Hydrogen Bonding in the Carboxyl-Terminal Half-Fragment 78–148 of Calmodulin As Studied by Two-Dimensional Nuclear Magnetic Resonance†

Mitsuhiko Ikura, Osamu Minowa, and Kunio Hikichi*

High-Resolution NMR Laboratory and Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060, Japan

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ABSTRACT: The C-terminal half-fragment (residues 78–148) of scallop testis calmodulin was investigated by 500-MHz two-dimensional proton NMR in order to clarify the structure and the structural change accompanying Ca^{2+} binding. The sequential resonance assignment to individual amino acid residues was made in part (27 out of 71 residues) by a combination of correlated spectroscopy and nuclear Overhauser effect spectroscopy of a 90% H_2O solution. In the Ca^{2+}-bound state, resonances of backbone amide protons of Gly-98, Gly-134, Ile-100, Asn-137, and Val-136 appear at extremely low fields. These findings suggest that amide protons of these residues are hydrogen bonded. In the Ca^{2+}-free state, the amide resonances of Ile-100 and Gly-134 disappear into the crowded normal shift region. This observation indicates that two hydrogen bonds of Ile-100 and Gly-134 are destroyed (or weakened) as Ca^{2+} ions are removed from two Ca^{2+}-binding sites. Chemical shifts of amide and α-protons of residues located in the Ca^{2+}-binding loop of domain III are similar to those of domain IV. These results suggest that the conformations of the two loops are very similar. The present results can be interpreted in terms of a structure predicted by Kretsinger [Kretsinger, R. H. (1980) *Ann. N.Y. Acad. Sci. 356*, 14].

Calmodulin (CaM)[1] is a member of a family of structurally related Ca^{2+}-binding proteins. It has four Ca^{2+}-binding domains named I, II, III, and IV from the N-terminus [for reviews, see Kretsinger (1980b) and Klee & Vanaman (1982)]. To date, information on the three-dimensional structure of the Ca^{2+}-binding protein family comes from only two proteins, carp muscle parvalbumin (Kretsinger & Nockolds, 1973; Moews & Kretsinger, 1975) and intestinal Ca^{2+}-binding protein, ICBP (Szebenyi et al., 1981). Kretsinger proposed predicted structures for CaM (Kretsinger, 1980a) and for troponin C (Kretsinger & Barry, 1975) on the basis of the parvalbumin crystal structure and the energy minimization calculation. Little experimental evidence has been reported for the predicted structure at atomic resolution.[2]

NMR is becoming the most powerful technique for studying protein tertiary structure in solution at the atomic level. ^1H NMR spectra of CaM have been observed by several groups (Seamon, 1980; Krebs & Carafoli, 1982; Ikura et al., 1983a,b). Our recent paper (Ikura et al., 1984) showed that Ca^{2+} binding to CaM induces the conformational transition in two steps; the first occurs mainly in the C-terminal half-region in a slow-exchange manner and the second in the N-terminal half-region in a fast-exchange manner. In addition, ^1H NMR of tryptic fragments of CaM indicated that the N-terminal half-fragment F12 (residues 1–75 containing domains I and II) and the C-terminal half-fragment F34 (residues 78–148 containing domains III and IV) maintain most of the tertiary structure observed in the intact protein (Ikura et al., 1984; Aulabaugh et al., 1984a; Dalgarno et al., 1984; Klevit et al., 1984; Thulin et al., 1984). The structural conservation of the fragments was also demonstrated by circular dichroism and fluorescence experiments (Drabikowski et al., 1982), ^{113}Cd NMR spectroscopy (Andersson et al., 1983), and Ca^{2+}-binding measurements (Minowa & Yagi, 1984).

In the present work, we performed two-dimensional (2D) ^1H NMR studies on the C-terminal half-fragment of CaM (F34) both in the Ca^{2+}-free and in the Ca^{2+}-bound states and assigned resonance lines to specific protons. A combination of COSY and NOESY (Wüthrich et al., 1982) was used to assign both backbone amide and α-protons of individual residues in the amino acid sequence. The problem of peak overlapping in 2D spectra was partly overcome by RCT (Eich et al., 1982) and DECSY (Ikura & Hikichi, 1984). A total of 27 amino acid residues out of 71 in the sequence were identified. The scheme of hydrogen bonding in loop areas of this fragment will be discussed in terms of an empirical rule of conformation-dependent secondary shifts of backbone amide and α-protons (Pardi et al., 1983; Wagner et al., 1983; Dalgarno et al., 1983b). The difference in structure between the Ca^{2+}-free and the Ca^{2+}-bound states will be disclosed.

