

Metal-binding studies for a *de novo* designed calcium-binding protein

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To understand the key determinants in calcium-binding affinity, a calcium-binding site with pentagonal bipyramid geometry was designed into a non-calcium-binding protein, domain 1 of CD2. This metal-binding protein has five mutations with a net charge in the coordination sphere of -5 and is termed DEEEE. Fluorescence resonance energy transfer was used to determine the metal-binding affinity of DEEEE to the calcium analog terbium. The addition of protein concentration to Tb(III) solution results in a large enhancement of Tb(III) fluorescence due to energy transfer between terbium ions and aromatic residues in CD2-D1. In addition, both calcium and lanthanum compete with terbium for the same desired metal binding pocket. Our designed protein exhibits a stronger affinity for Tb(III), with a K_d of 21 μ M, than natural calcium-binding proteins with a similar Greek key scaffold.

Keywords: calcium-binding protein/CD2/design/fluorescence energy transfer/Tb(III) binding

Introduction

Calcium controls numerous physiological functions both within the cell and in the extracellular matrix through interaction with proteins with different affinities. Improper calcium-binding affinity of calcium-binding proteins is one of the major causes of many human diseases related to the overloading or depleting cellular calcium concentration (Falke *et al.*, 1994; Kawasaki and Kretsinger, 1995; Berridge *et al.*, 1998). Local structural factors, electrostatic interactions and protein environment have been shown to contribute to calcium-binding affinity for the calcium-binding proteins (Linse *et al.*, 1988; Linse and Forsen, 1995). Calcium-binding proteins have been shown to exhibit from μ M to mM range affinities depending upon the environment (Linse and Forsen, 1995). For example, extracellular calcium-binding proteins such as the calcium-dependent cell adhesion molecule cadherin and growth factors have calcium-binding affinities in the range from 0.1 μ M to mM (Downing *et al.*, 1992; Pokutta *et al.*, 1994; Koch *et al.*, 1997). To date, relating calcium affinity with structural aspects of proteins has been difficult owing to the complex dynamic properties of the natural calcium-binding proteins, such as the intrinsic irregularity of the calcium-binding sites and the metal-metal interactions between sites in proteins. Protein design has been shown to be very useful in understanding protein folding and to develop novel materials (Regan, 1995; Lu and Valentine, 1997; Pinto *et al.*, 1997; DeGrado *et al.*, 1999). Previously,

the study of calcium binding has utilized peptide models (Marsden *et al.*, 1990), mutagenesis in natural calcium-binding proteins (Linse and Forsen, 1995; Shea *et al.*, 1996; Wu and Reid, 1997) and grafting a calcium-binding loop into a structural homologue (Toma *et al.*, 1991; Ye *et al.*, 2001). Here we report a new approach using the *de novo* design of a calcium-binding site in a host protein domain, one of the rat cell adhesion protein CD2 (CD2-D1), with the objective of elucidating key determinants for calcium binding in a single site system.

Previous work with the cell adhesion protein CD2-D1 has demonstrated that this protein is an excellent choice as a host system for engineering a calcium-binding site (Yang, J.J. *et al.*, 2000a,b). CD2-D1 is a small β -sheet protein (99 amino acids) with a common IgG fold (Figure 1). Large conformational changes are not observed in the pH range 1–10 (Yang, J.J. *et al.*, 2000a). Moreover, it produces a high expression yield in BL-21. CD2-D1 can also be reversibly refolded after chemical (guanidinium.HCl) (Tanford, 1968) and thermal denaturation (Yang, J.J. *et al.*, 2000b). NMR assignments (Davis and van der Merwe, 1996) and X-ray structures (Jones *et al.*, 1992) are available. CD2-D1 has been shown to accommodate a calcium-binding pocket (Ye *et al.*, 2001). CD2-D1 has been shown to withstand up to ~ 40 separate mutations without changing conformation (Arulanandam *et al.*, 1993).

