Chapter IV

"Evidence for Calmodulin Inter-domain Compaction in Solution Induced by W-7 Binding"

IV-1 Summary

Small-angle X-ray scattering and nuclear magnetic resonance were used to investigate the structural change of Ca^{2+}/CaM in solution upon binding to its antagonist, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7). The radius of gyration was 20.3 ± 0.7 Å for Ca^{2+}/CaM and 17.4 ± 0.3 Å for Ca^{2+}/CaM–W-7 with a molar ratio of 1:5. 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 of Ca^{2+}/CaM to form a globular structure in solution, inducible by a small compound like W-7.

IV-2 Introduction

In solution, Ca^{2+}/CaM adopts an 'elongated' structure in which the two globular domains are connected by a highly flexible linker (Barbato *et al.*, 1992; Finn *et al.*, 1995; Heidorn *et al.*, 1989; Kretsinger *et al.*, 1986; Persechini & Kretsinger, 1988; Seaton *et al.*, 1985; Spoel *et al.*, 1996), while the structures of Ca^{2+}/CaM complexed with a peptide from target enzymes is in a compact globular shape caused by bending of the domain linker (Ikura *et al.*, 1992; Meador *et al.*, 1992; Meador *et al.*, 1993). The conformational change from an 'elongated' form to a globular structure, which is due to the high flexibility of the domain linkers, is important for the high affinity (K_d = ~10^{-9} M) with target peptides.

Such global structural change was observed for the crystal structures of the Ca^{2+}/CaM–TFP complexes (CaM:TFP = 1:1 and 1:4), in which CaM adopt a globular structure similar to that of Ca^{2+}/CaM-target peptide complexes (Figures I-5 and I-6, Cook *et al.*, 1994; Ikura *et al.*, 1992; Meador *et al.*, 1992; Meador *et al.*, 1993; Vandonselaar *et al.*, 1994). NMR analyses demonstrated that two antagonist molecules bind to one Ca^{2+}/CaM molecule with high affinity (K_d = ~10^{-6} M, Craven *et al.*, 1996; Osawa *et al.*, 1998), although additional binding with lower affinity was observed. However, the lack of NOEs between the two domains precluded the determination of their relative orientation (Osawa *et al.*, 1998). In order to investigate whether the binding of W-7 induces a globular structure similar to the Ca^{2+}/CaM–TFP complex, small-angle X-ray scattering (SAXS) as well as NMR spectroscopy was applied. 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.

IV-3 Materials and Methods

IV-3-1 Sample Preparation

W-7 was synthesized in bulk in the previous study and has been carefully stored in Hidaka's laboratory (Hidaka *et al.*, 1978). 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 (Ikura *et al.*, 1990b). 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 (Bradford, 1976). 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, the solutions for 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.

IV-3-2 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 (Ueki *et al.*, 1982/1983). An X-ray wavelength of 1.488 Å was selected. The samples were contained in a quartz cell with a volume of 80 µl in volume, and the temperature was maintained at 35 ± 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 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 (Guinier, 1939) which gives the radius of gyration, R_g. The range of Q used for Guinier plots was 3.44×10^{-2} to 6.88×10^{-2} Å^{-1}. 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 (Glatter, 1982). The *p(r)* is calculated by a direct Fourier transformation (Glatter, 1982). Data up to Q = 0.7 Å^{-1} 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 (Matsushima *et al.*, 1998; Matsushima *et al.*, 1989).

IV-3-3 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 (Kay *et al.*, 1992; Palmer III *et al.*, 1991), and 2D ^{13}C-^{1}H CT-HSQC (Vuister & Bax, 1992) 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 (Osawa *et al.*, 1998).