Experimental Procedures

Scallop testis CaM and the tryptic fragment were prepared

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* Address correspondence to this author at the High-Resolution NMR Laboratory, Hokkaido University.

[1] Abbreviations: CaM, calmodulin; F12, calmodulin tryptic fragment (residues 1–75) containing calcium-binding domains I and II; F34, calmodulin tryptic fragment (residues 78–148) containing calcium-binding domains III and IV; ICBP, vitamin D dependent calcium-binding protein from bovine intestine; 2D, two dimensional; COSY, correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; RCT, relayed coherence transfer in COSY; DECSY, double-quantum coherence echo correlated spectroscopy; COCONOSY, combined COSY/NOESY experiment; Cl_3CCOOH, trichloroacetic acid; TSP, (trimethylsilyl)-propionic-d_4 acid; NOE, nuclear Overhauser enhancement; BPTI, basic pancreatic trypsin inhibitor from bovine organs; inhibitor E, trypsin inhibitor E from the venom of *Dendroaspis polylepis polylepis*; inhibitor K, trypsin inhibitor homologue K from the venom of *Dendroaspis polylepis polylepis*.

[2] The X-ray crystal structure of skeletal muscle troponin C at 2.8–3.0-Å resolution was recently reported independently by two groups (Herzberg & James, 1985; Sundaralingam et al., 1985). Both groups show that the predicted structure is mostly correct except for a long helical linker between the N-terminal half and the C-terminal half.

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as reported previously (Toda et al., 1981; Minowa & Yagi, 1984). The purity of CaM and F34 was checked by polyacrylamide gel electrophoresis in alkaline urea, high-performance liquid chromatography, and amino acid analysis. Removal of contaminating Ca^{2+} ion from CaM and F34 was achieved by Cl_3CCOOH precipitation. NMR measurements were made for 6 mM solutions in the following solvent systems: (1) D_2O–0.2 M KCl, pH 7.4 (all exchangeable protons were replaced with deuteriums at room temperature, and residual water protons were minimized by repeated lyophilization of D_2O solution); (2) H_2O–0.2 M KCl, pH 6.3. The pH values were adjusted by adding 4% KOD stock solution. The pH values are the direct meter readings without correction of isotope effects.

^1H NMR spectra were obtained at a frequency of 500 MHz on a JEOL GX-500 spectrometer. 2D COSY, NOESY, RCT, and DECSY spectra were obtained by pulse sequences described previously (Bax & Freeman, 1981; Kumar et al., 1980; Eich et al., 1982; Ikura & Hikichi, 1984). In most cases, COSY and NOESY spectra were simultaneously observed by the COCONOSY method (Haasnoot et al., 1984; Gurevich et al., 1984). The mixing time was 260–300 ms for NOESY and 30 ms for RCT. Quadrature detection was used in both the t_1 and t_2 dimensions. A 45° phase shifter was used for quadrature detection in the t_1 dimension of DECSY. A time domain data matrix of 256 × 2048 points was in most cases expanded to 1024 × 2048 (or 512 × 2048) points by "zero filling" in the t_1 dimension. Prior to Fourier transformation, the time domain data matrix was multiplied in both dimensions with a sine-bell window function. 2D spectra are shown in the absolute value representation. Chemical shifts are reported in parts per million from the internal standard TSP.

Results

The procedure for analyzing the ^1H NMR spectrum of F34 is divided into two steps: (1) complete analysis of amino acid spin systems by COSY, NOESY, RCT, and DECSY of a D_2O solution; (2) sequential individual resonance assignment of the polypeptide backbone protons by a combined use of COSY and NOESY of a H_2O solution (Wüthrich et al., 1982).

Analysis of Amino Acid Spin Systems. Nine kinds of amino acid residues were identified from their unique spin–spin coupling connectivities: Gly, Ala, Thr, Val, Leu, Ile, Phe, His, and Tyr. COSY was used for this purpose. NOESY was useful to identify the through-space connectivity between two self-closed spin systems such as aromatic ring protons and their α-methine and β-methylene protons. RCT was helpful (i) to distinguish spin systems of Thr from those of Ala on the ground of RCT cross-peaks between the α-proton and γ-methyl protons of Thr (King & Wright, 1983) and (ii) to assign Val and Ile spin systems through RCT cross-peaks between the α-proton and γ-methyl protons. DECSY was employed (i) to distinguish methine protons of Ala and Thr from others through "magnetic equivalence" cross-peaks and (ii) to assign aromatic protons of Phe, His, and Tyr through "direct connectivity" cross-peaks and additional "remote connectivity" cross-peaks. In the Ca^{2+}-free state, we assigned four glycines out of five, four alanines out of five, all five threonines, all five valines, three leucines out of five, all four isoleucines, all four phenylalanines, one histidine, and one tyrosine. The latter two are contained as a single amino acid, i.e., His-107 and Tyr-138. In the Ca^{2+}-bound state, one more glycine was identified while one leucine failed to be assigned. Since Asp, Asn, and Ser belong to the same spin system of so-called AMX type, distinction of these amino acids was difficult at this stage. Spectra for Glu, Gln, Pro, Met, Arg, and Lys having long side chains were too complex to be assigned completely.

