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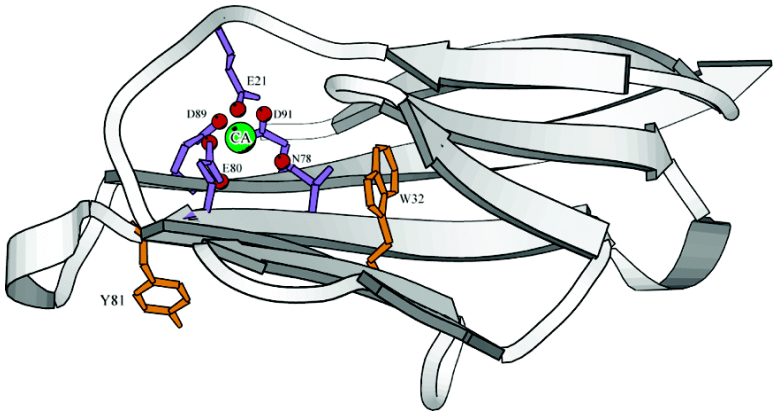

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Rational Design of a Calcium-Binding Protein

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Abstract: Calcium ions play key roles as structural components in biomineralization and as a second messenger in signaling pathways. We have introduced a de novo designed calcium-binding site into the framework of a non-calcium-binding protein, domain 1 of CD2. The resulting protein selectively binds calcium over magnesium with calcium-binding affinity comparable to that of natural extracellular calcium-binding proteins (K_d of 50 μM). This experiment is the first successful metalloprotein design that has a high coordination number (seven) metal-binding site constructed into a β-sheet protein. Our results demonstrate the feasibility of designing a single calcium-binding site into a host protein, taking into account only local properties of a calcium-binding site obtained by a survey of natural calcium-binding proteins and chelators. The resulting site exhibits strong metal selectivity, suggesting that it should now be feasible to understand and manipulate signaling processes by designing novel calcium-modulated proteins with specifically desired functions and to affect their stability.

Introduction

The intracellular concentrations of magnesium and potassium are about 1.0 and 150 mM, respectively. In the resting eukaryotic cell, the calcium concentration is approximately 10^4-10^6-fold lower than that of magnesium and potassium, respectively.[1] Yet, calcium, and only calcium, is able to be not only an essential structural component in the biomineralization of teeth, bones, and shells but also a "second messenger" that controls numerous cellular processes such as cell division and growth, secretion, ion transport, and muscle contraction through calcium-induced conformational change.[2−4] Signal proteins with different Ca(II)-binding affinities mediate and regulate Ca(II)-dependent functions both extracellularly and intracellularly. Calcium ions couple tightly with the calcium-binding receptors on the outside of cell membranes to regulate the movement of calcium into a cell, as well as activate/trigger a wide range of hydrolytic enzymes and cell adhesion molecules.[5] The strong metal selectivity of calcium-binding proteins over other metal ions appears to be closely related to physiological processes.[2,6,7] Understanding key determinants contributing to calcium affinity and selectivity and calcium-dependent conformational change is the essential step for unraveling the process of calcium signaling.

The rational design of novel proteins is a benchmark for our insight into the structure, folding, and function of proteins. Since its elementary beginnings in 1986,[8−11] this field has progressed rapidly to the point that it is now possible to consider tertiary and quaternary structures and even functional elements, moving toward constructing new biomaterials, sensors, catalysts, and pharmaceutical drugs.[12−18] Designing calcium-binding sites in a non-calcium-binding scaffold protein contributes to our understanding of the protein design process in general and the

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biological role of calcium ions in particular. It allows us to define the key determinants for calcium-binding affinity and metal selectivity by establishing the required metal coordination ligands and geometry for calcium binding in a scaffold that lacks the complication of global conformational changes and the cooperative effect of multiple calcium-binding sites in proteins, which are frequently observed in natural calcium-binding proteins.[6,19] However, the rational design of calcium-binding sites into proteins with a strong selectivity is extremely challenging and daunting for several reasons.[20−22] First, calcium-binding sites commonly adopt pentagonal bipyramidal or distorted octahedral geometries with a large range of deviation in bond lengths and angles. Second, the calcium coordination spheres observed in naturally evolved proteins vary in coordination number from 6 to 9 with one to three coordinating ligands contributed by solvent molecules. If waters are identified and counted, the coordination number of calcium is almost 7 in proteins. Calcium ions are predominantly chelated with oxygens from several types of groups such as carboxylates (bi- and monodentate interactions), carbonyls (main-chain or amide side-chain), and hydroxyls (either from protein side-chains or solvent hydroxyls). Because of the complexity and irregularity in the calcium-binding sites, the rational design of calcium-binding sites into proteins with a strong selectivity is extremely challenging, even, according to some, impossible.[20−22]

