

## Metal binding affinity and structural properties of an isolated EF-loop in a scaffold protein

Yiming Ye<sup>1</sup>, Hsiau-Wei Lee<sup>1</sup>, Wei Yang<sup>2</sup>, Sarah J. Shealy<sup>1,3</sup>, Anna L. Wilkins<sup>1</sup>, Zhi-ren Liu<sup>4</sup>, Ivan Torshin<sup>2</sup>, Robert Harrison<sup>5</sup>, Robert Wohlhueter<sup>6</sup> and Jenny J. Yang<sup>1,7</sup>

<sup>1</sup>Department of Chemistry and <sup>2</sup>Department of Biology, Center of Drug Design and <sup>3</sup>Department of Computer Science, Georgia State University, Atlanta, GA 30303, <sup>3</sup>Department of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, <sup>4</sup>Department of Animal and Dairy Sciences, Auburn University, Auburn, AL 36849, and <sup>6</sup>Biotechnology Core Facilities Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

<sup>7</sup>To whom correspondence should be addressed.  
E-mail: chejyy@panther.gsu.edu

**To establish an approach to obtain the site-specific calcium binding affinity of EF-hand proteins, we have successfully designed a series of model proteins, each containing the EF-hand calcium-binding loop 3 of calmodulin, but with increasing numbers of Gly residues linking the loop to domain 1 of CD2. Structural analyses, using different spectroscopic methods, have shown that the host protein is able to retain its native structure after insertion of the 12-residue calcium-binding loop and retains a native thermal stability and thermal unfolding behavior. In addition, calcium binding to the engineered CD2 variants does not result in a significant change from native CD2 conformation. The CD2 variant with two Gly linkers has been shown to have the strongest metal binding affinity to Ca(II) and La(III). These experimental results are consistent with our molecular modeling studies, which suggest that this protein with the engineered EF-loop has a calmodulin-like calcium binding geometry and backbone conformation. The addition of two Gly linkers increases the flexibility of the inserted EF-loop 3 from calmodulin, which is essential for the proper binding of metal ions.**

**Keywords:** calcium binding proteins/EF-hand calcium binding motifs/metal-binding affinity/NMR/protein engineering

### Introduction

EF-hand proteins use the ubiquitous EF-hand calcium-binding motif to coordinate diverse cellular functions ranging from muscle control and neuronal signaling to apoptosis and cell cycle control (Falke *et al.*, 1994; Kawasaki and Kretsinger, 1995; Ikura, 1996; Carafoli and Klee, 1999). Such widespread use arises partially from the changeable architecture of the motif, which has enabled its calcium-binding parameters, such as calcium-binding affinity, to be specialized for different biological roles (Kawasaki and Kretsinger, 1995; Miyawaki *et al.*, 1997). According to their different biological roles, the calcium-binding affinities of EF-hand proteins vary over a range of 10<sup>6</sup>-fold or more (Seamon and Kretsinger, 1983; Falke *et al.*, 1994; Kawasaki and Kretsinger, 1995; Linse and Forsén, 1995). Upon calcium binding, the trigger proteins

(calcium-modulated protein or sensor proteins such as calmodulin and troponin C) undergo a large conformational change and in turn regulate a vast number of target proteins (Nelson and Chazin, 1998a; She *et al.*, 1998). Extensive studies have demonstrated that the formation of a calcium-induced molecular surface for the activation of target proteins requires trigger proteins with finely tuned calcium binding affinities. Another class of calcium binding proteins is called non-trigger proteins (or buffer proteins); examples include parvalbumin and calbindin<sub>D9K</sub> (Linse *et al.*, 1988; Kawasaki and Kretsinger, 1995; Nelson and Chazin, 1998b; Cox *et al.*, 1999). The non-trigger proteins also exhibit a range of calcium-binding affinities which influences their roles in calcium homeostatic functions, such as uptake, transport and maintenance of proper calcium concentration in the cellular environment (Nelson and Chazin, 1998a).

The term EF-hand motif was first used by Dr Robert Kretsinger to describe the calcium-binding sites in parvalbumin (Kretsinger and Nockolds, 1973; Kawasaki and Kretsinger, 1995; Kawasaki *et al.*, 1998). A classic EF-hand motif spans 29 residues and consists of a highly conserved calcium-binding loop flanked by two helices. A 12-residue loop (EF-hand loop) contains all of the calcium-binding ligands. Seven oxygen atoms from the side chains of Asp, Asn and Glu, the main chain and water at sequence positions 1, 3, 5, 7, 9 and 12 of the EF-hand calcium-binding loop coordinate the calcium ion in a pentagonal bipyramidal arrangement (Falke *et al.*, 1994; Kawasaki and Kretsinger, 1995). In a typical geometry, the side chain of Asp serves as a ligand on the *x*-axis in the calcium-binding loop (position 1). The  $-x$ -axis (position 9) is filled by a bridged water molecule connecting the side chains of Asp, Ser and Asn (Herzberg *et al.*, 1986; Babu *et al.*, 1988; Strynadka and James, 1989). The  $-z$ -axis is shared by the two carboxyl oxygen atoms of a glutamate side chain at position 12 that binds in a bidentate mode to Ca(II). In natural proteins, two EF-hand motifs linked by a small anti-parallel  $\beta$ -sheet are often associated in the same domain of the protein to yield highly cooperative calcium binding systems (Falke *et al.*, 1994). Calmodulin, for example, contains four EF-hand calcium-binding sites in two domains.

A major barrier to understanding the molecular mechanism of calcium-dependent biological function is the lack of site-specific information about the calcium-binding properties of individual EF-hand motifs. Therefore, the sequence for the calcium-binding process and the contribution of each EF-hand motif to the conformational change of the intact protein currently cannot be defined. Understanding the calcium-binding properties of calmodulin has been the focus of numerous studies, since the activation of calmodulin is directly related to calcium binding (Jarrett and Kyte, 1979; Haiech *et al.*, 1981; Keller *et al.*, 1982; Burger *et al.*, 1984; Ogawa and Tanokura, 1984; Iida and Potter, 1986; Linse *et al.*, 1991; Linse and Forsén, 1995). The interaction among multiple calcium-binding sites for  $K_d$  complicates the understanding of

calcium-binding properties of calmodulin and other EF-hand proteins. The establishment of firm rules relating calcium-binding affinity to structural aspects of EF-hand proteins and the identification of determinants for calcium binding affinity are hampered by the complexities encountered in cooperative, multi-site systems of natural EF-hand proteins and the use of calcium-binding energy in the conformational change of coupled EF-hand motifs. On the other hand, studies of the intrinsic calcium-binding properties of isolated EF hand motifs are limited by a marked decrease in calcium affinity associated with peptide models.

In this paper, we report our investigation of the intrinsic calcium-binding affinity of isolated EF-hand motifs by engineering a single EF-hand calcium-binding site in a scaffold protein, domain 1 of CD2. This novel approach allowed us to focus specifically on the intrinsic binding ability of an individual EF-hand motif while avoiding simplistic peptide models. We have successfully designed a series of model proteins, each containing a single EF-hand calcium-binding loop but with increasing numbers of Gly residues linking the loop to domain 1 of CD2. Specifically, the 12-residue EF-hand calcium-binding loop 3 of calmodulin (CaM) with zero, one or two Gly linkers attached at both the N- and C-termini has been inserted at the junction of two  $\beta$ -strands (C'D) of CD2 (denoted CaM-CD2, CaM-CD2-3G and CaM-CD2-5G). We demonstrate that the host protein is able to retain its native structure after engineering of the 12-residue calcium-binding loop. The modified protein with Gly linkers on both ends of the loop (CaM-CD2-5G) has a native-like thermal stability and the strongest metal affinity to Ca(II) and La(III). In contrast to the large conformational change of calmodulin with the calcium-binding loop flanked by two helices (E and F), calcium binding to the CD2 with the insertion of a calcium-binding loop does not result in a significant change in conformation or thermal stability of the connected protein frame. This suggests that the addition of Gly linkers allows the loop a degree of flexibility required for the binding of calcium by the EF-loop without disturbing the core structure. Our approach creates the opportunity to investigate the intrinsic calcium-binding affinity of EF-hand proteins at a site-specific level.

## Materials and methods

### *Protein engineering, expression and purification of modified CD2*

Clones for expression of CaM-CD2, CaM-CD2-3G and CaM-CD2-5G have been obtained in three steps. Nucleotide sequences coding calcium-binding loop III (12 amino acids) of calmodulin were first inserted into CD2 (CaM-CD2). Subsequently, nucleotide sequences coding a Gly linker (3Gly) were inserted into CaM-CD2 at the 5' end of the loop (CaM-CD2-3G). Finally, nucleotide sequences coding a Gly linker (2 additional Gly) were inserted into CaM-CD2-3G at the 3' end of the loop (CaM-CD2-5G). All three steps were completed using the polymerase chain reaction technique. The newly synthesized DNA (linearized mutant) was then ligated with T4 DNA ligase and subsequently transformed into *Escherichia coli*. After verification by DNA sequence analysis and sub-cloning into expression vector pGEX-2T, the fused GST:CaM-CD2 proteins (~38 kDa) were well expressed using the BL 21

cell line in LB<sub>AMP</sub>. Expression yields were about 30 mg/l. The GST fusion proteins were then cleaved by thrombin and the cleavage protein fragments were further purified by FPLC, using modified purification protocols based on those for w.t. CD2 (Driscoll *et al.*, 1991; Yang *et al.*, 2000a, 2001a). The masses of the purified proteins were verified by mass spectrometry. All three proteins have solubilities >2 mM. Protein concentrations were estimated using an extinction coefficient at 280 nm of 11 700 cm<sup>-1</sup> M<sup>-1</sup> (Driscoll *et al.*, 1991; Yang *et al.*, 2000a).