Sequential Assignment. The second step of individual resonance assignments is to identify sequentially neighboring residues by through-space NOE connectivities in NOESY spectra. Information on the primary structure is useful for this purpose. The sequence of F34 (C-terminal region of scallop testis calmodulin) (Toda et al., 1981; M. Yazawa, private communication) is DTD^{80}SEEEIREAFR^{90}VFDKDGDGFI^{100}SAAELRHVMT^{110}NLGEK'LTDEE^{120}VDEMIREADI^{130}DGDGQVNYEE^{140}FVTMMTSK^{148}.[3] Two types of NOE connectivity were used for the analysis of F34. These are d_1 (from NH_{i+1} to $C^{\alpha}H_i$) and d_2 (from NH_{i+1} to NH_i) connectivities according to the notation used by Wüthrich and others. d_3 (from NH_{i+1} to $C^{\beta}H_i$) connectivity was also observed but was less useful because of severe peak overlapping in NOESY spectra. The following sequences were identified through d_1 connectivities: Val-Phe, Phe-Ile-AMX-Ala-Ala, Thr-AMX, xxx-Val-AMX-Tyr-xxx, and Val-Thr (AMX is any one of Asp, Asn, and Ser; xxx is any one of Glu, Gln, Pro, Met, Arg, and Lys). On the basis of the primary structure indicated above, these can be assigned to specific positions in the sequence as follows: Val^{91}-Phe^{92}, Phe^{99}-Ile-Ser-Ala-Ala^{103}, Thr^{117}-Asp^{108}, Gln^{135}-Val-Asn-Tyr-Gln^{139}, and Val^{142}-Thr^{143}. In a similar manner, the following sequences were identified through d_2 connectivities: Asp^{95}-Gly-Asp-Gly-Phe^{99}, Ala^{102}-Ala^{103}, and Gly^{132}-Asp-Gly-Gln^{135}. At present, 27 amino acid residues out of 71 were assigned in the Ca^{2+}-free state and/or in the Ca^{2+}-bound state. Although chemical shifts of individual α and amide protons are significantly perturbed upon Ca^{2+} binding, d_1 and/or d_2 connectivities are observed at similar positions in the two states Ca^{2+} free and Ca^{2+} bound. Complete sequential assignment could not be made yet because of severe overlapping of the 2D spectra. Figures 1 and 2 show fingerprint regions of COSY spectra of F34 in the Ca^{2+}-free state and in the Ca^{2+}-bound state, respectively. Assignments of individual cross-peaks are indicated. The chemical shifts of assigned resonances are listed in Table I.

Calcium Titration of Backbone Amide Protons. We reported (Ikura et al., 1984) that the conformational exchange between the Ca^{2+}-free and the Ca^{2+}-bound states of F34 ($\sim$5 s^{-1}) is slower than that of F12 ($\sim$500 s^{-1}) when side-chain protons such as aromatic protons and ring current shifted methyl protons are monitored. We obtained here additional evidence that backbone amide protons also exhibit the slow-exchange behavior. Two amide proton resonances at the lowest fields assignable to Gly-98 and Val-136 in the Ca^{2+}-free state decrease in intensity with increasing Ca^{2+} content in parallel with the increase of newly appearing amide proton resonances of Gly-98, Gly-134, Ile-100, and Asn-137 in the Ca^{2+}-bound state (data not shown). The results strongly suggest that the backbone conformation of the two predicted Ca^{2+}-binding loops of F34 varies slowly between the two states, because the amino acid residues mentioned above are located in the loops.

Discussion

Since CaM has no disulfide bridge, only noncovalent forces act to stabilize the native structure. They are (1) electrostatic interaction, (2) hydrogen bonding, (3) hydrophobic interaction, and (4) van der Walls interaction (Schultz & Schirmer, 1979). The occurrence of the hydrophobic interaction in CaM has been demonstrated by NOE effects between ring current

[3] The IUB–IUPAC one-letter symbol was used. K' is ϵ-trimethyllysine.