In this paper, we present the de novo introduction of a single calcium-binding site into a protein scaffold that is devoid of such sites. Unlike previously published regional swapping experiments based on the strong structural similarity,[23−26] our design strategy does not rely on sequence or structural similarity between the recipient scaffold and a naturally evolved donor protein. Instead, the strategy takes into account the general local calcium-binding properties such as ligand types, charge, and the geometry of the primary coordination sphere and then identifies a constellation of backbone positions in the recipient scaffold that allows the introduction of the metal-binding site by mutations with potential ligand residues. We have chosen as the scaffold the N-terminal domain of the rat cell surface adhesion receptor CD2. CD2 has a β-sheet structure with the common IG-fold similar to the other 3000 in the family. CD2 is one of the best-characterized cell adhesion molecules in the immunoglobulin superfamily. It was chosen because several structures are available,[27,28] the domain is small (99 amino acids), and it has a predominantly β-sheet architecture with a buried Trp-32. In addition, the expression, solubility, and structural integrity [29−31] of this domain with single or double mutations remains unchanged. Our lab has shown that grafting the EF-loop III from calmodulin into CD2 does not alter the tertiary structure of the protein.[32]

Materials and Methods

Design Process. The calcium-binding protein design was carried out on an SGI O2 computer using the Dezymer program and following the established procedure.[33−35] A geometric description of the ligands around a metal, the three-dimensional structure of the backbone of a protein, and a library of side-chain rotamers of amino acids (or atoms from the main-chain) were input into the Dezymer algorithm to identify a set of potential metal-binding sites. The first residue located in the calculation (called anchor) defines the relative position of the calcium atom to the protein backbone and is used as a starting point to construct a calcium-binding site. After attaching the anchor residue to the backbone of the protein along the protein sequence, the calcium-binding geometry or positions of other ligands are then defined around the anchor. The CD2 structure (1hng)[28] was chosen as the host structure for the construction of a new calcium-binding site. One bidentate glutamate and four unidentate ligands from glutamate, aspartate, asparagine, and/or glutamine were used for the calculations. The parameters derived from the ideal pentagonal bipyramidal geometry with allowed floating ranges for Ca−O lengths (2.0−3.0 Å, ideal is 2.4 Å), O−Ca−O angles (30−120°, 90−180°, and 45−135° for the ideal values of 72°, 144°, and 99°, respectively), and C−O−Ca−O dihedral angles (0−45° for those on the plane and 45−135° for those off the plane) were used in the first step of the Site-search. The constructed sites were minimized on the basis of the ideal geometry in the second step of Refinement. CD2.Ca1 was selected after further analysis of the location of calcium-binding sites, side-chain clash induced by mutations,[36,37] and the number of charged residues in the binding coordination shell.[38]

Protein Engineering and Purification. CD2.Ca1 was cloned by two stages of standard PCR from wild-type CD2 DNA. The first stage mutated F21E and the second stage mutated the other four residues (V78N, V80E, L89D, and K91D). The four primers used were ATCCCTAACGAGCAAATGACTGAT, GTTCAGGTTGATGCCATGACC, GGGACACGTATCGATAACGATGCACTGGAC, and ATTTGTGCGTATTCCGTGTTATTATAGGT manufactured by Life Technology. The mutations were confirmed by auto-sequencing at the Advanced Biotechnology Core Facilities at Georgia State University. The DNA was ligated into vector pGEX-2TKM, which contained the GST tag sequence and a PreScission cleavage sequence. The protein was expressed in the *E. coli* BL21 strain and purified using Glutathione Sepharose 4B beads (Pharmacia). The fusion protein was cleaved on a Glutathione Sepharose 4B column using PreScission protease (Pharmacia) in 50 mM Tris, 150 mM NaCl, 1 mM DTT, pH 7.0. GST and the PreScission enzyme remained on the column, and only the desired cleaved protein flowed through the column. The protein concentration was calculated on the basis of the extinction coefficient of 11 700 cm^{-1} M^{-1} for w.t. CD2.[27]

Terbium Fluorescence. All Tyr/Trp-sensitized fluorescent resonance energy transfer experiments were performed on a PTI fluorimeter with

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slit widths of 8 and 12 nm for excitation and emission, respectively. A glass filter with cutoff of 320 nm was used to avoid Raleigh scattering. The emission spectra were collected from 520 to 570 nm with an excitation wavelength at 282 nm. The terbium titration was performed in 100 mM MOPS pH 6.9 by gradually adding terbium stock solution (1 mM) into 2.2 μM CD2.Ca1 solution. The same concentration of protein was incorporated into the metal stock solution to avoid dilution of the protein concentration due to titration. Thirty minutes of equilibrium time was allowed between each point. For the metal competition study, the solution containing 30 μM of terbium and 2.2 μM of protein was used as the starting point. The stock solutions of each metal (La(III), Ca(II), and Mg(II)) containing the same amounts of terbium and protein were gradually added in. The contribution of Tb(III) background to the emission at 545 nm was determined using blank metal solutions with 30 μM Tb(III) in the absence of protein for every metal concentration.