### *Homology modeling and molecular dynamics simulations*

Models of the modified CD2s (CaM-CD2, CaM-CD2-3G and CaM-CD2-5G) were prepared using rat testis calmodulin (3cln) and rat CD2 (1hng) taken from the Protein Data Bank as templates. For each calmodulin fragment to be included in each model, a set of restraints was produced on the basis of the crystal structure of calmodulin. Application of these restraints during building procedures allows for the preservation of the exact conformation of the calcium-binding site. The initial models were subjected to 10 separate runs of the building procedures: a Kohonen network and/or an analytical building procedure (Harrison, 1999). These 10 separate models were then averaged into one and the structure was minimized. Minimization included short runs of molecular dynamics (I.Weber, I.Torshin, D.Cavanough and R.Harrison, unpublished results). After preparation of the models, the above-mentioned restraints were removed. Homology modeling procedures were implemented using algorithms implemented in AMMP (a modern full-featured molecular mechanics, dynamics and modeling program with SP4 potential set) and using AMMP scripting (Harrison *et al.*, 1995).

The built models were subjected to molecular dynamics (MD) simulations in water. Each of the built models was placed in a spherical water box (about 3000 water molecules) with a radius of 30 Å to prevent 'evaporation' of the solvent molecules. Simulation was performed at 300 K using 0.1 fs steps to obtain smooth MD trajectories and each model was subjected to a 100 ps MD run (700 MHz PC, 1 week/model).

The comparison of the deviation of the C $\alpha$  backbone of the inserted EF-loop in engineered proteins with that in calmodulin was carried out using SYBYL 6.5 with an SGI Indigo 2 IRIX 6.5. Ligand atoms for calcium-binding in the EF-loop were superpositioned.

### *CD spectroscopy*

The CD spectra were measured using a Jasco-710 spectropolarimeter equipped with a temperature-controlled water-bath (Neslab 110). CD cells with a 10 mm path length were used for both far- and near-UV CD spectra. All spectra were the average of eight scans with a scan rate of 50 nm/min. The protein concentrations were 6 and 120  $\mu$ M for both the far- and near-UV CD measurements, respectively. All solutions were prepared using water that was pre-purified on a Chelex-100 (Bio-Rad) chelating column. For thermal denaturation, the temperatures in the CD cells were calibrated using a temperature probe (VWR) inserted into the reference cell containing PBS buffer (pH 7.3).

### *Trp fluorescence spectroscopy*

Fluorescence experiments were performed using a PTI lifetime fluorimeter equipped with a temperature-controlled water-bath (Neslab 110). A fluorescence cell with 1 cm pathlength was

used. The concentration used for fluorescence was 6  $\mu\text{M}$  for all CD2 variants. The scan wavelength for the emission spectrum was from 300 to 400 nm with an excitation wavelength of 283 nm.

#### Data analysis of chemical and thermal denaturation curves

The experimental data from thermal denaturations in far-UV CD were first converted to fractional native with corrected baselines (Santoro and Bolen, 1988; Oliveberg *et al.*, 1994). The data obtained at 25°C and 85°C were used as references for 100% native state (N-state) and 100% high-temperature state (H-state), respectively. Raw data were converted to fractional native and baselines were corrected by the methods of Santoro and Bolen (Santoro and Bolen, 1988). The thermal denaturation profiles were fitted to a two-state model with pre-transition and post-transition baseline corrections (Oliveberg *et al.*, 1994; Myers *et al.*, 1995; Kuhlman *et al.*, 1998).

#### NMR spectroscopy

NMR samples were prepared by diluting proteins in 10.0 mM Tris-KCl, 10% D<sub>2</sub>O at pH 7.5 with either 1.0 mM EGTA or 1.0 mM CaCl<sub>2</sub>. Protein concentrations were limited to ~0.8 mM to avoid aggregation. Dioxane was used as an internal reference for the NMR spectra (3.743 p.p.m.). All NMR spectra were recorded using Varian Unity plus 500 MHz and 600 MHz NMR spectrometers. Spectral widths of 6600 and 8000 Hz were used for NMR at 500 MHz and 600 MHz, respectively. A modified WATERGATE pulse sequence (Piotto *et al.*, 1992; Kay, 1995) was used for 1D NMR with 16K complex data points. DPGFSE-TOCSY (mixing time, 40–90 ms) and wg-NOESY (mixing time, 30–300 ms) (Kay, 1995; Sheng and van Halbeek, 1998) were used for 2D NMR at 5°C. Complex data points of 2K in the  $t_2$  dimension, 300–600  $t_1$  increments and 64 scans for each  $t_1$  increment were used.

The data were processed with the program FELIX95 (MSI). Typically, a squared sine-bell window function shifted over 75° was used. For 2D experiments, the data were zero-filled to yield 2K×2K ( $t_2$ ,  $t_1$ ) data matrices. Post-acquisition suppression of the water signal was achieved by a convolution of a Gaussian function with a function width of 60–90.

#### Metal titration

Metal titrations were carried out using the CD signals at 215 nm and resolved peaks in 1D NMR. Titrations were performed in 10 mM Tris buffer, 10 mM KCl at pH 7.4. For CD measurements, a protein concentration of 6  $\mu\text{M}$  was pre-equilibrated with different Ca(II) or La(III) concentrations. To increase the signal-to-noise ratio, 600 CD scans were averaged at 215 nm. For the NMR experiments, a protein concentration of 0.150 mM was used. Different amounts of metal ions (10–40  $\mu\text{l}$  of 1 mM, 10 mM, 100 mM and 1 M CaCl<sub>2</sub> or LaCl<sub>3</sub> stock solutions at pH 7.4) were gradually injected into the NMR tube using a needle with a long plastic tube. 1D NMR spectra of the proteins were obtained using a modified WATERGATE pulse sequence with 1024 scans at different calcium concentrations.

#### Calculation of $K_d$

Calcium-binding affinities of proteins were calculated using data obtained from calcium titrations by CD and NMR. For CD titrations, the ellipticity of the proteins at 215 nm in 1 mM EGTA was used as  $E_0$  with 0% calcium-binding. The ellipticity at 215 nm in 10 mM CaCl<sub>2</sub> was used as  $E_{100}$  with 100% calcium binding. The fractional change values,  $f$ , at different calcium concentrations were calculated using the equation  $f =$

$(E - E_0)/(E_{100} - E_0)$ . For NMR titrations, the chemical shift values of the resolved peaks in the absence of metal ions were used as  $S_0$  with 0% calcium-binding and the chemical shift values of resolved peaks in 10 mM CaCl<sub>2</sub> or LaCl<sub>3</sub> were used as  $S_{100}$  with 100% metal binding. The fractional change values,  $f$ , at different metal concentrations were calculated using the equation  $f = (S - S_0)/(S_{100} - S_0)$ .

$K_d$  values of proteins were also calculated by fitting the titration curves from CD signals and NMR chemical shift changes using the following equation:

$$F = \frac{([P]_T + [M]_T + K_d) - \sqrt{([P]_T + [M]_T + K_d)^2 - 4[P]_T[M]_T}}{2[P]_T} \quad (1)$$

where  $[M]_T$  and  $[P]_T$  are the total concentration of calcium and protein, respectively (Bagshaw and Harris, 1987; Yang *et al.*, 2000b).

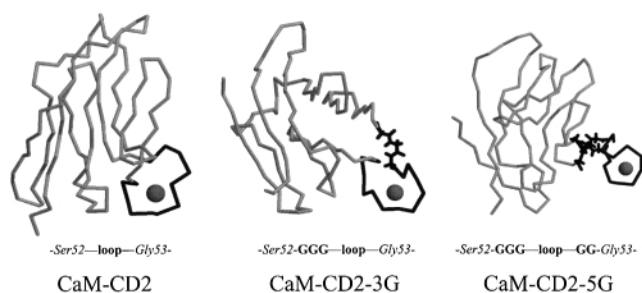
#### Mass spectrometry

Metal-binding uptake by the proteins was analyzed by mass spectrometry using the electrospray technique. A Micromass Quattro LC instrument was used to acquire the data in the positive ion mode by syringe pump infusion of the protein solutions at a flow-rate of 10  $\mu\text{l}/\text{min}$ . All samples were run in either 100% water or 1.0 mM Tris buffer at pH 7.4 to maintain non-denaturing conditions. Calcium (CaCl<sub>2</sub>) or lanthanum (LaCl<sub>3</sub>) was added in 0–200 molar excess over the the protein concentration to observe specific binding. Both ions bind with 1:1 stoichiometry such that the metal-bound mass peak was observed at either  $M + 37$  for calcium or  $M + 137$  for lanthanum. The molecular masses of the apo-form of CaM-CD2, CaM-CD2-3G and CaM-CD2-5G are 12 508, 12 679 and 12 793 Da, respectively. The corresponding molecular masses of Ca(II)-loaded forms are 12 546, 12 679 and 12 793 Da and the corresponding molecular masses of La(III)-loaded forms are 12 645, 12 816 and 12 930 Da.

## Results

#### Design of CD2 variants with different Gly linkers

Our choice for a ‘host system’ is the N-terminal domain of the cell surface adhesion receptor, CD2 from rat (Arulanandam *et al.*, 1993; Davis *et al.*, 1998). CD2 is a very well characterized cell adhesion molecule in the immunoglobulin superfamily (IgSF), with a  $\beta$ -sheet architecture similar to the calcium-dependent cell adhesion molecule, cadherin (Arulanandam *et al.*, 1993; Shapiro *et al.*, 1995; Nagar *et al.*, 1996; Davis *et al.*, 1998; Yang *et al.*, 2000b). Domain 1 of rat CD2 was shown to be an excellent model protein for the engineering of EF-hand motifs (Yang *et al.*, 2000a, b), for several reasons. First, domain 1 of CD2 is small (99 amino acids) with several structures solved at high resolution (Driscoll *et al.*, 1991; Jones *et al.*, 1992; Withka *et al.*, 1993; Bodian *et al.*, 1994; Wyss *et al.*, 1995). Second, CD2 can tolerate protein engineering. More than 40 mutations have been generated to identify key residues required for cell adhesion and these mutations have not exhibited any effect on the expression, solubility and structural integrity of CD2 (Arulanandam *et al.*, 1993; Davis *et al.*, 1998; Yang *et al.*, 2000a,b, 2001). Third, far-UV circular dichroism (CD) and high-resolution NMR studies indicate that three surface charged mutants, K41A, K45E and R87E, do not change the secondary and tertiary structures of CD2. Fourth, high-resolution NMR studies show that changing the pH from 1 to 10 and increasing the KCl concentration from 0.1 mM to

