

Isolated EF-loop III of calmodulin in a scaffold protein remains unpaired in solution using pulsed-field-gradient NMR spectroscopy

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Abstract

Calmodulin (CaM) is a trigger calcium-dependent protein that regulates many biological processes. We have successfully engineered a series of model proteins, each containing a single EF-hand loop but with increasing numbers of Gly residues linking the EF-hand loop to a scaffold protein, cluster of differentiation 2 (CD2), to obtain the site-specific calcium-binding ability of a protein with EF-hand motifs without the interference of cooperativity. Loop III of calmodulin with two Gly linkers in CD2 (CaM-CD2-III-5G) has metal affinities with K_d values of 1.86×10^{-4} and 5.8×10^{-5} M for calcium and lanthanum, respectively. The oligomeric states of the CD2 variants were examined by pulsed-field-gradient nuclear magnetic resonance (PFG NMR). The diffusion coefficient values of CD2 variants are about 11.1×10^{-7} cm²/s both in the presence and absence of metal ions, which are the same as that of wild-type CD2. This suggests that the isolated EF-loop III of calmodulin inserted in the scaffold protein is able to bind calcium and lanthanum as a monomer, which is in contrast to the previous observation of the EF-hand motif. Our results imply that additional factors that reside outside of the EF-loop III may contribute to the pairing of EF-hand motifs of calmodulin. This result is of interest as it opens up the way for studying the ion-binding properties of isolated EF-hands, which in turn can answer important questions about the properties of EF-hands, the large and important group of calcium-binding signaling proteins. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

EF-hand proteins use the paired EF-hand calcium-binding motifs to regulate many cellular functions such as muscle contraction, neuronal signaling, apoptosis, and cell cycle control [1–5]. Upon calcium binding, the trigger proteins (calcium-modulated protein or sensor proteins) undergo a large conformational change and, in turn, regulate a vast number of target proteins [6,7]. Calmodulin (CaM), one of the many Ca (II)-dependent regulatory proteins, is an ubiquitous, multifunctional protein that can interact with

and regulate many (>50) different target enzymes and proteins including phosphodiesterase, myosin light chain kinase, calmodulin kinase, calcineurin, and nitric oxide synthase [5,8–13]. Another trigger protein, troponin C (TnC), is ~51% homologous to calmodulin in its amino acid sequence. TnC serves as a calcium sensor in muscle cells [7,14–19]. Both calmodulin and TnC consist of two domains with two pairs of EF-hand motifs.

As one of the most popular motifs in calcium-binding proteins, all of the EF-hand motifs consist of a highly conserved 12-residue EF-loop flanked by two helices (helix–loop–helix) [20,21]. Flanking helices contain several conserved hydrophobic residues involved in the calcium-induced conformational change. Seven oxygen atoms from the side chains of Asp, Asn, and Glu, the main chain, and water at the sequence positions of the EF-hand calcium-binding loop of 1, 3, 5, 7, 9, and 12 coordinate the calcium ion in a pentagonal bipyramidal arrangement. Two closely packed helix–loop–helix modules formed within a single

Abbreviations: PFG NMR, Pulsed-field-gradient nuclear magnetic resonance; PFG-SLED, Pulsed-field-gradient stimulated echo longitudinal encode–decode; CaM, calmodulin; TnC, troponin C; CD2, cluster of differentiation 2

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globular domain constitute the basic calcium-binding unit of EF-hand proteins. Two EF-hand motifs arranged with respect to each other in a pseudo-two-fold symmetry in the same protein domain yield highly cooperative calcium-binding systems [3]. The distance between two calcium ions in two paired EF-hand motifs is usually about 11 Å, and the coordination shells of two calcium-binding motifs can be completely overlapped in almost all EF-hand proteins. Obtaining the site-specific calcium-binding affinity of each

EF-hand loop is hampered by the cooperativity and calcium-induced conformational changes of proteins.

