Ca²⁺-bound calmodulin forms a compact globular structure on binding four trifluoperazine molecules in solution

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Small-angle X-ray scattering (SAXS), which determines the radius of gyration, R_g , and the pair distance distribution function, was used to investigate the conformational changes of calmodulin (CaM) on binding to an antagonist, trifluoperazine (TFP), with or without Ca²⁺ in solution. We previously applied this SAXS method to CaM complexed with *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide (W-7) [Osawa, Kuwamoto, Izumi, Yap, Ikura, Shibamura, Yokokura, Hidaka and Matsushima (1999) FEBS Lett. 442, 173–177] and found that the binding of two W-7 TFP molecules to one Ca²⁺-saturated CaM molecule induces structural changes from a ‘dumb-bell’ shape to a compact

globular shape. We report here that the most compact globular shape whose size is consistent with that of the 1:2 Ca²⁺-saturated CaM–W-7 complex is formed by the binding of four TFP molecules to one Ca²⁺-saturated CaM molecule. Even in the absence of Ca²⁺, the conformational changes of CaM occur on TFP binding, giving a slightly smaller R_g than Ca²⁺-free CaM alone.

Key words: antagonist, Ca²⁺-binding protein, globular form, solution X-ray scattering, structural change.

INTRODUCTION

Calmodulin (CaM) is a ubiquitous Ca²⁺-binding protein of 148 residues that regulates a variety of physiological processes in a Ca²⁺-dependent manner [1]. The regulation is achieved through the interaction of Ca²⁺-bound CaM with a large number of target enzymes [2–4]. The CaM molecule in both the Ca²⁺-bound and Ca²⁺-free states adopts an ‘elongated’ structure [5–8] in which the two globular domains are connected by a highly flexible linker [9–12], whereas the structures of Ca²⁺-bound CaM complexed with a peptide from target enzymes adopt a compact globular shape caused by the bending of the domain linker [13–15]. These structural studies suggest that the flexibility of the domain linker is important in target recognition.

CaM antagonists such as 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10*H*-phenothiazine (trifluoperazine; TFP) and *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide (W-7) have been used extensively to study the Ca²⁺-bound CaM-dependent activation of various enzymes (Figure 1). However, there is some controversy over the stoichiometry of

binding of TFP and its analogue to CaM and their affinities. Equilibrium dialysis experiments indicate that the two primary TFP-binding sites in the presence of Ca²⁺ display similar affinities [16,17]. An automated HPLC binding assay indicated that Ca²⁺-bound CaM binds independently 4 equiv. of TFP with no significantly different affinity [18]. Gel-filtration experiments indicated that chlorpromazine, which is structurally similar to TFP, binds at not four but five binding sites [19]. Photolabelling of CaM with a reactive phenothiazine derivative indicated two binding sites, one in each globular domain [20]. ¹H-, ⁴³Ca- and ¹¹³Cd-NMR studies indicated the presence of two distinct binding sites for TFP in which the affinities differ by one or two orders of magnitude [21–23].

The stoichiometry of binding of W-7 to CaM is also similarly complicated. Gel-filtration experiments indicated that three W-7 molecules bind to Ca²⁺-bound CaM with a dissociation constant of 11 μM [24]. In contrast, NMR studies including NOESY results indicated that two molecules of W-7 and its analogue J-8 bind to one CaM molecule with high affinity [25,26]. Moreover, distinct binding sites of W-7 and TFP were reported to exist in the N-terminal and C-terminal domains of CaM [26,27].

Furthermore, it has been suggested that overall conformational changes of Ca²⁺-bound CaM might occur on binding TFP [21,23,27]. It has been reported that a major conformational change occurs on the binding of TFP to Ca²⁺-bound CaM [28]. X-ray crystal structure analysis [29–31] revealed that Ca²⁺-bound CaM–TFP complexes with three molar ratios of 1:1, 1:2 and 1:4 are very similar and adopt a globular structure similar to those of Ca²⁺-bound CaM–target-peptide complexes. Small-angle X-ray scattering (SAXS) can be useful in exploring changes in the size and overall shape of molecules in solution. A recent SAXS study provided evidence that, in solution, the structure of Ca²⁺-bound CaM changes from an elongated shape to a compact globular shape on binding two W-7 molecules [32]. The main aim

Figure 1 Chemical structures of W-7 (left) and TFP (right)

Abbreviations used: CaM, calmodulin; cTnC, cardiac troponin C; $p(r)$, pair distance distribution function; R_g , radius of gyration; SAXS, small-angle X-ray scattering; TFP, trifluoperazine {10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10*H*-phenothiazine}; W-7, *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide.

