Acta Cryst. (1996). D**52**, 916–922

X-ray Structures of Mn, Cd and Tb Metal Complexes of Troponin C

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(*Received 9 October 1995; accepted 13 May 1996*)

Abstract

The crystal structures of three metal complexes of troponin C (TnC) have been determined and refined where the two occupied structural Ca^{2+} sites in the C domain have been substituted by Mn^{2+}, Cd^{2+} and Tb^{3+}. The X-ray intensity data were collected to 2.1, 1.8 and 1.8 Å resolution, respectively, on the three metal complexes, which are isomorphous with Ca-TnC. The three complexes have r.m.s. deviations of 0.27, 0.25 and 0.35 Å, respectively, for all protein atoms, from Ca-TnC. Irrespective of the charge on the metal (+2 or +3), the occupied sites 3 and 4 exhibit a distorted pentagonal bipyramidal coordination, like Ca-TnC, with seven ligands, six from the 12-residue binding loop and the seventh from a water molecule. Mn^{2+} at site 4 seems to display a longer distance to one of the carboxyl bidentate ligands representing an intermediate coordination simulating the six-coordinate Mg^{2+}. The carboxyl O atoms of the bidentate Glu12 are displaced on the side of the equatorial plane passing through the remaining three ligands with one O atom closer to the plane (Δ of 0.11 to 0.76 Å) than the other (Δ of 0.93 to 1.38 Å). The two axial ligands are an aspartic carboxyl O atom and a water molecule. The metal is displaced (0.18 to 0.56 Å) towards the water facing the water channel.

1. Introduction

Troponin C (TnC) is a subunit of the trimeric complex troponin. TnC undergoes a conformational change upon calcium binding and triggers muscle contraction (Leavis & Gergeley, 1984). The crystal structure of calcium-TnC (Ca-TnC) from turkey skeletal muscle was first determined at 3 Å resolution (Herzberg & James, 1985) and later refined at 2 Å resolution (Herzberg & James, 1988). The crystal structure of the corresponding Ca-TnC from chicken has been determined at 3 Å (Sundaralingam *et al.*, 1985) and refined at 2 Å (Satyshur *et al.*, 1988) and at 1.78 Å (Satyshur, Pyzalska, Greaser, Rao & Sundaralingam, 1994). The structures revealed the molecule to be dumbbell shaped with the N and C domains at each termini acting as the 'balls' with the long central helix acting as the 'bar' (Fig. 1). Of the four potential calcium-binding sites, the two N-domain regulatory sites 1 and 2 have a high specificity to Ca^{2+} but low affinity. The K_M for Ca^{2+} is $\simeq 10^5\ M^{-1}$, while for the other metals, such as Mg^{2+}, it is two orders lower (Potter & Gergely, 1975). These N-domain sites are unoccupied in crystals of both chicken and turkey TnC. On the other hand, the two C-domain structural sites 3 and 4 have a low specificity, but high affinity for Ca^{2+} ($K_M \simeq 10^7\ M^{-1}$) and are occupied in TnC crystals. The two Ca atoms at the C domain have a pentagonal bipyramidal coordination

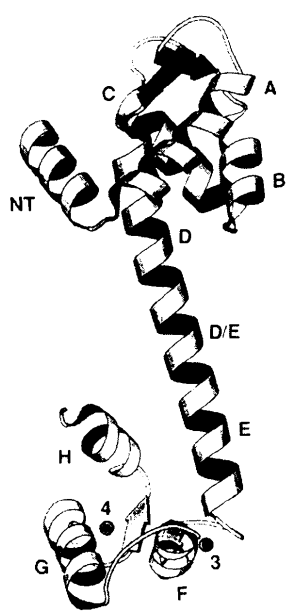

Fig. 1. A ribbon diagram produced using *Molscript* (Kraulis, 1991) showing the various helices in TnC from chicken skeletal muscle. The structural Ca^{2+}/Mg^{2+} metal binding sites 3 and 4 in the C domain are shown as filled circles. These structural sites are occupied by Ca^{2+}/Mg^{2+} *in vivo* and can also bind other divalent and trivalent ions. In the present study, these sites are occupied by Mn^{2+}, Cd^{2+} and Tb^{3+}.