Whether an isolated EF-hand motif can function as an individual unit has been a hot debate for several decades. Peptide models have been used extensively to understand the mechanism of metal binding and calcium-induced conformational change of EF-hand proteins. Previous work carried out by Shaw et al. [22] has shown that peptide fragments encompassing the EF-hand motifs III and IV with

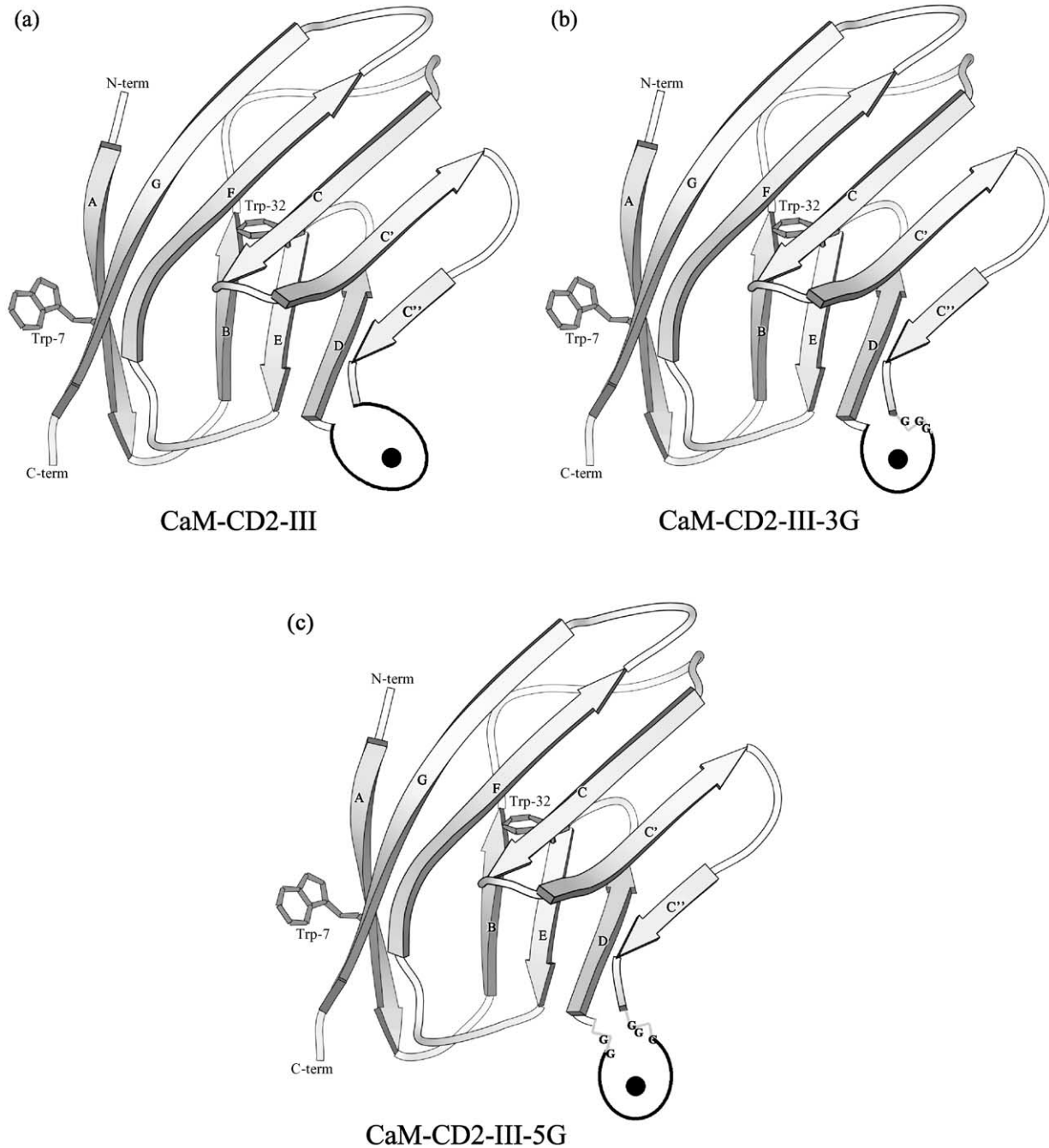


Fig. 1. Schematic model structures of CaM-CD2-III, CaM-CD2-III-3G and CaM-CD2-III-5G. They are constructed based on the pdb file of 1hng of wild-type rat CD2 [27,28].