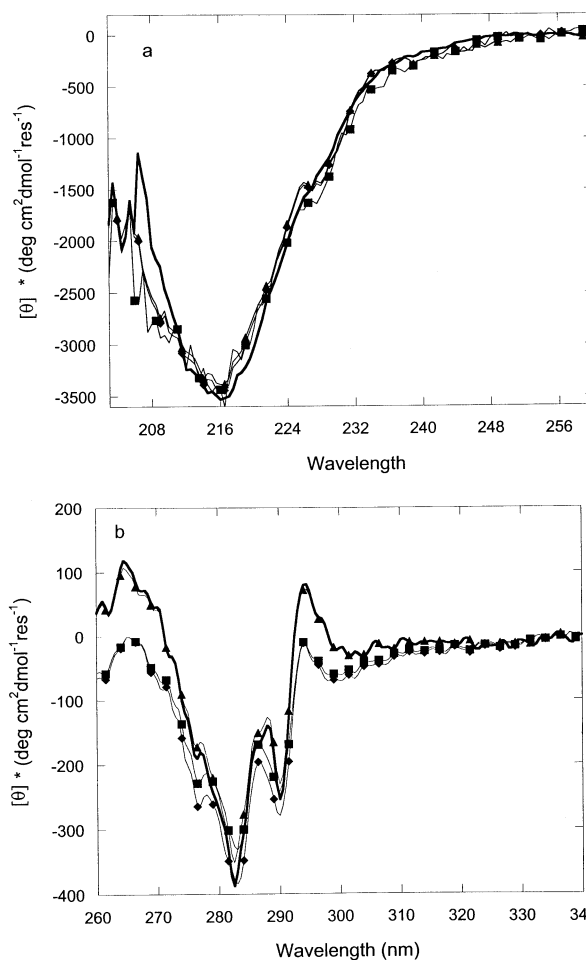


**Fig. 1.** The modeled structures of CaM-CD2, CaM-CD2-3G and CaM-CD2-5G of 100 ps of molecular dynamics in water at 300 K. The inserted calcium-binding loop III from calmodulin and the Gly linker(s) are highlighted.

4.5 M do not lead to any significant change in the native structure, suggesting that electrostatic interactions play a minor role in the folding of CD2 (Yang *et al.*, 2000a). In addition, CD2 contains an exposed Trp at position 7 and a buried Trp at position 32. Therefore, the intrinsic Trp fluorescence and near-UV CD signals can be used to monitor calcium binding and the conformational change of the protein.

A solvent-exposed loop region, C''D (residues 52–53, SG) in CD2, was selected for the insertion of an EF-loop (Figure 1), in order to minimize disruption to the host protein's hydrogen bonding network, hydrophobic core packing and likelihood of interactions between the calcium-binding motif and the host protein. Previous studies have shown that mutations at position 52 of CD2 did not change the adhesion affinity significantly, suggesting that the integrity of the protein is not disrupted by protein engineering at this location (Recny *et al.*, 1990; Davis *et al.*, 1998). Therefore, the EF-loop III of calmodulin has been inserted at position G53 of CD2, between strands C'' and D (CaM-CD2) to dissect specifically the contribution of the EF-loop to calcium-binding affinity.

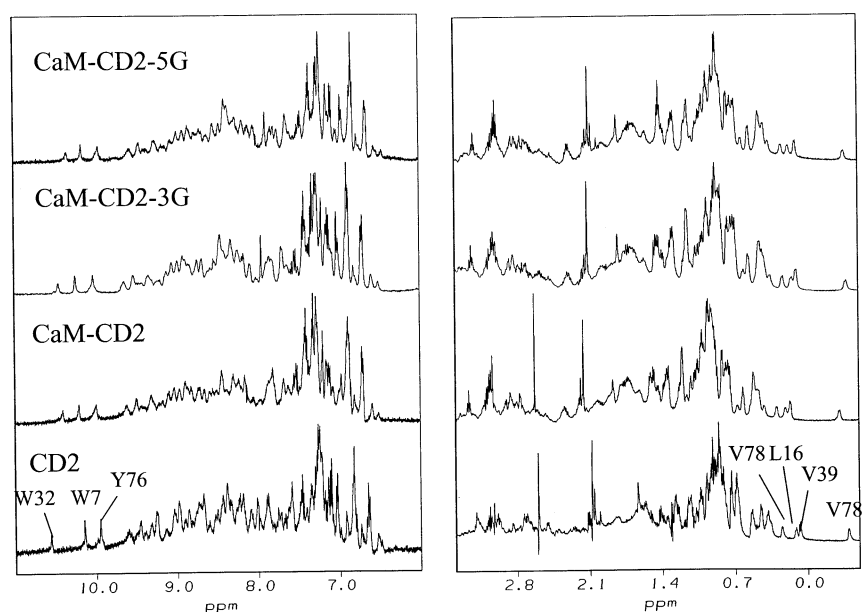
The calcium-binding loop III (site III) of calmodulin was chosen to investigate the intrinsic calcium-binding affinity because extensive studies using peptide/fragments and mutagenesis on the intrinsic calcium-binding affinity of this EF-loop are available (Borin *et al.*, 1989; Marsden *et al.*, 1990; Dadlez *et al.*, 1991; Linse *et al.*, 1991; Falke *et al.*, 1994; Linse and Forsén, 1995; Wojcik *et al.*, 1997; Wu and Reid, 1997). The distances between the two terminal C $\alpha$  atoms of the calcium-binding loop III in calcium-loaded (3cln.pdb) and calcium-free (1cfd.pdb) forms are 6.62 and 9.01 Å, respectively, while the distance between the termini of the C'' (Ser52) and D'' (Gly53) of CD2 (1hng.pdb) that will connect to the calcium-binding loop is only 3.83 Å. Therefore, Gly linkers were added at the termini of inserted EF-loop to avoid the alteration of the required local geometry for calcium binding at the same loop position of CD2. Two additional CD2 variants were engineered with the insertion of the EF-loop III of calmodulin with a Gly linker at its N-terminus (CaM-CD2-3G) or with Gly linkers at both ends (CaM-CD2-5G) to investigate further the influence of the loop flexibility on the calcium-binding affinity (Figure 1). The attachment of different numbers of Gly linkers is proposed to affect the ability of the inserted EF-loop to adopt the required conformation for calcium-binding by reducing conformational constraints imposed by the host protein.



**Fig. 2.** Far-UV CD (a) and near-UV CD (b) spectra of w.t. (solid line), CaM-CD2 (squares), CaM-CD2-3G (diamonds) and CaM-CD2-5G (triangles) in 10 mM Tris, 10 mM KCl at pH 7.4 at 25°C.

#### Conformational analysis of CD2 variants with inserted EF-loop

Several spectroscopic methods were applied to probe the different levels of the structures of the engineered proteins. This was completed in order to investigate the conformation of the host protein after insertion of the calcium-binding loop. The far-UV CD, and near-UV CD spectra of three CD2 variants and wild-type (w.t.) in 10 mM Tris at pH 7.5 are displayed in Figure 2. Both the far-UV and near-UV CD spectra of CaM-CD2, CaM-CD2-3G and CaM-CD2-5G are very similar to that of w.t., suggesting that the secondary and tertiary structures of these modified proteins are well folded and the native structure of CD2 is retained after the insertion of the EF-loop. This is further supported by the close similarity of the 1D  $^1\text{H}$  NMR spectra at both the amide and side chain regions of three CD2 variants to that of w.t.. As shown in Figure 3, the chemical shifts of the dispersed NMR signals for the aromatic ring protons of Trp32, Trp7 and Tyr76 and the signals for the methyl protons of the side chains of Val78, Val39 and Leu16 of the three CD2 variants are almost identical with those of w.t. CD2. The majority of the dispersive signals in the fingerprint regions of the TOCSY and NOESY spectra of the CD2 variants are located at the same positions as that of w.t. (data not shown), suggesting that the integrity and



**Fig. 3.** 1D  $^1\text{H}$  NMR spectra at amide (left) and side chain (right) regions of 0.150 mM w.t. CD2, CaM-CD2, CaM-CD2-3G and CaM-CD2-5G in 10 mM Tris, 10 mM KCl at pH 7.4 at 25°C.

packing of the host protein frame are maintained after the insertion of the calcium-binding loop from calmodulin.

#### *Effect of Gly linkers on the thermal stability of CD2 variants*

Our previous studies have shown that a partially folded intermediate with an increased molar ellipticity at 222 nm can be induced for w.t. CD2 by thermal denaturation (Yang *et al.*, 2000a,b). The change in the CD spectra of CD2 variants as a function of temperature was measured to investigate if introducing the EF-hand calcium-binding loop with different Gly linkers places different conformational constraints on the host protein CD2 or even perturbs the folding behavior of CD2. As shown in Figure 4, with increasing temperature in the presence of 1 mM EGTA, 10 mM Tris at pH 7.5, all three CD2 variants increase their molar ellipticity at 222 nm, similar to that of the w.t. protein. Further increasing the temperature to 90°C does not induce any additional change in the far-UV CD spectrum of the CD2 proteins. These data suggest that the insertion of an EF-loop into CD2 does not alter the thermal denaturation behavior of the host protein CD2. Like w.t. CD2, a non-native folding intermediate has been induced at the high temperature for the three CD2 variants (Yang *et al.*, 2000a). Estimated by the fractional changes of CD signal at 220 nm as a function of temperature of w.t. CD2 and its three CD2 variants, CaM-CD2 without the Gly linker has the lowest  $T_m$  of 42°C whereas CaM-CD2-3G and CaM-CD2-5G with one and two Gly linkers, respectively, have significantly higher thermal stability ( $T_m = 55^\circ\text{C}$ ), which is similar to that of the w.t. protein (56°C).

#### *Contribution of calcium binding to the thermal stability*

The effect of calcium binding on thermal stability was further investigated by monitoring the CD signal as a function of temperature in the presence of 1.0 mM  $\text{CaCl}_2$ . As shown in Table I, the thermal stability of CaM-CD2-5G is increased by 7°C in the presence of 1 mM  $\text{CaCl}_2$  compared with that obtained in the presence of 1 mM EGTA. On the other hand, the  $T_m$  values of CaM-CD2, CaM-CD2-3G and w.t. CD2 remain unchanged in the presence of calcium ions.