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ISSN 0907-4449

geometry. It is believed that the structural sites are occupied by Mg^{2+} ($K_M \simeq 10^5\ M^{-1}$) in the absence of Ca^{2+}. In this respect, the crystal structure of TnC differs from calmodulin (CaM), which has a similar dumbbell-shaped structure with a slightly shortened central helix. All the four Ca^{2+} sites have nearly equal affinity and in CaM crystals are all occupied. The substituents in the 12-residue loop are involved in the calcium binding, the loop is flanked by two helices that are nearly perpendicular to each other (EF-hand, Kretsinger, 1980). The orientation of the helices is different depending upon whether the metal is bound or not. It is thought that this change in the helix orientation upon metal binding is the conformational trigger for muscle contraction.

Since Ca^{2+} is spectroscopically silent, other divalent and trivalent metals are used to probe the structure of the binding sites in solution. Mn^{2+} can exchange with Ca^{2+} (Fuchs, 1971) and is used in electron-spin resonance studies. Cd^{2+} also exchanges with Ca^{2+} (Fuchs, 1971) and ^{113}Cd is used to probe the environment of the metal-binding sites using NMR (Ellis, Strang & Potter, 1984; Ellis, Marchetti, Strang & Potter, 1988). Cd^{2+} can substitute for Ca^{2+} and retain the myofibril ATPase activity (Chao, Bu & Cheung, 1990). Tb^{3+} can exchange with Ca^{2+} (Wang, Leavis & Gergley, 1983) and is employed in fluorescence energy transfer studies using Tb^{3+} as donor and an organic chromophore as acceptor to determine the distance between them. To study the structural perturbation caused by the substitution of these divalent and trivalent metals for Ca^{2+} at the structural sites, we have prepared crystals of three metal complexes of TnC.

In our 3 Å study we had used manganese TnC (Mn-TnC) (Sundaralingam *et al.*, 1985) as our native. In this paper, we have extended the Mn-TnC work to 2.1 Å resolution. Cadmium TnC (Cd-TnC) was prepared by soaking pregrown crystals of Ca-TnC in solutions containing the heavy-atom reagent. The 3 Å structure was initially solved (Sundaralingam *et al.*, 1985) by using a single heavy-atom derivative of the lanthanide Nd^{3+}. However, the derivative data could not be used beyond 3.8 Å because of the lack of isomorphism. The phases were extended to 3 Å by using the 3.8 Å electron-density map obtained using the iterative single isomorphous replacement (ISIR) method (Wang, 1985). In the Nd^{3+} derivative, site 4 had a considerably lower occupancy compared to site 3. Therefore, we were prompted to use another lanthanide, Tb^{3+}. Tb-TnC crystals diffracted to 1.8 Å resolution. In this paper we report the crystal structures of TnC complexed with the three metals Mn^{2+}, Cd^{2+} and Tb^{3+}, and compare the effects of increasing the charge on metal-binding and metal-coordination geometries with that of the isomorphous Ca-TnC.

Table 1. *Intensity data for metal TnC complexes*

Resolution range (Å)	Number possible	Number of reflections with $F > 3\sigma$ (completeness, %) Mn-TnC	Cd-TnC	Tb-TnC
8.00–3.00	3135	2558 (82)	2828 (90)	3060 (98)
3.00–2.50	2359	1686 (71)	2302 (98)	2156 (91)
2.50–2.20	2569	1620 (63)	2467 (96)	2145 (83)
2.20–2.10	1185	516 (44)	1104 (93)	871 (74)
2.10–2.00	1478	—	1340 (91)	958 (65)
2.00–1.90	1758	—	1376 (78)	934 (53)
1.90–1.80	2174	—	1345 (62)	950 (44)
Overall				
8.00–2.10	9248	6380 (69)	8701 (94)	8232 (89)
8.00–1.80	14658	—	12762 (87)	11074 (76)

2. Experimental methods

2.1. *Crystals of metal complexes of TnC and intensity data*

Ca-TnC from chicken skeletal muscle was extracted, purified and crystallized using procedures already published (Strasburg, Greaser & Sundaralingam, 1980).