flanking helices of TnC associate in solution with a native-like structure of the C-terminal domain of the protein. Hydrophobic residues on the flanking helices of the EF-hand motif were shown to be essential for the dimerization [23]. In contrast, the shorter peptide fragment composed of only a 12-residue EF-loop III of TnC remains as monomeric both in the presence and absence of metal ions [24]. Siedlecka et al. [25] and Wojcik et al. [26] have shown that the isolated 12-residue peptide with both ends blocked does not dimerize in the presence of calcium but dimerizes to form a native-like structure in the presence of Ln(III) that has a similar ionic radius and coordination properties as Ca(II). Based on their observation, they concluded that local interactions between the EF-hand calcium-binding loops alone could be responsible for the observed cooperativity of calcium binding to EF-hand protein domains. Whether an isolated EF-loop is stable as an unpaired monomer at physiologic conditions in solution is still questionable. The mechanism for calcium-induced conformational change is not clear.

To obtain site-specific information about EF-hand proteins and to correlate the measured calcium-binding properties with the local structural elements of the EF-hand motif without the interference of cooperativity, we have developed a new strategy of using a scaffold protein to avoid the undefined structure of short peptides and fragments in solution. The N-terminal domain of rat cluster of differentiation 2 (CD2) was chosen as the ‘host system’ because of its small size, high expression yield, high solubility, and tolerance to mutations. CD2 from the immunoglobulin superfamily is comprised of antibodies and Ig-like domains. It plays a fundamental role not only in the immune system, but also in the interactions between cells, specifically in cell–cell adhesion [27]. As shown in Fig. 1, the X-ray structure of the domain 1 of CD2 contains nine beta-strands in two layers. Aromatic residue Trp-32 is sandwiched between these two layers [27,28]. We have successfully designed and engineered a series of CD2 variants, each containing a single EF-hand calcium-binding loop from calmodulin with zero (CaM-CD2-III), one (CaM-CD2-III-3G), and two (CaM-CD2-III-5G) Gly linkers at the end(s) of the loop. As shown in Fig. 1, the EF-hand loop was inserted at the loop position C''D of CD2 to avoid the disruption of the hydrogen bonding network and the packing and hydrophobic interactions of the host protein (Fig. 1). We reported that the host protein is able to retain its native structure after insertion of the EF-loop. An EF-loop with two Gly linkers (CaM-CD2-III-5G) in CD2 has the strongest metal binding affinity to Ca(II) and La(III) [29].

Pulsed-field-gradient nuclear magnetic resonance (PFG NMR) has been proven valuable for the study of molecular motions and the effective dimensions of native, unfolded, and oligomeric states of proteins in solution [30–36]. The translational motion of well-packed spherical-like molecules in solution has a direct correlation with the hydrodynamic radius and molecular size according to the equation

$D = K_B T / 6\pi a \eta$ [37,38]. At a given temperature (T) and solvent viscosity (η), the diffusion constant (D) of this particular molecule decreases as the radius (a) of the molecule increases. This method is extremely useful for the characterization of the oligomeric state of proteins in solution. Since most well-packed globular proteins usually can be well described as a spherical shape, the molecular size of a protein and the oligomeric state of the protein can be estimated by measuring the diffusion constant [37,39]. The PFG NMR technique is advantageous for our studies since the diffusion constant can be measured under the same conditions as for metal binding or structural determination [40–42]. In this paper, we present results of our NMR studies of the EF-loop III of calmodulin grafted in the scaffold protein CD2. To investigate the oligomeric state of the CD2 variants, we have conducted PFG NMR experiments in solution. In contrast to the previously observed dimeric form, this grafted EF-loop in the scaffold protein remains unpaired both in the presence and absence of metal ions.