The fluorescence intensity at 545 nm was first normalized by subtracting the contribution of the baseline slope. The contribution of intrinsic Tb(III) background (blank) was then removed from that of fluorescence intensity of the protein sample. The Tb(III)-binding affinity of CD2.Ca1 was obtained by fitting the Tb(III) titration data using the 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 f is the fractional change, K_d is the dissociation constant, and $[P]_T$ and $[M]_T$ are the total concentrations of protein and metal, respectively.

The metal competition data of CD2.Ca1 was analyzed using the apparent dissociate constant of the competitive metal ion obtained by eq 1. Because CD2.Ca1 is almost saturated with Tb(III) at the starting point of the competition, this apparent binding affinity has the relationship with the true binding affinities and Tb(III) concentration as

$$K_{d2} = K_{app} \times \frac{K_{d1}}{K_{d1} + [M_1]} \quad (2)$$

where K_{d1} and K_{d2} are the dissociation constants of Tb(III) and the competing metal ion, respectively, K_{app} is the apparent dissociation constant, and $[M_1]$ is the Tb(III) concentration.[39]

NMR Spectra. One-dimensional ^{1}H NMR spectra were recorded on Varian Inova 500 and 600 MHz NMR spectrometers with a spectral width of 8000 Hz (for 800 MHz) or 6600 Hz (for 500 MHz) and complex data points of 16K using a modified WATERGATE pulse sequence at 25 °C. CD2.Ca1 (200 μM) was in 10 mM MOPS pH 6.8 with 5% D_2O. Metal titrations were carried out by gradually adding 5−10 μL of the metal stock solutions (10 and 100 mM for calcium, 10 mM for lanthanum, and 100 mM and 4 M for potassium) to the samples with less than 10% dilution of the protein concentration for the final metal concentration. The data were processed with the program FELIX98 (MSI) with an exponential line broadening of 2 Hz window function (EM2).

ICP-MS. The designed protein (0.1 mM) was dialyzed (3K cutoff membrane) against 10 mM Tris pH 7.4 with 10 μM of Ca(II) at 4 °C for 72 h. The metal contents of the dialysis buffer and the dialyzed protein solution after acidification with 1% HNO_3 were determined using inductively coupled plasma-mass spectrometry (ICP-MS) to give free metal and total metal concentrations, respectively. The ICP-MS instrument used was a Finnigan MAT Element2, a double-focusing instrument with reverse Nier-Johnson geometry. Three isotopes, ^{42}Ca, ^{43}Ca, and ^{44}Ca, were used for calcium. Raw data was produced in counts per second for each isotopic species. External standards were used to determine an absolute concentration calibration curve for all isotopes of interest. The elemental concentrations in the standard solutions have an uncertainty of approximately ±2.5%. A concentration of 1 ppb ^{115}In was added as an internal standard in all samples and standard solutions to compensate for sample-to-sample variations in instrument performance. Calibration curves are established for each isotope using blank-subtracted intensities from the standard solutions. After a sample was run, the blank-subtracted intensities were used to determine absolute concentrations of each isotope from the calibration curves.

CD Study. The far-UV CD spectra of CD2.Ca1 were obtained from 190 to 260 nm with a bandwidth of 0.5 nm at room temperature using a Jasco 810 spectropolarimeter. A rectangular cell with a light path length of 1 cm was used. The protein (2.5 μM) was in 1 mM MOPS pH 6.8. The metal titration was carried out by gradually adding the stock solutions preequilibrated with the same protein concentration. To increase the sensitivity, 10 min collection of 600 points at 205 nm was averaged at each metal concentration. The dissociation constants were obtained by fitting the data using eq 1.

Results and Discussion

Common Features of Natural Calcium-Binding Proteins Were Used To Design and Filter Calcium-Binding Sites. To achieve the task of designing de novo calcium-binding sites in proteins, we have carried out detailed structural analysis of more than 500 calcium-binding sites in small chelators and natural calcium-binding proteins as well as extensive folding studies of the host protein CD2. After surveying several classes of naturally evolved calcium-binding proteins, we chose the most common geometry of pentagonal bipyramid for the coordination shell of the target calcium-binding site in CD2 (CD2.Ca1). Parameters describing the pentagonal bipyramidal geometry of calcium-binding sites such as the Ca−O bond lengths (2.0−3.0 Å), O−Ca−O angles (30−120°), and O−Ca−O−Cδ dihedral angles (0−45° for the oxygen on the pentagonal plane and 45−135° for that off the plane) in proteins were established and tested on naturally evolved calcium-binding proteins.[33−35] Analogous to the natural sites, one of the seven coordination sites was left open to allow solvent to access and to reduce steric crowding in our description. Two oxygen atoms are from the bidentate Glu. The remaining four positions were occupied by unidentate ligand oxygen atoms contributed by the carboxyl groups of Asp, Asn, Glu, and/or main-chain carbonyls. Approximately 7000 potential sites were generated by the program.