Materials and methods

All enzymes and $10\times$ enzyme buffers for site-directed mutagenesis were purchased from Promega. The vector plasmid pGEM-4Z used during site-directed mutagenesis was transformed into the *Escherichia coli* strain DH5- α for purification of the DNA. The CD2 variant was cloned into the pGEX-PKT vector plasmid (a gift from Dr Hillary Godwin of Northwestern University) and transformed into the *E. coli* strain BL-21 using Luria-Bertani (LB) broth with ampicillin (100 μ g/ml) plates.

Computational design of calcium-binding site

The protein is designed using established methods based on the local geometry of the calcium-binding site. CD2-D1 (1hng) was used as the host protein. The designed sites were screened based on their geometric deviations, solvent accessibility, side chain clashes and charge numbers.

Protein expression and purification

The designed DEEEE protein was made using site-directed mutagenesis. Automated DNA sequencing confirmed that the mutant had been successfully made.

LB broth with ampicillin was used for growth and expression of the GST-fusion CD2 variant. The variant was grown until an OD₆₀₀ of 0.8 and induced with 0.1 mM isopropyl- β -D-thiogalactoside (IPTG). Purification was completed using affinity chromatography with GS4B beads (Amersham-Pharmacia) and the GST-tag was cleaved on the column with 40 units/ml of PreScission protease (Amersham-Pharmacia) to obtain pure CD2 variant.

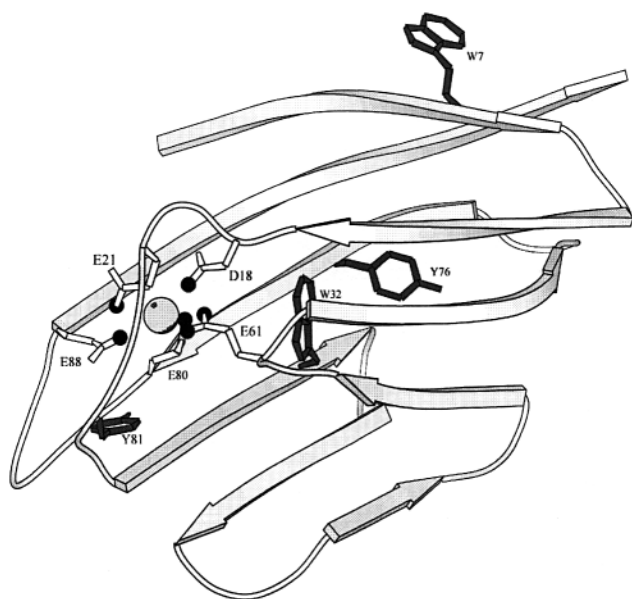


Fig. 1. Schematic representation of CD2-D1, with the designed calcium-binding site (Site_4047) (DEEEE), modified after the crystal structure (1hng) using Molscript (Kraulis, 1991). Calcium is shown as a large sphere in the center of the binding site. The calcium-binding ligand residues (I18D, F21E, G61E, V80E and I88E) are highlighted with the respective oxygen ligands from the mutated residues shown as small black spheres. The aromatic residues are also highlighted (W7, W32, Y76 and Y81).

Both electrospray ionization (ESI) and the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry were used to verify the correct molecular mass and purity of the DEEEE protein. SDS-PAGE analysis was also used to measure the purity.

The protein was dialyzed against 10 mM Tris-HCl, pH 7.4. The concentration was measured using a UV-1601 spectrophotometer (Shimadzu) interfaced to a PC at 280 nm ($\epsilon = 11\,700$) (Driscoll *et al.*, 1991).

Fluorescence resonance energy transfer (FRET)

A PTI fluorimeter was used with an excitation slit width of 6 nm and an emission slit width of 25 nm. A glass filter was placed in front of the exit slit of the emission to filter out the secondary Raleigh reflection. The excitation wavelength was set at 283 nm and the emission scan was from 500 to 600 nm, with an expected emission peak at 545 nm. An average of two scans was taken. A sample of 30 μM Tb(III) and a stock solution of 20 μM DEEEE with 30 μM Tb(III) were prepared using 10 mM Tris, pH 7.4. FRET with increasing protein concentration was measured by adding 100 μl aliquots of the stock solution directly to 30 μM Tb(III) with an equilibration time of 15 min. The emission fluorescence was measured. This procedure was continued until a final concentration of ~ 8.0 μM protein was reached.

