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Sequence-specific assignments of downfield-shifted amide proton resonances of calmodulin

Use of two-dimensional NMR analysis of its tryptic fragments

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Two-dimensional NMR methods were applied to assign the extremely downfield-shifted amide-proton resonances in the 500-MHz ^{1}H-NMR spectra of the NH_2-terminal fragment of residues 1–75 of calmodulin. The low-field resonances of the ^{1}H-NMR spectra of intact calmodulin were assigned to specific amino acid residues by comparison with spectra of the tryptic fragments of residues 1–75 and 78–148, in both the Ca^{2+}-free and Ca^{2+}-bound states. The hydrogen bonding of glycine residues connecting the two amino acid residues at the Z and $-Y$ positions in the octahedral Ca^{2+} coordination site was investigated. The Gly 134 in site IV showed a different property from the other glycines, 25, 61 and 98, involved in sites I, II and III, respectively.

Calmodulin; ^{1}H-NMR; 2D NMR; Hydrogen bonding

1. INTRODUCTION

We showed in a previous paper [1] that the extremely low-field region of the ^{1}H-NMR spectra of tryptic fragment F34 (residues 78–148) of calmodulin in H_2O exhibits resonances from the hydrogen-bonded backbone amide protons. These amide protons are located in the loop regions of Ca^{2+}-binding sites III and IV in the COOH-terminal domain (C-domain), and provide information on the Ca^{2+}-induced conformational change around the Ca^{2+} coordination site. However, another tryptic fragment, F12 (residues 1–75), which possesses the two Ca^{2+}-binding sites I and II in the NH_2-terminal domain (N-domain), has remained to be studied.

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Here, resonances in the low-field region of the ^{1}H-NMR spectra of F12 were assigned using two-dimensional (2D) NMR methods including COSY [2], NOESY [3], relayed COSY [4], and DECSY [5]. The spectra of intact calmodulin were analyzed by comparison with the spectra of F12 and F34. The hydrogen bonding of Gly 25, Gly 61, Gly 98 and Gly 134 is discussed on the basis of an empirical role for the secondary shift of amide protons [6].

2. MATERIALS AND METHODS

Calmodulin was isolated and purified from scallop testis as described [7]. F12 and F34 were prepared by limited proteolysis of purified calmodulin with trypsin in the presence of Ca^{2+}, followed by separation of fragments by DEAE-cellulose column chromatography, as described by Minowa and Yagi [8]. Calmodulin and fragments F12 and F34 were shown to be homologous by polyacrylamide gel electrophoresis in the presence

