Evidence for calmodulin inter-domain compaction in solution induced by W-7 binding

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Abstract Small-angle X-ray scattering and nuclear magnetic resonance were used to investigate the structural change of calcium-bound calmodulin (Ca^{2+}/CaM) in solution upon binding to its antagonist, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7). The radius of gyration was 17.4 ± 0.3 Å for Ca^{2+}/CaM-W-7 with a molar ratio of 1:5 and 20.3 ± 0.7 Å for Ca^{2+}/CaM. Comparison of the radius of gyration and the pair distance distribution function of the Ca^{2+}/CaM-W-7 complex with those of other complexes indicates that binding of two W-7 molecules induces a globular shape for Ca^{2+}/CaM, probably caused by an inter-domain compaction. The results suggest a tendency for Ca^{2+}/CaM to form a globular structure in solution, which is inducible by a small compound like W-7.

Key words: Calmodulin-W-7 complex; Small-angle X-ray scattering; Conformational change; Globular structure

1. Introduction

Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein of 148 residues that regulates a variety of physiological processes in a Ca^{2+}-dependent manner [1]. The regulation is achieved through the interaction of Ca^{2+}-bound CaM (Ca^{2+}/CaM) with a large number of target enzymes [2–4]. The Ca^{2+}/CaM molecule adopts an 'elongated' structure in which the two globular domains are connected by a highly flexible linker [5–11], while the structures of Ca^{2+}/CaM complexed with a peptide from target enzymes assume a compact globular shape caused by the bending of the domain linker [12–14]. These structural studies suggest that the flexibility of the domain linker plays an important role in the target recognition.

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Abbreviations: CaM, calmodulin; Ca^{2+}/CaM, calcium-bound CaM; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; SAXS, small-angle X-ray scattering; NMR, nuclear magnetic resonance; TFP, trifluoperazine, 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10*H*-phenothiazine; DMSO, dimethyl sulfoxide; R_g, radius of gyration; $p(r)$, pair distance distribution function; HSQC, heteronuclear single quantum coherence; CT-HSQC, constant time HSQC; NOE, nuclear Overhauser enhancement; MLCK, myosin light chain kinase; CaM-KII, Ca^{2+}/CaM-dependent protein kinase II

CaM antagonists such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10*H*-phenothiazine (trifluoperazine, TFP) have been used extensively to study Ca^{2+}/CaM-dependent activation of various enzymes (Fig. 1). X-ray crystal structure analysis revealed that Ca^{2+}/CaM-TFP complexes (CaM:TFP = 1:1 and 1:4) adopt a globular structure similar to that of Ca^{2+}/CaM-target peptide complexes [12–16]. NMR analyses demonstrated that two antagonist molecules bind to one Ca^{2+}/CaM molecule with high affinity [17,18]. However, the lack of NOEs between the two domains precluded the determination of their relative orientation [18]. In order to investigate whether the binding of W-7 induces a globular structure similar to the Ca^{2+}/CaM-TFP complex, we applied small-angle X-ray scattering (SAXS) as well as NMR spectroscopy. The results provide evidence that a small organic compound such as W-7 can induce inter-domain compaction of Ca^{2+}/CaM even in solution.

