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Refined crystal structure of ytterbium-substituted carp parvalbumin 4.25 at 1.5 Å, and its comparison with the native and cadmium-substituted structures

Vinod D. Kumar[1], Lana Lee[2] and Brian F.P. Edwards[3]

[1]*Macromolecular Structure Laboratory, NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, PO Box B, Frederick, MD 21702-1201, USA,* [2]*Department of Chemistry and Biochemistry, University of Windsor, Canada N9B 3P4* and [3]*Department of Biochemistry, Wayne State University School of Medicine, Detroit, MI 48202, USA*

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The crystal structure of carp parvalbumin 4.25 containing a 1:1 molar ratio of ytterbium chloride to protein has been refined at 1.5 Å resolution by restrained least-squares methods to a crystallographic *R* value of 0.199. The crystal structure confirms the NMR studies, which suggest that low concentrations of ytterbium cause an extensive displacement of calcium from the EF metal binding site. A comparison of the ytterbium-substituted model with the native and cadmium-substituted structure show no significant differences, except around the substituted EF metal-binding region. The displacement of calcium by ytterbium at the EF site has caused a movement in the polypeptide backbone of Ser-91 and Asp-92. This movement resulted in an increase in the number of oxygen ligands bound to ytterbium in the EF site from seven to eight.

Calcium binding protein; Lanthanide; Ytterbium-substituted; X-ray structure

1. INTRODUCTION

Lanthanides from lanthanum to lutetium, form trivalent ions in aqueous solution and have ionic radii [1] which are comparable to the radius of divalent calcium (0.99 Å); thus they have frequently been used to mimic the role of calcium in proteins [2,3]. Lanthanides are of biological interest because their optical and paramagnetic properties can be exploited to obtain information about the structure and function of calcium-binding proteins [4]. The replacement of calcium by lanthanide ions has been observed in a number of proteins, such as α-amylase [5], trypsinogen [6] and thermolysin [7].

The 3-D structure of parvalbumin was originally established by Kretsinger and Nockolds [8]. They described the unique structural configuration of a helix–loop–helix calcium-binding site as an EF hand, a conformation which is a common structural feature for all members of a superfamily of calcium-binding proteins which includes troponin-C and calmodulin. The two such calcium-binding sites in parvalbumin are termed CD and EF. Lanthanide ions have been used to study the affinities by which each of the helix–loop–helix structures in this unique class of proteins binds metals.

Correspondence address: V.D. Kumar, Macromolecular Structure Laboratory, NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, PO Box B, Frederick, MD 21702-1201, USA

Up to this point, controversy remains concerning the binding affinities of the metals for the two metal-binding sites in parvalbumin. The original X-ray crystallographic studies on the isomorphous replacement of calcium with terbium [9] showed an increase in electron density only at the EF site at low molar ratios of terbium to parvalbumin, implying a sequential replacement of calcium in the CD and EF sites by terbium. The terbium fluorescence data [10–12] as a function of terbium concentration showed a maximum upon the addition of 1.4–1.8 equivalents of terbium with quenching at higher terbium ratios, which suggests an equal displacement of calcium from two sites followed by binding of terbium to a third weaker site. Cavé et al. [13] used proton relaxation enhancement methods to study the binding of gadolinium and reported equal affinities. Rhee et al. [14] used europium and terbium luminescence to study the binding of these metals and reported relatively equal displacement for the two sites. Lee and Sykes [15] studied ytterbium-shifted ^{1}H-NMR resonances as a function of ytterbium concentration and found sequential displacement of the calcium from the two sites. Corson et al. [16] who used optical stopped-flow kinetics and cadmium NMR to study the displacement of calcium and cadmium by ytterbium also observed sequential displacement in the two sites. The nearly identical binding affinities of the middle-weight lanthanides for parvalbumin was considered reasonable in view of the crossover in relative CD/EF site affinities across the lanthanides. Further NMR investigations suggest that

the CD hand of parvalbumin exhibits selectively across the lanthanide series whereas the EF hand exhibits no selectivity [17].

In this paper we present: (a) the refinement of the crystal structure of carp parvalbumin 4.25 containing a 1:1 molar ratio of ytterbium chloride to protein at 1.5 Å resolution by a restrained least squares procedure [18], (b) evidence of isomorphous replacement of calcium with ytterbium occurring only at the EF metal binding site, and (c) a comparison of the model with native [19], and cadmium-substituted models [20].

