Lanthanide-Binding Peptides

Structural Origin of the High Affinity of a Chemically Evolved Lanthanide-Binding Peptide**

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Lanthanide-binding tags (LBTs) have been developed to enable the efficient production of fusion proteins that contain a high-affinity lanthanide-binding site.[1] These multitasking tags, which comprise only encoded amino acids, are easily incorporated, at the DNA level, into any recombinant protein. Upon complexation of a lanthanide ion, the LBT in a fusion protein provides a powerful and versatile handle to facilitate investigations involving luminescence,[1–4] NMR spectroscopy,[5–9] and X-ray crystallography.[10,11] Previously, these biophysical techniques have been implemented by binding lanthanides to the EF-hand motifs of calcium-binding proteins.[2,4,12] Ca^{2+}-binding loops are stabilized in EF-hand proteins by intraprotein interactions across the flanking helices of the helix–loop–helix motif. Achieving the nM–pM level of affinity of native calcium-binding proteins in an LBT for lanthanide ions necessitated the optimization of short peptides that bind lanthanides tightly without relying on the stabilizing forces of the native protein scaffold. Another design consideration was to build a lanthanide-binding site that satisfies the coordination chemistry solely from peptide-based ligands, thereby excluding water molecules from the primary coordination sphere, which would lead to luminescence quenching.[13,14] LBTs with these attributes were obtained through a combination of peptide design, targeted combinatorial synthesis, and a powerful screening protocol that utilized Tb^{3+}-based luminescence.[1,15]

Herein we present the 2.0-Å resolution X-ray crystal structure of a Tb^{3+}–LBT complex that achieves the design goals by using only 17 amino acids to maintain an overall structural integrity that is similar to an EF-hand motif found in calcium-binding proteins. The closed-shell coordination of the Tb^{3+} ion observed in the crystal structure is maintained in solution, as confirmed by time-resolved luminescence measurements. These studies provide insight into the origins of the enhanced luminescence of the LBT and its selectivity and high affinity for Tb^{3+} ions.

Figure 1A shows a ribbon diagram of the Tb^{3+}–LBT complex, together with the metal-binding residues. The eight-coordinate Tb^{3+} complex is formed by the monodentate oxygen ligands of Asp1, Asn3, and Asp5, bidentate carboxylate ligands from Glu9 and Glu12, and the backbone carbonyl group of Trp7, which also provides the indole group that sensitizes the Tb^{3+} luminescence. All the metal–oxygen bond lengths are within the expected range of 2.3–2.5 Å commonly found in eight-coordinate terbium complexes.[16,17] Subtle but significant differences are evident between this structure and the corresponding site (Figure 1B) from the structure of loop 3 of troponin C bound to Tb^{3+},.[18] An overlay of the backbones of the Tb^{3+}–LBT complex and Tb^{3+}-loaded troponin C gives a root-mean square deviation of only 1.18 Å over all main-chain atoms (Figure 1C) despite the *absence* of a supporting protein scaffold for the troponin C loop. Additionally, new side-chain contacts have been created both to the Tb^{3+} ion and between noncontiguous residues within the LBT. Most importantly, the chemical evolution achieved a coordination sphere for the Tb^{3+} ion that was provided exclusively by peptide-based ligands in the LBT, whereas an ordered water molecule provides one of the seven ligands in the troponin C structure (Figure 1B).

The most noticeable deviation between the structures occurs at residues 9 and 10 at the top of the loop (Figure 1C). This part of the backbone has moved closer to the metal

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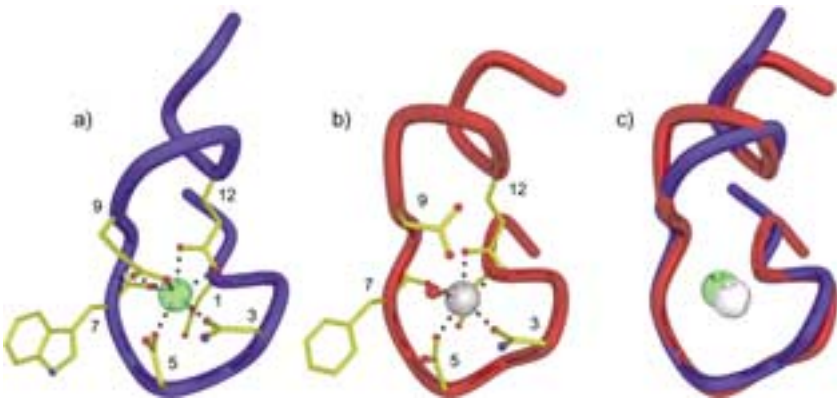