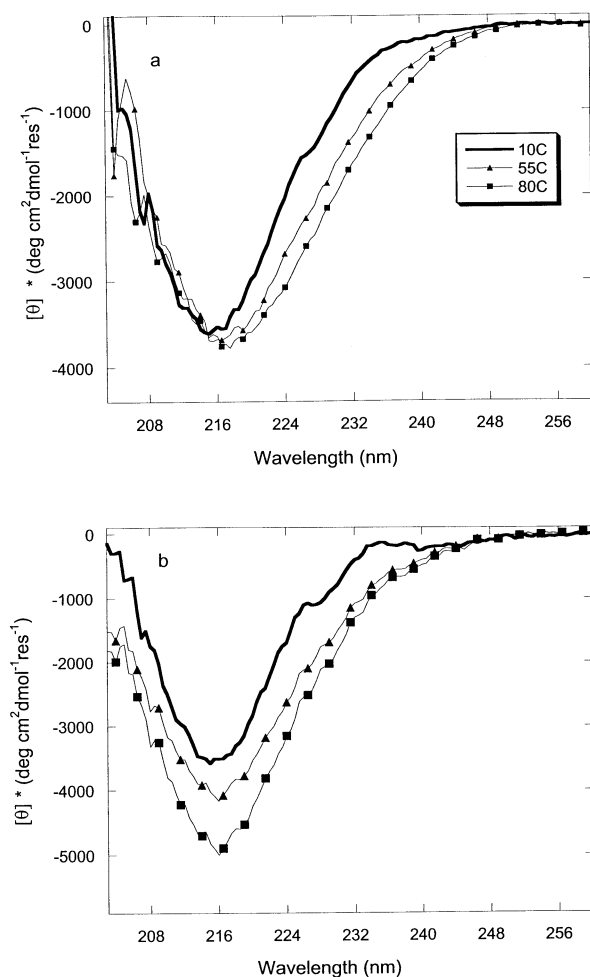
#### *Monitoring metal binding by mass spectrometry*

Figure 5 shows the electrospray mass spectra of CaM-CD2, CaM-CD2-3G and CaM-CD2-5G in the presence of  $\text{CaCl}_2$  or  $\text{LaCl}_3$ . In the absence of metal ions, the observed molecular masses of these three proteins are the same as calculated based on their corresponding primary sequences (see Materials and methods, and visible as the lower-mass peak in these spectra). In the presence of a 20–200-fold molar excess of Ca(II) or La(III), all three modified CD2 proteins with an inserted calcium-binding loop show an additional peak (Figure 5) with molecular masses of  $38 \pm 2$  or  $137 \pm 2$  Da greater than those for the corresponding apo-proteins. The increased molecular masses of 38 and 138 Da are consistent with the formation of a calcium- or lanthanum-protein complex (Veenstra *et al.*, 1997). W.t. CD2, on the other hand, has an identical molecular mass in the absence and presence of the same amount of metal ions (data not shown). These results suggest that the CD2 variants with the insertion of an EF-loop have the ability to bind Ca(II) and La(III). The appearance of a single dominant metal adduct in the metal-bound form indicates the formation of a 1:1 metal-protein complex.

#### *Monitoring metal binding affinity by spectroscopic methods*

The calcium-binding affinities of these three engineered proteins were first measured by monitoring Trp emission fluorescence as a function of metal concentrations after the removal of trace amounts of metal ions by a Chelex 100 column (Figure 6, Table II). W.t. CD2 and free Trp amino acid were used as negative controls. As shown in Figure 6, the Trp emission fluorescence intensity at 327 nm of CaM-CD2-5G (6  $\mu\text{M}$ ) gradually decreases with the addition of metal ions without changing the emission maximum greatly. The fractional bound curves of this protein can be well fitted by Equation 1 (see Materials and methods), assuming the formation of a 1:1 metal-protein complex (Bagshaw and Harris, 1987; Yang *et al.*, 2000a). The best-fitting  $K_d$  value for Ca(II) is  $(1.86 \pm 0.4^\circ) \times 10^{-4}$  M. Table II summarizes the  $K_d$  values determined for the three CD2 variants.

These calcium-binding constants were further verified by monitoring the CD and NMR signal changes as a function of metal ions (Figures 6 and 7). The addition of metal ions does not change the shape of the CD spectra, but does decrease the molar ellipticity by about 5–10%, suggesting that the conformations of these CD2 variants are not changed significantly by the binding of metal ions. This conclusion is supported also by the 1D NMR studies. As shown in Figure 7, the overall pattern of NMR spectra of CaM-CD2-5G at both side chain and amide regions remains unchanged in the presence and absence of metal ions. On the other hand, the chemical shift values of several resonances such as at 6.94, 6.96 and



**Fig. 4.** Far-UV CD spectra of w.t. CD2 (a) and CaM-CD2-5G (b) at 10 (solid line), 55 (triangles) and 80°C (squares) in 10 mM Tris, 1 mM EGTA at pH 7.4.

**Table I.**  $T_m$  of CaM-CD2, CaM-CD2-3G and CaM-CD2-5G compared with that of w.t. CD2 in 10 mM Tris, 10 mM KCl at pH 7.4

Protein	$T_m$ (°C)	
	In 1 mM EGTA	In 1 mM CaCl <sub>2</sub>
w.t. CD2	56 ± 2	58 ± 1
CaM-CD2	43 ± 2	42 ± 2
CaM-CD2-3G	55 ± 2	53 ± 2
CaM-CD2-5G	55 ± 1	62 ± 1

7.89 p.p.m. (marked by arrows) gradually move with the increase in metal ions. By monitoring the fractional changes at 222 nm for the CD signal and chemical shifts at 6.94, 6.96 and 7.89 p.p.m. in the 1D <sup>1</sup>H NMR spectra, the calcium-binding affinities of three CD2 variants with the inserted EF-loop were also determined and are summarized in Table II. All the  $K_d$  values obtained by Trp fluorescence at 328 nm, the CD signal at 215 nm and NMR chemical shifts agree very well. CaM-CD2-5G, with two linkers, has the highest calcium-binding affinity with a  $K_d$  of  $1.86 \times 10^{-4}$  M, whereas CaM-CD2, without a Gly linker, has a relatively weaker metal binding affinity with  $K_d$  between 1.7 and 5.1 mM. Such a weak binding ability of CaM-CD2 excludes the accurate measurement of its  $K_d$  values.

La(III) has an ionic radius similar to that of Ca(II) and it is commonly used as a probe for calcium binding of proteins (Garipey *et al.*, 1983; Dadlez *et al.*, 1991). Using the same spectroscopic methods, we examined the metal affinity of CD2 variants for La(III). As shown in Table II, under identical conditions, CD2 with inserted EF-loops exhibits stronger metal binding affinities for La(III) than for Ca(II), which is consistent with many observations that La(III) ions bind more strongly to calcium-binding proteins than Ca(II) ions because of their relative higher positive charge. As with calcium binding, the Trp emission maximum, the shape of the far-UV CD spectra (Figure 6) and the overall pattern of 1D NMR resonances at both amide and side chain regions are not changed greatly by the addition of La(III) for all three CD2 variants (data not shown). The chemical shifts of limited resonances such as at 6.94, 6.96 and 7.89 p.p.m. are changed by the addition of metal ions. Our results suggest that metal binding to the calcium-binding loop III from calmodulin in CD2 does not exhibit a significant conformational change as observed in calmodulin in which EF-hand loops are flanked by two EF-hand helices.

#### Effect of 5% TFE on the calcium affinity

As sketched in Figure 1, the last four residues of the EF-loop participate in the first turn of the flanking F-helix. To examine if a low TFE concentration is able to enhance the metal binding affinity of the inserted EF-loop by encouraging/stabilizing the native secondary structure of proteins, we measured metal binding affinity of the three CD2 variants for Ca(II) and La(III) in the presence of 5% TFE (Table II). In the presence of 5% TFE, the far-UV CD, Trp fluorescence and NMR spectra of all three CD2 variants are identical with those in the absence of the organic solvent, suggesting that these proteins are able to retain their native conformation. Under the same conditions, these spectra are not altered by the addition of Ca(II) and La(III). As shown in Table II, the addition of 5% TFE results in a decrease in metal binding affinity of CaM-CD2-5G for Ca(II) and La(III) of about 3- and 2-fold, respectively.

## Discussion

*The structure and thermal unfolding of the engineered protein is not changed upon insertion of a calcium-binding loop*

As shown in Figures 2 and 3, conformational analyses of CD2 with inserted EF-loop variants using different biophysical methods suggest that engineering an EF-loop from calmodulin into CD2 does not alter the native structure of the protein. The thermal stability of the modified protein without the Gly linker (CaM-CD2) is reduced about 13°C after directly attaching the EF-loop III from calmodulin. Apparently the

added flexibility conferred by one or two Gly linkers leads to the preservation of the thermal stability of the native CD2 (Figure 4, Table I) in the absence of metal ions.

