is remarkably different from that with the peptides. In all cases, it was noted that 70% of the changes occurred when 2 mol of Ca\(^{2+}\)/mol of calmodulin were added.

**DISCUSSION**

Earlier work showed the binding of 1 mol of substance P to a mol of calmodulin (K\(_D\) \sim 2 \mu M) (21, 22). Our data shown in
**Fig. 5.** Inhibitory effect of substance P and mastoparan on the trypsin digestion of calmodulin analyzed by urea-PAGE. Panel A, lane 1 is undigested calmodulin, and lanes 2–9 are calmodulin digested by trypsin for 60 min in the presence of 3 mM Ca²⁺ and various concentrations of substance P at molar ratios of 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, and 3.0 to calmodulin, respectively. Lane 10 is calmodulin digested in the presence of 1 mM EDTA and a 2-fold molar excess of substance P to calmodulin. Other conditions of the digestion reaction are described under “Materials and Methods.” The positions of F12 and F34 fragment (corresponding to the N-lobe and C-lobe, respectively) on the urea-PAGE, based on Ref. 34, are indicated.  

Panel B, the changes in degradation of calmodulin depending on the added molar ratio of substance P to calmodulin (●, △) are shown. The ratio of the readings obtained by densitometry of the digested calmodulin (lanes 2–9 in panel A) to the undigested calmodulin (lane 1 in panel A) is represented as the percentage degradation. The results obtained in the presence of various concentration of mastoparan are also indicated (○, □) (urea-PAGE patterns are not shown). Digestion times for circles were 60 min, and those for triangles were 90 min.

Fig. 5 support the specific binding of substance P to calmodulin. Therefore, the $R_e$ changes shown in Table I and Fig. 3 indicate that the Ca²⁺-dependent binding of substance P induces a large shape change in calmodulin. The intrinsic $R_e$ change of calmodulin induced by the binding of substance P under Ca²⁺-saturated conditions is 4.2 Å, which is similar to that induced by mastoparan.

Table I also indicates that the intrinsic $R_e$ values of calmodulin with or without Ca²⁺ are basically independent of pH between 7.6 and 6.0. The apparent pH effect on the $R_e$ of calmodulin seen in Fig. 1 is due to the change of interparticle interference between calmodulin molecules at different pHs.

In previous papers we concluded that the complex of calmodulin with mastoparan was not a dumbbell but a compact globular structure in which the distance between the two lobes of calmodulin becomes closer because of a bend in central linker region (7, 8). Recently, NMR and crystallographic studies for the structure of calmodulin-M13 or calmodulin-smMLCK peptide (a synthetic peptide corresponding to the calmodulin-binding domain of smooth muscle myosin light chain kinase) complex gave solid support to the bending model (32, 33).
In this paper, therefore, we can discuss the structure of the calmodulin-substance P complex in comparison to the compact structure of the calmodulin-mastoparan complex. As discussed already, the behavior of Guinier plot at small-to-moderate angles could be a useful criterion for a dumbbell shape of scattering particles (31). It is clear that the Guinier plot of calmodulin without peptides has the characteristic behavior of a dumbbell-shaped particle as shown by traces 1–3 in Fig. 4. On the other hand, the Guinier plot of calmodulin-Ca\(^{2+}\)-mastoparan complex, which is a compact globular structure as described, is clearly a single straight line as shown by trace 5 in Fig. 4. In this situation the molecular shape of the complex of calmodulin with substance P should be a compact globular structure as well, since the Guinier plot shown in trace 4 can be superimposed on that of trace 5 in Fig. 4.

Under conditions used here, Ca\(^{2+}\)-saturated calmodulin was proteolysed by trypsin at Lys-75 and Lys-77 of central linker region (34). The desensitization of the limited trypsinolysis of calmodulin due to substance P and mastoparan binding indicate that 1 mol of the peptide bound to calmodulin alters the microenvironments of Lys-75 and Lys-77 (Fig. 5). A similar inhibition of proteolysis was occurred by the presence of melittin (35).

In the absence of Ca\(^{2+}\), calmodulin has high sensitivity to trypsin digestion. The binding of Ca\(^{2+}\) to the first and second sites, presumably on the C-terminal lobe of calmodulin, decreases the susceptibility to trypsinolysis by 20% (Fig. 6B, triangles). It has been well established that the high affinity Ca\(^{2+}\) binding sites are located in the C-lobe (36, 37). The 20% decrease of the degradation of calmodulin might due to a reduced cleavage rate of Arg residues in the both N-lobe and C-lobe, but not due to a change of the cleavage rate of Lys-75 and Lys-77 (38). In the presence of substance P or mastoparan, however, the sensitivity to trypsin digestion decreased more than 60% when the C-lobe was occupied by Ca\(^{2+}\) (Fig. 6B, circles). Therefore, Ca\(^{2+}\) occupancy of the C-lobe led to the binding of the peptides and caused a change in the microenvironments around Lys-75 and Lys-77, to protect against attack by trypsin. The profile of the change in degradation shown in Fig. 6 cannot be interpreted by assuming a mixture of Ca\(^{2+}\)-free and Ca\(^{2+}\)-saturated calmodulin at 2 mol of Ca\(^{2+}\)/mol of calmodulin with or without the peptides.