2. Materials and methods

2.1. Sample preparation

W-7 was synthesized in bulk in the previous study and was carefully stored in our laboratory [19]. Uniformly $^{13}C/^{15}N$-labeled or non-labeled recombinant *Xenopus laevis* CaM was expressed in *Escherichia coli* and purified to homogeneity as previously described [20]. For SAXS experiments, non-labeled CaM was dissolved in PIPES buffer (50 mM PIPES-NaOH, pH 6.5), followed by dialysis against the buffer containing 10 mM $CaCl_2$. The protein concentration was examined by the method of Bradford [21]. The W-7 powder was dissolved in dimethyl sulfoxide (DMSO) and added to the CaM solution. The final concentration of DMSO was set to 1% (v/v) for all samples. The solutions for Ca^{2+}/CaM and Ca^{2+}/CaM complexed with five equivalents of W-7 were prepared at protein concentrations of 6.0, 9.0, 12.0, and 16.2 mg/ml. Moreover, solutions for the Ca^{2+}/CaM-W-7 mixtures with molar ratios of 1:1, 1:2, 1:3, and 1:4 were each prepared at a protein concentration of 9.0 mg/ml. For NMR experiments, $^{13}C/^{15}N$-labeled CaM was dissolved in unbuffered 0.4 ml 95% H_2O/5% D_2O or 99.99% D_2O solution containing 0.1 M KCl and 10.6 mM $CaCl_2$. The pH/pD values of the samples were 6.8 without consideration of isotope effects. The protein concentration was 1.5 mM.

2.2. Small-angle X-ray scattering

The measurements were performed using synchrotron orbital radiation with an instrument for SAXS installed at BL-10C of Photon Factory, Tsukuba [22]. An X-ray wavelength of 1.488 Å was selected. The samples were contained in a quartz cell with a volume of 80 μl, and the temperature was maintained at 35 ± 0.1°C by circulating water

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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 600 s at individual protein concentrations and for 1800 s at only 6.0 mg/ml.

Two methods of data analysis were used. The first method is that of Guinier [23] which gives the radius of gyration, R_g. The range of Q ($Å^{-1}$) used for Guinier plots was 3.44×10^{-2} to 6.88×10^{-2}. The second method is 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 [24]. The $p(r)$ is calculated by a direct Fourier transformation [24]. Data to Q ($Å^{-1}$) = 0.7 were used for $p(r)$ analysis. The maximal pair distance, d_{max}, was also estimated from the $p(r)$ function; $p(r)$ becomes zero at values of r equal to or greater than the maximum d_{max} of the particle. Furthermore, R_g and $p(r)$ were calculated from atomic coordinates of the Ca^{2+}/CaM-TFP complexes in order to compare the X-ray values for the Ca^{2+}/CaM-W-7 complex. Details of the calculation method are given elsewhere [25,26].

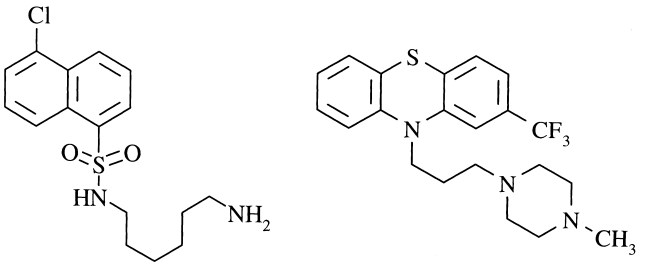
2.3. NMR spectroscopy

All of the NMR spectra were measured at 35°C on a Bruker AMX-600 spectrometer. W-7 was titrated in aliquots of 0.33 protein equivalent into a uniformly $^{13}C/^{15}N$-labeled sample of the protein. After the addition of each aliquot of W-7, 1D ^{1}H, 2D ^{15}N-^{1}H HSQC [27,28], and 2D ^{13}C-^{1}H CT-HSQC [29] spectra were acquired. Finally, spectra with 0, 0.33, 0.66, 1.0, 1.33, 1.66, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, and 6.0 equivalents of W-7 to CaM were recorded. Spectral changes for 2D ^{13}C-^{1}H CT-HSQC were previously reported [18].