IV-4 Results

IV-4-1 The Radius of Gyration and the Distance Distribution Function

Figure IV-1 shows the Guinier plots for Ca^{2+}/CaM alone and Ca^{2+}/CaM in the presence of W-7 with a molar ratio of 1:5 at four protein concentrations. The radius of gyration, R_g, as a function of protein is shown in Figure IV-2. R_g values of the Ca^{2+}/CaM–W-7 complex and Ca^{2+}/CaM at zero concentration are given in Table IV-1. For comparison, Table IV-1 also contains R_g values for other Ca^{2+}/CaM complexes reported in other studies. The R_g value (17.4 ± 0.3 Å) for Ca^{2+}/CaM–W-7 complex is comparable to the calculated R_g from the 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 (Vandonselaar *et al.*, 1994). It is also consistent or comparable to R_g values of other Ca^{2+}/CaM complexes containing mastoparan (Matsushima *et al.*, 1989; Yoshino *et al.*, 1989), melittin (Kataoka *et al.*, 1989), cyclosporin A (Knott *et al.*, 1994), substance P (Yoshino *et al.*, 1993) and the synthetic peptide corresponding to the calmodulin-binding domains of MLCK (M13, Heidorn *et al.*, 1989), phosphorylase kinase (RhK5, Trewhella *et al.*, 1990) and Ca^{2+}-pump (C24W, Kataoka *et al.*, 1991). The R_g value for Ca^{2+}/CaM (20.3 ± 0.7Å) is also comparable to values reported previously (Heidorn *et al.*, 1989; Kataoka *et al.*, 1991; Kataoka *et al.*, 1989; Knott *et al.*, 1994; Matsushima *et al.*, 1989; Trewhella *et al.*, 1990; Yoshino *et al.*, 1989; Yoshino *et al.*, 1993). Figure IV-3 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 R_g upon W-7 binding to Ca^{2+}/CaM finishes at the ratio of 1:2.

Figure IV-4 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 Ca^{2+}/CaM alone has a peak near 20 Å (principally representing interatomic distances within each domain of Ca^{2+}/CaM) and a shoulder near 40 Å (mainly representing interdomain

distances, Matsushima *et al.*, 1989; Seaton *et al.*, 1985). In 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 (Cook *et al.*, 1994; Vandonselaar *et al.*, 1994) and from the SAXS profiles of other complexes including Ca^{2+}/CaM-M13 complex (Heidorn *et al.*, 1989; Kataoka *et al.*, 1991; Kataoka *et al.*, 1989; Knott *et al.*, 1994; Matsushima *et al.*, 1989; Trewhella *et al.*, 1990). 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 other complexes.

IV-4-2 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. Figure IV-5 shows the selected portions of the spectra for Ca^{2+}/CaM:W-7 molar ratio from 1:0 to 1:3. Most of the HSQC peaks in each of the two CaM domains (Ala1 to Lys75, Glu82 to Lys148) 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.

IV-5 Discussion

IV-5-1 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 in solution; the overall shape changes from an elongated structure to a compact globular structure. My previous NMR analysis (Osawa *et al.*, 1998) showed that the backbone conformation in each CaM domain remains essentially unchanged upon binding of 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 (Cook *et al.*, 1994; Vandonselaar *et al.*, 1994), and in both solution and crystal structures of Ca^{2+}/CaM complexed with a peptide from skeletal muscle and smooth muscle MLCK and brain CaMKII (Ikura *et al.*, 1992; Meador *et al.*, 1992; Meador *et al.*, 1993). The previous NMR study (Osawa *et al.*, 1998) 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 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 (Osawa *et al.*, 1998). The present results show that binding of two W-7 molecules induces the 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.

IV-5-2 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 structure of the Ca^{2+}/CaM-TFP complex (Figure I-6, Cook *et al.*, 1994; Vandonselaar *et al.*, 1994). Although the structure of W-7 differs from that of TFP, both contain a hydrophobic aromatic group with positively charged group via the aliphatic chain (Figure II-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 (Osawa *et al.*, 1998), whereas only one end of TFP phenothiazine ring is inserted into the pocket of the Ca^{2+}/CaM–TFP complex (Cook *et al.*, 1994; Vandonselaar *et al.*, 1994). This hydrophobic pocket can accommodate such chemically different groups through van der Waals interactions. This high adaptability originates from highly abundant methionine residues with the flexible and polarizable side-chain (Osawa *et al.*, 1998).