2.1.1. *Mn-TnC.* Ca-TnC was used to prepare Mn-TnC. The protein was dissolved in 6 *M* urea, 50 m*M* Tris–HCl, pH 8.0 and twice dialyzed against 200 vol of 50 m*M* sodium acetate, pH 4.9, 5 m*M* $MnCl_2$, 1 m*M* NaN_3, 0.1 m*M* EGTA and the concentration was adjusted to 35 mg ml^{-1}. Crystals were grown by vapor diffusion, the droplets contained the protein in 30% saturated ammonium sulfate and the reservoir buffered with 50 m*M* sodium acetate, pH 4.9, 5 m*M* $MnCl_2$ 1 m*M* NaN_3 and 43% ammonium sulfate. Large crystals grew in 2–3 weeks (Strasburg *et al.*, 1980). The intensity data were collected to 3 Å resolution on an Enraf–Nonius diffractometer later extended to 2.1 Å resolution with oscillation film data using the synchrotron source at CHESS. R_{merge} of the data was 0.05 on intensities. The diffractometer and oscillation data sets were scaled by using the common reflections between 4 and 3 Å resolution [742 reflections, $R_{merge}(F) = 0.04$]. In all, there were 6380 reflections (69% of total) with $F > 3\sigma(F)$ (Table 1).

2.1.2. *Cd-TnC.* Cd-TnC crystals were prepared by removing Ca-TnC crystals from the droplet and placing in a buffered droplet of soaking solution containing 5 m*M* $CdCl_2$. After 5 min, the crystal was removed and placed in a second droplet for 2 d. Using a Cd-TnC crystal of dimensions $0.4 \times 0.25 \times 0.2$ mm, 1.8 Å intensity data were collected on a multiwire area detector at Charlottesville. 29532 reflections were measured, yielding 12762 independent reflections with $F > 3\sigma(F)$ (87% of total) with an $R_{merge}(I)$ of 0.06 (Table 1).

2.1.3. *Tb-TnC.* Tb-TnC crystals were made similar to the procedure used to produce Cd-TnC crystals by using 0.25 m*M* $TbCl_3$ instead of 5 m*M* $CdCl_2$ in the soaking solution. The intensity data to 1.8 Å resolution were collected from a crystal measuring

Table 2. *Summary of geometrical parameters for the three TnC structures*

	Target sigma	R.m.s. values observed in Mn-TnC	Cd-TnC	Tb-TnC
Bond lengths (Å)	0.02	0.013	0.018	0.020
Bond-angle lengths (Å)	0.04	0.046	0.049	0.056
Planarity (Å)	0.01	0.004	0.005	0.006
Chiral volume (Å^3)	0.05	0.033	0.042	0.048
Peptide planarity (°)	5.0	2.5	1.8	1.8
B restraints 1-2 dist (Å^2) (bonded atoms)	4.0	3.3	3.9	3.7
B restraints 1-3 dist (Å^2) (pendent atoms in a bond angle)	6.0	5.6	5.9	6.1

$0.4 \times 0.3 \times 0.25$ mm on an Siemens/Nicolet area detector at Argonne. 26 721 reflections were measured, yielding 11 074 independent reflections with $F > 3\sigma(F)$ (76% of total) with an $R_{merge}(I)$ of 0.05 (Table 1).

Crystals of Mn-, Cd- and Tb-TnC are isomorphous to Ca-TnC, space group $P3_221$ and $a = 66.7$, $c = 60.8$ Å.