3. Results

3.1. The radius of gyration and the p(r) function

Fig. 2 shows the Guinier plots for Ca^{2+}/CaM alone and Ca^{2+}/CaM in the presence of W-7 with the molar ratio of 1:5 at four protein concentrations. R_g as a function of protein is shown in Fig. 3. R_g values of the Ca^{2+}/CaM-W-7 complex and Ca^{2+}/CaM at zero concentration are given in Table 1. For comparison, Table 1 also contains R_g values for other Ca^{2+}/CaM complexes reported by other researchers. The R_g value for Ca^{2+}/CaM-W-7 complex (17.4 ± 0.3 Å) is comparable to the calculated R_g from atomic coordinates of Ca^{2+}/CaM-TFP complex (1:4) which is smaller due to the lack of atomic coordinates for the first two residues [16]. It is also consistent or comparable to R_g values of other Ca^{2+}/CaM complexes containing mastoparan [25,30], melittin [31], cyclosporin-A [32], substance P [33] and respective synthetic peptide corresponding to the calmodulin-binding domains of MLCK (M13) [34], phosphorylase kinase (RhK5) [35] and Ca^{2+} pump (C24W) [36]. The R_g value for Ca^{2+}/CaM (20.3 ± 0.7 Å) is also comparable to the values reported previously [25,31–36]. Fig. 4 shows R_g values as a function of molar ratio of Ca^{2+}/CaM and W-7 at 9.0 mg/ml. A drastic decrease in the R_g upon W-7 binding to Ca^{2+}/CaM finishes at the ratio of 1:2.



(a) W-7 (b) TFP

Fig. 1. Chemical structures of W-7 and TFP.