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 (Vandonselaar *et al.*, 1994). 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 (Osawa *et al.*, 1998). However, it is noted that the number of methylene groups in W-7 derivatives is proportional to their binding affinity to Ca^{2+}/CaM (Figure II-6, Tanaka *et al.*, 1982). 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.

IV-5-3 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 CaMKII are stabilized by extensive van der Waals interactions as well as electrostatic interactions, where the target peptide forming α-helix binds to both CaM domains simultaneously (Ikura *et al.*, 1992; Meador *et al.*, 1992; Meador *et al.*, 1993). 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 (Blumenthal *et al.*, 1985; Hidaka *et al.*, 1979; Levin & Weiss, 1977; Massom *et al.*, 1990). 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.

IV-6 Concluding Remarks

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 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, while 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.

a

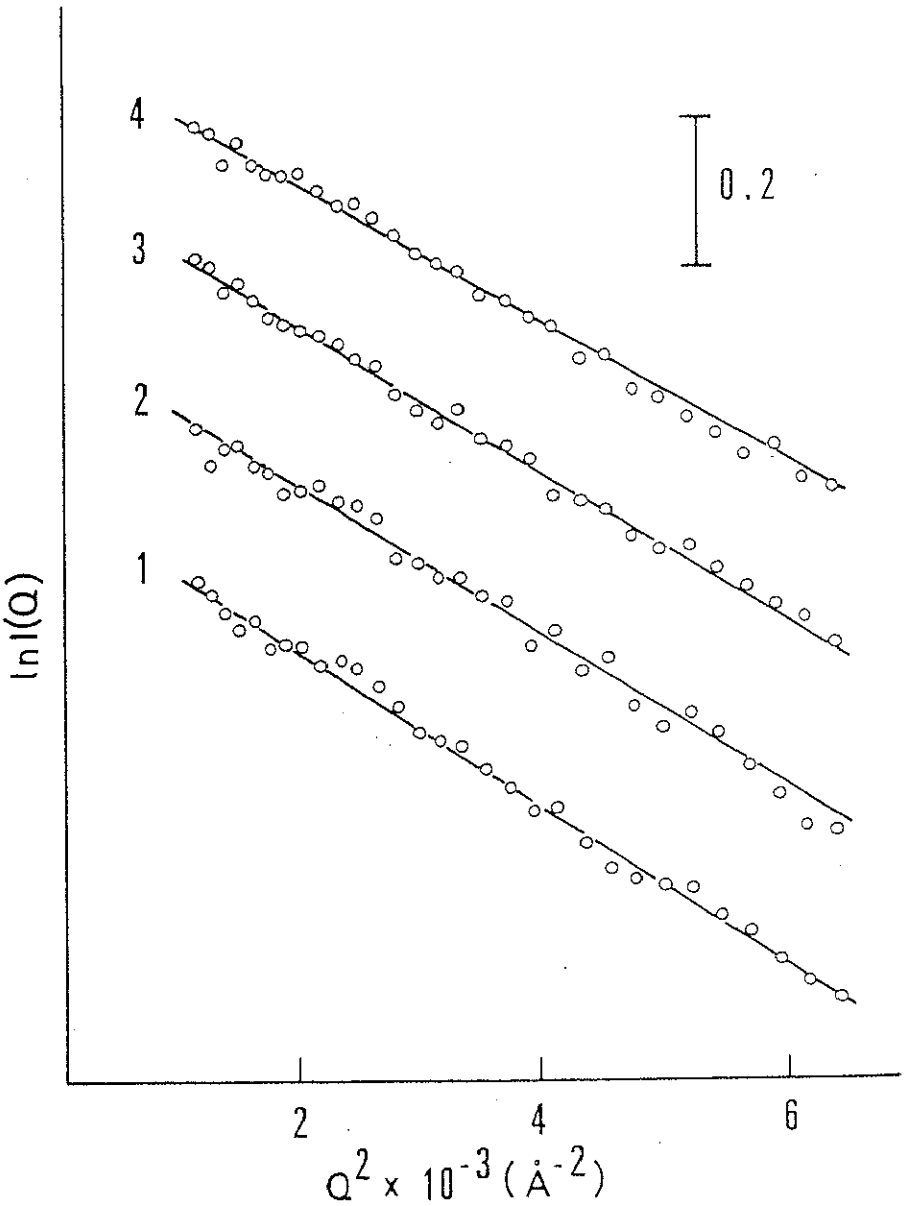


Figure IV-1. a, Guinier plots for the Ca^{2+}/CaM–W-7 complex (Ca^{2+}/CaM:W-7=1:5) at various protein concentrations.
1, 6.0 mg/ml; 2, 9.0 mg/ml; 3, 12.0 mg/ml; 4, 16.2 mg/ml.