2. MATERIALS AND METHODS

Crystals of ytterbium-substituted parvalbumin were grown from a 1:1 molar solution of ytterbium chloride (114 mM) and parvalbumin (20.8 mg/ml) using the hanging drop method as previously described for the native protein [19]. The crystals were stabilized by adding 0.1 volume of 65%-saturated ammonium sulfate to the protein drop containing the crystals. The space group of the crystals was determined from precission photographs and the unit cell dimensions from 14 high-angle reflections refined on the diffractometer. The crystals belong to space group C2 with one molecule in the asymmetric unit and have unit cell dimensions a = 28.5 Å, b = 61.0 Å and c = 54.5 Å and β = 95°.

Crystals of ytterbium-substituted carp parvalbumin were found to be quite stable in X-rays. The complete 1.5 Å resolution data were collected from two crystals on a Nicolet/Syntex P2$_1$ four circle diffractometer, which had been modified for protein data collection with an extended detector arm and a 385-nm-long, helium-filled beam tunnel. The first crystal was used to record 7544 reflections with $I > 2\sigma(I)$ from ∞–1.9 Å resolution. The second crystal was used to record 6684 reflections with $I > 2\sigma(I)$ in the 2.0–1.5 Å range. Radiation damage on each crystal was monitored by the measurement of 5 reflections distributed evenly through the two-θ range after every 200 reflections. The intensity measurements were discontinued when the average intensities for the standard reflections had decreased by 10% for the first crystal and 16% for the second crystal. The symmetry residual was 0.049 for 362 symmetry-related observations on crystal 1 and 0.092 for 302 observations on crystal 2. The absorption-corrected data from the two crystals was merged with the ROCKS programs [21] and placed on a common scale using the reflections in the overlapping range between 2.0–1.9 Å range. The merging residual value for the 966 reflections in the overlapping range was 0.084. The final data set contained 12646 reflections in the 10–1.5 Å range with $I > 2\sigma$ of a possible 14318 reflections.

Electron density maps were interpreted on an MMSX graphics system using the M3 software package [22].

3. RESULTS AND DISCUSSION

3.1. *Refinement*

The parvalbumin coordinate set 1CPV obtained from the Protein Data Bank [23], was chosen as the starting point for the refinement because it had the lowest residual while still retaining ideal geometry. The calcium atom in the EF site was substituted by ytterbium in the atomic coordinate file before running PROTIN [18]. Restrained least-squares refinement was initiated with 5–3.0 Å data with an overall temperature factor B = 14 Å^2. Twenty-six cycles of refinement brought the R value from 0.406 to 0.213. At this point individual temperature factors were included in the refinement scheme. Thirty-five cycles (27–61) of refinement were performed with data from 3.0–1.5 Å added sequentially in 6 shells to give an R value of 0.266. Two additional cycles of refinement were performed after including intensity data in the 10.0–5.0 Å to give an R value of 0.27 for the complete 10.0–1.5 Å resolution data. Fourier maps using $2F_o$-F_c coefficients were calculated and displayed on the graphics system. The electron-density map revealed poor density for the residues 1–5, and the side chain atoms of residues Ser-39, Asp-41, Asp-79, Lys-83 and Lys-87. An F_o-F_c electron-density map calculated after three cycles of refinement (64–66) without these atoms showed unambiguous density for the residues 1–5. Four cycles (67–71) of refinement with the correctly positioned N-terminal region reduced the R value to 0.259.

At this stage peaks in the F_o-F_c map that had heights greater than 3 times the standard deviation of the map and were within 2.5–3.5 Å of a hydrogen-bonding atom of the protein or a previously included water molecule were located. A total of 86 acceptable peaks were found. They were included in the next round of structure factor and least-square calculations. Nineteen cycles (72–90) of refinement reduced the R value to 0.206. Because changes were observed in the coordination of the metal ions in the CD and EF sites, three cycles (91–93) of refinement were performed omitting the side-chain atoms beyond the Cβ position for all residues that directly liganded to the metal ions. Analysis of the resulting F_o-F_c density map showed that these atoms were correctly fit to the observed density. In addition the N-acetyl group and some of the side chain atoms of residues Ser-39, Asp-41, Asp-79, Lys-83, Lys-87 could be located from the map and were fit into density. A check for contacts for the 86 water molecules revealed that eight of them were symmetry-related molecules and subsequently removed. A total of twenty more cycles (94–113) of refinement, were performed to give an R value of 0.200. In order to determine if ytterbium caused an equal or sequential displacement of the calcium, an F_o-F_c electron density map was calculated, omitting the two metal ions. The relative peak height for the metal ion at the EF site was found to be two times higher than the corresponding metal position at the CD site. As a last step the occupancies of the 78 water molecules were refined in three cycles to give an R value of 0.199.