The designed proteins were filtered using several criteria. First, the calcium should be solvent accessible. Second, the mutations should introduce little or no side-chain steric conflicts with the preexisting atoms. Third, minimal disruption of hydrogen bonding and hydrophobic interactions is required. Fourth, three or four negatively charged residues at the primary coordination shell are preferred.[38] On the basis of these criteria, we identified a site (designated CD2.Ca1) involving five mutations to construct the primary coordination sphere. As shown in Figure 1, the designed calcium-binding site is constructed by ligand residues from three different sequence regions with a total of four negatively charged ligands. The bidentate oxygen from Glu (V80E) is located at the end of the F strand, while the four side-chain oxygens come from strand F (V78N), the G strand (I89D and K91D), and the flexible BC loop (F21E). As with many natural calcium-binding proteins, this designed calcium-binding site is partially exposed to solvent. The interference of the hydrogen-bonding network and van der

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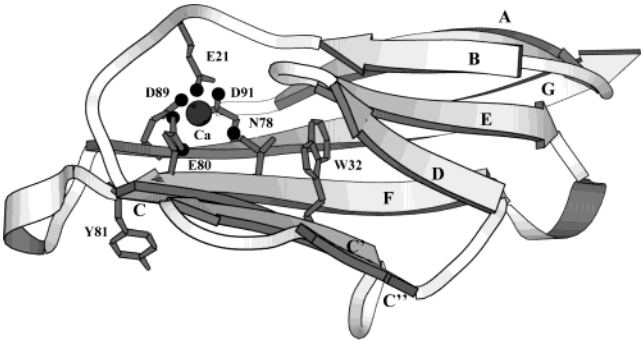

Figure 1. Model structure of the N-terminal domain of rat CD2 (1hng) with a designed calcium-binding site (CD2.Ca1). The designed calcium-binding residues at positions 21, 78, 80, 89, and 91 are shown in ball-and-stick representation. Glu-80 is a bidentate ligand. The aromatic residues Trp-32 and Tyr-81 located near the metal-binding sites are highlighted. The calcium-binding sites were designed using the Dezymer program.[33,35]

Waals and hydrophobic interactions of these designed sites are minimized. This designed calcium-binding site is in close proximity with several aromatic residues (Tyr-81 and Trp-32) that allow us to use fluorescence resonance energy transfer to monitor calcium binding.

Monitoring Formation of Calcium-Binding Site by Terbium Fluorescence Resonance Energy Transfer (Tb(III)-FRET). Direct binding of lanthanides was established by demonstrating FRET between aromatic residues and a lanthanide. Lanthanides are commonly used to probe calcium-binding sites because of their similar ionic radii and metal coordination chemistry.[39,40] For example, the ionic radii for Ca(II), Tb(III), and La(III) are 1.00, 0.92, and 1.03 Å, respectively.[41] Close proximity of Trp or Tyr residues to a calcium-binding site allows for the observation of fluorescence resonance energy transfer from aromatic residues to bound Tb(III) ions.[40] The metal-binding site in CD2.Ca1 is 7.7 and 9.4 Å from Trp-32 and Tyr-81, respectively. As shown in Figure 2, the addition of CD2.Ca1 to 30 μM Tb(III) solution results in an increase of Tb(III) fluorescence emission at 545 nm when excited at 282 nm, suggesting the formation of a metal:protein complex. Furthermore, when the protein concentration is unchanged, Tb(III) fluorescence enhancement is gradually increased with the addition of Tb(III). In contrast, the addition of wild-type CD2 leads to a much smaller change of the signal (Figure 2b), although it has the same two Trp (7 and 32) and Tyr (76 and 81) residues. The Tb(III)-binding affinity of CD2.Ca1 was obtained by monitoring the change of Tb(III) fluorescence enhancement by the protein as a function of Tb(III) concentration. The fractional changes of Tb(III) fluorescence enhancement can be well-fit using eq 1 assuming the formation of a 1:1 metal:protein complex (Materials and Methods). The dissociation constant of Tb(III) to CD2.Ca1 is 3 ± 2 μM in 100 mM MOPS pH 6.9.