b

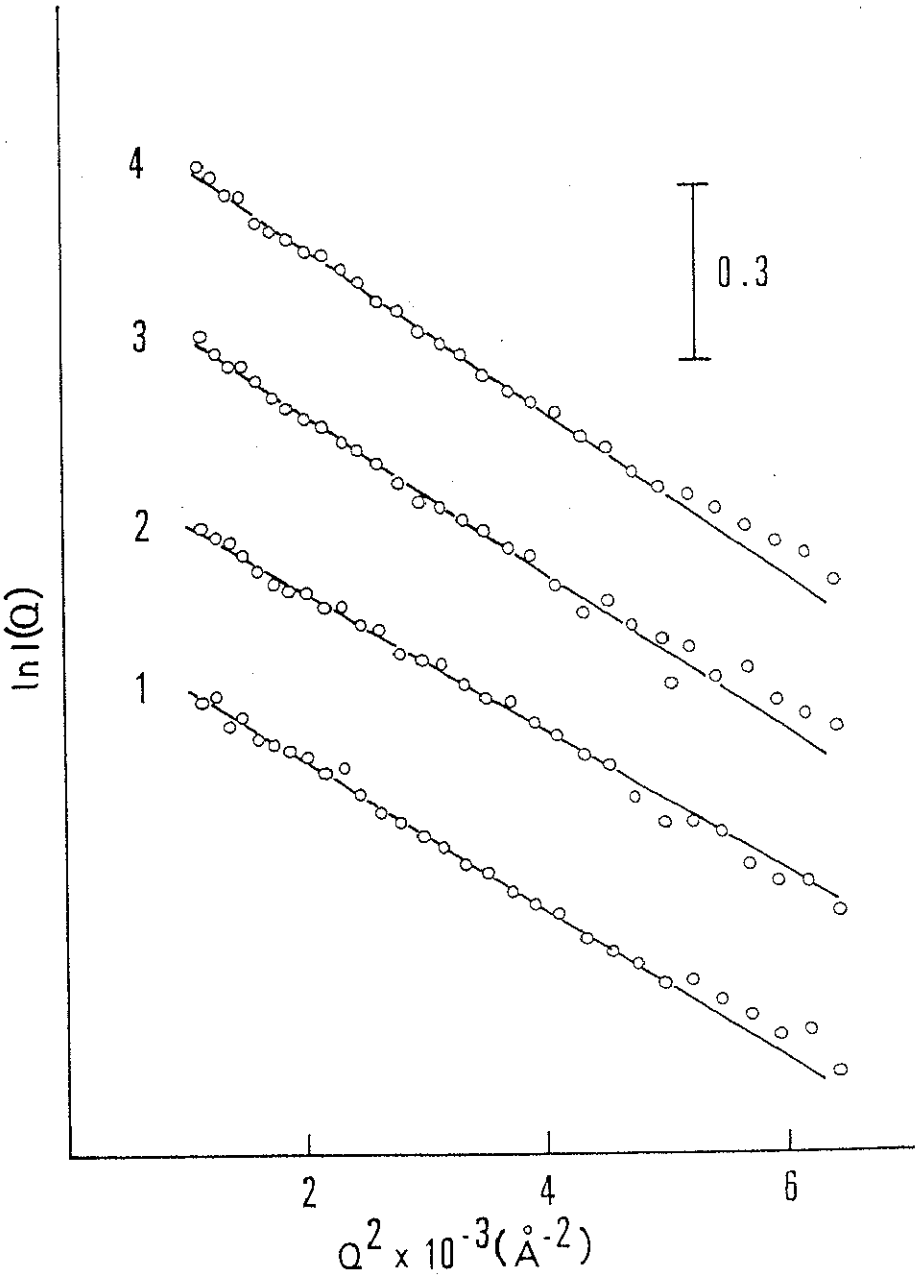


Figure IV-1. b, Guinier plots for the Ca^{2+}/CaM at various protein concentrations.

1, 6.0 mg/ml; 2, 9.0 mg/ml; 3, 12.0 mg/ml; 4, 16.2 mg/ml.

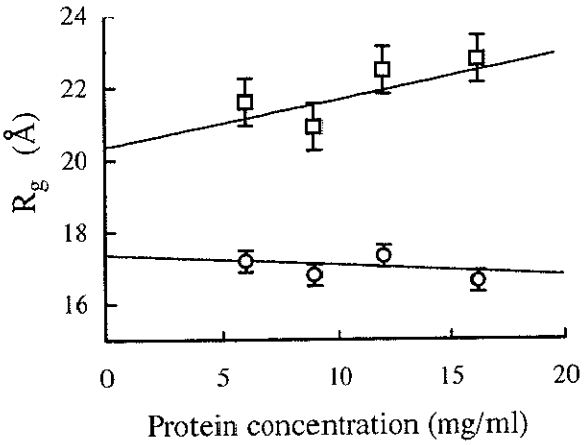


Figure IV-2. The radius of gyration, R_g, for Ca^{2+}/CaM–W-7 complex (Ca^{2+}/CaM:W-7=1:5) and Ca^{2+}/CaM as a function of the protein concentration.

○, Ca^{2+}/CaM–W-7 complex; □, Ca^{2+}/CaM.

Table IV-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] (Kataoka *et al.*, 1989)	20.17±0.16	62.5
Ca^{2+}/CaM[a] (Matsushima *et al.*, 1989)	21.5 ± 0.3	69
Ca^{2+}/CaM[a] (Kataoka *et al.*, 1991)	21.36±0.10	62.5 ± 2.5