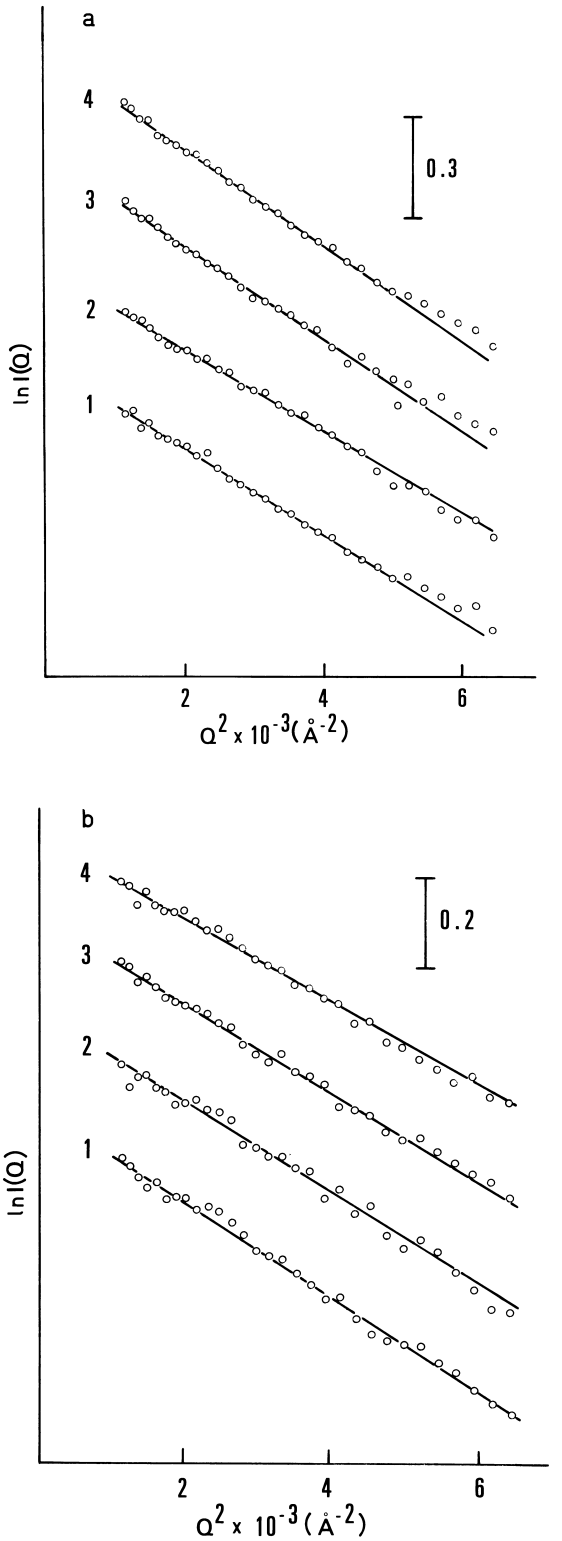


Fig. 2. Guinier plots for Ca^{2+}/CaM-W-7 complex (Ca^{2+}/CaM:W-7 = 1:5) and Ca^{2+}/CaM at various protein concentrations. a: Ca^{2+}/CaM-W-7 complex. b: Ca^{2+}/CaM. 1, 6.0 mg/ml; 2, 9.0 mg/ml; 3, 12.0 mg/ml; 4, 16.2 mg/ml.

Fig. 5 shows the $p(r)$ functions for Ca^{2+}/CaM alone and Ca^{2+}/CaM in the presence of W-7. The $p(r)$ function for the

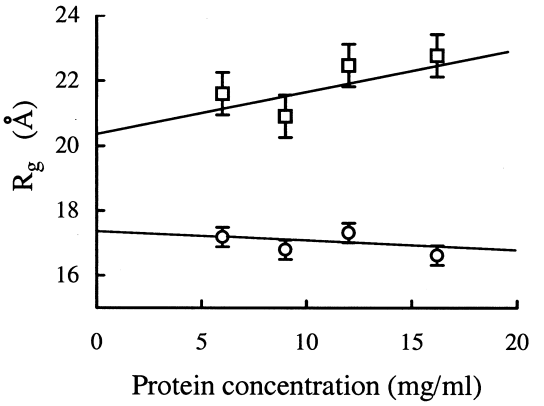


Fig. 3. The radius of gyration, R_g, for Ca^{2+}/CaM-W-7 complex (Ca^{2+}/CaM:W-7 = 1:5) and Ca^{2+}/CaM as a function of the protein concentration. ○, Ca^{2+}/CaM-W-7 complex; □, Ca^{2+}/CaM.

Ca^{2+}/CaM alone has a peak near 20 Å (principally representing interatomic distances within each domain of Ca^{2+}/CaM) and a shoulder at near 40 Å (mainly representing inter-domain distances) [7,25]. By contrast, the shoulder near 40 Å disappears for the Ca^{2+}/CaM-W-7 complex. Its d_{max} is about 14 Å smaller than that for the Ca^{2+}/CaM alone. These characteristic behaviors are also seen in the $p(r)$ function calculated from the atomic coordinates of the crystal structure of Ca^{2+}/CaM-TFP complex [15,16] and from the SAXS profiles of other complexes including Ca^{2+}/CaM-M13 complex [25,31,32,34–36]. The determination of the three-dimensional structure indicated that Ca^{2+}/CaM-TFP and -M13 complexes are in a globular form. Thus, the present data indicate that Ca^{2+}/CaM complexed with W-7 adopts a globular structure similar to those of other complexes.

3.2. NMR spectral changes

The NMR spectral changes in the ^{15}N-^{1}H HSQC spectra of uniformly $^{13}C/^{15}N$-labeled CaM were monitored upon addition of unlabeled W-7. Fig. 6 shows the selected portions of the spectra for Ca^{2+}/CaM:W-7 molar ratios from 1:0 to 1:3. Most of the HSQC peaks in each of the two CaM domains (Ala-1 to Lys-75, Glu-82 to Lys-148) were gradually shifted with little change in their intensities, indicating that the conformational exchange rate between W-7 bound state and unbound state is fast on the NMR time scale. In contrast, the signals from Met-76, Asp-78, Thr-79, Asp-80, Ser-81 and Glu-82 in the domain linker are broadened upon addition of W-7. This broadening indicates that the conformational exchange rate of the linker is slower than that in each domain.