CD2.Ca1 Exhibits Metal Selectivity. The metal selectivity of CD2.Ca1 for calcium ions over magnesium and potassium was first revealed by the competitive binding of a metal-binding pocket by monitoring fluorescence resonance energy transfer (FRET) between tryptophan and terbium. As shown in Figure 3, the enhancement of the Tb(III) fluorescence signal at 545 nm was significantly decreased upon the addition of 10 mM Ca(II) or 0.1 mM La(III) to the Tb−protein complex, which suggests that calcium and lanthanum competitively displaced the bound Tb(III) ion from the metal-binding pocket, while the addition of 10 mM Mg(II) or 100 mM K(I) reduced the Tb(III) fluorescence signal only by approximately 10−20%. Further, all of these metal ions decrease Tb(III) fluorescence by only about 5−10% in the absence of the protein. Therefore, the decrease observed in the Tb(III) fluorescence enhancement due to addition of metal ions is directly related to the relative binding affinity of CD2.Ca1 to these metal ions instead of nonspecific quenching.

This strong metal selectivity is further supported by ^{1}H NMR used to monitor calcium-induced chemical shift changes in the presence of different metal concentrations. First of all, resonances in the region corresponding to the side-chain of ligand residues, such as side-chain amide protons of Asn (at 6.7−7.7 ppm) and the methylene next to the carboxylates (at 2.4 ppm), experience a large change upon the addition of calcium ions. For example, chemical resonances at 6.81, 6.91, and 7.09 ppm in Figure 4a shift and broaden at calcium concentrations lower than 0.5 mM. They gradually shift and sharpen upon further addition of calcium ions. In addition, the small triplet at about 7.45 ppm becomes a sharp doublet. A new resonance at 6.99 ppm is observed upon addition of calcium concentrations above 0.5 mM. Furthermore, in a similar manner, resonances in the 2.4 ppm region also gradually changed upon the addition of calcium or lanthanum (data not shown). Some other nearby resonances (6.94 and 7.24 ppm in Figure 4a and methyl groups for Ala, Val, and Ile at 0−1.5 ppm) do not exhibit a significant change with the addition of excess calcium ions. Therefore, these changes in the chemical shifts specifically respond to the calcium ions and are not induced by nonspecific salt effects. The line-broadening effect observed for several resonances at 6.81, 6.91, and 7.09 ppm is likely to be a result of intermediate chemical exchange between the calcium-loaded and calcium-free forms at partially saturated calcium concentrations. The appearance of a new resonance at 6.99 ppm implies that the calcium-binding process is in a slow to intermediate exchange regime. The binding of calcium to CD2.Ca1 is relatively strong, which corresponds to calcium affinity in the μM range (K_d) under these conditions. A similar effect on the chemical shifts of CD2.Ca1 was also observed for the addition of the La(III).

^{1}H NMR was further used to test whether our designed calcium-binding protein has the ability to selectively bind calcium over a large excess of potassium or magnesium under physiological cellular conditions. The addition of 130 mM KCl does not result in significant changes for any of the resonances (data not shown). In the presence of magnesium concentrations at 0.6−2.0 mM, only a small broadening effect of resonances at 6.7 to 7.7 ppm is observed (Figure 4b). The addition of magnesium up to 10 mM does not lead to any significant change for most resonances, except the broadened resonances were slightly sharpened. However, the addition of 1.0 mM calcium to the protein preloaded with magnesium converts the ^{1}H NMR spectrum to one that is almost identical to that of the calcium-loaded form (Figure 4b top) with the appearance of new proton signals at 6.99 and 7.47 ppm. On the other hand, no significant change of chemical shifts of w.t. protein (as a negative control) upon the addition of calcium ions was