The final R value for the model, including all residues, and 78 water molecules is 0.199 for 12646 reflections with $I > 2\sigma(I)$, in the 10.0–1.5 Å resolution range. The distribution of the agreement index R, with resolution ranges for the data is shown in Table I. In the final model the rms deviation of bond lengths from ideality is 0.026 Å, the rms deviation from planarity for 146 planar groups is 0.01 Å, and the rms deviation for the 108 peptide bonds in the molecule is 1.9°. Only 88

Table I

Summary of the refinement statistics

Resolution (Å)	No. of reflections		R factor
	Observed[a]	%[b]	
10.00–5.00	359	93.4	0.269
5.00–3.00	1479	96.5	0.179
3.00–2.50	1347	96.8	0.208
2.50–2.00	3010	96.2	0.198
2.00–1.80	2118	90.1	0.205
1.80–1.65	1991	76.1	0.215
1.65–1.50	2345	63.6	0.231

[a] Reflections with $F > 2\sigma$

[b] Percent of the total possible reflections in the shell that were observed with $F > 2\sigma$

of 2188 bond distances deviate by more than 0.03 Å from ideal values.

3.2. *Description of structure*

The refined model has the same overall structural features as the native parvalbumin structure. The observed peak height at the EF site was twice that of the CD site in a F_o-F_c map in which the metals were omitted. This increase in electron density at the EF site at low molar ratios of ytterbium to parvalbumin implies a sequential replacement of the calcium by ytterbium. Based on the number of electrons present in ytterbium and calcium, the anticipated peak height for the EF site should be 3.5 times higher than CD site. However, the discrepancy in the peak heights could be attributed to (1) a partial substitution of calcium by ytterbium at the CD site, or (2) an incomplete substitution of calcium by ytterbium at the EF site, or (3) differences in the temperature factors (B's) for the two metals (EF – 21 $Å^2$, CD – 9 $Å^2$). Fig. 1, which was calculated using the program OVERLAP [24], shows a least-squares superposition of the Cα atoms of the native, cadmium-substituted, and ytterbium-substituted structures. The rms difference in atomic positions (Cα) between the ytterbium-substituted structure and native structure and between the ytterbium-substituted structure and the cadmium-substituted structure is 0.10 Å and 0.39 Å, respectively. The results from this analysis showed that differences greater than twice the standard deviation are distributed throughout the molecule, and except in the immediate vicinity of the two metal sites, do not appear to be correlated with the replacement of calcium with ytterbium or cadmium at the metal binding site.

Since the present work provides the first refined structural description of a lanthanide-substituted parvalbumin it is instructive to examine the relative strengths of the metal–ligand bonds insofar as they can be inferred from the metal–ligand bond lengths in both CD and EF sites. The CD metal ion in all three structures is coordinated by 7 oxygens in a distorted octahedral arrangement. Fig. 2 shows the superposition of the CD metal-binding site for the three refined structures. The rms difference in bond length was 0.04 Å for the metal–oxygen bonds between the ytterbium-substituted and native structure, and 0.122 Å between the ytterbium-substituted and cadmium-substituted structure. As it is evident from the small rms difference in the atom positions that the relative strengths of the metal–ligand bonds at the CD site are very similar in all three structures. Table II compares the bond distances for the CD metal-binding sites for the three structures. The metal–oxygen distances for the three models range

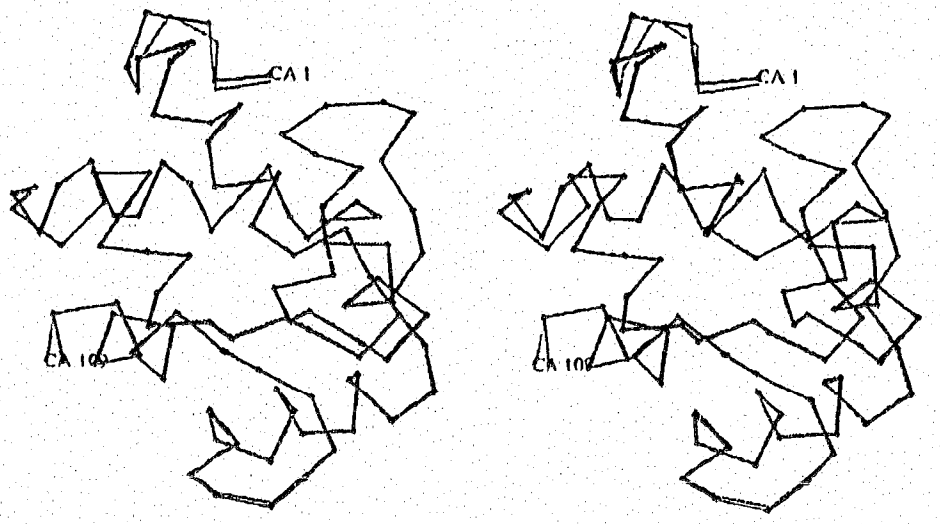

Fig. 1. Stereo view of the Cα superposition of Carp parvalbumin (solid lines), the cadmium-substituted structure (dashed lines) and the ytterbium-substituted structure (dotted lines).

between 2.12 Å and 2.77 Å with an average value for the 7 oxygen atoms ranging between 2.38 Å and 2.41 Å.