Since Ca\(^{2+}\) occupancy of the C-lobe alone is enough to form trypsin-resistant complexes, binding of the first two Ca\(^{2+}\) sites on the C-lobe is assumed to be the principal role of the binding of peptides to calmodulin. On the other hand, the \(R_g\) change of calmodulin with substance P (Fig. 3) or with mastoparan (8) was not completed solely by the occupancy of Ca\(^{2+}\) on the C-lobe. Consequently, the structural change of calmodulin from a dumbbell to a compact globular shape had been accomplished by the binding of Ca\(^{2+}\) to the N-lobe. These considerations led us to the idea of a two-step mechanism of activation by calmodulin. First, calmodulin binds the target protein and the C-lobe is occupied by Ca\(^{2+}\). Second, further structural change of calmodulin from a dumbbell to a globular shape is completed by the Ca\(^{2+}\) binding to N-lobe, and that structural change triggers the activation of the target protein. Indeed, the shape parameters obtained from the SOXS profiles of calmodulin with mastoparan at intermediate Ca\(^{2+}\) concentration still have characteristic features of a dumbbell shape (8). The isolated C-lobe could bind mastoparan without a significant change in the domain structure (39).

There have been studies that support such a two-step mechanism of activation. Calmodulin bound to the phosphoprotein phosphatase calcineurin upon saturation of the two higher Ca\(^{2+}\) binding sites, whereas additional Ca\(^{2+}\) binding was needed to activate the enzyme (40). Additionally, both phosphodiesterase and myosin light chain kinase were activated by the third and fourth Ca\(^{2+}\) binding to calmodulin, and the whole molecule of calmodulin was needed for activation (34, 41, 42). Furthermore, ruthenium red inhibited the Ca\(^{2+}\)-calmodulin-dependent activation of myosin light chain kinase without dissociation of the enzyme from calmodulin (43).

Since SOXS technique is a fruitful approach to understand the solution structure of calmodulin as a free monomer as well as when it is complexed with peptide, much effort has been devoted to the SOXS study so far (44). The structural parameters of calmodulin and Ca\(^{2+}\)-calmodulin at neutral pH reported in the literature are summarized in Table II. The \(R_g\) values of Ca\(^{2+}\)-calmodulin are basically consistent among them, permitting us to compare directly the \(R_g\) values for Ca\(^{2+}\)-saturated calmodulin obtained in the different laboratories. However, our \(R_g\) value for Ca\(^{2+}\)-free calmodulin (20.9 Å) is slightly larger than the other values. Possibly, this discrepancy was caused by the effect of ionic strength on the scattering measurements. In our preliminary experiments the SOXS data from Ca\(^{2+}\)-free calmodulin was very sensitive to ionic strength of the solution between 0 and 0.2 M NaCl (at a fixed calmodulin concentration, for instance, the \(R_g\) value changed from 18 to 20 Å with increase an ionic strength from 0 to 0.2 M NaCl). Our SOXS measurements were performed in 117 mM NaCl, whereas that of the other measurements were performed in 100 mM KCl. The effect of ionic strength on the SOXS experiments may also cause the differences in \(d_{max}\) values, since the pair distribution function for two-lobed molecule is quite sensitive to the relative orientation and mean separation of the two lobes (44). Moreover, our \(d_{max}\) value was calculated by a method of direct Fourier transformation (7), whereas other values were calculated by an indirect method (5, 6).

The intrinsic \(R_g\) value of the calmodulin-M13 complex is 16.4 Å (8), which is close to the value of the calmodulin-substance P complex (17.2 Å). Possibly, the overall shape of the calmodulin-substance P complex is similar to that of calmodulin-M13, which had a globular shape in solution (32). The globular structure of calmodulin-M13 complex in solution is stabilized by the hydrophobic interactions between the long helical cylinder of M13 (~28 Å) and both the N- and C-lobes. The predicted helical cylinder of substance P (less than 10 Å), however, seems to be too short to interact with the two lobes, as M13 does. Hence, the key residues on calmodulin that interact with substance P might be quite different from that with M13. The proposed structure of the calmodulin-substance P complex emphasizes the flexibility of calmodulin in binding peptides of different lengths and structures.

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<td>(R_g) (d_{max})</td>
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* The \(R_g\) values presented in this table were obtained by the Guinier method (30).
* The \(d_{max}\) is the maximum linear dimension of calmodulin molecule calculated from the pair distribution function, \(P(r)\) (see Ref. 44).
* The data were obtained at the Photon Factory in the National Laboratory for High Energy Physics at Tsukuba, Japan.
* The data were obtained at the Photop Factory in the National Laboratory for High Energy Physics at Tsukuba, Japan.
modeling study for the structure of calmodulin-\( \alpha \)-purothionin reported recently also suggested the flexibility of the calmodulin molecule (45). We do not know yet the minimum size of peptide, nor the determinants, required to bind calmodulin in the contracted, or nearly spherical, form.

SOXS investigation of calmodulin with peptides closely related to substance P might give us the answer, although the physiological significance of the binding of substance P to calmodulin is unknown.

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REFERENCES