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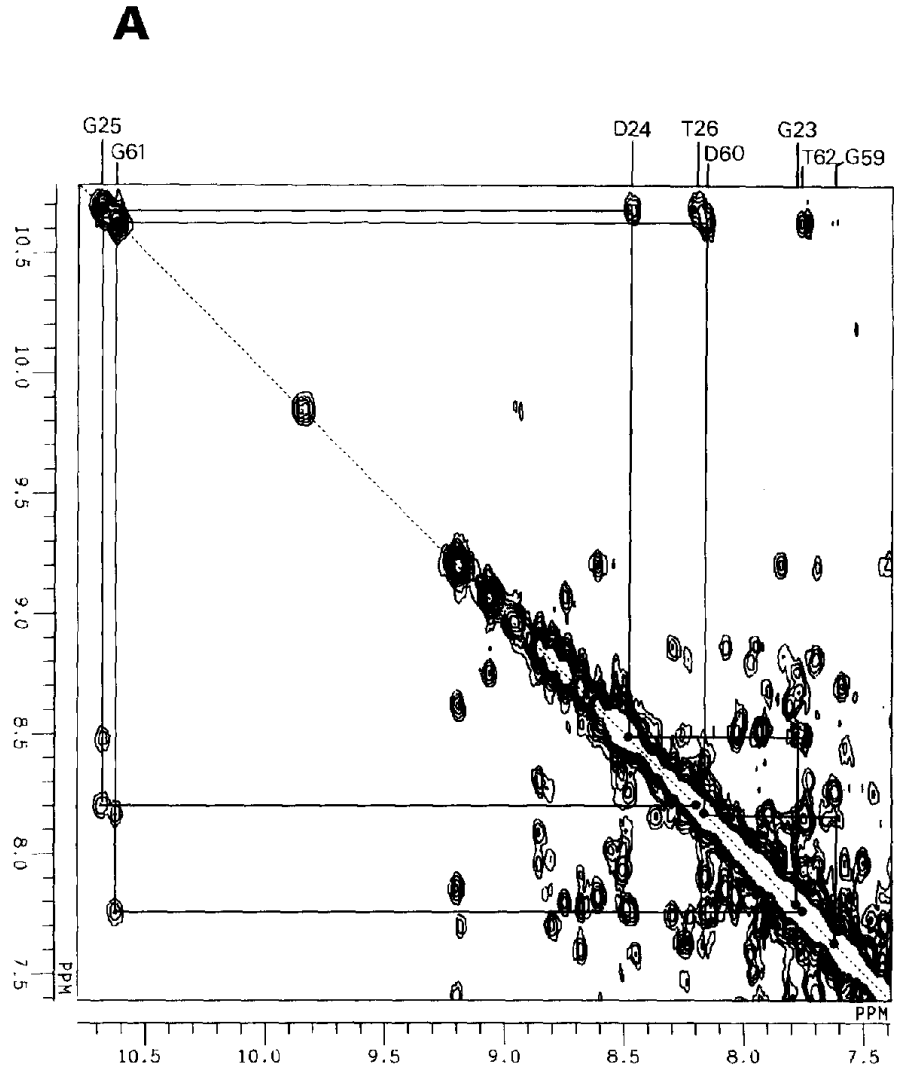
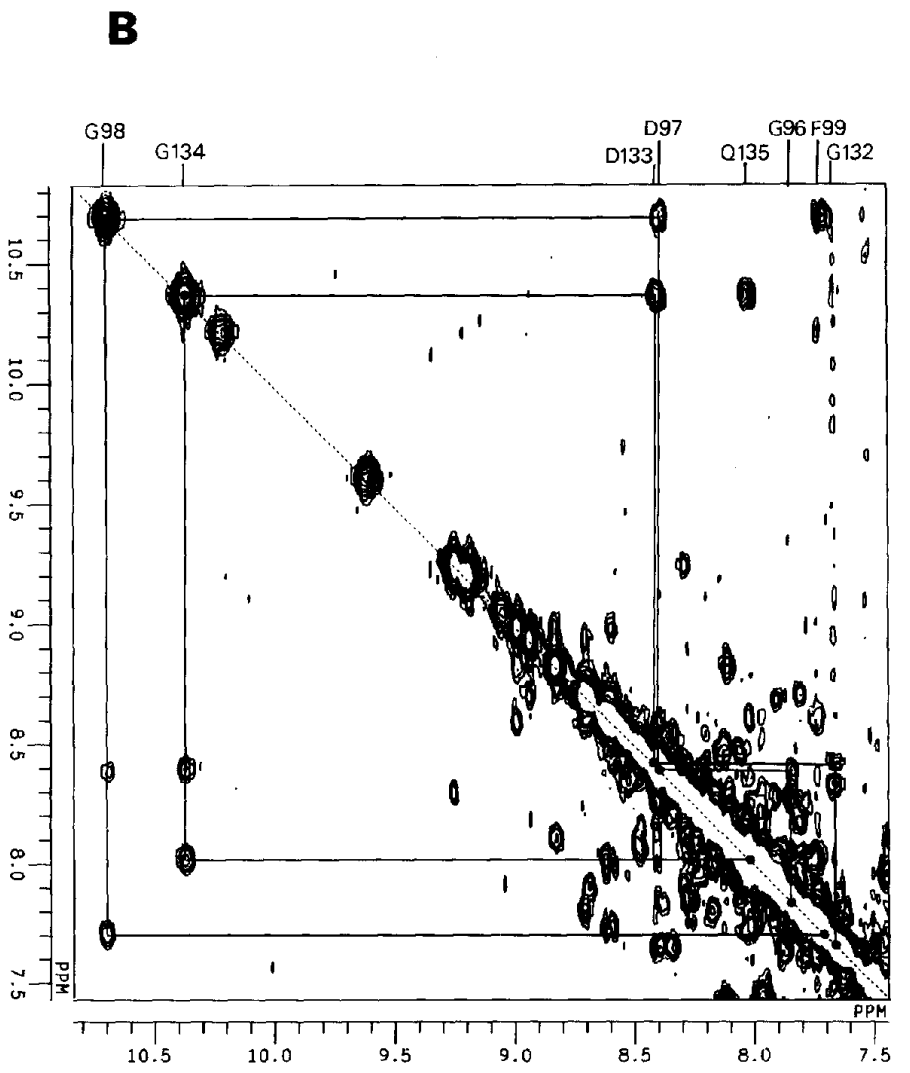


Fig.1. (A) Low-field region of NOESY spectrum of F12 in the presence of Ca^{2+}; (B; facing page) low-field region of NOESY spectrum of F34 in the presence of Ca^{2+}. Mixing time was 260 ms.

of SDS or alkaline urea, HPLC, and amino acid analysis. Removal of contaminating Ca^{2+} from calmodulin and its fragments was achieved by trichloroacetic acid precipitation and renaturation [7]. The sample was dissolved to 3.0 and 6.0 mM in a mixture of H_2O and D_2O (90:10) for 1D and 2D measurements, respectively. All samples contained 0.2 M KCl. The pH was adjusted to 6.4–6.6 by addition of NaOH.