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of the present study was to investigate with the SAXS method whether, in solution, TFP binding to Ca^{2+} -bound CaM induces an inter-domain compaction of CaM and, if so, how many TFP molecules are required for the compaction. This work is expected to eliminate some ambiguity regarding the stoichiometry of TFP as shown in the CaM-W-7 complex [32].

There is also some controversy over TFP binding to CaM in the absence of Ca^{2+} . Very little binding was observed by equilibrium dialysis experiments [18]. This differs from the results of Levin and Weiss [16], which displayed only low-affinity, high-capacity binding. A ^{19}F -NMR study suggested that TFP bound to Ca^{2+} -free CaM [33,34]; however, it remains to be clarified whether this binding really takes place. The other aim of the present study was to investigate this subject through measurable structural changes of Ca^{2+} -free CaM.

The present results indicate that structural changes of CaM occur on TFP binding irrespective of Ca^{2+} . In Ca^{2+} -saturated CaM-TFP complex the most compact globular structure is formed by the binding of four TFP molecules to one CaM molecule.

MATERIALS AND METHODS

Sample preparation

Recombinant rat CaM was expressed in *Escherichia coli* and purified to homogeneity as described previously [35]. For SAXS experiments, the recombinant CaM was dissolved in Tris buffer (50 mM Tris/HCl, pH 7.6) containing 120 mM NaCl. The protein concentration was determined by quantitative amino acid analysis. The TFP powder was dissolved in water containing 1% (v/v) DMSO and added to the CaM solution. At every stage the concentration of DMSO was kept at 1.0%. Three series of solutions were prepared. The first were solutions for Ca^{2+} -saturated CaM and Ca^{2+} -saturated CaM complexed with 5 equiv. of TFP at protein concentrations of 6.0, 9.0, 12.0, 15.0 and 18.0 mg/ml. The second were solutions corresponding to the first in the Ca^{2+} -free state. The third were solutions of Ca^{2+} -saturated CaM in the presence of TFP with molar ratios of 1:0, 1:1, 1:2, 1:3, 1:4 and 1:5 at a protein concentration of 9.0 mg/ml.

SAXS

The measurements were performed with synchrotron orbital radiation on an instrument for SAXS installed at station BL-10C of Photon Factory (Tsukuba, Japan) [36]. An X-ray wavelength of 1.488 Å (1 Å = 0.1 nm) was selected. The samples were contained in a quartz cell with a volume of 80 µl; the temperature was maintained at 25 ± 0.1 °C by circulating water through the sample holder. The reciprocal parameter, Q , equal to $4\pi\sin\theta/\lambda$, was calibrated by the observation of peaks from dried chicken collagen, where 2θ is the scattering angle and λ is the X-ray wavelength. Scattering data were collected for 250 or 300 s at various protein concentrations.

Two methods of data analysis were used. The first method was that of Guinier [37], which gives the radius of gyration, R_g . The range of Q used for Guinier plots was 0.02 – 0.09 Å $^{-1}$, except for Ca^{2+} -saturated CaM alone and in the presence of TFP with a ratio of 1:1, in which a range of 0.02 – 0.07 Å $^{-1}$ was used. The second method was the calculation of pair distance distribution function, $p(r)$, which is the frequency of the distances r within a macromolecule obtained by combining any volume element with any other volume element [36]. The $p(r)$ was calculated by a direct Fourier transformation [38]. Data to $Q = 0.7$ Å $^{-1}$ were used for $p(r)$ analysis. The maximal pair distance, d_{max} , was also

estimated from $p(r)$; $p(r)$ becomes zero at values of r equal to or greater than the maximum d_{max} of the particle. Details of the calculation method are given elsewhere [39]. As reported previously [32], R_g and $p(r)$ were also calculated from atomic coordinates of the Ca^{2+} -bound CaM-TFP complexes [39].

RESULTS

R_g and $p(r)$ of Ca^{2+} -saturated CaM with TFP

Four representative Guinier plots for Ca^{2+} -saturated CaM and Ca^{2+} -free CaM with or without TFP are shown in Figure 2.

Figure 2 Representative Guinier plots for CaM with TFP and for CaM without TFP at a protein concentration of 9.0 mg/ml

Curve 1, Ca^{2+} -saturated CaM with TFP (CaM-to-TFP ratio 1:5); curve 2, Ca^{2+} -saturated CaM alone; curve 3, Ca^{2+} -free CaM with TFP (CaM-to-TFP ratio 1:5); curve 4, Ca^{2+} -free CaM alone.

