Crystallographic determination of lanthanide ion binding to troponin C

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X-ray intensity data to 2.8 Å resolution were collected from each of three crystals of turkey skeletal troponin C (TnC) that had been soaked individually in solutions containing the lanthanide ions europium, thulium and lutetium. Each of the resulting difference electron density maps computed for the three data sets showed three lanthanide ion-binding sites on the TnC molecule. Two of the sites are approximately coincident with the Ca^{2+} positions in binding loops III and IV and the third site is near the Ca^{2+}-free loop I. The mode of ion binding in loop I is different from that commonly observed for Ca^{2+} in the Ca^{2+}-binding proteins.

(Muscle) Troponin C Crystallography X-ray analysis Lanthanide binding

1. INTRODUCTION

The contraction process in skeletal muscle is regulated by troponin, a complex of 3 protein molecules (review [1]). One of them, troponin C (TnC), is the subunit that binds Ca^{2+}; two are bound with high affinity ($K_{assoc} \simeq 10^7\ M^{-1}$) to the C-terminal domain and two with lower affinity ($K_{assoc} \simeq 10^5\ M^{-1}$) to the N-terminal domain [2]. It is generally accepted that the low-affinity sites of the N-domain are responsible for the regulatory function of TnC.

Trivalent lanthanide ions have been used as probes of structure and conformation for many Ca^{2+}-binding proteins because they can replace bound Ca^{2+}. The binding of Tb^{3+} or Eu^{3+} to either the high- or low-affinity sites of TnC has been shown to be 10- and 100-fold tighter, respectively, than that of Ca^{2+} [3]. Fluorescence changes upon binding these ions have been useful in monitoring possible conformational changes that TnC undergoes. With reference to this, it is important to establish whether lanthanide ions and Ca^{2+} bind in similar ways to the protein.

The crystal structure of turkey skeletal TnC at 2.8 Å resolution has been determined [4], and a 3 Å resolution α-carbon tracing of chicken skeletal muscle TnC with Mn^{2+} replacing Ca^{2+} has been reported [5]. In both structures two metal ions are bound to the high-affinity domain, whereas the regulatory domain is Ca^{2+}-free. We have extended and refined the initial 2.8 Å resolution structure to 2.2 Å, a process that has currently yielded a model with an R factor of 0.155. The high quality of the refined structure has afforded an interpretation of the intricate hydrogen-bonding network and the molecular framework of the Ca^{2+}-binding loops [6]. At this stage it is appropriate to analyse in detail data from crystals soaked in various lanthanide solutions that were used for resolving the phase ambiguity necessary for the initial structure determination. The resultant difference electron density maps provide an estimate of the extent of isomorphism between Ca^{2+} and lanthanide ion binding to TnC.

2. EXPERIMENTAL

Crystals of turkey skeletal muscle TnC were grown as in [7]. Lanthanide ion-doped crystals were prepared by soaking native crystals for 1 h in a solution of similar composition but omitting

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$CaCl_2$ and polyethylene glycol 200. The crystals were then soaked in an equivalent solution to which 1 mM $EuCl_3$, 1 mM $LuCl_3$ or 5 mM $Tm_2(SO_4)_3$ had been added allowing 14–16 h soaking time as listed in [4].

X-ray intensity data were collected on an Enraf-Nonius CAD4 single-crystal diffractometer. Two native TnC crystals were used to obtain data to a resolution limit of 2.0 Å, and single crystals of each lanthanide ion derivative provided 2.8 Å resolution data. These data were corrected for intensity decay due to radiation exposure, for crystal absorption profile and for Lorentz and polarization factors.

The native data set has been refined at 2.2 Å resolution using a restrained-parameter, reciprocal space least-squares refinement process [8]. A total of 6529 reflections (resolution range 10–2.2 Å, with the intensity, I, greater than twice the standard deviation in measurement of I) was used. The present agreement factor R ($= \Sigma||F_o| - |F_c||/|F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively) after 157 cycles of refinement is 0.155, and the molecular structure has only relatively small root mean square (r.m.s.) deviations from expected geometry (0.017 Å for covalent bond lengths, 0.038 Å for inter-bond bond angles and 2.6° r.m.s. deviation from 180° of the trans-planar peptide bond). The phases obtained by this refinement process were used to phase 2.8 Å resolution difference Fourier maps calculated for each lanthanide data set. The Fourier coefficients were of the form ($|F_L| - |F_N|$), where $|F_N|$ and $|F_L|$ are the observed structure factor amplitudes of the native and lanthanide data, respectively. The difference maps together with the TnC model were displayed and inspected on an MMS-X interactive graphics system using the program M3 [9].