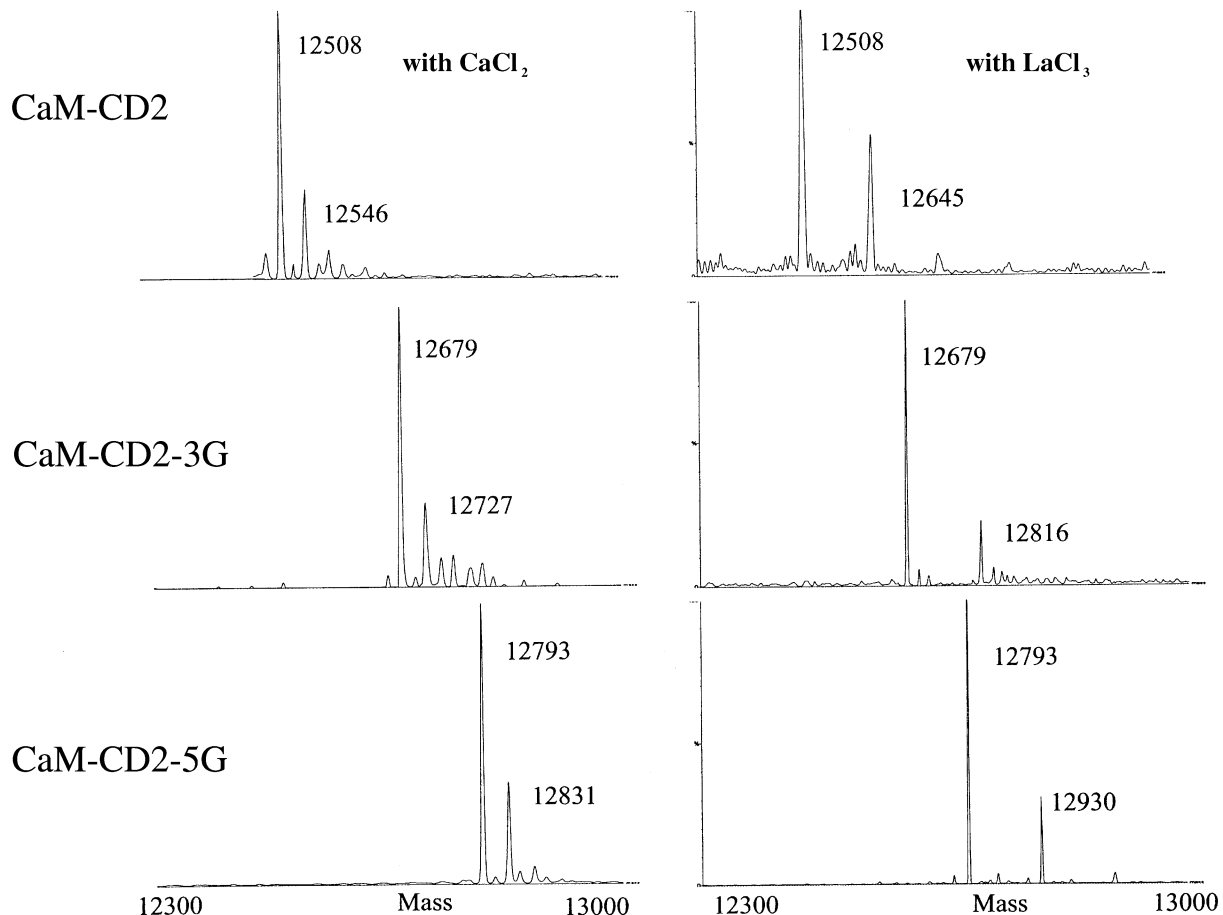
Our previous equilibrium unfolding studies have shown that at high temperatures, a partially folded state with increased negative molar ellipticity at 222 nm is formed for w.t. CD2 (Yang *et al.*, 2000ab, 2001). About 11% of the  $\beta$ -structure is converted to an  $\alpha$ -helical conformation as estimated by both FT-IR and CD methods, indicating the formation of a non-native helical conformation. The existence of this non-native high-temperature intermediate is consistent with relatively high intrinsic helical propensities in the primary sequence of w.t. CD2. Like w.t. CD2, all three CD2 variants inserted with zero, one and two Gly linkers joining the EF-loop of calmodulin to the CD2 backbone, exhibit increased CD signal at high temperature, suggesting that the insertion of an EF-loop from calmodulin does not perturb the thermal unfolding behavior of the host protein (Yang *et al.*, 2000a). The reduced  $T_m$  value of CaM-CD2 compared with that of the w.t. protein is probably due to the conformational constraints introduced by direct connection of the EF-loop to the host protein.

*EF-loop with different numbers of Gly linkers in CD2 exhibits different metal binding ability*

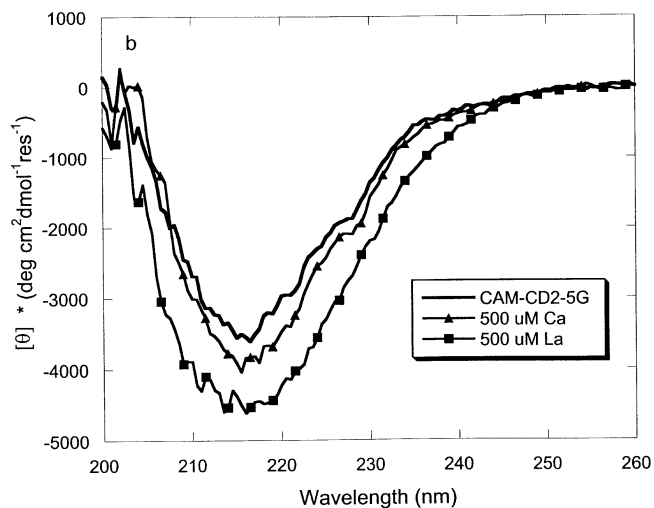
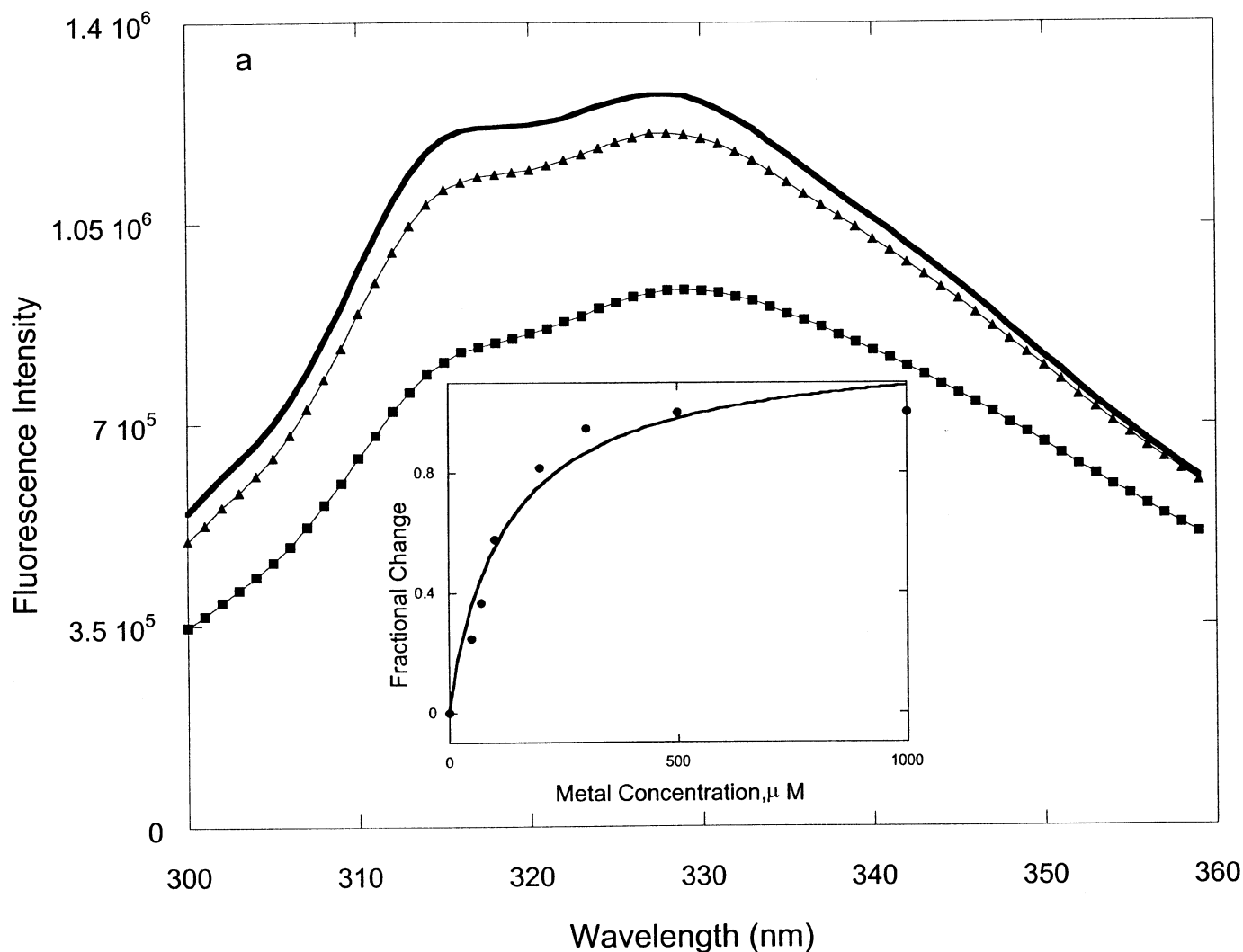
As shown in Table II, comparing the CD2 variant with two Gly linkers (CaM-CD2-5G) with one without a Gly linker (CaM-CD2) reveals at least a 10-fold greater affinity for both Ca(II) and La(III). Since the binding of Ca(II) or La(III) does not result in a significant change in far-UV, Trp fluorescence

and NMR spectra (Figures 6 and 7), the host protein CD2 is able to maintain its native-like secondary and tertiary structure. This structural stability contrasts dramatically with the trigger proteins calmodulin and troponin C, which undergo large conformational change upon binding calcium. In the case of the CD2 variants, where large free energy contributions from conformational entropy do not complicate the picture, the measured calcium-binding affinity of the protein should directly reflect its intrinsic metal binding ability (Voordouw *et al.*, 1976; Veltman *et al.*, 1998). Therefore, strong metal binding in the site should also lead to an increase in thermal stability ( $T_m$ ) in the presence of 1 mM CaCl<sub>2</sub> compared with that in the presence of 1 mM EGTA. For example, the  $T_m$  of CaM-CD2-5G in 1 mM CaCl<sub>2</sub> is about 7°C greater than the  $T_m$  in the absence of metal ions, which correlates with its strongest metal binding affinity. On the other hand, the  $T_m$  values of CaM-CD2 and CaM-CD2-3G in 1 mM CaCl<sub>2</sub> and in 1 mM EGTA, which have at least 10-fold weaker metal binding affinities than that for CaM-CD2-5G, are about the same.

It is interesting that NMR resonances, such as at 6.94, 6.96 and 7.89 p.p.m., whose chemical shifts change with the addition of Ca(II) and La(III), do not exist in the NMR spectrum of the w.t. protein. In addition, the  $K_d$  values obtained by monitoring the fractional change of chemical shifts at these three different resonances are identical, implying that residues involved in metal binding probably arise from the inserted EF-loop 3 of calmodulin. More detailed structural analysis of the engineered CD2 variants using high-resolution NMR is under way.