NMR experiments were performed at 30°C on a JEOL JNM-GX500 spectrometer (^1H 500 MHz). COSY and NOESY spectra were simultaneously obtained by the COCONOSY method [9,10]. Relayed COSY and DECSY spectra were obtained using the pulse sequences of Eich et al. [4] and Ikura et al. [5], respectively. The H_2O peak was suppressed by the gated-decoupling method. All the 2D spectra were measured with 256 × 1024 data points and spectral width of 5500 Hz. 256 scans were accumulated for each t_1 and the total measurement time was about 40 h. After zero filling in the t_1 dimension, a 1024 × 1024 data matrix was processed. Sine bell was applied for both t_1 and t_2 dimensions as an apodization function. The



contour display of the 2D spectrum is presented in the absolute-value mode. The chemical shifts were measured in ppm relative to an internal standard, (trimethylsilyl)propionic-d_4 acid (TSP).

The assignment strategy is based on an established procedure proposed by Wuthrich and coworkers [11]. To identify the spin systems of amino acids we used COSY and relayed COSY spectra with some assistance from NOESY spectra. Spin systems of neighboring residues in the polypeptide chain were identified by NOEs between backbone NH resonances of one residue and resonances of NH and/or αCH groups of the preceding residue.

3. RESULTS AND DISCUSSION

Fig.1A shows the low-field amide region of the NOESY spectrum of F12 in the Ca^{2+}-saturated state. Sequential NOEs between NH_i and NH_{i+1} (type d_{NN}) are illustrated for the two sequences, Gly 23-Asp 24-Gly 25-Thr 26 and Gly 59-Asp 60-Gly 61-Thr 62. Through sequential NOEs between αCH_i and NH_{i+1} (type $d\alpha_N$), the two sequences can be further extended to Thr 26-Ile 27-Thr 28-Thr 29 and Thr 62-Ile 63-Asp 64-Phe 65, respectively (not shown). The former sequence (Gly 23 to Thr 29) is involved in Ca^{2+}-binding site I and the latter (Gly 59 to Phe 65) in site II.

Fig.1B shows the low-field amide region of the NOESY spectrum of F34 in the Ca^{2+}-saturated state. The assignments are reported in our previous paper [1]. The patterns of d_{NN}-type NOE connectivity in the sequences of Gly 23-Asp 24-Gly 25-Thr 26 (site I) and Gly 59-Asp 60-Gly 61-Thr 62 (site II), which is shown in fig.1A, considerably resemble those of Gly 96-Asp 97-Gly 98-Phe 99 (site III) and Gly 132-Asp 133-Gly 134-Gln 135 (site IV). The resemblance of the NOE connectivity patterns in this region indicates that the four Ca^{2+}-binding sites have a similar backbone conformation.

Wagner et al. [6] indicated that large low-field shifts of amide protons in bovine pancreatic trypsin inhibitor are well correlated with the length of the hydrogen bond between the amide proton and carbonyl oxygen. Here, we found that the amide proton resonances of Gly 23 and Gly 61 in F12 appear at extremely low fields. This indicates that Gly 23 and Gly 61 in F12 form hydrogen bonds in the same manner as Gly 98 and Gly 134 in F34 [1]. The present NMR analysis could not determine the acceptors of the hydrogen bonds of Gly 23 and Gly 61, but we suggest that those are the side chain carboxyl groups of Asp 18 and Asp 56, respectively. This suggestion is based on the model of carp parvalbumin determined by X-ray crystallography at 1.5 Å resolution proposed by Moews and Kretsinger [12]. The low resolution (3.0 Å) of the crystal structure of rat testis calmodulin [13] precludes analysis for these hydrogen-bonding patterns.

The abnormal chemical shift of these glycines may be a common character of the 'EF-hand'-type Ca^{2+}-binding site, because EF-hand proteins generally possess this glycine at the position connecting the two amino acid residues at the Z and $-Y$ positions in the octahedral-coordination site (review [14]). Furthermore, the aspartic acid that is the most probable candidate for the acceptor of the Gly hydrogen bond is also well conserved among many different EF-hand proteins. The bent-backbone conformation suitable for the octahedral coordination of Ca^{2+} should be stabilized by the hydrogen bonding of this characteristic glycine. However, Herzberg and James [15] recently reported a refined crystal structure at 2.2 Å resolution of skeletal-muscle troponin-C, suggesting that the corresponding glycines in the Ca^{2+}-binding loops do not form hydrogen bonds. It remains to be determined whether the characteristic NMR feature for the hydrogen bonding of the glycines found in calmodulin is seen for skeletal-muscle troponin-C, as well as for other EF-hand proteins.