3. Refinement

The Mn-, Cd- and Tb-TnC data sets were scaled with that from Ca-TnC yielding *R* values, based on $|F|$, of 0.10, 0.14 and 0.16, respectively. Three difference electron-density maps were calculated using $|F_{metal}| - |F_{Ca}|$ as coefficients and the model phases of Ca-TnC (Satyshur *et al.*, 1988) where $|F_{metal}|$ and $|F_{Ca}|$ are the structure amplitudes of the three metal complexes and Ca-TnC, respectively. In each map the two highest peaks, between 6σ and 20σ, corresponded to the two metal sites. The remaining features in the difference maps were under 2σ and were confined to the solvent regions. In the metal-binding loops, there were no significant electron densities, suggesting that the structural perturbations were small.

The three protein structures were refined following the same procedure. The water sites were removed retaining only the protein atoms and the two metal sites in the starting model (Satyshur *et al.*, 1994). The distances between the metal and the ligands were not restrained. Several rounds of refinement were performed using *PROLSQ* (Hendrickson, 1985). The solvents were added if they had electron densities $> 3\sigma$ in difference electron-density maps and were within 3.4 Å of a polar atom of the protein or other solvent molecules (Satyshur *et al.*, 1994). $2F_o - F_c$ omit maps were used to check the protein trace and minor adjustments were made. The waters with *B* values > 70 Å^2 were removed from the model at the end of each refinement cycle.

The final models for Mn-, Cd- and Tb-TnC included two metal sites and 129, 160 and 174 solvent sites and had *R* values of 0.13, 0.16 and 0.19, respectively. All three complexes contained a sulfate ion lodged in the *B* helix, bridging the protein *via* two inserted water molecules and directly interacting with the side chain of Arg47, as observed in Ca-TnC (Satyshur, *et al.*, 1988). No evidence was found for metal-ion binding at the regulatory sites in the N domain in any of the three complexes. The refinement statistics for the three models is presented in Table 2. The estimated coordinate errors in these models (Luzzati, 1952) are in the range 0.18–0.21 Å (given along the diagonal in Table 3). The coordinates and structure factors have been deposited with the Brookhaven Protein Data Bank* (Bernstein *et al.*, 1977).

Table 3. *R.m.s. deviations (Å) between the three TnC structures and Ca-TnC*

The metals at sites 3 and 4 for each complex are shown in the corresponding column and row, respectively. The coordinate errors in the models are given along the main diagonal (in bold). The values above the main diagonal are deviations between all protein atoms in the metal complexes. The values below the main diagonal are deviations for all common atoms in the two 12-residue metal-binding loops (sites 3 and 4).

	Ca	Mn	Cd	Tb
Ca	**0.18**	0.27	0.25	0.35
Mn	0.33	**0.20**	0.14	0.32
Cd	0.30	0.16	**0.18**	0.29
Tb	0.32	0.22	0.20	**0.21**

4. Discussion

The three TnC metal complexes have structures very similar to Ca-TnC (Table 3). The r.m.s. deviations for all atoms, between the complexes and Ca-TnC are 0.14–0.35 Å, while the deviations are 0.09–0.27 Å when only the backbone atoms are considered. The smallest deviation is for Cd-TnC and the largest deviation is for Tb-TnC containing the trivalent cation Tb^{3+}, with a +3 charge.

The hydration patterns in the three metal complexes are very similar to what is observed Ca-TnC. 114 sites out of (total of 129) in Mn^{2+}, 140 (total of 160) in Cd^{2+} and 131 (total of 174) in Tb^{3+} complexes are within 1 Å of the corresponding water sites in Ca-TnC with mean deviations of 0.31, 0.34 and 0.42 Å, respectively.