3. RESULTS AND DISCUSSION

The difference Fourier maps for the 3 lanthanide data sets gave rise in each case to 3 peaks, indicating 3 ion-binding sites: two replacing the Ca^{2+} in loops III and IV of the high-affinity domain and the third binding to the Ca^{2+}-free loop I in the

Table 1

Binding of lanthanide ions to TnC

Lanthanide	Site occupancy[a]	I	III	IV	Effective ionic radius (Å)[e]
Lu^{3+}	from heavy atom refinement[b]	0.2	0.8	0.4	0.86
	normalized from difference Fourier[c]	0.2	1	0.4	
Tm^{3+}	from heavy atom refinement	0.8	0.6	[d]	0.88
	normalized from difference Fourier	0.7	0.7	0.3	
Eu^{3+}	from heavy atom refinement	0.4	0.9	0.9	0.95
	normalized from difference Fourier	0.4	0.8	1	

[a] Occupancies are calculated based on the atomic number (Lu = 71; Tm = 69; Eu = 63) and with the assumption that in sites III and IV the occupancy represents the difference between the scattering of the lanthanide and calcium whereas in site I it wholly represents the lanthanide scattering

[b] Heavy atom refinement was carried out as described in [4] and occupancies were determined on absolute scale

[c] Occupancies were estimated from the peak height of difference Fourier maps and normalized such that the highest peak (Lu^{3+} site III) has the value 1

[d] The binding of Tm^{3+} to loop IV was not determined from the initial heavy atom refinement process

[e] The effective ionic radii are according to [10]. They are quoted for coordination number 6 and are linearly dependent on the rise in coordination number. The corresponding value for Ca^{2+} is 1.0 Å

regulatory domain (see [4] for nomenclature of the loops in TnC). Each of the lanthanide ions binds to these sites with different relative occupancies, as shown in table 1. Note that Tm^{3+} binding to loop IV was not identified in the initial 2.8 Å structure determination work. We attribute this to the relatively low occupancy of that site (table 1) and the phase errors associated with the multiple isomorphous replacement method.

The lanthanide ions each bind to the high-affinity sites III and IV in much the same manner as Ca^{2+}. The calculated distances between the Ca^{2+} and lanthanide ion positions in sites III and IV average 0.3 and 0.8 Å, respectively. There is no indication that the protein undergoes substantial conformational changes in the vicinity of the lanthanide ions. These results should be taken with caution since the difference Fourier maps are biased by the phases that were derived from the Ca^{2+}-occupied native structure. Accurate lanthanide positions and subtle conformational changes may be determined only by extending the resolution of the lanthanide data and by independently refining these structures. However, the overall properties of the binding are not likely to change in substance.

The noise level of the difference electron density in the region Eu^{3+} bound in loops III and IV is larger than that for the Tm^{3+} and Lu^{3+} derivatives. This suggests that the binding of Eu^{3+} is accompanied by somewhat larger shifts of the protein, and may explain the lack of isomorphism of this derivative which allowed us to use its phasing information only to 4.5 Å resolution [4].

Lanthanide ions bind to loop I in a different fashion. This can be seen for Tm^{3+} in fig.1. The difference electron density indicates that the metal ion binds to the following residues: Asp-30 O^{δ}; Asp-34 O^{δ}; Asp-36 O^{δ}; Asp-36 O. The carboxylate of Glu-41 is too far away to coordinate to the metal; it is involved in an ion pair with the N^{ϵ} atom of Lys-40 (fig.1). Thus, this mode of binding is different from that expected for Ca^{2+} binding based on our analysis [6] in two major aspects: (i) residues in the C-terminal part of the loop do not interact with the ion and (ii) Asp-36 contributes both its main chain carboxyl oxygen atom and its side chain carboxylate to the lanthanide ion ligand field.