Fig.2 shows a comparison of the low-field amide region of the ^{1}H-NMR spectra of intact calmodulin with those of F12 and F34. As demonstrated previously for aromatic and methyl resonances [16–19], the superposition of the spectra of F12 and F34 apparently yields the spectrum of intact calmodulin. This 'additivity' is also seen in this work in the low-field amide region, and permitted us to assign the amide proton resonances of calmodulin using the resonance identities of F12 and F34. The lowest-field peak of calmodulin indicates three-proton intensity and is assigned to the amide protons of Gly 25, Gly 61, and Gly 98. The four peaks which appear from 9.5 to 10.5 ppm were assigned to Asn 137, Ile 27, Ile 100 and Gly 134.

The same procedure as described above was employed for completely decalcified samples of F12, F34 and intact calmodulin. Fig.3 shows a comparison of the low-field amide region of the ^{1}H-NMR spectrum of intact calmodulin with spectra of the tryptic fragments F12 and F34 in the Ca^{2+}-free state. The three peaks of calmodulin appearing in the range 9.5–10.5 ppm were assigned to Gly 61, Gly 98 and Gly 25 by spectral comparison with fragments F12 (a) and F34 (b), although the deviations in chemical shift between these residues are more noticeable than those obtained for the Ca^{2+}-saturated state. A peak at

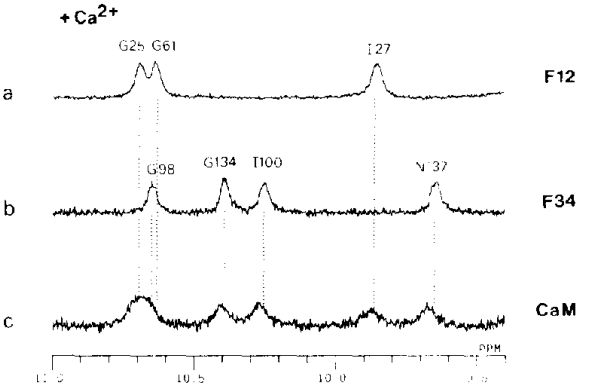


Fig.2. Low-field region of the normal ^{1}H spectra of F12 (a), F34 (b), and calmodulin (c) in the presence of Ca^{2+}.

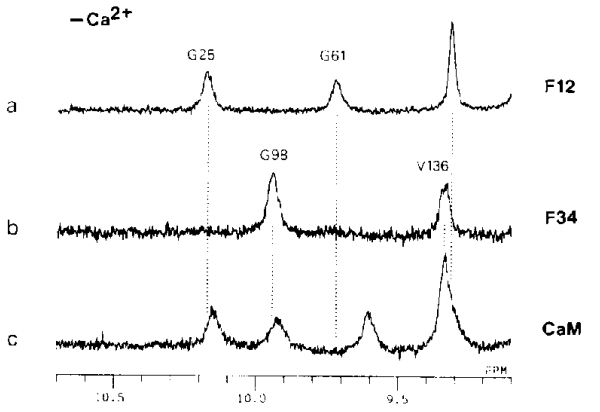


Fig.3. Low-field region of the normal ^1H spectra of F12 (a), F34 (b), and calmodulin (c) in the absence of Ca^{2+}.

9.33 ppm in the calmodulin spectrum has two-proton intensity; one is contributed by the amide proton of Val 136 of F34 and the other from a probable amide proton of an unidentified residue of F12. It should be noted that the resonances of the amide protons of Gly 25, Gly 61 and Gly 98 appear in this low-field region in the Ca^{2+}-free state as well as in the Ca^{2+}-bound state, but the resonance of Gly 134 disappears from this low-field region.

From these observations, we conclude that, when calmodulin is bound with Ca^{2+}, the hydrogen bonds of Gly 25, Gly 61 and Gly 98 are probably tightened (as indicated by the downfield shifts of these resonances compared to the Ca^{2+}-free state) thus stabilizing the structures of the Ca^{2+}-binding loops. In contrast to Gly 25, Gly 61 and Gly 98, Gly 134 in site IV does not form the hydrogen bond until Ca^{2+} binds to calmodulin. The gain in structural stability due to the formation of the glycine hydrogen bonding in the Ca^{2+}-bound state is significant for site IV relative to the other three sites. The exceptional character of site IV possibly relates to the positive Ca^{2+}-binding cooperation between sites III and IV that has been characterized by flow-dialysis experiments [8] and by kinetic studies of ^1H- [20], ^{113}Cd- [21] and ^{43}Ca-NMR [22].

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