4.1. *Metal coordination*

The metal is seven-coordinate, with six ligands coming from the side-chain carboxyl O atom and the backbone carbonyl atoms of the 12-residue metal-binding loop and the seventh is a water molecule. The carboxyl group of Glu, the 12th residue in the loop,

* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory [Reference: 1NCX, R1NCXSF (Cd-TnC); 1NCY, R1NCYSF (Mn-TnC) and 1NCZ, R1NCZSF (Tb-TnC)]. Free copies may be obtained through The Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Reference: GR0488).

forms a bidentate coordination, except possibly at site 4 of Mn-TnC (see below). Apart from the liganded water, the additional water molecules surrounding this water and the loop residues are also similar to that found in Ca-TnC (Satyshur *et al.*, 1988).

4.1.1. *Coordination polyhedron.* Two regular polyhedra, a pentagonal bipyramid and a trigonal biprism were considered. The r.m.s. deviations between the ligands in the three complexes and the apices of the two regular polyhedra are in the range of 0.39–0.48 Å. The differences between the two polyhedra are not significant. In Fig. 2 the site 3 Cd ligands are shown superposed on the two regular polyhedra. However, the metal is closer to the center in the pentagonal bipyramid, distances from 0.3 to 0.4 Å, compared with the trigonal biprism, with distances of 0.7–1.0 Å and is probably a reason for the coordination to be regarded as a distorted pentagonal bipyramid. However, in parvalbumin which contains two metal binding sites, one site (CD-site) is designated as a pentagonal bipyramid and the other site (EF-site) as a capped trigonal prism or a split-vertex octahedron (Swain, *et al.*, 1989). However, we find that the r.m.s. deviations of the metal-binding ligands from the apices of the two polyhedra are not significantly different and the polyhedra could be designated as pentagonal bipyramid. This is consistent with the results of Cd-NMR studies that the environments of the two Cd sites in parvalbumin are very similar (Drakenberg, Lindman, Cave & Parello, 1978).

4.1.2. *Coordination geometry.* In the pentagonal bipyramid, the five equatorial plane ligands are the side-chain atoms of residues 3 and 5, the carbonyl O atom of residue 7 and the two carboxyl group O atoms of residue 12. The two axial ligands are the side-chain O atom of residue 1 and a water molecule exposed to the solvent channel. The ligand distances in Mn-TnC, Cd-TnC and Tb-TnC, are listed in Table 4 and compared with those for Ca-TnC, and are also shown in Fig. 3. The average metal–ligand distances at the two sites are quite similar. The bidentate distances to the two O atoms of