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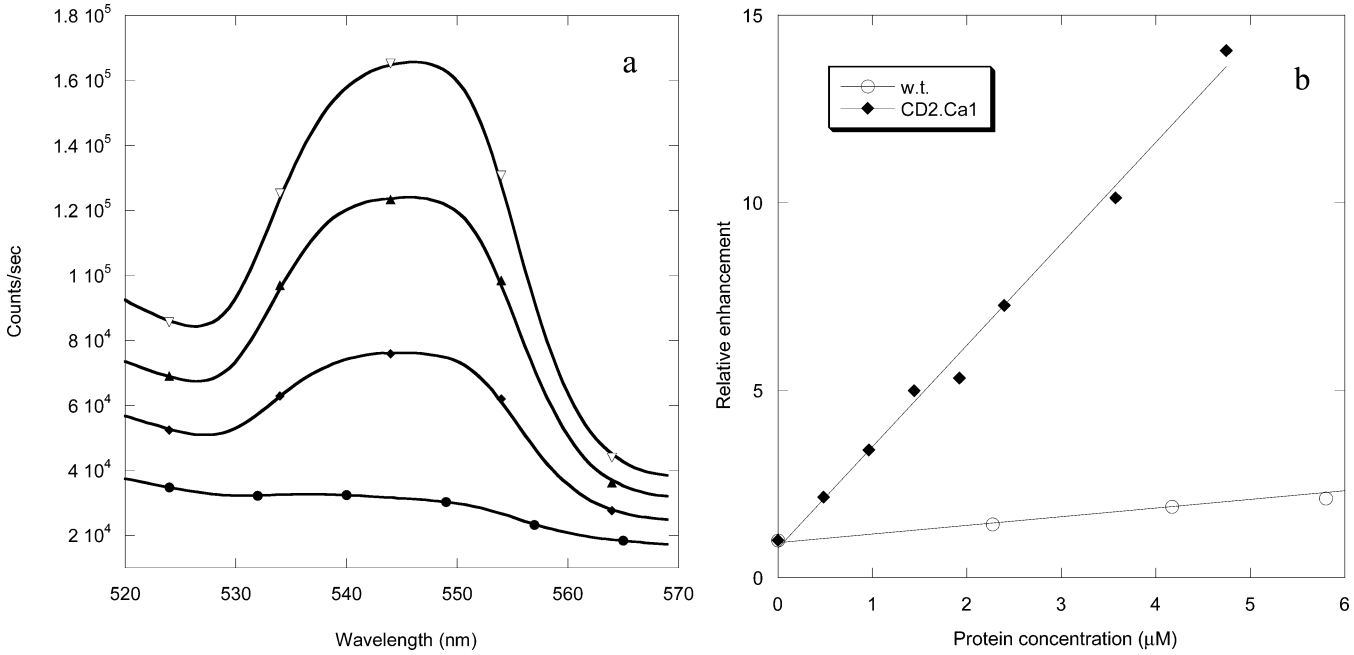

Figure 2. (a) Emission spectra of Tb(III) fluorescence excited at 282 nm with increasing protein concentration in 100 mM MOPS, pH 6.9. The Tb(III) concentration is 30 μM, and the protein concentrations of CD2.Ca1 from bottom to top are 0, 1.9, 3.6, and 4.8 μM, respectively. (b) Tb(III) fluorescence enhancement at 545 nm due to resonance energy transfer (excited at 282 nm) as a function of protein concentrations of CD2.Ca1 (squares) and w.t. CD2 (circles), in 100 mM MOPS, pH 6.9. The value of fluorescence enhancement was normalized by the Tb(III) signal in the absence of the protein.

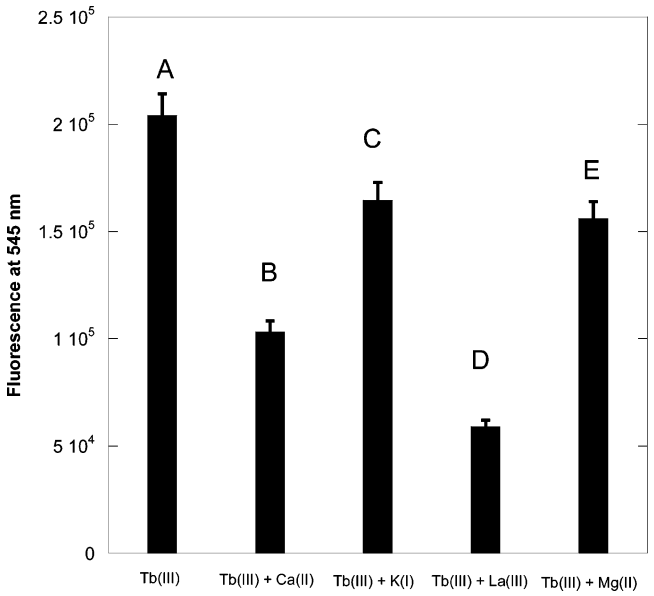

Figure 3. Tb(III) fluorescence enhancement at 545 nm of 10 μM Tb(III) and 6 μM CD2.Ca1 (A) and in the presence of (B) 10 mM Ca(II), (C) 100 mM K(I), (D) 0.10 mM La(III), and 10 mM Mg(II) in100 mM MOPS, pH 6.9.

observed. These results are clearly significant since they suggest that our designed calcium-binding protein is able to bind specifically to calcium.