Figure 1. a) Ribbon structure of the LBT–Tb^{3+} complex showing metal-ligating residues and the terbium ion (green sphere). b) Ribbon structure of the Tb^{3+}-bound troponin C EF-hand motif (residues 104–120, PDB accession code: 1NCZ) showing metal-ligating residues, the terbium ion (gray sphere), and the ligating water molecule (red sphere). c) Overlay of the Tb^{3+} complexes of LBT (blue) and troponin C (red) complexes.

center in the LBT, thereby allowing bidentate chelation by Glu9. In fact, position 9 is the only metal-binding residue that differs between the two peptides, as shown in Table 1. In

Table 1: Sequence alignment of the LBT and the corresponding EF-hand motif of troponin C.

	Position														
	−1	1	3	5	7	9	11	13	15						
troponin C	I	F	**D**	K	**N**	A	**D**	G	**F**	I	**D**	I	E	**E**	L	G	E
LBT	Y	I	**D**	T	**N**	N	**D**	G	**W**	Y	**E**	G	D	**E**	L	L	A

troponin C, the aspartate residue at position 9 does not coordinate the metal directly, rather it forms a hydrogen bond to a metal-ligating water molecule. So it is the Asp9Glu mutation, together with the translation of the backbone, that allows the exclusion of water from the Tb^{3+} coordination sphere. Bidentate coordination has not been observed previously at position 9 of EF-hand motifs. In the parvalbumins, a glutamate residue occupies this position, but it cannot easily adopt the correct conformation for bidentate ligation.[19] The dihedral angles of Gly10($\phi = 43.7°$, $\psi = -123.4°$) of the LBT complex are uniquely allowed for glycine residues and it is this conformation that allows translation of the peptide backbone to position the glutamate residue at position 9 so as to achieve the bidentate coordination that is key to the LBT structure.

Measurements of the terbium luminescence lifetime in solution were performed to correlate the crystallographic results with the solution-state biophysical properties.[14] The number of water ligands (q) to the Tb^{3+} ion can be measured by determining the luminescence lifetime of the complex in H_2O and D_2O since the lanthanide-excited state is quenched by the vibrational overtones of H_2O. The lifetimes of the LBT complex were fitted to a single exponential decay and were determined to be exceptionally long (2.6 ms in H_2O and 3.4 ms in D_2O; Figure 2). These rates lead to a q value of 0.3, thereby reinforcing the crystal-structure results indicating that there are no water ligands in the first coordination sphere of the Tb^{3+} center. Uncertainties in the q value arise from errors in the empirical proportionality constant relating the number of ligating water molecules to the luminescence decay rates and are estimated to be considerably less than 0.5 water molecules.[14] The closed-shell coordination of the Tb^{3+} ion is the major source of the improved luminescence intensities and long lifetimes, which have not previously been observed with peptide ligands.

Terbium ligation by the Trp7 backbone carbonyl group observed in the crystal structure is consistent with the early work in the Szabo research group who established that the tryptophan residue at position 7 of a calcium-binding loop is optimal for sensitizing Tb^{3+} lumines-