The EF metal is also coordinated by oxygen atoms in a distorted octahedral geometry. Fig. 3 shows the superposition of the EF metal-binding site for all three refined structures. The rms difference in the metal–oxygen bond-lengths was 0.14 Å and 0.204 Å for the native and cadmium-substituted proteins respectively. The most drastic change is in the relative positions of the polypeptide backbone atoms of Ser-91 and Asp-92. As a result of this change the carboxyl oxygen atom (OD2) of Asp-92 is now closer to the ytterbium ion by 1.12 Å compared with the corresponding metal–oxygen distance in the native structure. The major difference between the three structures is the number of oxygen ligands of the metal ion: in ytterbium-substituted parvalbumin there are 8 oxygen ligands as compared to 7 oxygen ligands in both the native and cadmium-substituted proteins. The increase from 7 to 8 is as a result of both bidentate carboxylate oxygens of Asp-92 now forming ligands with the ytterbium. Table III compares the bond distances for the EF site for all three models. The average metal–oxygen distances for the three models are between 2.35 Å and 2.42 Å. Coordination numbers of 8 or 9 are not unexpected with oxygen ligands and have been observed for rare earth metals [25,26]. Although the average metal–oxygen distance (2.37 Å) for the EF metal site in the ytterbium-substituted model did not differ from the other models significantly (2.42 Å and 2.35 Å for the native and cadmium-substituted models), the metal binding pocket appears to be slightly more compact as a result of the movement of certain oxygen ligands. These shifts are more pronounced in the X (Asp-90) and –X (Water-128) direction. Another interesting observation is a shift of 0.11 Å in the position of the metal ion relative to the native structure. Such shifts in the metal ion have been observed in thermolysin when a calcium ion is replaced by lanthanides, and were found to vary with their atomic numbers [7]. The shifts in position were the smallest for the lanthanides of higher atomic number, presumably because of the closer similarity of their ionic radii to that of calcium ion.

Table II

Comparison of the metal–oxygen distances (Å) in the CD metal-binding site

Ligand	Calcium	Cadmium	Ytterbium
Asp-51 OD1	2.21	2.12	2.21
Asp-53 OD1	2.34	2.36	2.20
Ser-55 OG	2.61	2.58	2.53
Phe-57 O	2.37	2.25	2.33
Glu-59 OE1	2.34	2.34	2.43
Glu-62 OE1	2.61	2.77	2.51
Glu-62 OE2	2.41	2.36	2.42
Average	2.41	2.40	2.38

The average temperature factor (B) for the main chain atoms (20.28 ± 7.0 Å^2) of the ytterbium-substituted structure is higher than for the native or the cadmium-substituted structure (18.0 and 16.7 Å^2), but they follow the same trend as the native and cadmium-substituted structures. The average temperature factor for the ligands around the EF metal-binding site in all three models is higher than that for the ligands around the CD metal binding site, suggesting the EF site may be a more flexible environment. In addition, a comparison of the temperature factor of the two metal ions in each of the three models shows higher B's for the metal ion in the EF site, suggesting that it is in a less constrained environment and therefore subject to greater movement.