**Fig. 5.** Mass spectra of CaM-CD2 (200-fold molar excess of metal ions), CaM-CD2-3G and CaM-CD2-5G in the presence of a 20-fold molar excess of CaCl<sub>2</sub> (left) and LaCl<sub>3</sub> (right).



**Fig. 6.** (a) Trp fluorescence emission spectra (excited at 283 nm) and (b) far-UV CD spectra of 6  $\mu\text{M}$  of CaM-CD2-5G in 0 (solid line), 0.5 mM  $\text{CaCl}_2$  (triangles) and 0.10 mM  $\text{LaCl}_3$  (squares) in 10 mM Tris buffer at pH 7.4. The fractional changes of Trp emission fluorescence signal at 327 nm as a function of  $\text{LaCl}_3$  concentration is shown as an inset in (a).

Molecular modeling studies suggest that CD2 variants have a different local structure around the EF-loop

The structural basis for the differences in the calcium-binding affinity of three CD2 variants inserted with the same EF-hand loop from site III of calmodulin with different Gly linkers was analyzed by molecular modeling. Homology models were built for the three CD2 variants. Since homology models cannot in themselves define structural variation as they are a single model, molecular dynamics was used to estimate the relative stability of the three CD2 variants. The modeled structures of CaM-CD2, CaM-CD2-3G and CaM-CD2-5G of 100 ps of molecular dynamics in water at 300 K for these CD2 variants are shown in Figure 1. As shown in Figure 8, like the EF-loop III in calmodulin, the amino acid residues at loop positions 1, 3, 5, 7 and 12 are also used as direct calcium ligands in the modeled structure of CaM-CD2-5G. The Ca–O distances to the side chains of Asp at loop positions 1 and 3, Asn at position 5 and to Glu at position 12 and the main chain

carbonyl of Tyr at position 7 are  $2.4 \pm 1.0 \text{ \AA}$  (Yang *et al.*, 2000c), suggesting that the calcium-binding geometry of the loop with two Gly linkers in this protein is well retained. On the other hand, the model structures of the protein with no (CaM-CD2) and one Gly linker (CaM-CD2-3G) were shown to have a distorted calcium-binding geometry. CaM-CD2 has two Asp residues (at positions 1 and 3) functioning as bidentate ligands instead of a unidentate ligand. CaM-CD2-3G has one Asp at position 3 serving as a bidentate ligand. In addition, residues Asn and Tyr (at positions 5 and 7) of CaM-CD2 were not able to coordinate calcium because they were forced to have longer Ca–O distances, 6.0 and 7.8  $\text{\AA}$ , respectively. As shown in Figure 8A, the modeling results suggest that the engineered proteins approximate the calmodulin calcium-binding site (based on the Ca–O bond lengths) as CaM-CD2-5G  $\gg$  CaM-CD2-3G  $>$  CaM-CD2, an order which correlates very well with the metal binding affinity and the metal-induced increase in thermal stability of CaM-CD2-5G (Tables II and I, respectively).

Additional evidence supporting the idea that the loop flexibility provided by two Gly linkers is crucial for calcium binding comes from the detailed comparison of the backbone of the calmodulin EF-loop III in the CD2 variants with that in native calmodulin. As shown in Figure 8B, the deviation of the C $\alpha$  backbone of CaM-CD2-5G from that of calmodulin for each residue in the EF-loop III is  $<1.6 \text{ \AA}$ . In contrast, CaM-CD2 and CaM-CD2-3G have a much greater deviation in the backbone C $\alpha$  from that of calmodulin (up to 5.8  $\text{\AA}$ ). Interestingly, CaM-CD2-5G has the highest calcium-binding affinity and is stabilized most by the addition of calcium, suggesting that our modeling study is consistent with the experimental data.

#### Comparison with calmodulin and peptide studies

Examining a host protein CD2 by three different methods, we have shown that the calcium-binding affinity ( $K_d$ ) of the

**Table II.** Summary of metal binding affinities of CD2 variants in 10 mM Tris, 10 mM KCl at pH 7.4

Protein	$K_d$ Ca(II) ( $\mu\text{M}$ )	$K_d$ La(III) ( $\mu\text{M}$ )
CaM-CD2-5G	$186 \pm 40$ $600 \pm 50^a$	$58 \pm 9$ $770 \pm 40^a$
CaM-CD2-3G	$>3000$	$420 \pm 5$
CaM-CD2	1700–5100	570–1500
EF-loop peptide	$1400^b$ – $5000^c$	$6.7^{c,d}$
Calmodulin (site III/site IV)	$0.31^e$ – $3.1^f$	

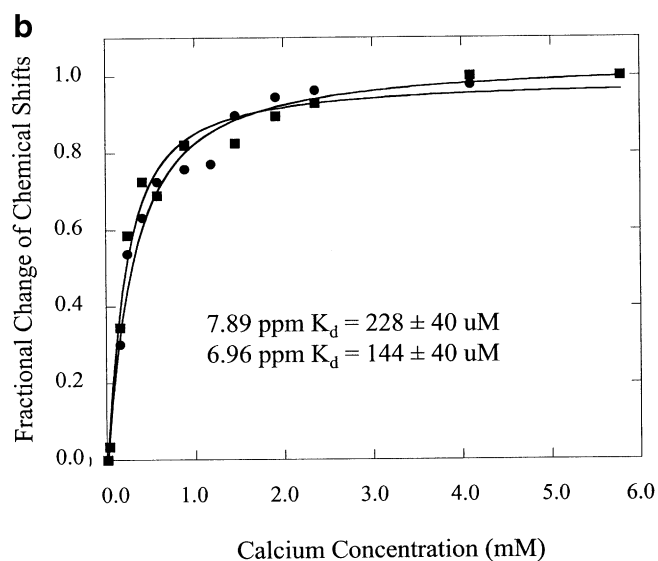
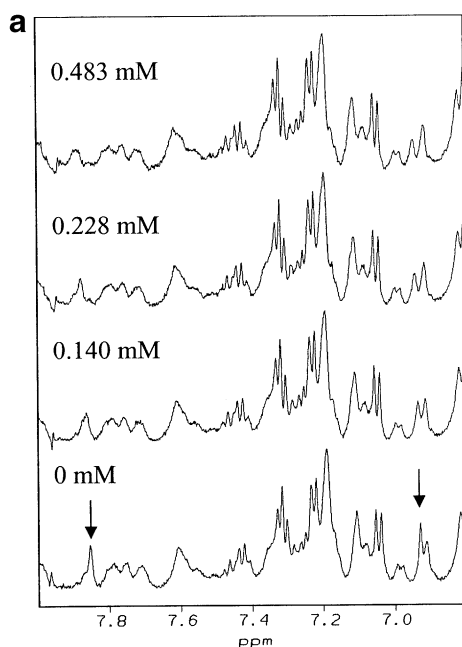
<sup>a</sup>In 2 mM Tris at pH 7.4 with 5% TFE.

<sup>b</sup>Borin *et al.* (1989).

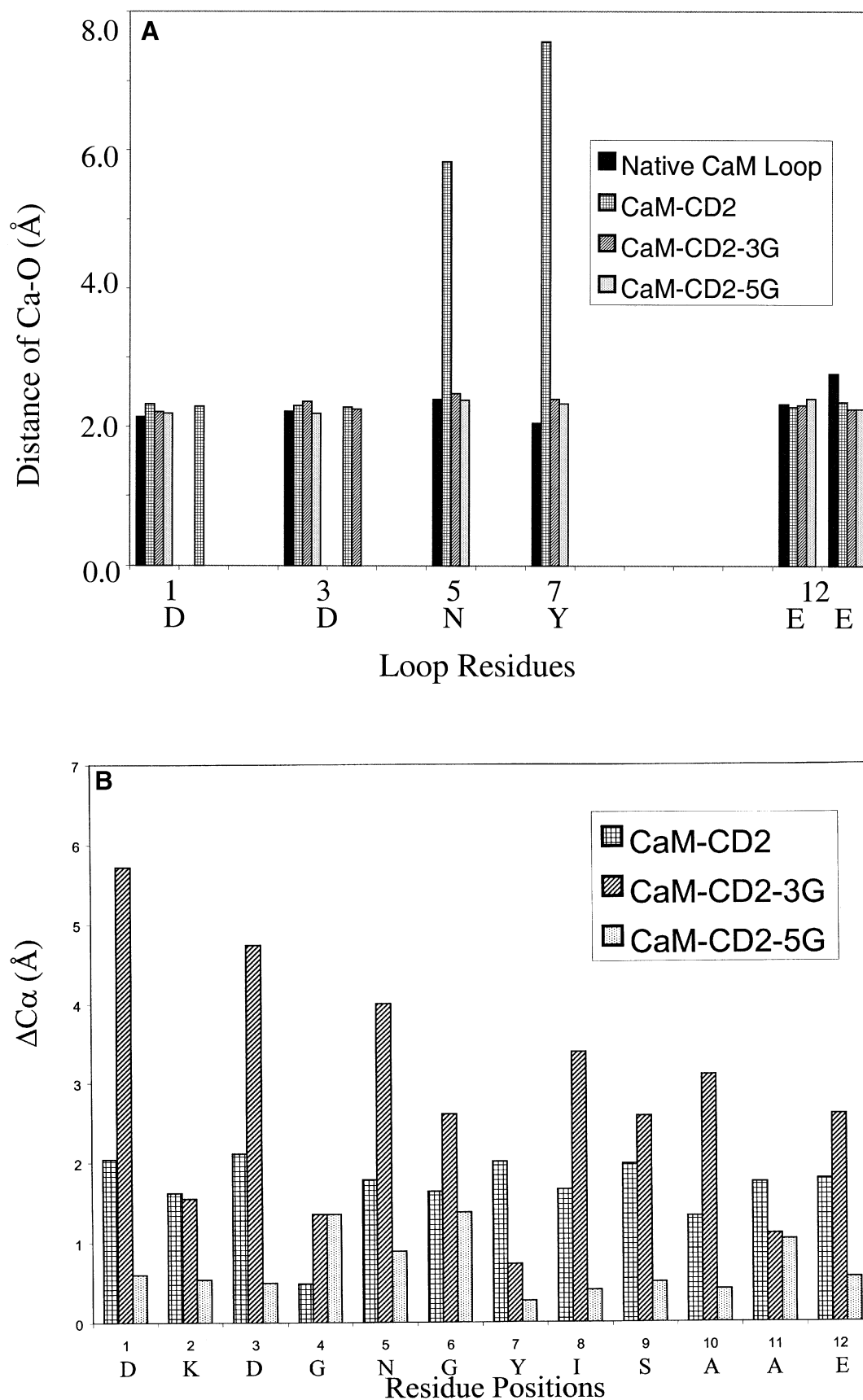
<sup>c</sup>Dadlez *et al.* (1991).