Table I: Chemical Shifts[a] (ppm) of Assigned Resonances of Calmodulin Fragment F34

residue	no.	NH	α		β		others			
Val	91	7.19	3.59		1.77		0.86[b]	0.41[b]		
		–	–		–		–	–		
Phe	92	7.32	4.50		3.48	2.82	7.29[c]	7.11[c]		
		–	–		–	–	–	–		
Asp	95	8.58	4.78		2.76	1.96				
		–	–		–	–				
Gly	96	8.08	3.99	3.83						
		7.84	3.92	3.83						
		(−0.29)	(−0.07)	(0.00)						
Asp	97	9.02	4.84		3.09	2.82				
		8.40	4.11		2.60	2.32				
		(−0.62)	(−0.73)		(−0.49)	(−0.50)				
Gly	98	9.98	3.90	3.12						
		10.70	4.03	3.46						
		(0.72)	(0.13)	(0.34)						
Phe	99	7.81	5.61		2.97	2.89	6.97[c]	7.35[c]		
		7.72	5.20		2.38	2.20	6.95	7.48		
		(−0.09)	(−0.41)		(−0.59)	(−0.69)	(−0.03)	(0.13)		
Ile	100	8.69	5.04		2.49		1.57[d]	1.20[d]	1.01[d]	0.71[d]
		10.22	4.93		1.96		1.20	0.47	1.02	0.31
		(1.53)	(−0.11)		(−0.53)		(−0.37)	(−0.73)	(0.01)	(−0.40)
Ser	101	8.95	4.89		4.38	4.02				
		9.07	4.89		4.48	4.05				
		(0.12)	(0.00)		(0.10)	(0.03)				
Ala	102	8.80	3.90		1.43					
		9.25	3.99		1.53					
		(0.45)	(0.09)		(0.10)					
Ala	103	8.25	4.07		1.41					
		8.30	4.10		1.48					
		(0.15)	(0.03)		(0.07)					
His	107	–	4.18		3.26	3.05	7.71[e]	6.81[e]		
		–	–		–	–	7.76	6.98		
Gly	113	–	–	–						
		7.93	4.28	3.78						
Leu	116	–	–		–	–	–	–	–	
		7.17	4.80		3.04	–	1.64[f]	0.87[f]	0.85[f]	
Thr	117	8.97	4.51		4.73		1.39[g]			
		9.20	4.52		4.79		1.39			
		(0.23)	(0.01)		(0.06)		(0.00)			
Asp	118	8.81	4.22		2.73	2.60				
		8.94	4.26		2.76	2.63				
		(0.13)	(0.04)		(0.03)	(0.03)				
Gly	132	–	–	–						
		7.66	4.02	3.86						
Asp	133	–	–		–	–				
		8.40	4.52		2.97	2.51				
Gly	134	–	–	–						
		10.37	4.07	3.47						
Gln	135	8.30	4.78		2.12	–	–	–		
		8.03	4.90		2.02	–	–	–		
		(−0.27)	(0.12)		(−0.10)					
Val	136	9.41	4.58		1.93		0.86[b]	0.84[b]		
		9.21	5.29		2.38		1.34	0.99		
		(−0.20)	(0.71)		(0.45)		(0.48)	(0.15)		
Asn	137	8.82	4.37		2.85	2.62				
		9.62	5.28		3.37	–				
		(0.80)	(0.91)		(0.52)					
Tyr	138	7.83	5.08		3.21	2.61	6.72[c]	6.67[c]		
		8.50	3.50		2.44	2.13	6.35	6.56		
		(0.68)	(−1.58)		(−0.77)	(−0.48)	(−0.37)	(−0.11)		
Glu	139	8.42	4.14		1.94	–	–	–		
		–	–		–	–	–	–		
Phe	141	–	–		–	–	–	–		
		8.99	4.06		3.43	3.27	6.98[c]	7.18[c]		
Val	142	8.17	3.60		2.39		1.18[b]	1.01[b]		
		8.61	3.21		1.97		0.82	0.61		
		(0.44)	(−0.39)		(−0.42)		(−0.36)	(−0.40)		
Thr	143	7.92	3.92		4.22		1.23[g]			
		7.76	3.82	4.28			1.27			
		(−0.16)	(−0.10)		(0.06)		(0.04)			