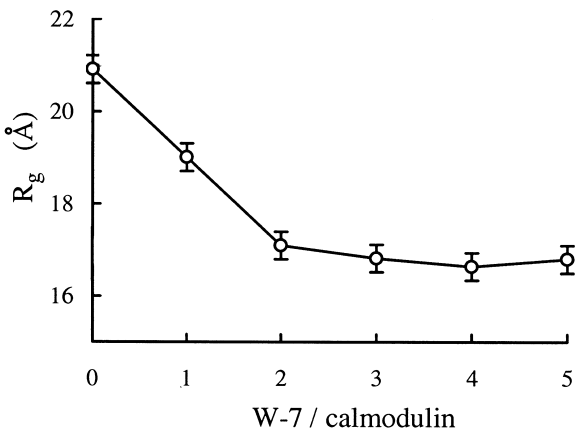


Fig. 4. The radius of gyration, R_g, as a function of the molar ratio of W-7 to Ca^{2+}/CaM at the protein concentration of 9.0 mg/ml.

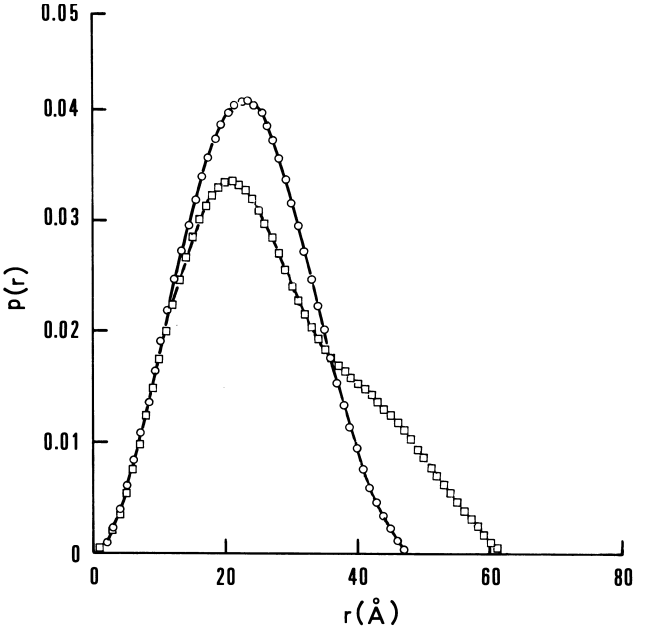


Fig. 5. Pair distance distribution function, $p(r)$, for Ca^{2+}/CaM-W-7 complex (Ca^{2+}/CaM:W-7 = 1:5) and Ca^{2+}/CaM. ○, Ca^{2+}/CaM-W-7 complex; □, Ca^{2+}/CaM.

4. Discussion

4.1. Globular structure of Ca^{2+}/CaM-W-7 complex

The SAXS analysis shows that the binding of two W-7 molecules induces drastic structural change in Ca^{2+}/CaM; the overall shape changes from an elongated structure to a compact globular structure in solution. Our previous NMR analysis [18] showed that the backbone conformation in each CaM domain remains essentially unchanged upon binding of

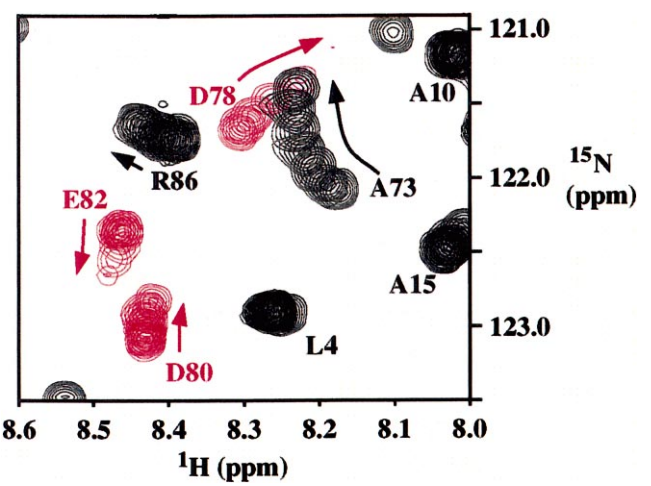


Fig. 6. NMR spectral changes for Ca^{2+}/CaM amide groups upon W-7 binding. Spectra of the same region, which are for Ca^{2+}/CaM-W-7 = 1:0, 1:0.33, 1:0.66, 1:1, 1:2, 1:3, were superimposed. NMR signals in each CaM domain and in the domain linker are drawn in black and red, respectively.