Figure 3 R_g for Ca^{2+} -saturated CaM with TFP and for Ca^{2+} -saturated CaM without TFP, as a function of protein concentration

Symbols: ○, Ca^{2+} -saturated CaM with TFP (CaM-to-TFP ratio 1:5); □, Ca^{2+} -saturated CaM alone. The values are means $\pm$ S.E.M.

Table 1 R_g (means $\pm$ S.E.M.) and maximum dimension, $d_{\max}$, for Ca^{2+} -saturated CaM, Ca^{2+} -free CaM saturated CaM with TFP, Ca^{2+} -free CaM and Ca^{2+} -free CaM with TFP

Condition	R_g (Å)	$d_{\max}$ (Å)
Ca^{2+} -saturated CaM*	21.9 ± 0.3	61
Ca^{2+} -saturated CaM with TFP (CaM-to-TFP ratio 1:5)*	17.6 ± 0.3	48
Ca^{2+} -free CaM*	21.5 ± 0.3	60
Ca^{2+} -free CaM with TFP (CaM-to-TFP ratio 1:5)*	20.5 ± 0.3	57
Ca^{2+} -bound CaM-TFP complex† [30]	15.92	46.0
Ca^{2+} -saturated CaM-W-7 complex* [32]	17.4 ± 0.3	47

* X-ray values are at zero protein concentration.
† Calculated values are from atomic coordinates of the crystal structure.

Figure 4 $p(r)$ for Ca^{2+} -saturated CaM with TFP and for Ca^{2+} -saturated CaM without TFP

Symbols: $\circ$, Ca^{2+} -saturated CaM with TFP (CaM-to-TFP ratio 1:5); $\square$, Ca^{2+} -saturated CaM alone.

Figure 3 shows the R_g for the Ca^{2+} -saturated CaM-TFP complex (CaM-to-TFP ratio 1:5) and Ca^{2+} -saturated CaM alone as a function of protein concentration. Table 1 gives the R_g values of both proteins at zero concentration, which, for comparison, contained the R_g for the Ca^{2+} -saturated CaM-W-7 complex [32] and the calculated R_g from the crystal structure of the 1:4 Ca^{2+} -bound CaM-TFP complex [30]. The X-ray R_g value for the TFP complex was consistent with that for the Ca^{2+} -saturated CaM-W-7 complex and was comparable with the calculated value, which was smaller owing to the lack of atomic coordinates for the N-terminal first two residues. It was also consistent or comparable with those of Ca^{2+} -bound CaM complexed with mastoparan [40,41], melittin [42], cyclosporin A [43], substance P [44] and respective synthetic peptide corresponding to the CaM-binding domains of myosin light-chain kinase [45], phosphorylase kinase [46] and Ca^{2+} pump [47].

The $p(r)$ for the Ca^{2+} -saturated CaM-TFP complex was very similar to that for the Ca^{2+} -saturated CaM-W-7 complex. However, they clearly differed from that for the Ca^{2+} -saturated CaM alone, as shown in Figure 4. The $p(r)$ for Ca^{2+} -saturated CaM alone might have two main features; one was a peak near

Figure 5 R_g as a function of the molar ratio of TFP to Ca^{2+} -saturated CaM at a protein concentration of 9.0 mg/ml

Figure 6 R_g for Ca^{2+} -free CaM with TFP and for Ca^{2+} -free CaM without TFP, as a function of protein concentration

Symbols: $\circ$, Ca^{2+} -free CaM with TFP (CaM-to-TFP ratio 1:5); $\square$, Ca^{2+} -free CaM alone.

20 Å (principally representing interatomic distances within each domain of Ca^{2+} -bound CaM) and the other was a shoulder near 40 Å (mainly representing interdomain distances) [9,40]. In contrast, the peak near 20 Å shifted to near 22 Å in the Ca^{2+} -saturated CaM-TFP complex and the shoulder near 40 Å disappeared completely. The $d_{\max}$ for the Ca^{2+} -saturated CaM-TFP complex was approx. 13 Å smaller than that for Ca^{2+} -saturated CaM alone, and consequently its $p(r)$ was relatively symmetrical, suggesting that the structure is spherical [38]. These characteristic behaviours are also seen in the $p(r)$ of the other complexes, including Ca^{2+} -bound CaM-target-peptide complexes [45–47]. The present results obviously indicate that the Ca^{2+} -saturated CaM-TFP complex adopts a globular structure similar to those of the other complexes.