2. Materials and methods

2.1. Protein expression

All proteins were expressed as GST-fusion proteins in *E. coli* BL21 strain. They were induced by IPTG (0.1–0.3 mM) and purified by Glutathione Sepharose 4B affinity column (Pharmacia Biotech). The fused proteins were cleaved by thrombin (Sigma) and separated by FPLC. The purities of these proteins were confirmed by SDS-PAGE and mass spectrometry. The protein concentration was estimated by absorbance with an extinction coefficient at 280 nm of $17700 \text{ M}^{-1} \text{ cm}^{-1}$ [29].

2.2. Gradient diffusion experiments

Protein concentrations varied from 0.15 to 1.2 mM. Spectra widths of 6600 and 8000 Hz were used for diffusion experiments at 500 and 600 MHz NMR, respectively. The spectra were collected using a modified PG-SLED pulse sequence [38] with 16 or 8 K complex data points for each FID in 10 mM Tris–HCl pH 7.4 at 25 °C. The diffusion constants were obtained via fitting the desired integrated area of the resonances of each arrayed spectrum through Eq. (1) [37]

$$A = A_0 \exp[-(\gamma \delta G)^2 (\Delta - \delta/3) D] \quad (1)$$

where γ is the gyromagnetic ratio of proton. The time between PFG pulse (Δ) and the PFG duration time (δ) were 80.5 and 5 ms, respectively. The gradient strength (G) was arrayed from 0.2 G/cm to about 31.0 G/cm using 64 or 50 steps. A is the integrated area of desired resonances at each array spectrum after subtraction of baselines. A_0 is the

integrated area of the desired resonances when the PFG strength is zero. Using an internal reference with the known diffusion constant and simplifying Eq. (1) to

$$A = A_0 \exp(-CG^2) \quad (2)$$

the unknown diffusion constants can be measured by equation

$$D = D_0 C / C_0 \quad (3)$$

where C and C_0 are the combination constants at the studied molecule and the internal reference. The data were processed following the steps for the process of dimension 1 in the 2D NMR spectra using Felix98. The A values used in the calculation were obtained by integrating NMR signals of all the resonances in the identical regions at each 1D spectrum with the subtraction of the baseline. The diffusion constant D was obtained by fitting A as a function of gradient strength using Eq. (2,3) and KaleidaGraph 3.5 (Synergy). The baseline was corrected using a zero-order polynomial. Tris and dioxane were used as internal references and their signals were processed using the same procedure as for protein samples. All experiments were repeated three times to ensure the required accuracy.

2.3. Line-shape fitting

The resolved peaks of 1D NMR spectra both with and without metal ions were fitted using the 'peak optimize' function of Felix98, which gave the linewidth, position and intensity of each optimized peak.

3. Results

3.1. The grafted EF-loop III in CD2 remains unpaired in the absence of metal ions

By monitoring the fractional changes of chemical shifts at several positions in 1D ^1H NMR signal changes at several different wavelengths, the metal binding affinities of the inserted EF-loop III of calmodulin in CD2 variants were determined [29]. CaM-CD2-III-5G, with two linkers, has the highest calcium-binding affinity with a K_d of 1.86×10^{-4} M, while CaM-CD2-III and CaM-CD2-III-3G, have one-order of magnitude weaker calcium-binding affinities with K_d not smaller than 1 mM. CaM-CD2-III-5G also has the highest La(III) binding affinity with a K_d of 58 μM , which is about three-fold stronger than the calcium binding, possibly due to the higher charge number. CaM-CD2-III and CaM-CD2-III-3G again exhibit one-order lower binding affinities with K_d not smaller than 400 μM . All of the affinity values are in good agreement with those obtained by CD and fluorescence studies [29]. In our published paper [29], we show that a 1:1 metal/protein complex can be determined for both calcium and lanthanum.