Metal-Binding Constants. The metal-binding constants of CD2.Ca1 for five different metal ions were obtained using three different spectroscopic techniques, including a competition assay using fluorescence resonance energy transfer (FRET) between tryptophan and terbium, CD, and ICP-MS. The Ca(II)-, Mg-(II)-, and La(III)-binding properties of CD2.Ca1 were first examined by competitive binding with Tb(III) under saturated conditions.[6] CD2.Ca1 (2.2 μM) was preequilibrated with 30 μM

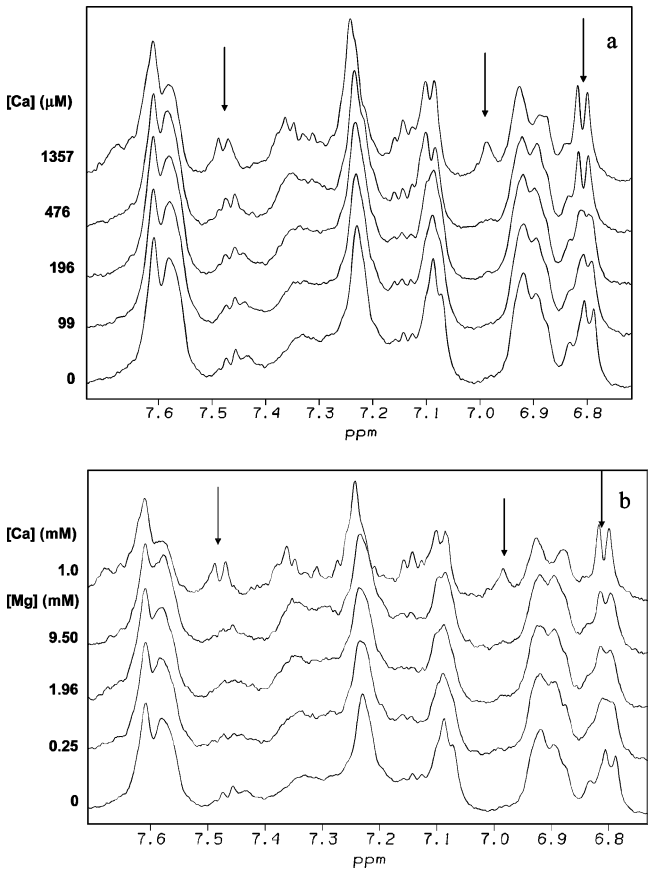

Figure 4. (a) 1D ^{1}H NMR spectra of CD2.Ca1 (0.21 mM) as a function of calcium concentration. The calcium concentrations from bottom to top are 0, 99, 196, 476, and 1357 μM, respectively. (b) 1D ^{1}H NMR spectra of CD2.Ca1 (0.21 mM) in the presence of 0, 0.50, 1.96, and 9.5 mM magnesium concentration (from bottom to top bottom four spectra). The 1D NMR spectrum of further addition of 1.0 mM Ca(II) to the protein sample with 10 mM magnesium is shown on the top. Resonances changed upon addition of calcium are highlighted by the arrows.

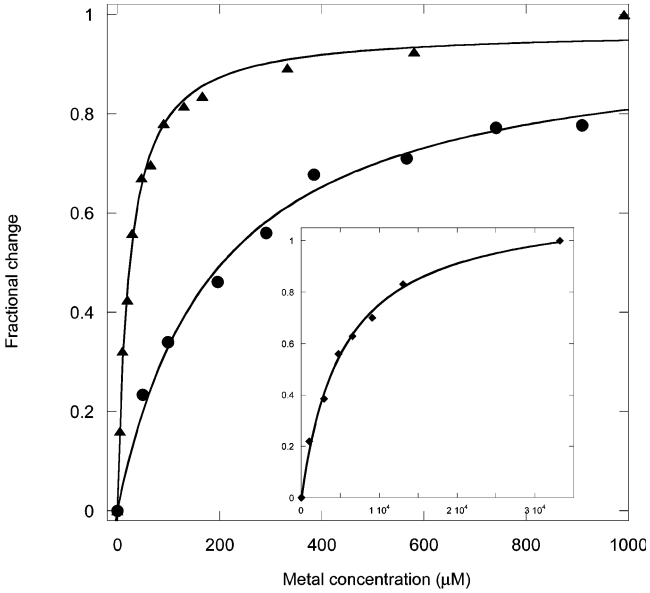

Figure 5. Fractional change of terbium FRET decay at 545 nm as a function of Ca(II) (circles), La(III) (triangles), and Mg(II) (inset, diamonds) concentrations. The solid lines are simulated using eq 1 assuming formation of a 1:1 metal−protein complex.[39]

Table 1. Summary of Metal-Binding Affinities of CD2.Ca1 to Different Metal Ions

metal ion	K_d (μM)[a]	K_d (μM)[b]	K_d (μM)[c]
La(III)	2 ± 1	2.5 ± 0.5	
Tb(III)	3 ± 2	2.3 ± 0.5	
Ca(II)	40 ± 10	55 ± 10	40 ± 5
Mg(II)	670 ± 70	500 ± 100	
K(I)	>10 000	>10 000	