Ca^{2+}/CaM–TFP[b] (Vandonselaar *et al.*, 1994)	15.92	46.0
Ca^{2+}/CaM–M13[a] (Heidorn *et al.*, 1989)	16.4 ± 0.2	49
Ca^{2+}/CaM–RhK5[a] (Trewhella *et al.*, 1990)	17.3 ± 0.2	49
Ca^{2+}/CaM–C24W[a] (Kataoka *et al.*, 1991)	17.2 ± 0.3	52.5 ± 2.5
Ca^{2+}/CaM–mastoparan[a] (Matsushima *et al.*, 1989)	17.8 ± 0.3	55
Ca^{2+}/CaM–melittin[a] (Kataoka *et al.*, 1989)	17.85±0.13	47.5
Ca^{2+}/CaM–substance P[a] (Yoshino *et al.*, 1993)	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.

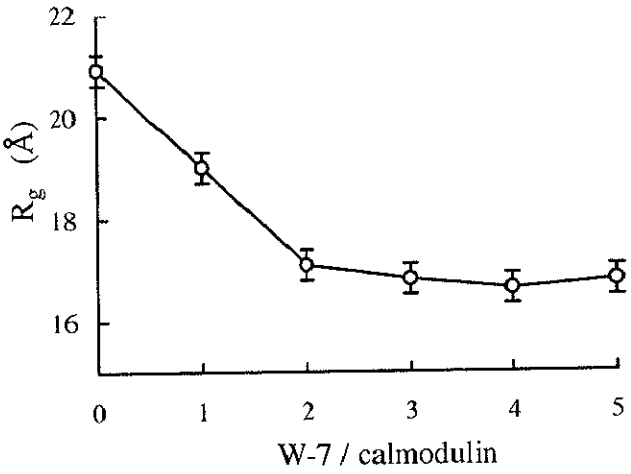


Figure IV-3. The radius of gyration, R_g, as a function of the molar ratio of W-7 to Ca^{2+}/CaM at the protein concentration of 9.0 mg/ml.