Figure 5 shows R_g values as a function of the molar ratio of Ca^{2+} -saturated CaM and TFP at 9.0 mg/ml. A decrease in the R_g on TFP binding to Ca^{2+} -saturated CaM began at a ratio of 1:1 and ended at a ratio of 1:4. This behaviour differed from that of

Figure 7 $p(r)$ for Ca^{2+} -free CaM with TFP and for Ca^{2+} -free CaM without TFP

Symbols: $\circ$, Ca^{2+} -free CaM with TFP (CaM-to-TFP ratio 1:5); $\square$, Ca^{2+} -free CaM alone.

the Ca^{2+} -saturated CaM–W-7 complex [32]. The R_g of the Ca^{2+} -saturated CaM–TFP complex with a ratio of 1:4 was consistent with that of the Ca^{2+} -saturated CaM–W-7 complex with a ratio of 1:2.

R_g and $p(r)$ of Ca^{2+} -free CaM with TFP

Figure 6 shows R_g as a function of protein concentration for Ca^{2+} -free CaM in the presence of TFP with a molar ratio of 1:5 and Ca^{2+} -free CaM alone. Table 1 gives the R_g values of both proteins at zero concentration. The R_g value for the Ca^{2+} -free CaM with TFP (CaM-to-TFP ratio 1:5) was approx. 1 Å smaller than that of Ca^{2+} -free CaM alone. The $p(r)$ of the Ca^{2+} -free CaM with TFP seemed to be similar to that of the Ca^{2+} -saturated CaM–TFP complex rather than the Ca^{2+} -free or Ca^{2+} -saturated CaM alone (Figure 7). Indeed, a peak near 22 Å in the Ca^{2+} -free CaM with TFP was consistent with that in the Ca^{2+} -saturated CaM–TFP complex. Moreover, the shoulder near 40 Å seen in Ca^{2+} -saturated CaM alone grew very weak or disappeared in the Ca^{2+} -free CaM with TFP. Consequently, the $p(r)$ of Ca^{2+} -free CaM with TFP was relatively symmetrical. This suggests that the Ca^{2+} -free CaM with TFP existed as an ellipsoid in solution. However, the structure was less compact than that of the Ca^{2+} -saturated CaM–TFP complex, because the d_{max} of the Ca^{2+} -free CaM with TFP was quite comparable to that of Ca^{2+} -free CaM alone. The present results provide evidence that the binding of TFP to Ca^{2+} -free CaM occurred, leading to the structural changes reported here.

DISCUSSION

Compact globular structure of Ca^{2+} -bound CaM–TFP complex

The SAXS data demonstrate that the binding of TFP molecules induces structural changes from an elongated structure to a compact globular structure in solution, as seen in the Ca^{2+} -saturated CaM–W-7 complex [32]. Such globular structures were also observed in both solution and crystal structures of Ca^{2+} -bound CaM complexed with various target peptides [13–15, 40–47]. X-ray structure analysis revealed that Ca^{2+} -bound

CaM–TFP complexes (CaM-to-TFP ratios of 1:1, 1:2 and 1:4) adopt a globular structure in the crystal [29–31]. It is obvious that the globular structure of the Ca^{2+} -saturated CaM–TFP complex and in the other complexes is caused by bending of the flexible linker in solution.

Interestingly, there is a difference in stoichiometry between the CaM–TFP and CaM–W-7 complexes in solutions. The binding of four TFP molecules induces the largest compaction between the two domains of CaM. A corresponding globular structure was observed in the 1:2 Ca^{2+} -saturated CaM–W-7 complex [32]. What causes this difference? A comparison of the structures of the CaM–TFP complex and the CaM–W-7 complex shows that there are at least two factors that contribute to the stabilization of the globular structure in these complexes: one is Van der Waals interactions, which contain an interaction between hydrophobic aromatic groups (the phenothiazine ring of the TFP molecule and the naphthalene ring of the W-7 molecule) and the hydrophobic pocket of the two CaM domains [26, 29–31]; the other is electrostatic interactions between the positively charged group of TFP or W-7 and negatively charged residues of CaM. Two similar interactions exist in the other CaM–target-peptide complexes. A difference in the strength of these stabilization factors might cause the difference in the stoichiometry observed.