In addition, the fractional changes of the fluorescence signal or NMR chemical shift can be well fitted with the equation assuming the formation of the 1:1 complex. Our recent equilibrium dialysis analysis using the atomic absorption method also shows that our engineered protein forms a 1:1 complex with calcium (Ye et al., unpublished data). That our measured calcium affinity of CaM-CD2-III-5G is weaker than the average value of the C-terminal domain of calmodulin is likely due to the lack of cooperative interactions between paired EF-hand motifs. As pointed out in several papers, the formation of pairs of EF-hand motif will lower the energy state of the calcium-binding form and increase the stability resulting from a mutual polarization of the associated groups. The binding of the first metal ion would enhance the binding of the second metal ion [16,43]. Isolated EF-hand motifs lack such stabilization, and, hence, its calcium-binding affinity is not increased. Our recent work also shows that the measured metal affinity is not affected by differing protein environments (Ye et al, submitted to *JACS*). Hence, the calcium-binding affinity we measured for the isolated EF-hand motif reflects its intrinsic calcium affinity.

The oligomeric states of CD2 variants with the inserted EF-loop in the absence of metal ions were investigated by the PFG NMR experiment. Amino acid Gly and proteins such as lysozyme (14.3 kDa), trypsinogen (24 kDa), and carbonic anhydrase (28 kDa) with different molecular weights were used as molecular standards to evaluate the accuracy of the PFG NMR method and to optimize experimental conditions. To minimize the contribution of other factors such as salt concentrations, protein concentrations, and temperature, all of the PFG diffusion experiments were carried out at identical conditions in 10 mM Tris–10 mM KCl, pH 7.4 at 25 °C. Signals from Tris buffer or dioxane were used as internal references to eliminate viscosity effects and to normalize diffusion constants of different NMR samples. Fig. 2a shows the 1D ^1H NMR signal decay of CaM-CD2-III-5G as a function of pulsed-field-gradient strength. Fig. 2b shows that of internal standards, dioxane, glycine and Tris buffer. All of the amplitudes of the NMR resonances gradually decrease with the increase of the gradient strength. NMR signals from the protein have a significantly slower decay than that of small molecule dioxane and Tris buffer. The integrated area of NMR signals of the protein, dioxane and Tris buffer were well fitted to Eqs. (1) and (2) with $R > 0.999$ (Fig. 3). The obtained diffusion constants of Tris and dioxane were 67.6 and 98.9×10^{-7} cm^2/s , respectively. Our measured diffusion constant for hen egg-white lysozyme is 10.6×10^{-7} cm^2/s , which is almost identical to that (10.8×10^{-7} cm^2/s) reported by Altieri et al. [37]. Under identical conditions, the diffusion constant of wild-type CD2 is 11.0×10^{-7} cm^2/s , which is very close to the value of lysozyme with similar molecular weight (14.3 kDa). As expected from Stokes–Einstein equation, larger proteins, such as trypsinogen (24 kDa) and carbonic anhydrase (28 kDa) have sig-

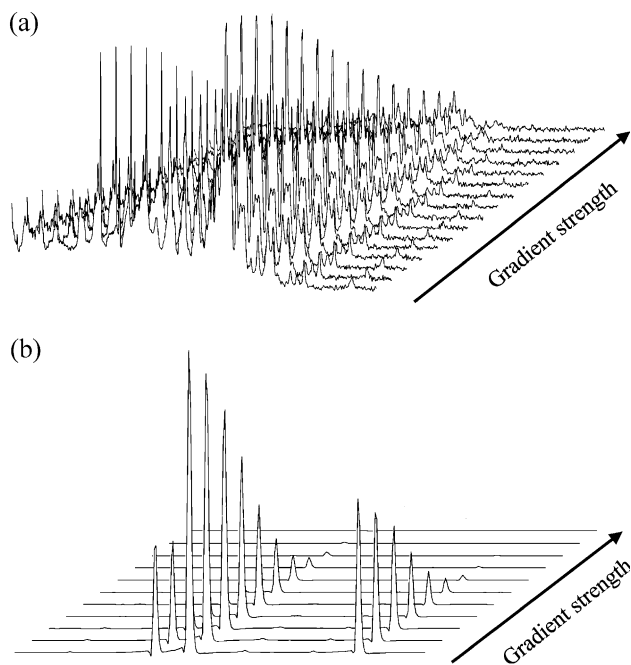


Fig. 2. Stack plot of 1D ^1H NMR spectra of (a) CaM-CD2-III-5G, and (b) Tris, Gly, and dioxane with the increase of PFG strength (indicated by an arrow). Each line represents the same region of 1D ^1H NMR spectra at different gradient strengths. From front to back, the PFG strengths are 0.20, 2.15, 4.10, 6.05, 8.01, 9.96, 11.91, 13.87, 15.82, 17.77, 19.73, 21.68, 23.63, 25.59, 27.54, and 29.49 G/cm in (a) and 0.20, 2.54, 4.88, 7.23, 9.57, 11.91, 14.26, 16.60, 18.95, 21.29, 23.63, and 25.98 G/cm in (b).