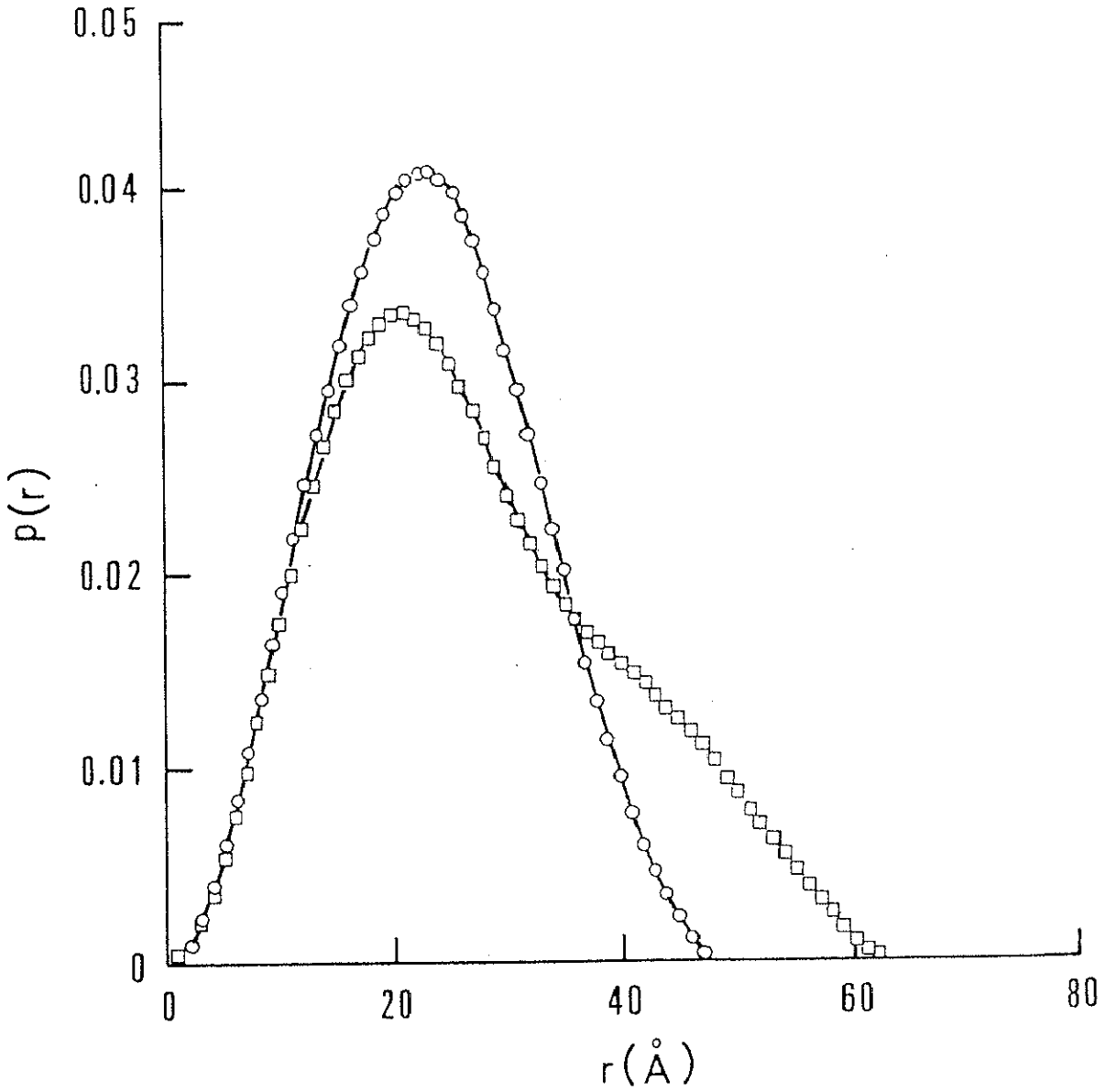


Figure IV-4. Pair distance distribution function, *p(r)*, for Ca^{2+}/CaM–W-7 complex (Ca^{2+}/CaM:W-7=1:5) and Ca^{2+}/CaM. ○, Ca^{2+}/CaM–W-7 complex; □, Ca^{2+}/CaM.