Two samples were prepared for competition binding studies, one containing 30 μM Tb(III) and 8 μM DEEEE with 100 μM La(III) and the other 30 μM Tb(III) and 8 μM DEEEE with 10 mM Ca(II). The samples were equilibrated at room temperature for ~ 2 h and measured by averaging two scans.

Once the intensity enhancement by protein had been determined at 545 nm, a titration with increasing terbium was performed. Samples of DEEEE with concentrations of 46 μM and samples of 1 mM Tb(III) with 4 and 6 μM DEEEE were prepared. The same parameters were used as indicated previously for analysis by energy transfer.

Data analysis

The fractional change versus the Tb(III) concentration was plotted with normalized fluorescence intensity at 545 nm. This was done by subtracting the intensity of free terbium from the sample of protein with terbium with baseline correction. The baseline shift was corrected by integrating areas between 527–568 nm. The obtained intensities of Tb(III) with protein were further subtracted from the intensity of free Tb(III) at the same concentrations.

The dissociation constant K_d was calculated using the following equation:

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

where F is the fractional change of Tb(III) fluorescence enhancement at 545 nm, $[P]_T$ is the total protein concentration and $[M]_T$ is the total metal concentration (Yang, W. *et al.*, 2000b).

Results and discussion

Structural features of the designed calcium-binding site

Using the established structural parameters from our previous studies on natural calcium-binding proteins, a single calcium-binding site with the common geometry of pentagonal bipyramid was designed in the host protein CD2 using the computer algorithm Dezymer (Hellenga and Richards, 1991; Yang, W. *et al.*, 2000a, 2001). Figure 1 shows a schematic representation of CD2-D1 containing the designed site (DEEEE), with the mutations G61E as the bidentate ligand and I18D, F21E, V80E and I88E as monodentate ligands in the loop regions and in two β -strands. One ligand position in the geometry remains open to permit water to act as a bridge and to avoid molecular crowding, which is similar to natural calcium-binding proteins (Falke *et al.*, 1994). The location of the designed site should allow for water accessibility and a way to monitor metal induced conformational change. Further, a net charge of -5 in the coordination sphere was chosen to ensure strong metal-binding affinity especially to lanthanide ions with similar ionic radii as calcium but with higher positive charge (Horrocks, 1993).

Metal-binding ability monitored by fluorescence resonance energy transfer

Tb(III) has been widely used to probe calcium binding because of its similar ionic radius and coordination properties to those of calcium (Horrocks, 1993; Drake *et al.*, 1997). Terbium directly competes for calcium-binding sites in natural calcium-binding proteins, such as calmodulin and galactose binding protein (Kilhoffer *et al.*, 1980; Wang *et al.*, 1982; Falke *et al.*, 1994). Moreover, terbium is intrinsically fluorescent, with a broad excitation spectrum and an emission maximum at ~ 545 nm. The excitation spectrum of terbium overlaps the emission spectrum of tryptophan and tyrosine, which allows for monitoring of terbium binding to CD2-D1 by energy transfer. As shown in Figure 1, fluorescence energy transfer was used since Tyr81 and the buried Trp32 of DEEEE are ~ 8.5 and ~ 8.4 \AA away from the binding pocket, respectively. The enhancement of the emission peak at 545 nm increases with the addition of protein to constant terbium, which suggests that terbium binds to the protein (Figure 2a). Furthermore, this enhancement is only observed with the CD2 variant with designed DEEEE site. Wild-type CD2-D1 does not show this enhancement