[a] The top row for each amino acid residue is for Ca^{2+}-free F34, and the middle row is for Ca^{2+}-bound F34 ([$CaCl_2$] = 15 mM). The bottom row (in parentheses) shows the difference in chemical shift value between the two: $\delta_{bound} - \delta_{free}$. Data for NH protons are obtained in 90% H_2O (pH 6.3), and those for α-CH and side-chain protons are obtained in D_2O (pH 7.4). Other experimental conditions were as follows: [F34] = 6 mM; [KCl] = 0.2 M; 30 °C. Chemical shifts are relative to internal TSP. [b] γ-Methyl protons and γ′-methyl protons. [c] Two δ-protons (left column) as well as two ε-protons (right column) are magnetically equivalent due to rapid flipping motion of the aromatic ring. [d] γ-Methylene proton, γ-methylene proton, γ′-methyl protons, and δ-methyl protons from left to right. [e] The left column is for the C2 ring proton and the right column for the C4 ring proton. [f] γ-Methine proton, δ-methyl protons, and δ′-methyl protons from left to right. [g] γ-Methyl protons.

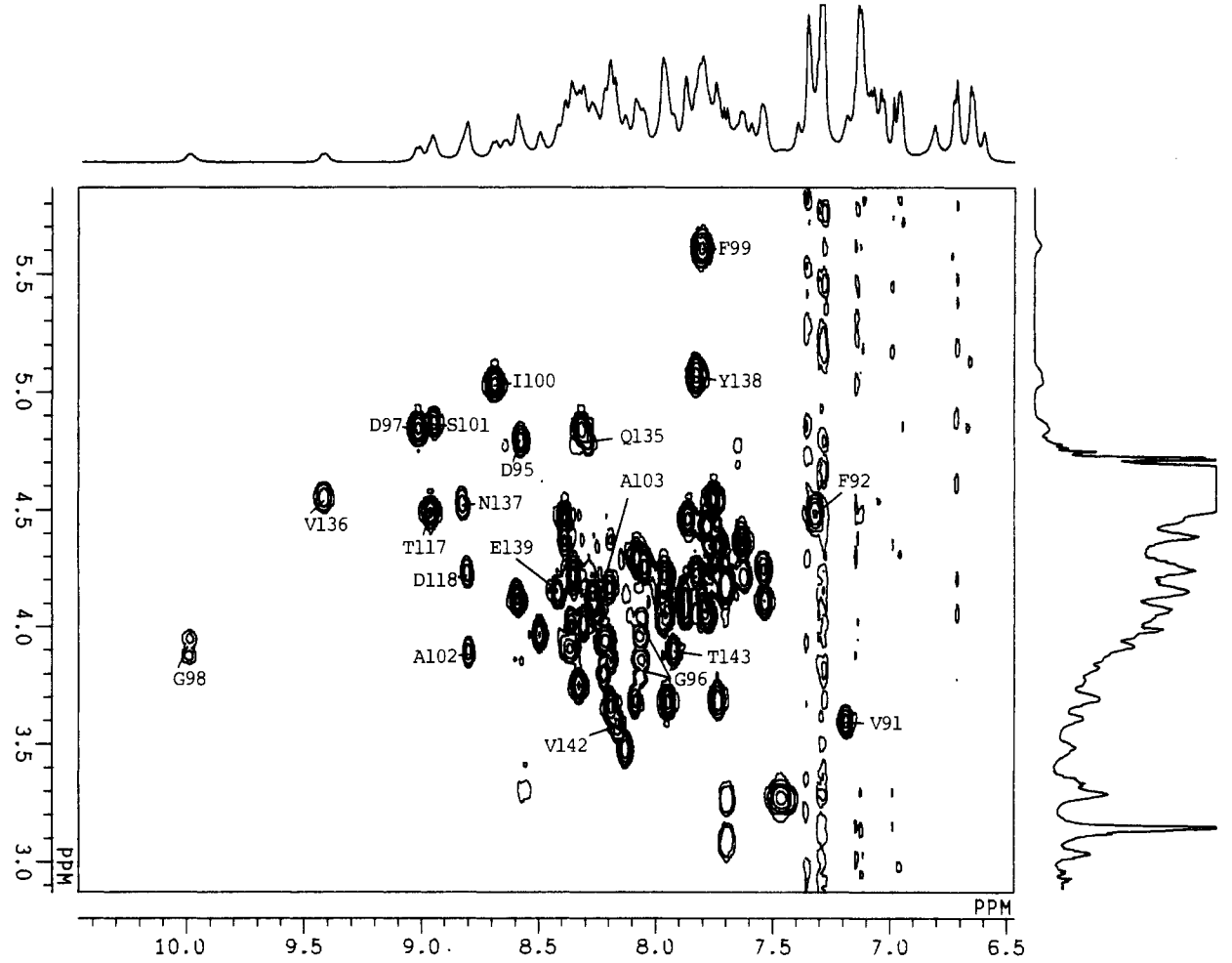

FIGURE 1: Expanded view of fingerprint region of 500-MHz COSY spectrum of Ca^{2+}-free F34 in 90% H_2O ([F34] = 6 mM; 10% D_2O–0.2 M KCl, pH 6.3, 30 °C). Assignable cross-peaks are indicated by the IUB–IUPAC one-letter symbol and the location in the sequence. One-dimensional spectra of the same region are shown at the top and at the right.

shifted methyl groups and aromatic side chains (Ikura et al., 1983a,b; Dalgarno et al., 1984; Aulabaugh et al., 1984b). In this paper, hydrogen bonding which may be important for structural stability of the C-terminal half-domain of CaM will be presented.

Pardi and others (Pardi et al., 1983; Wagner et al., 1983) and Dalgarno and others (Dalgarno et al., 1983b) analyzed ^1H NMR results of BPTI, inhibitor E, inhibitor K, egg white lysozyme, and a long neurotoxin and proposed an empirical rule of conformation-dependent secondary shifts that α and amide protons in the β-sheet structure will shift largely downfield. Dalgarno and others (Dalgarno et al., 1983a,b) assigned downfield-shifted α-proton resonances observed for parvalbumin and ICBP to residues located between –Y and –X coordination ligands of two Ca^{2+}-binding loops of CD and EF hands; only those residues are in the antiparallel β-sheet-like conformation as judged from the crystal structure (Moews & Kretsinger, 1975; Szebenyi et al., 1981). Furthermore, from comparison with the results of parvalbumin and ICBP, Dalgarno et al. (1984) assigned two downfield-shifted α-proton resonances of calmodulin fragment 1–75 to Thr-26 and Asp-64, which correspond to residues located between –Y and –X positions in the CD and EF hands of this fragment.