The shape of the electron density in fig.1 suggests that there are possibly two solvent molecules interacting with the lanthanide ion. It is apparent

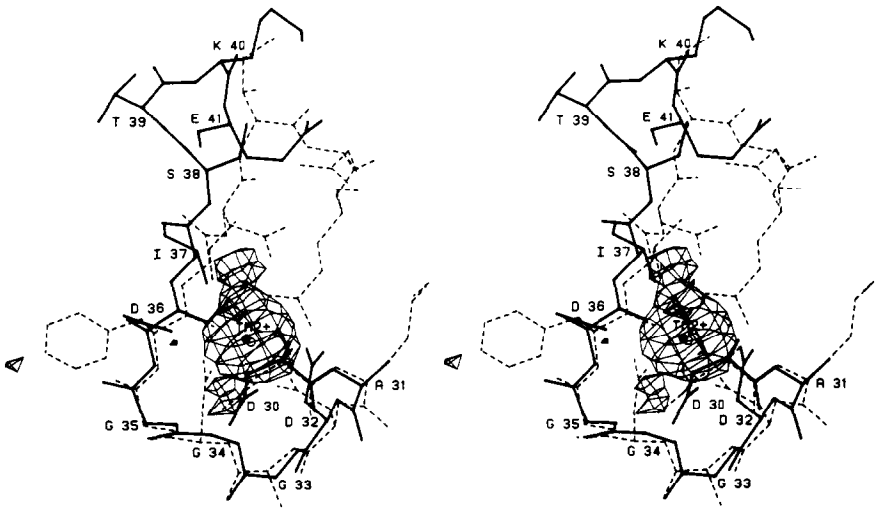

Fig.1. Stereoscopic representation of loop I (——) of TnC. Also shown is the difference electron density corresponding to the binding of Tm^{3+}. Superimposed on this loop is loop III (– – –). The superposition was done by a least-squares minimization using the main chain atoms of the 6 N-terminal residues of the loops. The r.m.s. difference in positions for these atoms is 0.42 Å. The resulting rotation matrix and translation vector were applied to all atoms of loop III including side chain and Ca^{2+}. The position of Tm^{3+} (●) is very near to that of Ca^{2+} (○).

by inspecting the conformation of the superposed loop III, that one of these postulated solvent molecules fulfills the function of the carboxylate of the third ligand in loop III (Asp-110). The other is equivalent to the solvent molecule that is the fifth ligand in both loops III and IV [6]. This arrangement makes up a binding mode with a coordination number of 6. Whether or not there are more solvent molecules interacting with the metal ion in loop I has to await the high-resolution refinement of these structures. No lanthanide ion is associated with loop II, whose conformation is similar to that of loop I. This may be correlated with the lack of an aspartic acid analogous to Asp-36 that can contribute both its main chain carbonyl and side chain carboxyl as ligands. In loop II the equivalent residue is Thr-72.

In the native structure there is no Ca^{2+} bound to the low-affinity loops (I and II). This suggests that the mode of ion binding to loop I depicted for lanthanides is not available for Ca^{2+}. We do not consider the lanthanides binding to loop I as physiologically significant. The specific packing of TnC molecules in the crystal occurs such that the regulatory domain is in a helix-loop-helix conformation different from that commonly observed at the Ca^{2+}-bound state. The transition to the bound state probably involves structural changes too large to be accommodated in the crystal [11]. In contrast to calcium, lanthanide ion affinity to loop I is high enough for binding even in the Ca^{2+}-free conformation. Presumably, in muscle, where the environment is not so restrictive as in the crystals and the transition from one state to the other is possible, lanthanides bind in the commonly observed conformation for all other Ca^{2+}-binding proteins [6].

It can be seen in table 1 that site I is occupied to a different extent by the three lanthanide ions. Unfortunately, the amount of data currently available and their accuracy do not permit systematic interpretation of binding properties to various lanthanides in the manner described for parvalbumin [12,13]. Sites III and IV show some tendency that may be significant. Whereas all 3 lanthanides apparently have full occupancy in site III, the occupancy of site IV appears to be more selective for the ion with the larger effective ionic radius (Eu^{3+}). This implies that there may be differences in the properties of sites III and IV, affecting the kinetics or the affinities of the lanthanide series. Systematic monitoring of their binding to TnC as performed for parvalbumin may contribute more information concerning this aspect.

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