nificantly smaller diffusion constants of 9.5 and 9.1×10^{-7} cm^2/s , respectively (Table 1). Wilkins et al. [30] reported that the hydrodynamic radius of dioxane is 2.12 Å [30]. Using dioxane as a reference of size, the effective hydrodynamic radii of CD2, lysozyme, trypsinogen, and carbonic anhydrase are 39.2 , 40.2 , 45.4 , and 47.4 Å, respectively. As shown in

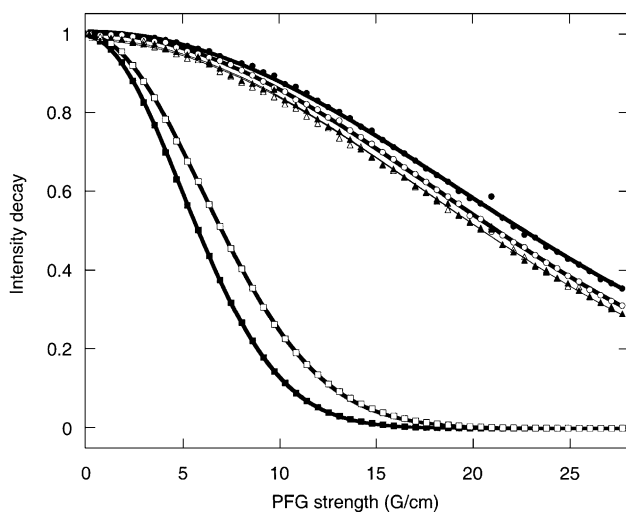


Fig. 3. NMR intensity decreases with the increase of PFG strength (■ dioxane, □ Tris, △ wild-type CD2, ▲ CaM-CD2-III-5G, ○ lysozyme, ● carbonic anhydrase). The solid lines are simulated based on Eq. (2) (Materials and Methods).

Table 1

Summary of the molecular weights, diffusion constants, and hydrodynamic radii of CD2 variants and standard molecules (10 mM Tris–HCl, pH 7.4, 25 °C)

Molecule	Molecular weight (kDa)	Diffusion constant ($\times 10^7$ cm^2/s)	Hydrodynamic radius (Å)
Dioxane	88	98.9 ± 0.9	2.12^a
Tris	121	67.6 ± 1.3	3.10 ± 0.06
Glycine	75	93.3^b	2.21 ± 0.02
Lysozyme	14.1	10.6 ± 0.2	40.2 ± 0.8
Trypsinogen	24	9.5 ± 0.2	45.4 ± 1.0
Carbonic anhydrase	28	9.1 ± 0.4	47.4 ± 2.1
CD2	11.3	11.0 ± 0.2	39.2 ± 0.7
CaM-CD2	12.3	10.9 ± 0.2	39.4 ± 0.7
CaM-CD2-III-3G	12.4	11.1 ± 0.3	38.8 ± 1.0
		11.4 ± 0.2^c	37.8 ± 0.7^c
CaM-CD2-III-5G	12.5	11.1 ± 0.2	38.9 ± 0.7
		11.4 ± 0.2^c	37.9 ± 0.6^c

The errors are given based on three different measurements.

^a Wilkins et al. [30].

^b 20 °C, Altieri et al. [37].

^c With 1 mM La(III).

Table 1, the relative sizes of these proteins are consistent with their relative molecular weights.