In this study, the α-proton resonance of Phe-99 in the Ca^{2+}-free state of F34 was found at the lowest field (5.61 ppm) among all α-protons. In the Ca^{2+}-bound state, the resonance shifts upfield but still remains at a relatively low-field position (5.20 ppm). Aulabaugh et al. (1984b) studied fragment 78–148 (F34) of bovine brain CaM. Their results show that the α-proton of Tyr-99 of bovine brain F34 resonates at positions as low as that of Phe-99 of scallop testis F34. Phe-99 as well as Tyr-99 is located between –Y and –X positions of the CD hand (domain III). The corresponding residue in the EF hand (domain IV) is Gln-135. The α-proton resonance of Gln-135 appeared at 4.78 ppm in the Ca^{2+}-free state and at 4.90 ppm in the Ca^{2+}-bound state. This resonance also remains at much lower positions than those of other α-protons, though not significant as compared with that of Phe-99. Figure 3 shows that in the Ca^{2+}-bound state large downfield shifts of α-proton resonances occur for Gln-135, Val-136, and Asn-137 in domain IV and for Phe-99, Ile-100, and Ser-101 in domain III. The empirical rule of conformation-dependent secondary shifts suggests that the β-sheet structure exists in the CD- and EF-hand loops of F34. The presence of d_1 connectivities observed for two sequences of Phe-99 to Ala-103 and Gln-135 to Glu-139 provides further evidence for the β-sheet structure [see Wüthrich et al. (1982) and subsequent papers]. In the Ca^{2+}-free state, α-protons of these residues give rise to resonances at different positions from those in the Ca^{2+}-bound state but still appear at relatively low fields. The results suggest that the β-sheet structure is conserved without regard to the slight rearrangement of the backbone conformation. A cross-strand NOE between amide protons of Ile-100 and Val-136 was observed in the Ca^{2+}-free state (unpublished results). This observation can be further evidence for the existence of a β-sheet hydrogen bond. Kretsinger (1980a) predicted the antiparallel β-sheet structure between