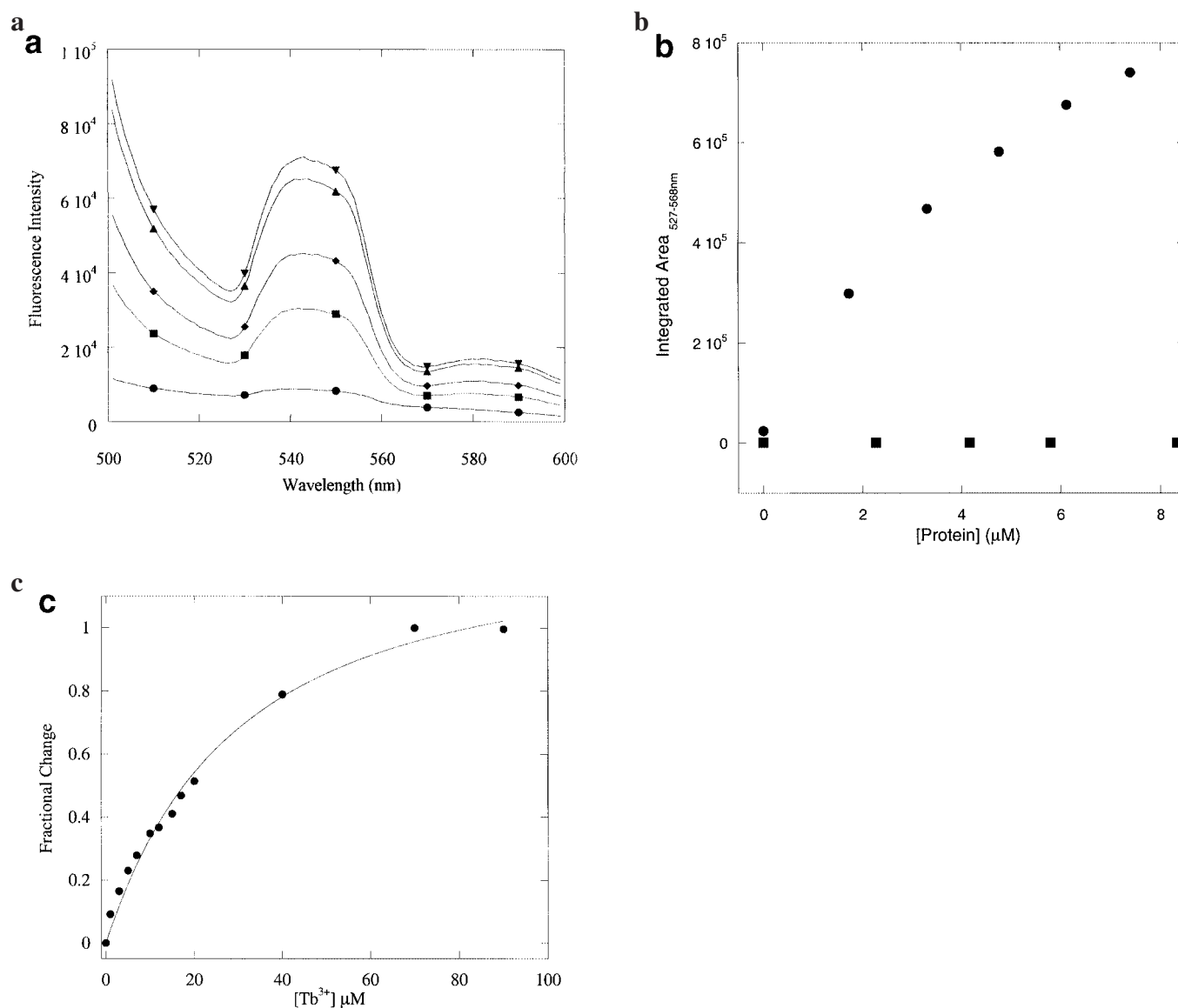


Fig. 2. (a) Emission spectra of 30 μM Tb³⁺ with increasing DEEEE concentrations of 0, 2, 4, 6 and 8 μM (from bottom to top) in 10 mM Tris-HCl, pH 7.4. Excitation was fixed at 283 nm. (b) Enhancement at 545 nm with respect to the protein concentration. DEEEE (●) compared with wild-type CD2-D1 (■). (c) Fractional change of Tb³⁺ fluorescence enhancement at 545 nm with respect to total terbium concentration. Fitted using a 1:1 binding equation involving total protein and terbium concentration.

although it has the same four aromatic residues, Trp7 and -32 and Tyr76 and -81 (Figure 2b).