The oligomeric states of three CD2 variants with the insertion of EF-hand loop III from calmodulin were investigated using NMR under the same conditions as for wild-type CD2. As shown in Fig. 3, increasing the gradient strength gradually attenuates NMR signals from three CD2 variants in a manner similar to that of wild type at pH 7.4 in 10 mM Tris, 10 mM KCl. The obtained diffusion constants of three proteins, CaM-CD2-III, CaM-CD2-III-3G and CaM-CD2-III-5G, are 10.9 , 11.1 , and 11.1×10^{-7} cm^2/s , respectively, which are very close to that of wild-type CD2 (Table 1). In addition, the CD2 variants' linewidths of the resolved NMR resonances from aromatic residues Trp-7 at 10.5 ppm and Val-78 at 0.3 ppm of CD2 variants with inserted EF-loop in 1D NMR spectra do not broaden noticeably compared to those of wild-type CD2 and lysozyme. Further increasing protein concentrations of CD2 variants from 0.11 to 0.80 mM does not result in a detectable increase in their linewidths at pH 7.4 in 10 mM Tris–10 mM KCl. The strong similarities in the diffusion constants and the linewidths of the CD2 variants to that of wild-type CD2 indicate that the insertion of EF-loop III of calmodulin into CD2 does not cause the dimerization of the protein in the absence of metal ions.

3.2. The grafted EF-loop III remains unpaired upon metal binding

To examine whether the isolated EF-loop III of calmodulin in CD2 dimerizes upon metal binding, the diffusion constants of the engineered proteins CaM-CD2-III-3G and CaM-CD2-III-5G have been measured in the presence of La(III) under physiological conditions using the PFG NMR experiment. At 1 mM La(III) concentration, the CD2

variants are expected to be predominantly metal-loaded. The obtained diffusion constant for CaM-CD2-III-3G and CaM-CD2-III-5G is $11.4 \times 10^{-7} \text{ cm}^2/\text{s}$. The corresponding hydrodynamic radius is 37.8 Å. These results are very surprising, since they are close to the corresponding values in the absence of metal ions with a slight increase of about 3%, which is within the experimental errors.

The NMR linewidths of the resolved NMR resonances from aromatic residues Trp-7 at 10.5 ppm and Val-78 at 0.3 ppm of wild-type CD2 and its variants were further measured in the presence of 1 mM La(III). No dramatic increase of linewidth was observed with the addition of metal ions. All linewidths range from 9.3 to 14.8 Hz, which is similar to those of metal-free proteins. Given the diffusion constant results, we conclude that the engineered proteins with the insertion of EF-loop from calmodulin are monomers in the absence and presence of metal ions.

4. Discussion

As shown in the Stokes–Einstein equation, any factors that affect the viscosity and temperature will introduce variations in the diffusion constants. The measured diffusion constants are likely affected by sample conditions, such as salt concentrations, protein concentrations, and/or buffer conditions. We have shown that these effects can be normalized/eliminated by using the relative values to the internal references dioxane and Tris. For example, lysozyme is known to be a monomer at protein concentrations up to 1.5 mM [36]. Baseline correction was applied to further overcome the baseline distortion. Using the two internal references, the measured diffusion constants or hydrodynamic radii of this protein remain the same from protein concentrations 0.1–1.2 mM (data not shown). The measured diffusion constant for the protein lysozyme ($10.6 \times 10^{-7} \text{ cm}^2/\text{s}$) agree with the reported value ($10.8 \times 10^{-7} \text{ cm}^2/\text{s}$) [37]. Chen et al. [39] reported that chemical exchange of the “bound” and “free” molecules could sometimes lead to a strong signal distortion. In our cases, we have not observed any signal distortions due to chemical exchanges of calcium binding and calcium-free forms. The errors in Table 1 are based on three different measurements of the same sample. We have shown that the error of all the experiments with CD2 variants is <3%. These data clearly demonstrate that the PFG NMR method with the internal reference has the accuracy and reproducibility to distinguish oligomeric states of proteins under physiological conditions.

