

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

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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] Ca²⁺-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 Tb³⁺-based luminescence.^[1,15]

Herein we present the 2.0-Å resolution X-ray crystal structure of a Tb³⁺-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 Tb³⁺ 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 Tb³⁺ ions.

Figure 1A shows a ribbon diagram of the Tb³⁺-LBT complex, together with the metal-binding residues. The eight-coordinate Tb³⁺ 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 Tb³⁺ 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 Tb³⁺.^[18] An overlay of the backbones of the Tb³⁺-LBT complex and Tb³⁺-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 Tb³⁺ ion and between noncontiguous residues within the LBT. Most importantly, the chemical evolution achieved a coordination sphere for the Tb³⁺ 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

cence.^[20] The structure of the LBT confirms that the center of the indole is positioned 7.0 Å from the Tb³⁺ 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 Tb³⁺ 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 Tb³⁺ 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 Tb³⁺-binding site in the troponin C is positively charged and favors water ligation (Figure 3).

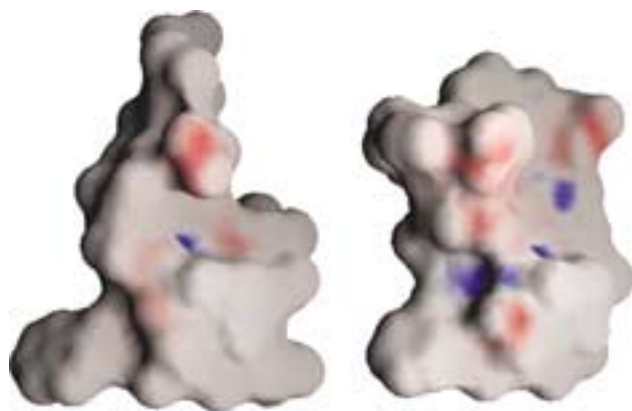


Figure 3. Electrostatic surface potential as calculated by the GRASP method.^[22] The LBT–Tb³⁺ complex is shown on the left and the troponin C EF-hand motif on the right. The electrostatic surface was contoured at –15 kT (red, negative) and +15 kT (blue, positive).

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 Tb³⁺ 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–Tb³⁺ complex with the interaction amongst hydrophobic residues Tyr1, Tyr8, and Leu13 emphasized (red).

reduce the affinity of the LBT for Tb³⁺ 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 La³⁺ ions to 57 nM for Tb³⁺ 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 Tb³⁺ 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

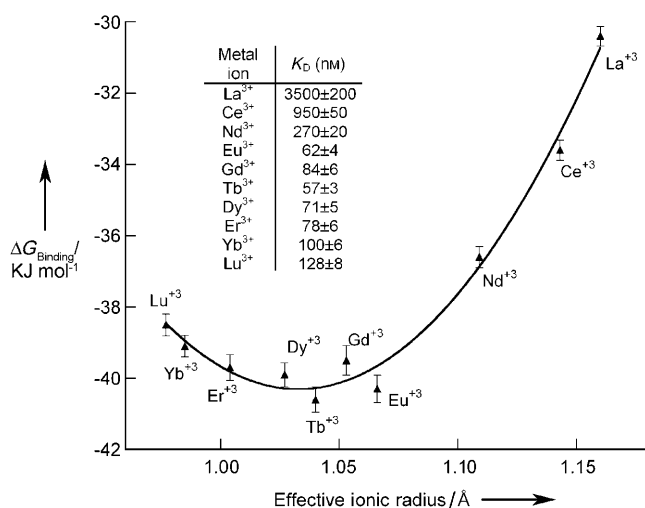


Figure 5. Dependence of binding free energy with the LBT on the effective ionic radii of rare-earth metals. The line is added to depict the trend rather than a theoretical correlation. Error bars indicate combined sources of error implicit in each competitive titration.

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

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 [26] Details regarding peptide synthesis, crystallization, data collection and refinement, and luminescence spectroscopy are available in the Supporting Information.