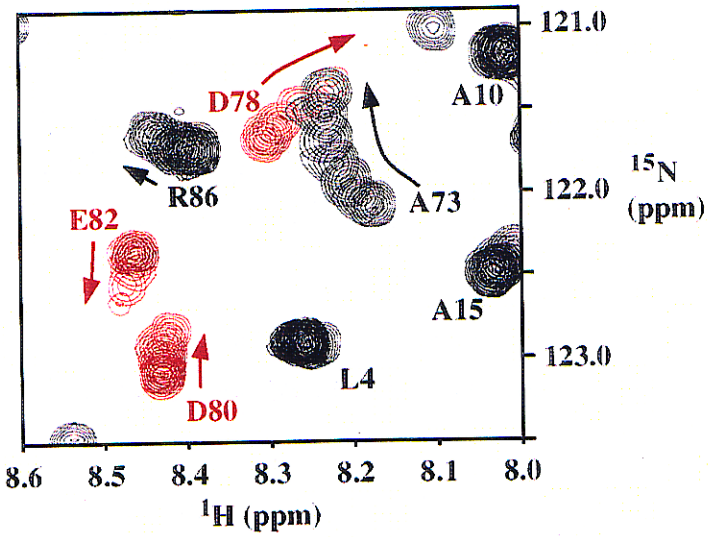
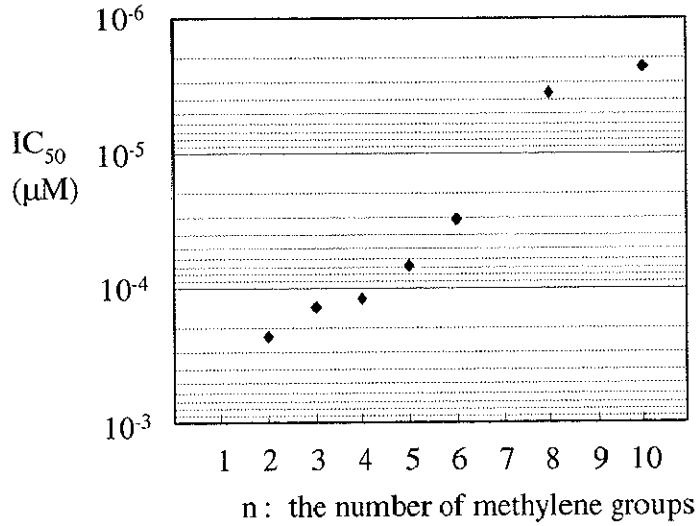


Figure IV–5. NMR spectral changes for Ca^{2+}/CaM amide groups upon W–7 binding. Spectra of the same region, each of which is for Ca^{2+}/CaM–W–7 = 1:0, 1:0.33, 1:0.66, 1:1, 1:2, 1:3 were superimposed. Signals from the residues in either domain and in the domain linker are shown in black and red, respectively.



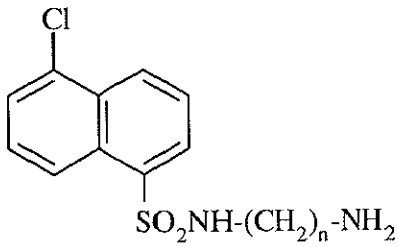


Figure IV-6 Correlation between the number of the methylene groups of the W-7 derivatives and the inhibition of ^{3}H-labeled W-7 to Ca^{2+}/CaM (Tanaka *et al.*, 1982).