The crystal structure of the 1:4 Ca^{2+} -bound CaM–TFP complex showed that two TFP molecules were located at the sequentially homologous hydrophobic pockets of the C-terminal and N-terminal halves termed ‘intradomain sites’, whereas the other two TFP molecules were found at the central linker region termed ‘interdomain sites’ [30]. Thus in a solution of the complex it is probable that two TFP molecules occupy the intradomain sites and the other two TFP molecules occupy the interdomain sites. Interestingly, in the crystal of the 1:2 Ca^{2+} -bound CaM–TFP complex both sites bind one TFP molecule per site [31]. The binding of TFP to both sites seems to be necessary to form a compact globular structure of CaM.

The binding of TFP to cardiac troponin C (cTnC) is of interest. The binding sites for TFP and bepridil on cTnC were identified by using methyl groups as site-specific structural markers [48]. According to the NMR study, TFP and bepridil bind to similar sites only in the presence of Ca^{2+} . There are three or four binding sites, in the shallow hydrophobic cup in the C-terminal domain and on two hydrophobic surfaces on the N-terminal domains. It therefore seems that the TFP-binding mode of cTnC is similar to that of Ca^{2+} -bound CaM.

Structural changes in Ca^{2+} -free CaM on binding TFP

The SAXS analyses indicate that structural changes in Ca^{2+} -free CaM occur in the presence of TFP under the conditions used here. The Ca^{2+} -free CaM in the presence of TFP (CaM-to-TFP ratio 1:5) adopts an ellipsoid rather than a ‘dumb-bell’ shape, although it is less compact than the Ca^{2+} -saturated CaM–TFP complex. This implies that such structural changes occur on the binding of TFP to Ca^{2+} -free CaM.

As noted, CaM has two types of TFP binding site, referred to as intradomain and interdomain sites [30, 31]. A ^{19}F -NMR study suggested that TFP is located near a hydrophilic region of the *Tetrahymena* CaM molecule [34]. It is likely that one or two TFP molecules bind to the interdomain site of CaM in the Ca^{2+} -free state. Does Ca^{2+} -free CaM inhibit the TFP binding to the intradomain site? It has been generally recognized that the N-terminal and C-terminal CaM domains (N-CaM and C-CaM) have two distinct structures that are referred to as the ‘open’ and ‘closed’ conformations. The ‘open’ conformations are seen in N-CaM, C-CaM and the C-terminal domain of troponin C (C-

TnC) in the Ca^{2+} -bound state, whereas the 'closed' conformations are seen in Ca^{2+} -free N-TnC domain. The structure analysis of CaM complexes revealed that an open form of Ca^{2+} -bound CaM recognizes target peptides [13–15]. From this observation it is clear that a closed conformation would be unable to bind peptides. A third distinct conformation (termed a 'semi-open' conformation) different from the open and closed conformations was identified in the C-terminal domains of the myosin regulatory and essential light chains (RLC and ELC) [49]. The C-RLC and C-ELC occupying the semi-open conformation bind to an IQ motif (IQXXRGXXR). In this connection it was suggested that, whereas Ca^{2+} -free N-CaM adopts the closed conformation, Ca^{2+} -free C-CaM adopts a semi-open conformation [50]. Although it was not confirmed that Ca^{2+} -free C-CaM adopts the semi-open conformation [51], Ca^{2+} -free CaM has been shown to interact with the IQ motif [49]. It is therefore reasonably presumed that Ca^{2+} -free C-CaM interacts with TFP as well as the IQ motif. In other words, TFP is bound to both the intradomain site of C-CaM and the interdomain sites in the Ca^{2+} -free state.

Finally, the observed conformational changes of Ca^{2+} -free CaM could arise from the binding of TFP molecules to both the interdomain sites and the intradomain site of C-CaM. Here, it might be significant to note the binding of TFP to fast myosin light chain. CD spectroscopy showed that TFP altered the secondary structural content of fast myosin light chain (LC) [52]. EPR spectroscopy also suggested that TFP binds to the hydrophobic core of the LCs.

Conclusion

The present SAXS data provide strong evidence that, in solution, the 1:4 Ca^{2+} -saturated CaM–TFP complex forms a compact globular structure whose size is consistent with that of the 1:2 Ca^{2+} -saturated CaM–W-7 complex. Even in the absence of Ca^{2+} , structural changes in CaM occur on the binding of TFP.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to N.M.), the Ryoichi Naito Foundation for Medical Research (to N.H.), the Tokai Science Foundation (to N.H.), the Sasakawa Scientific Research Grant (to N.H.) and the Aichi Cancer Research Foundation (to N.H.) and by a Grant-in-aid for Fujita Health University High-tech Research Center from the Ministry of Education, Science, Sports and Culture of Japan. SAXS measurements were performed with the approval of the Photon Factory Advisory Committee (proposal no. 98G202).

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