Table 1
Radius of gyration R_g and maximum dimension d_{max} for Ca^{2+}/CaM and its complexes

	R_g [Å]	d_{max} [Å]
Ca^{2+}/CaM[a]	20.3 ± 0.7	61
Ca^{2+}/CaM-W-7[a]	17.4 ± 0.3	47
Ca^{2+}/CaM[a] [31]	20.17 ± 0.16	62.5
Ca^{2+}/CaM[a] [25]	21.5 ± 0.3	69
Ca^{2+}/CaM[a] [36]	21.36 ± 0.10	62.5 ± 2.5
Ca^{2+}/CaM-TFP[b] [16]	15.92	46.0
Ca^{2+}/CaM-M13[a] [34]	16.4 ± 0.2	49
Ca^{2+}/CaM-RhK5[a] [35]	17.3 ± 0.2	49
Ca^{2+}/CaM-C24W[a] [36]	17.2 ± 0.3	52.5 ± 2.5
Ca^{2+}/CaM-mastoparan[a] [25]	17.8 ± 0.3	55
Ca^{2+}/CaM-melittin[a] [31]	17.85 ± 0.13	47.5
Ca^{2+}/CaM-substance P[a] [33]	17.2 ± 0.3	–

[a]Values at zero protein concentration obtained by SAXS experiment.
[b]Values calculated from the atomic coordinates of the crystal structure.

W-7. However, line broadening of NMR signals was observed for the residues of the domain linker (Met-76, Asp-78, Thr-79, Asp-80, Ser-81 and Glu-82) upon W-7 binding. Thus, it is likely that the globular structure is caused by bending of the flexible linker. A similar bending was observed in the crystal structure of Ca^{2+}/CaM-TFP complex [15,16], and in both solution and crystal structures of Ca^{2+}/CaM complexed with a peptide from skeletal muscle and smooth muscle MLCK and brain CaM-KII [12–14]. The previous NMR study [18] showed no inter-domain NOE in Ca^{2+}/CaM complexed with W-7, suggesting that the relative orientation of the two domains is not always fixed due to a conformational change between various orientations. However, the SAXS data indicate that the time- and spatially averaged shape of the Ca^{2+}/CaM-W-7 complex represents a compact, globular one. The lack of NOE is consistent with the notion that the inter-domain interaction is unstable and involves a rapid exchange between the associated and dissociated states. Evidence for the inter-domain interaction of CaM has been obtained by proteolytic footprinting studies [37], in which a conformational state with a short life-time could be trapped.

The NMR structure of Ca^{2+}/CaM-W-7 complex suggested that W-7 inhibits the CaM-mediated activation of target proteins by blocking the hydrophobic pocket [18]. The present results show that binding of two W-7 molecules induces compaction between the two domains of CaM. In addition to the direct interaction of W-7 with the hydrophobic pocket of Ca^{2+}/CaM, the induced globular structure of Ca^{2+}/CaM might also contribute to inhibition of activity, since the CaM binding region of the target enzyme becomes less accessible to Ca^{2+}/CaM in the compact conformation.