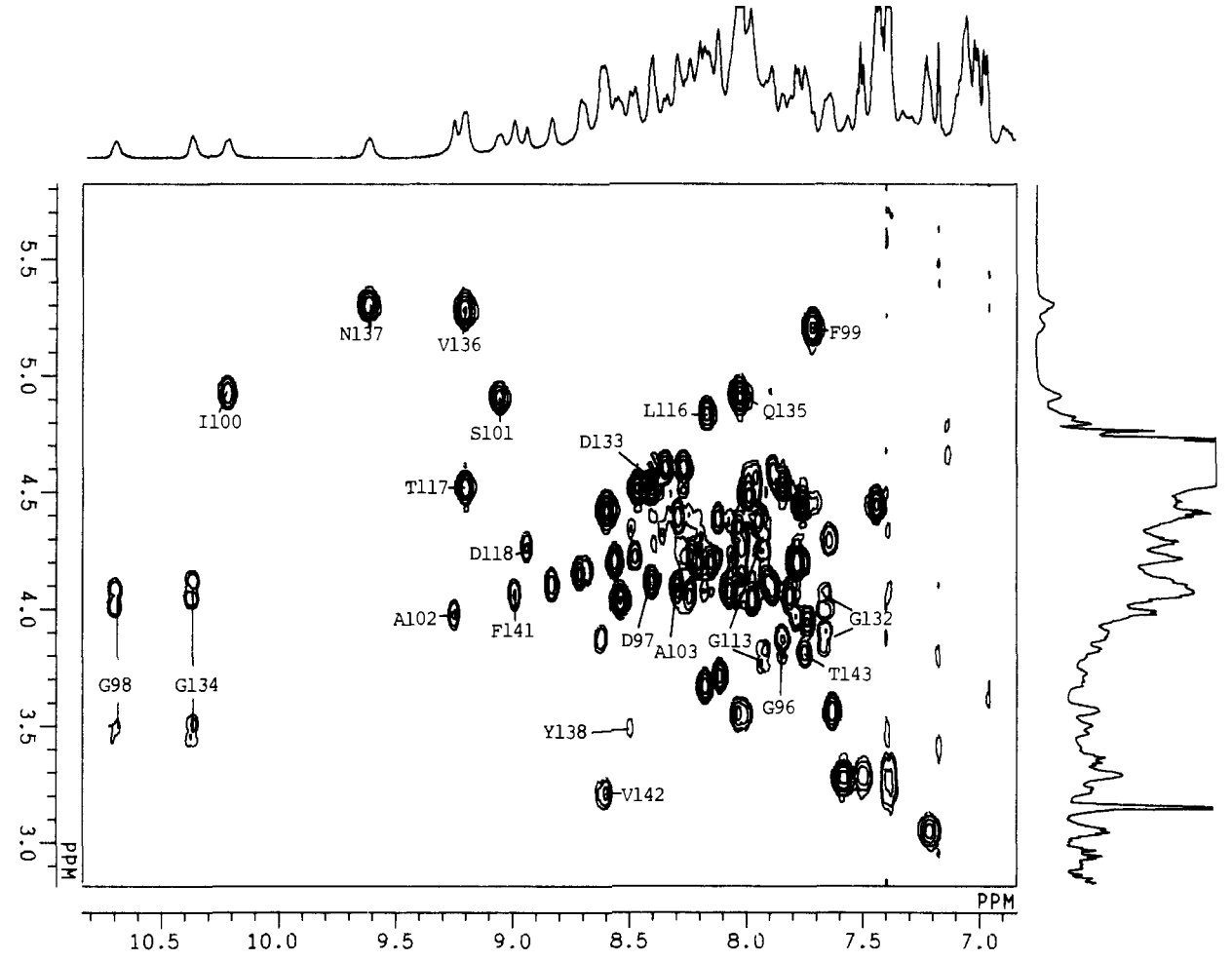

FIGURE 2: Expanded view of fingerprint region of 500-MHz COSY spectrum of Ca^{2+}-bound F34 in 90% H_2O. Experimental conditions are the same as those in Figure 1 except for the addition of $CaCl_2$ ($[Ca^{2+}]$ = 15 mM).

the two loops of domains III and IV; the amide group of Ile-100 is hydrogen bonded to the carbonyl group of Val-136 and the amide group of Val-136 to the carbonyl group of Ile-100.

As shown in Figures 2 and 3, amide proton resonances of Ile-100 and Val-136 largely shift downfield, implying that the two amide groups form hydrogen bonds in the Ca^{2+}-bound state. These findings are in agreement with the prediction by Kretsinger and can be regarded as experimental evidence for the antiparallel β-sheet structure in the Ca^{2+}-bound state. The results obtained in the Ca^{2+}-free state show that the hydrogen bond of the Ile-100 NH to the Val-136 CO is broken or weakened while another hydrogen bond of the Val-136 NH to the Ile-100 CO is retained.