In addition, terbium titrations were performed to determine the binding affinity of the metal to the protein. The terbium concentration was gradually increased at constant protein concentrations of both 4 and 6 μM. The observed enhancement of the terbium fluorescence suggests that the metal binds in the pocket of the designed binding site.

The fractional change versus the Tb(III) concentration was plotted by normalizing the fluorescence intensity at 545 nm and the baseline was corrected by using integrated areas between 527–568 nm. (Figure 2c). This was completed by subtracting the intensities at 525 and 565 nm from that at 545 nm. The Tb(III) titration curve can be fitted to a 1:1 binding model as expected. K_d for both terbium titrations was calculated and the average was 21 ± 3 μM. This work is very exciting since the metal binding ability of our designed protein is about 15-fold stronger than that for the natural calcium-binding

proteins with a similar Greek Key fold. Rajini *et al.* have reported that the K_d of γ -crystallin for terbium is ~ 300 μM (Rajini *et al.*, 2001). Now we can use this design approach to elucidate key determinants for calcium-binding affinity.

To test further the binding of terbium to the binding site, a competition of excess lanthanum (100 μM) and calcium (10 mM) with 30 μM terbium was performed. According to the published method for natural calcium-binding proteins such as calmodulin (Wang *et al.*, 1982) and galactose binding protein (Drake *et al.*, 1996), excess lanthanum and calcium were used to examine whether the calcium or lanthanum compete for the same binding pocket as terbium. A decrease in the fluorescence intensity at 545 nm for DEEEE was observed in the presence of both lanthanum and calcium (Figure 3), which suggests that these metals compete for the same binding pocket as terbium.

In summary, a calcium-binding site with five negatively charged ligand residues has been *de novo* designed and

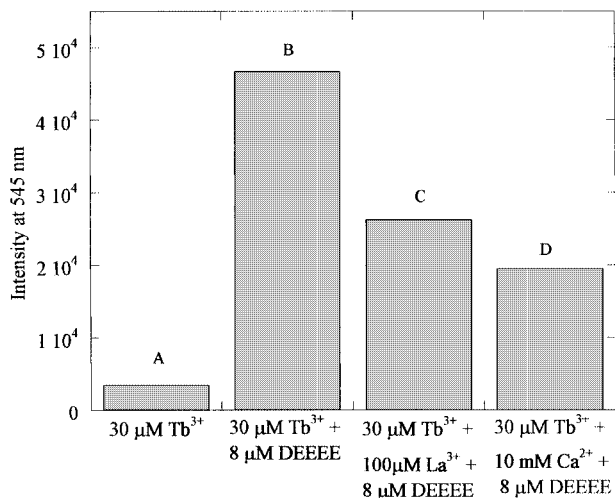


Fig. 3. Competition study of 30 μM Tb³⁺ (A) and ~8 μM DEEEE (B) in the presence of 100 μM La³⁺ (C) and 10 mM Ca²⁺ (D) in 10 mM Tris-HCl, pH 7.4.

engineered in the host protein CD2-D1. The CD2 variant DEEEE has been successfully engineered. The addition of protein concentration to Tb(III) solution results in a large enhancement of Tb(III) fluorescence due to energy transfer, suggesting that this designed protein has an affinity for terbium in a 1:1 ratio with an average K_d of 21 ± 3 μM. Further, the competition studies have shown that both calcium and lanthanum compete for the same desired binding pocket as terbium.

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