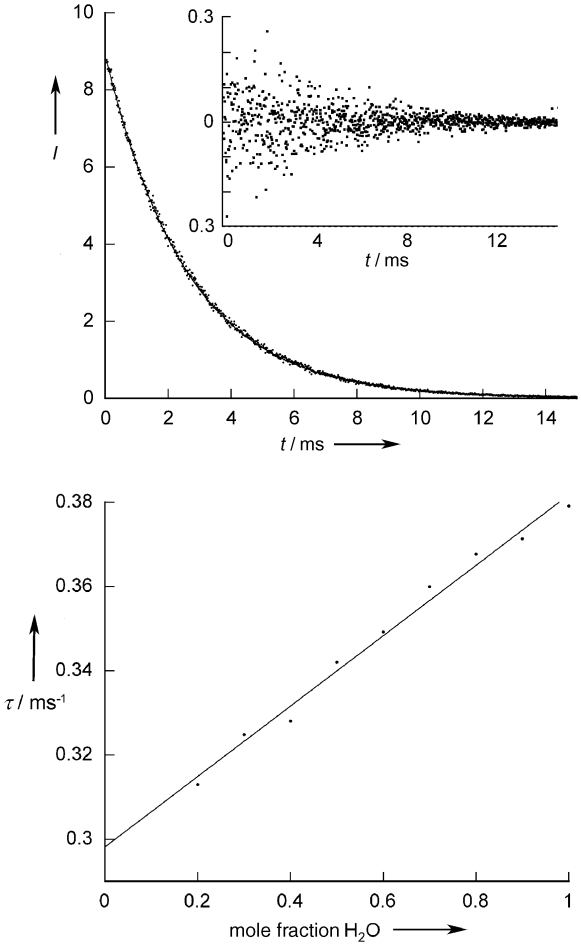

Figure 2. Top: Luminescent decay of a Tb^{3+} ion bound to the LBT. The excitation wavelength was 280 nm, and Tb^{3+}-sensitized emission was recorded at 544 nm. The line represents a single exponential curve fit of the experimentally determined intensities. Inset: Residuals (that is, deviation of the experimental intensities from the curve fit). Bottom: Luminescent decay rates with increasing H_2O concentrations. Extrapolation to an H_2O-free solution provides the rate constant in D_2O.

cence.[20] The structure of the LBT confirms that the center of the indole is positioned 7.0 Å from the Tb^{3+} center, thereby maximizing sensitization of the terbium luminescence.[21] In the LBT structure Tyr8 is 6.0 Å from the terbium center and may also contribute to the sensitized luminescence of the complex. To determine its contribution to the luminescence intensity, a mutant LBT peptide was synthesized in which Trp7 was replaced with phenylalanine, a residue that does not sensitize Tb^{3+} luminescence. Based on the integrated intensities of the major terbium emission peak (544 nm), the Trp7Phe mutant shows approximately 15% of the luminescence of the parent LBT. Therefore, while Tyr8 makes some contribution to the energy transfer to the Tb^{3+} ion, Trp7 provides the majority of the sensitizing power.

Analysis of the electrostatic potential of the LBT shows that the motif has been optimized to shield the terbium ion from water. The surface potential of the LBT is neutral and hydrophobic, despite the positive charge of the terbium ion and the negatively charged ligands. In contrast, the surface associated with the Tb^{3+}-binding site in the troponin C is positively charged and favors water ligation (Figure 3).

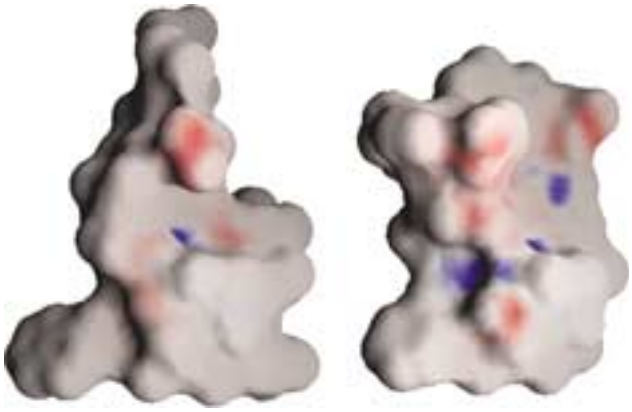

Figure 3. Electrostatic surface potential as calculated by the GRASP method.[22] The LBT–Tb^{+3} complex is shown on the left and the troponin C EF-hand motif on the right. The electrostatic surface was contoured at −15 kT (red, negative) and +15 kT (blue, positive).

In addition to improving the luminescence intensity of LBT peptides, the other goal of this study was to improve the lanthanide affinity. During the design process, hydrophobic amino acids were added at the C and N termini to promote hydrophobic interactions that would preorganize the loop for metal-ion binding. Examination of the structure reveals an N terminus with an extended conformation that allows it to pack against the C terminus. A hydrophobic cluster formed by Tyr1, Tyr8, and Leu13 (Figure 4) appears to "cinch" the loop closed, thereby creating a pseudo macrocycle that is primed for ion binding. The importance of these interactions is emphasized by the diminished affinity of LBT mutants that perturb this hydrophobic cluster for Tb^{3+} ions. Deletion of the N-terminal tyrosine residue reduces the terbium affinity 25-fold and the replacement of Tyr8 by an arginine residue reduces terbium affinity 120-fold. Replacement of Leu13 with an alanine residue reduces the affinity 36-fold. Conversely, deletion of the two C-terminal residues does not significantly