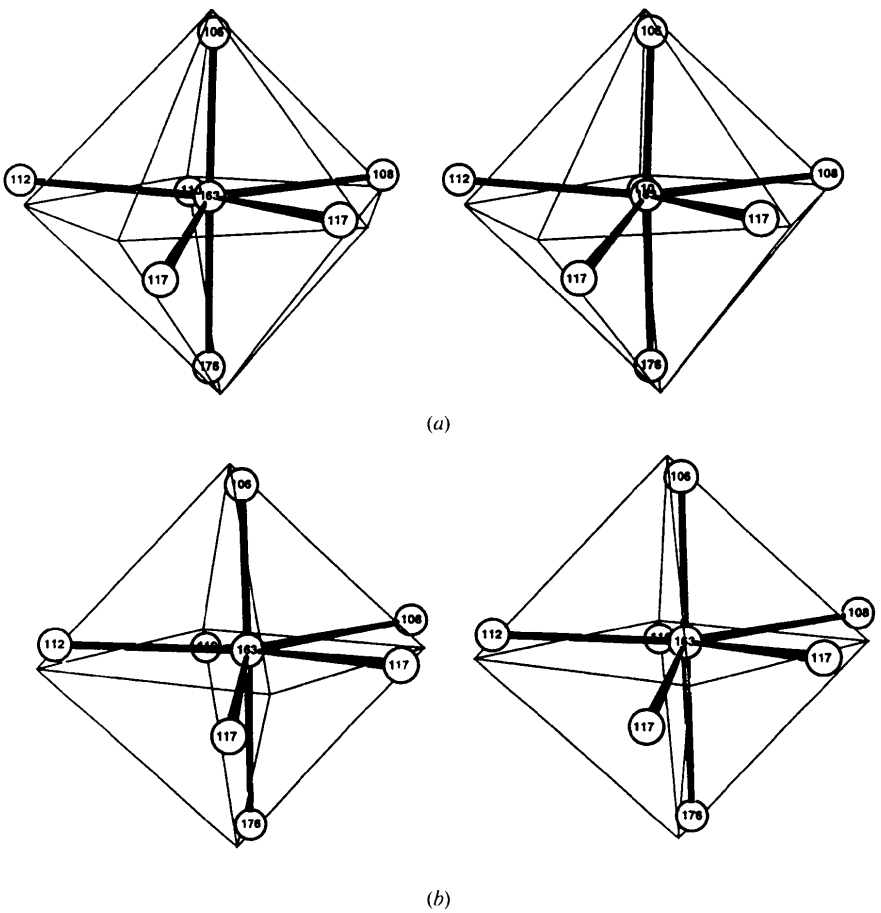

Fig. 2. Stereo figure superposing the seven ligands for site 3 of Cd-TnC on a (*a*) regular pentagonal biprism and (*b*) a regular octahedron. For the polyhedra, a metal-to-ligand distance of 2.4 Å was used.

Table 4. *Metal coordination distances in TnC complexes*

Ligand	Distance in metal complex (Å)			
	Ca	Mn	Cd	Tb
Site 3				
OD2, D106	2.22	2.15	2.08	2.26
OD1, N108	2.16	2.33	2.34	2.39
OD2, D110	2.43	2.26	2.30	2.43
O, F112	2.08	2.31	2.34	2.39
OE1,E117	2.53	2.52	2.47	2.44
OE2, E117	2.38	2.33	2.42	2.42
W, 176	2.41	2.38	2.22	2.39
Temperature factor				
$\langle B \rangle$ for liganded atoms (Å^2)	20	16	16	15
B for the metal (Å^2)	14	21	21	28
Ligand distance (Å)				
Mean	2.33	2.37	2.34	2.40
R.m.s.	0.16	0.20	0.19	0.08
Site 4				
OD2, D142	2.26	2.22	2.14	2.27
OD1, N144	2.24	2.26	2.35	2.35
OD2, D146	2.38	2.30	2.26	2.34
O, R148	2.05	2.28	2.30	2.37
OE1, E153	2.57	2.83*	2.66	2.47
OE2, E153	2.44	2.41	2.42	2.46
W, 209	2.40	2.28	2.23	2.53
Temperature factor				
$\langle B \rangle$ for liganded atoms (Å^2)	17	15	13	9
B for metal (Å^2)	12	20	18	34
Ligand distance (Å)				
Mean	2.33	2.29	2.32	2.39
R.m.s.	0.15	0.06	0.14	0.07

* Omitted from the average.

the carboxyl group of Glu at position 12 are nearly the same in all cases except at site 4 of Mn-TnC where the difference is 0.42 Å. Thus, Mn^{2+} is exhibiting a coordination between 6 and 7 and is trying to acquire a 6 coordination similar to Mg^{2+}. Indeed Mn^{2+} exhibits 6 coordination in pike parvalbumin (Declercq, Tinant, Parello & Rambaud, 1991) while at site 3 of Mn-TnC the coordination is still 7. Thus, Mn^{2+} behaves as an intermediate between Ca^{2+} and Mg^{2+} and can assume coordination of 6 or 7. It is interesting that the ionic radius of Mn^{2+} (0.80 Å) is also intermediate between that of Ca^{2+} (0.99 Å) and Mg^{2+} (0.65 Å).

The angles subtended by the ligands at the metal are listed in Table 5. The two axial ligands are significantly non-collinear with the subtended angles at the metal in the range 160–171 . The mean angles that the two axial ligands make with the five equatorial ligands are close to 90° but significant deviations are seen for the ligand from residue 3 and the bidentate ligand from residue 12. The angles subtended by the five equatorial legends at the metal atom are also given in Table 5. The sum of the five angles differs from 360 indicating that the five equatorial ligands and the metal are not coplanar.