4.2. Comparison with globular structure of Ca^{2+}/CaM-TFP complex

An inter-domain compaction of Ca^{2+}/CaM has been observed previously in the crystal structures of the Ca^{2+}/CaM-TFP complex [15,16]. There are differences in the TFP binding stoichiometry between the two structures (CaM:TFP = 1:1 and 1:4), probably due to differences in the crystallization conditions. On the other hand, the NMR signals of both CaM domains moved by similar amounts during the addition of the first two equivalents of TFP, suggesting that the binding affinities of either CaM domain to TFP is indistinguishable in solution [17]. Thus, it has been unclear yet how many TFP molecules induce an inter-domain compaction of CaM in solution. However, both crystal structures show a similar CaM conformation induced by TFP binding (backbone superposition of the two structures using CaM residues 9–73 and 84–145 gives a RMS deviation value of 0.39 Å), prompting us to use both structures for comparison.

Although the chemical structure of W-7 differs from that of TFP, both contain a hydrophobic aromatic group with positively charged group via the aliphatic chain (Fig. 1). In the Ca^{2+}/CaM-W-7 complex, the naphthalene ring of the two W-7 molecules interact intimately with the hydrophobic pocket of the two CaM domains [18], whereas only one end of TFP phenothiazine ring is inserted into the pocket of Ca^{2+}/CaM-TFP complex [15,16]. This hydrophobic pocket can accommodate van der Waals contacts with such chemically different groups. This high adaptability of the CaM hydrophobic pocket originates from highly abundant methionine residues with a flexible and polarizable side chain [18].

It has been suggested that positively charged nitrogen atoms of the TFP piperazine group participate in electrostatic interactions with negatively charged residues of Ca^{2+}/CaM such as Glu-127, reducing an electrostatic repulsion between both domains [16]. Similarly, electrostatic interactions between the positively charged nitrogen atom of the W-7 aminohexyl group and negatively charged residues within Ca^{2+}/CaM may contribute to the stabilization of the globular structure in the complex. Most probable partners in CaM include Glu-14 and Glu-54 in the N-terminal domain and Glu-87, Glu-114, and Glu-127 in the C-terminal domain.

The methylene groups of W-7 in the Ca^{2+}/CaM-W-7 complex were suggested to be unimportant in the specific interactions with Ca^{2+}/CaM [18]. However, it is noted that the number of the methylene groups in W-7 derivatives is proportional to their binding affinity to Ca^{2+}/CaM [38]. In the structure of Ca^{2+}/CaM-TFP complex, the methylene groups of TFP make contacts with the hydrophobic side chains of Ca^{2+}/CaM around the hydrophobic pockets. Similarly, the methylene groups of W-7 might contribute to the W-7 binding to Ca^{2+}/CaM by van der Waals interactions.

4.3. Comparison with Ca^{2+}/CaM-target peptide complex

The globular structures of Ca^{2+}/CaM in complex with its target peptide from skeletal/smooth muscle MLCK or brain CaM-KII are stabilized by extensive van der Waals interac-

tions in addition to electrostatic interactions, where the target peptide forming α-helix binds to both the CaM domains simultaneously [12–14]. In contrast, a similar globular structure of Ca^{2+}/CaM is induced by the main interaction of W-7 with the hydrophobic pocket of each CaM domain, though the relative orientation of the two domains is not always fixed. This is reflected in the binding affinity of W-7 to Ca^{2+}/CaM, which is about 10^3 times lower than the binding affinity of the target peptides [38–42]. Thus it is indicated that such weaker interactions as those of W-7 can induce the globular structure of Ca^{2+}/CaM in solution and, therefore, the bridging of both domains by a polypeptide chain of the target molecule is not necessary for the formation of the globular structure.

4.4. Conclusion

The present SAXS results indicate that the binding of small organic compound W-7 to Ca^{2+}/CaM induces a globular structure in solution, which is suggested to be caused by the bending of the flexible domain linker of Ca^{2+}/CaM. In contrast to the fixed orientation of the two CaM domains bound to a target peptide, the relative orientation of the CaM domains is flexible in the Ca^{2+}/CaM-W-7 complex, though the time-averaged shape remains globular and compact. These results indicate that the dynamics and relative orientation of two CaM domains can vary significantly in solution upon binding to various target molecules including antagonists such as W-7.

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