Another interesting observation is that in the Ca^{2+}-bound state (Figure 2) amide protons of Gly-98 and Gly-134 which are located between Z and −Y coordination ligands in domain III and domain IV, respectively, shift significantly downfield. Such large downfield shifts are also attributable to hydrogen bonding. The parvalbumin crystal structure (Moews & Kretsinger, 1975) indicates that Gly-95 of parvalbumin which corresponds to Gly-98 of F34 forms a hydrogen bond between its amide group and the side-chain carboxyl group of Asp-90 (X coordination ligand in the Ca^{2+}-binding loop) at a proton–oxygen distance of 1.7 Å. Taking into account the homology in amino acid sequence between parvalbumin and CaM, it seems most likely that acceptors of hydrogen bonds of Gly-98 and Gly-134 of F34 are oxygen atoms of side-chain carboxyl groups of Asp-93 and Asp-129, respectively. The other oxygen atoms of carboxyl groups of these Asp residues are considered to serve as Ca^{2+} ligands at X positions in domains III and IV. In the Ca^{2+}-free state, a large downfield shift of the amide proton was not observed for Gly-134 but only for Gly-98 as shown in Figure 1. This suggests that in the Ca^{2+}-free state the hydrogen bond between Gly-134 and Asp-129 is broken while that of Gly-98 and Asp-93 is preserved.

Figure 3 shows a remarkable resemblance in chemical shifts of amide and α-protons in the Ca^{2+}-bound state between Ca^{2+}-binding loops of domains III and IV, the amino acid sequences of which are different as shown by the top and bottom panels of the figure. This suggests that the conformations of Ca^{2+}-binding loops of domains III and IV are similar to each other and may be related by an approximate intramolecular 2-fold axis as similar as EF- and CD-hand domains in the parvalbumin crystal structure.

Conclusions obtained here are summarized as follows. (1) In the Ca^{2+}-bound state, two Ca^{2+}-binding loops (domains III and IV) are connected by two hydrogen bonds between Ile-100 and Val-136 in the antiparallel β-sheet conformation. (2) In the Ca^{2+}-free state, the loops are also in the β-sheet conformation, though one of the β-sheet hydrogen bonds from the Ile-100 NH to the Val-136 CO is destroyed (or weakened). (3) Backbone amide protons of Gly-98 and Gly-134 form hydrogen bonds in the Ca^{2+}-bound state, presumably to the side-chain carboxyl groups of Asp-93 and Asp-129, respectively. (4) In the Ca^{2+}-free state, the hydrogen bond between Gly-134 and Asp-129 is broken while that of Gly-98 and Asp-93 is preserved. (5) Backbone conformations of two Ca^{2+}-binding loops of domains III and IV are very similar to each other.

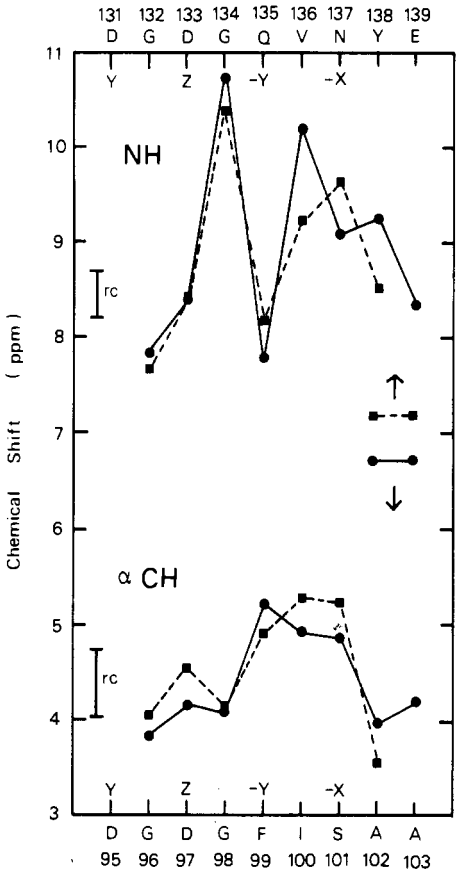

FIGURE 3: Chemical shift plots of backbone NH (amide) and α-CH protons in the Ca^{2+}-bound state against residue number. Solid lines represent the sequence of domain III (D95–A103) and broken lines that of domain IV (D131–E139). The letters Y, Z, –Y, and –X indicate Ca^{2+}-ligand residues at the octahedral coordination. The range labeled with "rc" indicates a chemical shift range in the "random-coil" state (Bundi & Wüthrich, 1979).

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