In the three cases, the carboxyl group of Glu at position 12 is twisted out of plane, with reference to the plane through the three ligands of residues 3, 5 and 7,

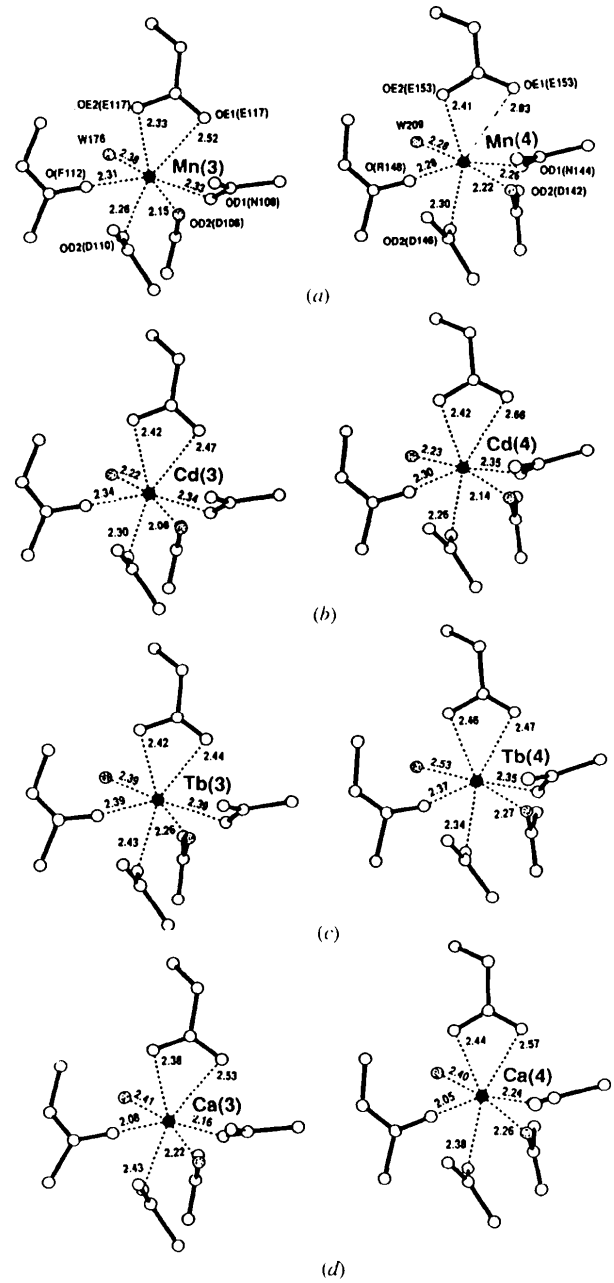

Fig. 3. Metal coordination in TnC. The numbers in parentheses following the metal refer to the metal-binding site. The columns correspond to sites 3 (left) and 4 (right). The rows correspond to (*a*) Mn, (*b*) Cd, (*c*) Tb and (*d*) Ca. The ligands are labelled in (*a*), the two axial ligands are shown as dotted circles and the metals as filled circles. The metal ligands are joined by dashed lines and the distances are also shown. The line with long dashes between OE1(153) and Mn(4) denotes the long distances of 2.83 Å.