[a] From Tb(III) FRET study in 100 mM MOPS pH 6.9. [b] From CD study in 1 mM MOPS pH 6.9. [c] From ICP-MS study in 10 mM Tris pH 7.4

Tb(III) in solution for over 14 h. Different metal ions were gradually added to the Tb(III)−protein solution. The replacement of Tb(III) by the competitive ions leads to a gradual decrease of the Tb(III) fluorescence signal at 545 nm (Figures 3 and 5). Metal concentrations up to 30 mM were required for magnesium to fully compete with the bound Tb(III), while 0.2 mM La(III) completely diminishes Tb(III) fluorescence enhancement. As shown in Figure 5, the competition process can be well fitted using eqs 1 and 2 (Materials and Methods). The obtained K_d's for La(III), Ca(II), and Mg(II) are 2 ± 1, 40 ± 10, and 670 ± 70 μM, respectively (Table 1). These metal-binding affinities agree with the results obtained by monitoring the CD signal change at 205 nm as a function of metal concentration (Table 1).

Equilibrium dialysis and ICP-MS were also developed to determine metal-binding constants. ICP-MS is a sensitive technique that has the advantages of large dynamic ranges, high speed of analysis, good signal-to-noise ratio, and low detection limits.[42] CD2.Ca1 and control proteins were dialyzed for 72 h against metal ions (5−10 μM) to reach equilibrium. To ensure complete equilibrium of dialysis and quantitative detection of metal ions by ICP-MS, the natural calcium-binding protein α-lactalbumin was used as a positive control and w.t. CD2, lysozyme, and GFP were used as negative controls. Solutions at both sides of the dialysis membrane were acidified and analyzed with an internal standard (^{115}In). The calcium concentration was then calculated using the natural abundance of the multiple isotopes ^{42}Ca, ^{43}Ca, and ^{44}Ca to avoid the interference of argon gas. For three different dialysis assays, all of these multiple isotopes, ^{42}Ca, ^{43}Ca, and ^{44}Ca, give data with good reproducibility, an error of < 5%, and K_d of 0.6 μM for calcium ions for the natural calcium-binding protein α-lactalbumin. These measured affinities agree with the published results using isothermal titration calorimetry[43] and fluorescence[44] under similar conditions. Further, no difference in the metal content was detected for buffer and w.t. CD2, lysozyme, and GFP (in 5% experimental error range). The measured calcium-binding affinities obtained using multiple isotopes of CD2.Ca1 is 40 μM, which is in good agreement with that obtained by FRET and CD methods.

As shown in Table 1, CD2.Ca1 exhibits strong affinities for Ca(II) and its analogues Tb(III) and La(III) with K_d's of 40, 2 and 3 μM, respectively, which are very similar to the natural calcium-binding proteins such as galactose-binding protein (GBP).[45] The Tb(III)-binding affinity of our designed protein is about 200-fold stronger than that for γ-crystallin with a similar Greek Key fold.[46] The greater affinity for Tb(III) and La(III) is likely a result of high positive charges for these metal ions.[45] On the other hand, it does not bind to potassium (K_d greater than 10 mM). More interestingly, CD2.Ca1 has a 10-fold weaker metal-binding affinity for Mg(II) (with a K_d of 500 μM), although both cations are divalent. High-resolution NMR has shown that magnesium is not able to induce similar structural changes as calcium. With several different methods, CD2.Ca1 protein was shown to have affinities varying about 200-fold (K_d from 3×10^{-6} to 6×10^{-4} M) among the four different divalent and trivalent metal ions tested. This strong metal selectivity property is one of the most important properties of intracellular proteins for controlling calcium level and responding to the dramatic change of calcium signaling in the presence of excess magnesium and potassium.[6]

Conclusions

We have introduced a de novo designed calcium-binding site into the framework of a non-calcium-binding protein, CD2. The resulting protein selectively binds calcium over magnesium with calcium-binding affinity comparable to that of natural calcium-binding proteins. This experiment is the first successful metalloprotein design that has a high coordination number (7) metal-binding site constructed into a β-sheet protein. Our results demonstrate the feasibility of designing a single calcium-binding site into a host protein, taking into account only local properties of a calcium-binding site obtained by a survey of natural calcium-binding proteins and chelators. The resulting site exhibited strong metal selectivity, suggesting that it should now be feasible to understand and manipulate signaling processes by designing novel calcium-modulated proteins with specifically desired functions and to affect their stability. In addition, our developed approach can be used to control cell adhesion by calcium binding since CD2 has a predominantly β-sheet

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architecture that mimics the topology of most extracellular calcium-dependent cell adhesion molecules such as C_2 and the cadherin families.

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