The radius and diffusion constant of a dimerized protein can be estimated by treating it as a protein with doubled molecular weight. Teller et al. [44] used the Stokes–Einstein equation to calculate the diffusion constant (D) after oligomerization for various geometries [44]. They suggested that the expected ratio of $D_{\text{dimer}}:D_{\text{monomer}}$ is 0.75 upon dimerization [44]. A further study done by Altieri et al. [37] on the dimerization of ubiquitin has shown that the

ratio of $D_{\text{dimer}}:D_{\text{monomer}}$ is 0.72 [37], which agrees with the monomer–dimer described above. Therefore, a globular well-folded protein with doubled molecular weight will increase its radius by 27% if the protein is assumed to be a well-packed hard sphere. Thus, the diffusion constant (D) is expected to be reduced by about 23%. The diffusion constants for all of the CD2 variants with the insertion of EF-loop III of calmodulin with different numbers of Gly linkers are the same as that of wild type within experimental errors (<3%). Further, these proteins do not have any decreased diffusion constants upon binding of La(III) (Table 1). This suggests that the engineered proteins do not dimerize either in the absence or presence of metal ions.

Siedlecka et al. [25] and Wojcik et al. [26] have shown that the isolated 12-residue peptide encompassing EF-loop III of calmodulin exhibits line broadening of some NMR resonances at 50 °C in the presence of high concentrations of La(III). Such broadening of NMR peaks can be fitted by assuming the peptide dimerized at high temperature. To support their hypothesis that the EF-loop is dimerized at high temperature and retains native EF-loop structure upon La(III) binding, they carried out another experiment using a frozen solution mixed with the EF-loops loaded with La(III) and Ho(III). Based on the observation of the energy transfer from Ho(III) to La(III) and the line broadening of some of the resonances at 50 °C, they concluded that local hydrophobic interactions between the EF-hand calcium-binding loops at loop positions 7 and 8 alone could be responsible for the observed cooperativity of calcium binding to EF-hand protein domains. The relatively strong La(III) binding affinity (6.7 μM) was attributed to the cooperative binding of the formed dimer.

Our PFG NMR experiments carried out at physiological conditions provide direct evidence of the size of the protein upon metal binding. All three proteins containing isolated EF-loop III of calmodulin remain monomeric with and without the bound metal. In contrast to their conclusion, our data unambiguously support our conclusion that the isolated EF-loop III is able to stabilize in solution without the pairing with another EF-hand motif. Our observed monomeric form of EF-loop III of calmodulin in an isolated environment agrees with previous work of the 12-residue peptide of this EF-loop by Borin et al. [45] and Kuboniwa et al. [46]. They did not observe any dimerization of the 12-residue peptide fragment of the calcium-binding loop III of calmodulin both in the presence and absence of Ca(III) or Tb(III). Therefore, additional factors that reside outside of the EF-loop III may contribute to the pairing of EF-hand motifs of calmodulin.

All of the other EF-hand peptide fragments from calmodulin, TnC, parvalbumin, and S100 proteins are reported to be dimers in solution containing several hydrophobic residues from the flanking helices [22,47–51]. Peptide fragments from TnC encompassing EF-hand motifs III and IV with both flanking helices were shown by NMR to be dimerized and to maintain their native structure by Slupsky et al. [52]. They

have observed large hydrogen bonding and hydrophobic interactions for the flanking residues in addition to the two hydrophobic residues in the EF-loop (positions 7 and 8) [22]. Mutation studies of the highly conserved residues of TnC, calmodulin, and S100 proteins have shown that hydrophobic residues on the flanking helices of the EF-hand motif are essential for dimerization [53,54]. Further investigation of the oligomeric state of an isolated EF-hand motif with flanking helices should provide further insight into the dominant forces that stabilize EF-hand structures.

In summary, the isolated EF-loop III of calmodulin in a scaffold host protein remains monomeric both in the presence and absence of metal ions. We have demonstrated that an approach for systematic study of the key determinants for the dimerization, calcium-binding affinity, and calcium-induced conformational changes has been established. Compared to the natural calcium binding in calmodulin and the isolated peptide model, the roles of the loop itself, helices at the ends of the loop, and the cooperativity between the loops can be further dissected without the complication of interactive metal ions, dimerization, and calcium-induced conformational change. Our results demonstrate that a new way to studying isolated EF-hand motifs of trigger proteins has been opened.

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