<sup>d</sup>Wojcik *et al.* (1997).

<sup>e,f</sup>Linse *et al.* (1991).



**Fig. 7.** (a) 1D  $^1\text{H}$  NMR spectra of CaM-CD2-5G at calcium concentrations of 0, 0.140, 0.228 and 0.483 mM (from bottom to top) at 6.8–8.0 p.p.m. in 10 mM Tris, 10 mM KCl at pH 7.4 at 25°C. (b) Fractional change of chemical shifts at 6.96 (squares) and 7.89 p.p.m. (circles) for CaM-CD2-5G as a function of calcium concentration. Both chemical shift changes are fitted by assuming 1:1 binding (solid line).



**Fig. 8.** (A) Comparison of the Ca-O distances of residues in EF-loop III of calmodulin (black) with that of modeled structures of CaM-CD2 (square hatched), CaM-CD2-3G (striped) and CaM-CD2-5G (dotted). (B) Comparison of the backbone structure of the inserted EF-loop 3 from calmodulin in CD2 from molecular dynamic studies with that in calmodulin. The r.m.s.d. difference in the  $C\alpha$  of CaM-CD2 (square hatched), CaM-CD2-3G (striped) and CaM-CD2-5G (dotted) is plotted as a function of amino acid positions of the EF-loop.

calmodulin EF-loop III with two Gly linkers (CaM-CD2-5G) is 0.186 mM in 10 mM Tris and 10 mM KCl at pH 7.4 (Table II). This affinity is about 10–30-fold greater than the published calcium-binding affinity of the isolated peptide of the same EF-loop (Table II,  $K_d = 1.4\text{--}5.0$  mM) under similar conditions. The relatively weaker calcium-binding affinity of a peptide fragment is probably due to the lack of a well-defined conformation of peptides in solutions (Malik *et al.*, 1987; Borin *et al.*, 1989; Dadlez *et al.*, 1991; Linse and Forsén, 1995; Kuhlman *et al.*, 1997; Yang *et al.*, 2000a). La(III) is shown to have similar coordination properties and higher positive charges. The metal-binding affinity of CaM-CD2-5G for La(III) is about 0.058 mM, which is about 3-fold stronger than that for Ca(II) in aqueous solution (Table II).

Low concentrations of TFE (5–10%, v/v) have been shown to stabilize native-like conformations of peptide fragments (Marsden *et al.*, 1990; Procyshyn and Reid, 1994; Kuhlman *et al.*, 1997; Yang *et al.*, 2000a) and to increase the dynamic flexibility of intact proteins without altering their native tertiary structures (Yang *et al.*, 1995; Buck, 1998). One would expect the metal-binding affinity of CD2 variants with the inserted EF-loop to increase with the addition of TFE. However, our studies here have shown that the Ca(II) and La(III) binding affinities decreased by 3- and 2-fold respectively on addition of 5% TFE compared with that in the absence of the organic solvent while no obvious change in the conformations of CD2 variants in the presence and absence of metal ions is observed. It is likely that the added Gly linkers provide enough flexibility to allow the calcium-binding loop to bind calcium properly in the presence of an organic solvent, which is in strong contrast to many calcium-binding peptide fragments (Marsden *et al.*, 1990; Procyshyn and Reid, 1994; Kuhlman *et al.*, 1997; Yang *et al.*, 2000a).

Bierzynski and colleagues have shown that the isolated 12-residue peptide with both ends blocked does not dimerize or show altered NMR resonances in the presence of calcium, possibly owing to much weaker calcium-binding affinity (about 10 mM) (Wojcik *et al.*, 1997; Siedlecka *et al.*, 1999). On the other hand, this synthetic peptide does dimerize in the presence of La(III) ions. The binding of La(III) to the peptide is highly cooperative with a strong La(III) binding affinity ( $K_d = 6.7$   $\mu$ M) under similar conditions. In contrast, we have not observed any metal-induced dimerization of the CD2 with an inserted EF-loop at protein concentrations ranging from 2 to 500  $\mu$ M. We have applied gradient-diffusion NMR experiments and far-UV CD methods as a function of calcium and protein concentrations to monitor the oligomeric state of CaM-CD2-5G (Altieri *et al.*, 1995; Jones *et al.*, 1997). The diffusion constants of CaM-CD2-5G in the presence and absence of metal ions are about the same as that of the w.t. protein and of proteins of similar size such as lysozyme. No significant change in the linewidth of NMR resonances on addition of Ca(II) and La(III) is observed, suggesting that CaM-CD2-5G remains a monomer at protein concentrations up to 0.6 mM and in the presence of Ca(II) or La(III) at concentrations up to 1 mM.

The measurement of the intrinsic calcium-binding affinity of an individual EF-hand motif requires a model system to overcome the limitation of dynamic properties and multiple interactive ions of natural EF-hand proteins. Using trypsin to cleave the calmodulin into two domains (TR<sub>1</sub>C and TR<sub>2</sub>C), Linse, Forsén and co-workers showed that the calcium-binding affinities of the two sites (sites III and IV)

in the C-terminal domain of calmodulin are, on average, 6-fold higher than those of the sites in the N-terminal domain (sites I and II) (Linse *et al.*, 1991). The separated domains retain the calcium-binding properties (macroscopic constant) that they have in the intact molecules, suggesting that there is no cooperativity between the two domains (Linse *et al.*, 1991). Unfortunately, owing to the lack of information about  $\eta = K_{II}/K_I$ , they could not provide an accurate estimation of the cooperativity between two coupled sites. By assuming that both sites in each of the two domains have equal calcium-binding affinities (i.e.  $\eta = 1$ ), they estimated that sites III and IV in the C-terminal domain of calmodulin exhibit relatively higher positive cooperativity than those in the N-terminal domain. However, the assumption that two sites in both the N- and C-terminal domains of calmodulin each have equal affinity is unlikely, since four calcium-binding loops of calmodulin have different ligand arrangements and charge distributions (Reid and Hodges, 1980; Marsden *et al.*, 1990; Falke *et al.*, 1994; Kawasaki and Kretsinger, 1995; Linse and Forsén, 1995). The average calcium-binding affinity for sites III and IV is about 0.31–3.1  $\mu$ M (Linse *et al.*, 1991; Falke *et al.*, 1994). Obtaining further site-specific calcium-binding constants by mutation of two coupled sites in N- and C-terminal domains is complicated by the strong interaction between two interactive metal ions and by the alteration of the stability of both the apo- and holo-forms of calcium-binding proteins. Any mutations that result in the change of the calcium-free state and the calcium-bound state of EF-hand proteins will lead to either an increase or a decrease in the calcium-binding affinity (Weinstein and Mehler, 1994; Kuboniwa *et al.*, 1995; Henzl *et al.*, 1998; Malmendal *et al.*, 1999; Tsuda *et al.*, 1999). Waltersson *et al.* have shown that the change of Asp to Asn at position 3 of site II in the loop leads to increases in both calcium-binding affinity and cooperativity of the N-terminal domain. However, the same mutation at site III leads to an opposite effect in the C-terminal domain (Waltersson *et al.*, 1993). Wu and Reid have shown that replacing Asp by Glu at position 5 of the site IV Ca(II)-binding loop drastically decreased the calcium-binding affinity of the site (2760-fold) and also significantly reduced that of site III in the same domain (24-fold) (Wu and Reid, 1997).

Our measured intrinsic calcium affinity of EF-loop III using a scaffold protein CD2 is about 60-fold weaker than the average macroscopic calcium affinities of calmodulin with sites III and IV (~0.3–3.1  $\mu$ M) (Linse *et al.*, 1991; Falke *et al.*, 1994). Such a difference in calcium affinity probably comes from the contributions of both flanking helices and cooperative interaction between two EF-loops in calmodulin. Our approach creates the opportunity to investigate the intrinsic calcium-binding affinity of EF-hand proteins at a site-specific level, especially from the current two-domain level toward the site-specific level. The relative calcium affinity of each calcium-binding loop of EF-hand proteins can be studied using this established approach. This novel approach also allows us systematically to introduce and study the role of the building blocks of EF-hand motifs. Currently we are using this approach to unravel the role of flanking helices in calcium-binding affinity of EF-hand motifs. Information obtained about the intrinsic calcium-binding affinity of isolated EF-hand motifs will help define the contribution of each EF-hand site to the overall calcium-binding ability of the intact protein and the sequence for calcium-binding in a multi-EF-hand protein.

Therefore, the contribution of the local protein context can be dissected and the minimum elements required for calcium-binding can be identified. This information is essential for us to construct further model systems to understand the molecular mechanism of calcium-induced conformational change and cooperative binding of EF-hand proteins.

In conclusion, by engineering isolated 12-residue EF-loop 3 from calmodulin into a scaffold protein CD2, we have shown that the structure and thermal unfolding of the engineered protein are not changed upon insertion of an EF-calcium-binding loop. The attachment of an inserted EF-loop with zero, one and two Gly linkers results in the host protein exhibiting different calcium-binding affinities. Modeling studies suggest that CD2 with the addition of two Gly linkers to the inserted EF-loop has a calmodulin-like calcium-binding geometry and backbone conformation, which is consistent with our experimental results that this CD2 variant has both the highest calcium-binding affinity and is stabilized by the addition of calcium. An approach for dissecting the contribution of the calcium-binding loop on the calcium-binding affinity of EF-hand proteins has been established.