Table 5. *Angles ( ) made by the ligands at the metal in four TnC structures*

	Site 3				Site 4			
Ligands*	Ca	Mn	Cd	Tb	Ca	Mn	Cd	Tb
Axial (1)-equatorial ligands								
1-3	80	82	81	78	83	83	81	78
1-5	83	85	86	80	81	85	84	84
1-7	81	83	86	77	85	87	87	85
1-12.2	113	113	115	106	117	108	113	111
1-12.1	93	97	98	92	94	87	90	88
Mean	90	93	93	87	92	90	91	89
Axial (*W*)-equatorial ligands								
W-3	99	96	92	112	88	92	93	102
W-5	78	78	77	84	87	87	79	82
W-7	94	92	94	88	99	95	91	90
W-12.2	83	82	82	83	78	80	84	81
W-12.1	104	98	96	107	96	98	105	105
Mean	90	89	88	95	90	90	90	92
Axial (1)-axial (*W*) ligand								
1-*W*	162	163	162	160	166	171	162	166
Equatorial ligands								
3-5	84	81	78	80	81	85	79	79
5-7	78	76	81	78	78	78	79	78
7-12.2	80	82	82	78	78	81	82	79
12.2-12.1	51	52	50	51	52	49	51	50
12.1-3	73	73	74	77	77	71	75	80
Sum	366	364	365	364	366	364	366	366

* The numbers denote the residue numbers of the O atoms in the 12-residue loop and *W* denotes the water molecule. The equatorial pentagonal ligands are the side-chain O atoms from residues 3 and 5, the backbone carbonyl of residue 7 and both O ligands of Glu at position 12 (denoted as 12.1 and 12.2). The axial ligands are the side-chain O atom to residue 1 and the water molecule.

one of the O atoms of Glu is more displaced than the other, similar to that observed in other Ca-bond EF-hand structures (Strynadka & James, 1989). It is interesting that in the bidentate ligand, the O atom that is more coplanar is at a longer distance from the metal. In all cases, the metal and the bidentate oxygen ligands are displaced towards the water ligand, which is exposed to the solvent channel. While the deviations of the two O atoms, OE1 and OE2 of the bidentate and the metal from the three-atom plane are similar at sites 3 and 4 for the metal complexes, the deviations are somewhat larger at site 3 (average 0.7 and 1.3 Å) than at site 4 (average 0.3 and 1.1 Å). The approach of the Glu side chain to coordinate with the metal is slightly different for the two sites.

The increased charge of +3 of Tb^{3+} does not result in significant shortening of any of its distances from the liganded or unliganded O atoms in the 12-residue loop. However, the average thermal parameters of the seven ligands (Table 4) are somewhat lower, suggesting lower mobility.

When the 7 coordinating oxygen ligands in the metal complexes are superposed, the r.m.s. deviations range from 0.11 to 0.19 Å, with the water ligand showing the largest deviations. The 12-residue metal-binding loops (sites 3 and 4) in each metal complex are more similar to each other (deviations of 0.11 to 0.25 Å) than for a given site among the complexes (deviations of 0.09 to 0.42 for site 3 and 0.10 to 0.41 Å for site 4). The atoms in the two 12-residue loops in the complexes have r.m.s. deviations in the range 0.16–0.42 Å, comparable to the r.m.s. deviations between the two loops in each complex.

5. Conclusions

Neither the charge (+2 or +3) nor the ionic radii of the metals substituting at the two structural sites in TnC have any substantial effect on the structure of either the EF-hands or the protein. However, the increased charge results in a reduced mobility (smaller thermal parameters) of the metal ligands. The largest structural variability in the coordinated ligands is found for the water molecule. At site 4 in Mn-TnC, Mn^{2+} seems to be intermediate between 6 and 7 coordinate and tending towards the hexa-coordinate Mg^{2+}. Cd^{2+} has an ionic radius (0.97 Å) very close to that of Ca^{2+} (0.99 Å) and causes very little perturbation in the structure. Cd^{2+} would therefore have been the best choice for the heavy-atom derivative. The replacement of the two Ca^{2+} by Cd^{2+} in carp parvalbumin also resulted in very little structural perturbation (Swain, Kretsinger & Amma, 1989). Thus, ^{113}Cd NMR should be an effective tool to probe the structure of calcium-binding sites in calcium-binding proteins.

The technical assistance of Ms Swati Shah is acknowledged. This research was supported by a NIH grant GM49547 and an Ohio Eminent Scholar Chair to MS.

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