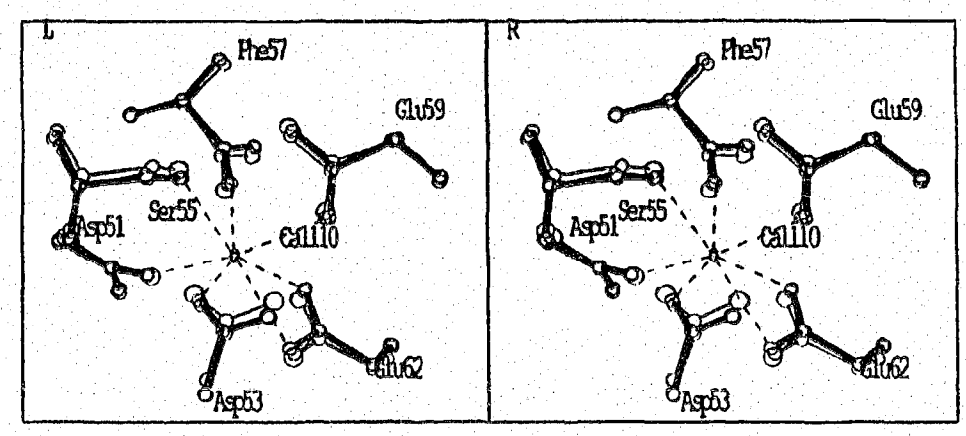

Fig. 2. Stereo view of the superposition of atoms around the CD metal-binding site for the native (open bonds), cadmium-substituted (single bonds) and ytterbium-substituted (filled bonds) parvalbumin structures. The metal atoms for all three models are indicated as open circles (Cal110) in the center. The bonds to the oxygen ligands are shown as dashed lines.

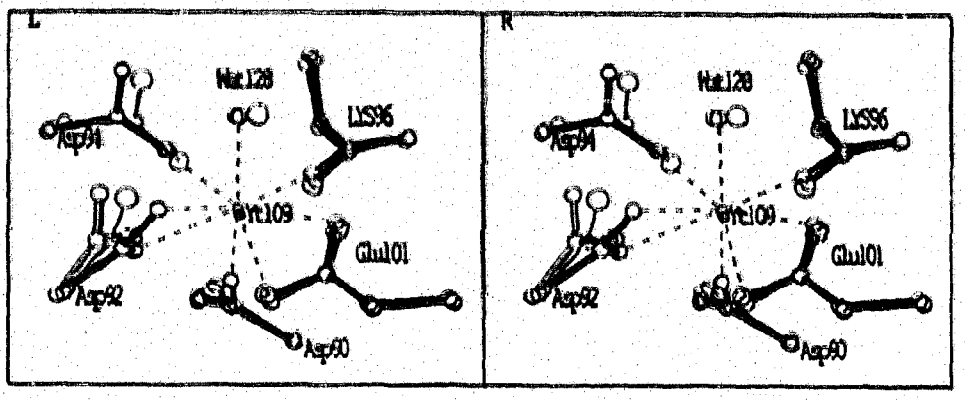

Fig. 3. Stereo view of the superposition of atoms around the EF metal binding site for the native (open bonds), the cadmium-substituted (single bonds) and the ytterbium-substituted (filled bonds) parvalbumin structures. In all three models, one water molecule (Wat128) is a ligand of the metal ion. The metal ion (Yt109) and the water molecule are represented as open circles in the center and the upper half of the figure respectively. The bonds to the oxygen ligands are shown as dashed lines.

The final refined model of ytterbium-substituted parvalbumin revealed 78 ordered water molecules, of which 43 have full occupancy, and the rest are partially occupied, the lowest occupancy being 0.74. Comparison of the ordered solvent structures with the native and the cadmium-substituted models show 48 of the 78 ordered water molecules are located at similar positions (i.e. within 1 Å), having the same hydrogen-bond partners.

The results from the refinement of the ytterbium-substituted parvalbumin structure indicate that at low molar ratios of ytterbium to parvalbumin (1 : 1) results in an extensive replacement of calcium by ytterbium at the EF site. This finding supports the NMR studies reported earlier by Lee and Sykes [15]. The metal displacement results in only minor perturbation of the overall structure, but significant changes around the substituted EF metal binding site. For instance, both carboxylate oxygens of Asp-92 are now bound by the ytterbium, resulting in an increase in the number of oxygen ligands to the EF metal from 7 to 8. There is a small displacement in the position of the substituted ytterbium ion, resulting in shorter metal–oxygen ligands with some residues. Higher temperature factors at the EF site suggest the EF site may be a more flexible environment than the CD metal site, thus making it more accessible for displacement. These results support NMR studies which suggest that the EF domain ligands are flexible whereas the CD domain ligands are rigid [27]. The present study on ytterbium-substituted carp parvalbumin shows the replacement of calcium by ytterbium results in only minor changes in the overall structure, thus supporting the ability of the lanthanide ions to mimic the biological function of calcium.

Table III

Comparison of the metal–oxygen distances (Å) in the EF metal-binding site

Ligand	Calcium	Cadmium	Ytterbium
Asp-90 OD2	2.24	2.26	2.14
Asp-92 OD1	2.37	2.39	2.29
Asp-92 OD2	–	–	2.62
Asp-94 OD1	2.41	2.17	2.48
Lys-96 O	2.42	2.30	2.28
Wat-128 O	2.35	2.39	2.13
Glu-101 OE1	2.381	2.26	2.35
Glu-101 OE2	2.69	2.66	2.69
Average	2.42	2.35	2.37

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