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### References

- Altieri, A.S., Hinton, D.P. and Byrd, R.A. (1995) *J. Am. Chem. Soc.*, **117**, 7566–7567.
- Arulanandam, A.R., Withka, J.M., Wyss, D.F., Wagner, G., Kister, A., Pallai, P., Recny, M.A. and Reinherz, E.L. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 11613–11617.
- Babu, Y.S., Bugg, C.E. and Cook, W.J. (1988) *J. Mol. Biol.*, **204**, 191–204.
- Bagshaw, C.R. and Harris, D.A. (1987) In Bagshaw, C.R. and Harris, D.A. (eds), *Spectrophotometry and Spectrofluorimetry: a Practical Approach*. IRL Press, Washington, DC, pp. 91–113.
- Bodian, D.L., Jones, E.Y., Harlos, K., Stuart, D.I. and Davis, S. (1994) *Structure*, **2**, 755–766.
- Borin, G., Ruzza, P., Rossi, M., Calderan, A., Marchiori, F. and Peggion, E. (1989) *Biopolymers*, **28**, 353–369.
- Buck, M. (1998) *Q. Rev. Biophys.*, **31**, 297–355.
- Burger, D., Cox, J.A., Comte, M. and Stein, E.A. (1984) *Biochemistry*, **23**, 1966–1971.
- Carafoli, E. and Klee, C.B. (1999) *Calcium as a Cellular Regulator*. Oxford University Press, Oxford.
- Cox, J.A., Durussel, I., Scott, D.J. and Berchtold, M.W. (1999) *Eur. J. Biochem.*, **264**, 790–799.
- Dadlez, M., Goral, J. and Bierzynski, A. (1991) *FEBS Lett.*, **282**, 143–146.
- Davis, S.J., Davies, E.A., Tucknott, M.G., Jones, E.Y. and van der Merwe, P.A. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 5490–5494.
- Driscoll, P.C., Cyster, J.G., Campbell, I.D. and Williams, A.F. (1991) *Nature*, **353**, 762–765.
- Falke, J.J., Drake, S.K., Hazard, A.L. and Peersen, O.B. (1994) *Q. Rev. Biophys.*, **27**, 219–290.
- Garipey, J., Sykes, B.D. and Hodges, R.S. (1983) *Biochemistry*, **22**, 1765–1772.
- Haiech, J., Klee, C.B. and Demaille, J.G. (1981) *Biochemistry*, **20**, 3890–3897.
- Harrison, R.W. (1999) *J. Math. Chem.*, **26**, 125–137.
- Harrison, R.W., Chatterjee, D. and Weber, I.T. (1995) *Proteins*, **23**, 463–471.
- Henzl, M.T., Hapak, R.C. and Likos, J.J. (1998) *Biochemistry*, **37**, 9101–9111.
- Herzberg, O., Moul, J. and James, M.N. (1986) *J. Biol. Chem.*, **261**, 2638–2644.
- Iida, S. and Potter, J.D. (1986) *J. Biochem. (Tokyo)*, **99**, 1765–1772.
- Ikura, M. (1996) *Trends Biochem. Sci.*, **21**, 14–17.
- Jarrett, H.W. and Kyte, J. (1979) *J. Biol. Chem.*, **254**, 8237–8244.
- Jones, E.Y., Davis, S.J., Williams, A.F., Harlos, K. and Stuart, D.I. (1992) *Nature*, **360**, 232–239.
- Jones, J.A., Wilkins, D.K., Smith, L.J. and Dobson, C.M. (1997) *J. Biomol. NMR*, **10**, 199–203.
- Kawasaki, H. and Kretsinger, R.H. (1995) *Protein Profile*, **2**, 297–490.
- Kawasaki, H., Nakayama, S. and Kretsinger, R.H. (1998) *Biometals*, **11**, 277–295.
- Kay, L.E. (1995) *Curr. Opin. Struct. Biol.*, **5**, 674–681.
- Keller, C.H., Olwin, B.B., LaPorte, D.C. and Storm, D.R. (1982) *Biochemistry*, **21**, 156–162.
- Kretsinger, R.H. and Nockolds, C.E. (1973) *J. Biol. Chem.*, **248**, 3313–3326.
- Kuboniwa, H., Tjandra, N., Grzesiek, S., Ren, H., Klee, C.B. and Bax, A. (1995) *Nature Struct. Biol.*, **2**, 768–776.
- Kuhlman, B., Boice, J.A., Wu, W.J., Fairman, R. and Raleigh, D.P. (1997) *Biochemistry*, **36**, 4607–15.
- Kuhlman, B., Boice, J.A., Fairman, R. and Raleigh, D.P. (1998) *Biochemistry*, **37**, 1025–1032.
- Linse, S. and Forsén, S. (1995) *Adv. Second Messenger Phosphoprotein Res.*, **30**, 89–151.
- Linse, S., Brodin, P., Johansson, C., Thulin, E., Grundstrom, T. and Forsén, S. (1988) *Nature*, **335**, 651–652.
- Linse, S., Helmersson, A. and Forsén, S. (1991) *J. Biol. Chem.*, **266**, 8050–8054.
- Malik, N.A., Anantharamaiah, G.M., Gawish, A. and Cheung, H.C. (1987) *Biochim. Biophys. Acta*, **911**, 221–230.
- Malmendal, A., Evenas, J., Forsén, S. and Akke, M. (1999) *J. Mol. Biol.*, **293**, 883–899.
- Marsden, B.J., Shaw, G.S. and Sykes, B.D. (1990) *Biochem. Cell Biol.*, **68**, 587–601.
- Miyawaki, A., Liopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M. and Tsien, R.Y. (1997) *Nature*, **388**, 882–887.
- Myers, J.K., Pace, C.N. and Scholtz, J.M. (1995) *Protein Sci.*, **4**, 2138–2148.
- Nagar, B., Overduin, M., Ikura, M. and Rini, J.M. (1996) *Nature*, **380**, 360–365.
- Nelson, M.R. and Chazin, W.J. (1998a) *Protein Sci.*, **7**, 270–282.
- Nelson, M.R. and Chazin, W.J. (1998b) *Biometals*, **11**, 297–318.
- Ogawa, Y. and Tanokura, M. (1984) *J. Biochem. (Tokyo)*, **95**, 19–28.
- Oliveberg, M., Vuilleumier, S. and Fersht, A.R. (1994) *Biochemistry*, **33**, 8826–8832.
- Piotto, M., Saudek, V. and Sklenar, V. (1992) *J. Biomol. NMR*, **2**, 661–665.
- Procyshyn, R.M. and Reid, R.E. (1994) *J. Biol. Chem.*, **269**, 1641–1647.
- Recny, M.A., Neidhardt, E.A., Sayre, P.H., Ciardelli, T.L. and Reinherz, E.L. (1990) *J. Biol. Chem.*, **265**, 8542–8549.
- Reid, R.E. and Hodges, R.S. (1980) *J. Theor. Biol.*, **84**, 401–444.
- Santoro, M.M. and Bolen, D.W. (1988) *Biochemistry*, **27**, 8063–8068.
- Seamon, K.B. and Kretsinger, R.H. (1983) *Calcium in Biology*. Wiley, New York, pp. 1–51.
- Shapiro, L., Fannon, A.M., Kwong, P.D., Thompson, A., Lehmann, M.S., Grubel, G., Legrand, J.F., Als-Nielsen, J., Colman, D.R. and Hendrickson, W.A. (1995) *Nature*, **374**, 327–335.
- She, M., Xing, J., Dong, W.J., Umeda, P.K. and Cheung, H.C. (1998) *J. Mol. Biol.*, **281**, 445–452.
- Sheng, S. and van Halbeek, H. (1998) *J. Magn. Reson.*, **130**, 296–299.
- Siedlecka, M., Goch, G., Ejchart, A., Sticht, H. and Bierzynski, A. (1999) *Proc. Natl Acad. Sci. USA*, **96**, 903–908.
- Strynadka, N.C. and James, M.N. (1989) *Annu. Rev. Biochem.*, **58**, 951–998.
- Tsuda, S., Miura, A., Gagne, S.M., Spyropoulos, L. and Sykes, B.D. (1999) *Biochemistry*, **38**, 5693–5700.
- Veenstra, T.D., Johnson, K.L., Tomlinson, A.J., Naylor, S. and Kumar, R. (1997) *Biochemistry*, **36**, 3535–3542.
- Veltman, O.R., Vriend, G., Berendsen, H.J., Van den Burg, B., Venema, G. and Eijssink, V.G. (1998) *Biochemistry*, **37**, 5312–5319.
- Voordouw, G., Milo, C. and Roche, R.S. (1976) *Biochemistry*, **15**, 3716–3724.
- Waltersson, Y., Linse, S., Brodin, P. and Grundstrom, T. (1993) *Biochemistry*, **32**, 7866–7871.
- Weinstein, H. and Mehler, E.L. (1994) *Annu. Rev. Physiol.*, **56**, 213–236.
- Withka, J.M., Wyss, D.F., Wagner, G., Arulanandam, A.R., Reinherz, E.L. and Recny, M.A. (1993) *Structure*, **1**, 69–81.
- Wojcik, J., Goral, J., Pawlowski, K. and Bierzynski, A. (1997) *Biochemistry*, **36**, 680–687.
- Wu, X. and Reid, R.E. (1997) *Biochemistry*, **36**, 8649–8656.
- Wyss, D.F., Choi, J.S., Li, J., Knoppers, M.H., Willis, K.J., Arulanandam, A.R., Smolyar, A., Reinherz, E.L. and Wagner, G. (1995) *Science*, **269**, 1273–1278.
- Yang, J.J., Buck, M., Pitkeathly, M., Kotik, M., Haynie, D.T., Dobson, C.M. and Radford, S.E. (1995) *J. Mol. Biol.*, **252**, 483–491.
- Yang, J.J., Carroll, A.R., Yang, W., Ye, Y.M. and Nguyen, C. (2000a) *Cell Biochem. Biophys.*, **33**, 253–273.

- Yang,J.J., Yang,H.D., Ye,Y.M., Hopkins,H. and Hastings,H. (2001a) *Cell Biochem. Biophys.*, in press.
- Yang,J.J., Ye,Y.M., Carroll,A.R., Yang,W. and Lee,H.W. (2001b) *Curr. Protein Pept. Sci.*, **2**, 1–17.
- Yang,W., Lee,H.W., Pu,M., Hellinga,H. and Yang,J.J. (2000c) *Computational Studies, Nanotechnology and Solution Thermodynamics of Polymer Systems*. Kluwer Academic/Plenum, New York, 127–138.
- Yang,W., Tsai,T., Kats,M. and Yang,J.J. (2000b) *J. Pept. Res.*, **55**, 203–215.

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