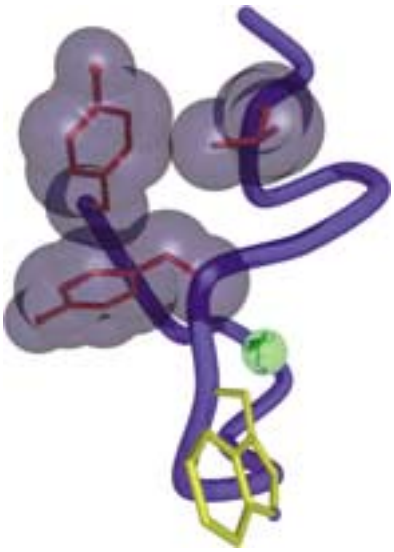

Figure 4. Ribbon structure of the LBT–Tb^{+3} complex with the interaction amongst hydrophobic residues Tyr1, Tyr8, and Leu13 emphasized (red).

reduce the affinity of the LBT for Tb^{3+} ions, a fact suggesting that these residues may have a minor role in predisposing the C terminus to a helical conformation.

The impetus for the design of the LBT sequences was to have a single tag that could take advantage of the range of useful physical properties possessed by lanthanide ions. Evaluation of the affinity of the LBT for the lanthanides across the series was carried out with competitive titrations.[23] The affinity of the LBT for lanthanides varies across the series in a harmonic relationship with the ionic radius, as previously observed for calcium-binding proteins.[23] The range in affinities varies from an apparent dissociation constant K_d of 4 μM for La^{3+} ions to 57 nM for Tb^{3+} ions. The range of affinities observed stems from the lanthanide contraction across the series, which leads to a 20% decrease in ionic radii with a concomitant increase in the Lewis acidity of the metal.[24] Thus, the size complementarity between the ion and the binding site (with bias for smaller lanthanides because of their higher Lewis acidity) is responsible for the range of affinities observed. With the minimum free energy of binding in the middle of the series, a range of useful high-affinity lanthanide complexes are readily accessed with the LBT (Figure 5).

The current crystallographic and luminescence studies indicate that the LBT binding environment for the Tb^{3+} ions has been optimized to provide a full complement of peptide-based ligands that exclude water from the first coordination sphere. In addition, the LBT has high affinity for a range of trivalent lanthanides, thereby allowing access to the varied physical properties displayed across the series. The LBT is independently folded, with the first and final residues proximal, which makes it compatible with insertion at the N terminus, at the C terminus, or within a loop of a target protein. The structure presented will facilitate the development of future generations of the LBT and provide a basis on which to model the LBT into known protein structures for

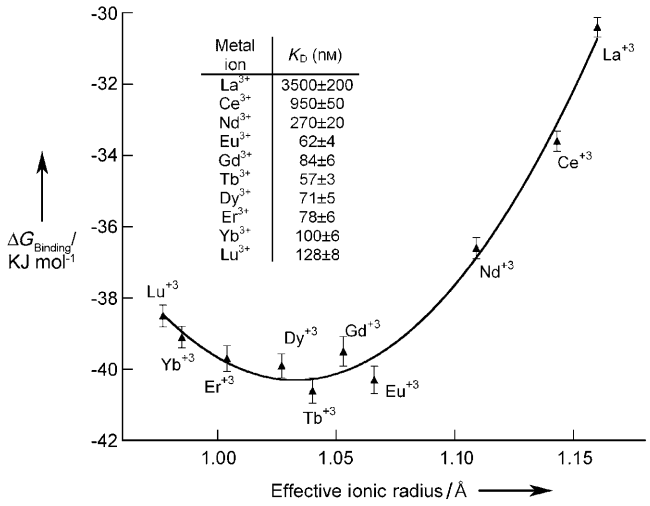

Figure 5. Dependence of binding free energy with the LBT on the effective ionic radii of rare-earth metals. The line is added to depict the trend rather than a theoretical correlation. Error bars indicate combined sources of error implicit in each competitive titration.

biophysical studies such as lanthanide-based resonance energy transfer (LRET).[12,25,26]

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