A Powerful Combinatorial Screen to Identify High-Affinity Terbium(III)-Binding Peptides

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Lanthanide-binding tags (LBTs) are protein fusion partners consisting of encoded amino acids that bind lanthanide ions with high affinity. Herein, we present a new screening methodology for the identification of new LBT sequences with high affinity for Tb^{3+} ions and intense luminescence properties. This methodology utilizes solid-phase split-and-pool combinatorial peptide synthesis. Orthogonally cleavable linkers allow an efficient two-step screening procedure. The initial screen avoids the interference caused by on-bead screening by photochemically releasing a portion of the peptides into an agarose matrix for evaluation. The secondary screen further characterizes each winning sequence in a defined aqueous solution. Employment of this methodology on a series of focused combinatorial libraries yielded a linear peptide sequence of 17 encoded amino acids that demonstrated a 140-fold increase in affinity (57 nM dissociation constant, K_D) over previously reported lanthanide-binding peptides. This linear sequence was macrocyclized by introducing a disulfide bond between flanking cysteine residues to produce a peptide with a 2-nM apparent dissociation constant for Tb^{3+} ions.

KEYWORDS:

combinatorial chemistry · fluorescent probes · lanthanides · peptides

Introduction

There is an ever-increasing need for chemical and physical probes that can be conveniently integrated into proteins to facilitate the analysis of structure and function. Lanthanide-binding tags (LBTs) are protein fusion partners of minimal dimensions capable of harnessing a vast array of lanthanide-dependent biophysical techniques, which include luminescence analyses with Tb^{3+} and Eu^{3+} species,[1, 2] NMR spectroscopy (through use of the paramagnetic lanthanides),[3] and X-ray crystallography, which utilizes the strong scattering power of the lanthanide ions.[4] Herein we demonstrate that a series of focused combinatorial libraries, coupled with a novel luminescent screening technique can be successfully implemented to identify peptide loops with low-nanomolar dissociation constants for Tb^{3+} ions. These sequences provide the next generation of LBT fusion partners for a broad range of biophysical applications.[5]

Incorporation of lanthanide ion binding sites into proteins has previously been accomplished through either chemical labeling of amino acid side chains with lanthanide chelators,[6] or by protein engineering to introduce calcium binding loops, such as the EF-hand motif.[7, 3h] Both of these approaches have limitations. Chemical labeling necessitates the presence of a uniquely reactive residue within the protein of interest, as well as the optimization of the labeling chemistry. Generation of a fusion protein with an EF-hand motif is an easier approach, however once the motif is removed from the context of the calcium-binding protein, the lanthanide affinity drops precipitously (10^{-8}–10^{-9} M to 10^{-5}–10^{-6} M). The loss of lanthanide affinity can lead to complications with nonspecific lanthanide binding, competing ligands, and lanthanide-induced protein aggregation.[7a, 8] In order to extend the utility of protein-bound lanthanide ions, we sought to optimize the Tb^{3+}-affinity and luminescent properties of short oligopeptides (including only the encoded amino acids) for use as LBT fusion partners. Two classes of peptide sequence were envisaged as useful for the intended eventual applications: disulfide-constrained peptide loops with maximal lanthanide affinity[1] and cysteine-free peptides for redox-sensitive applications. The studies described herein have resulted in rapid access to unconstrained peptides with 140-fold improvement in Tb^{3+} affinity over previously synthesized Tb^{3+}-binding loops, and a constrained peptide with an increase in affinity for terbium ions of over three orders of magnitude relative to the native peptide sequences.

Screening Methodology

The luminescence of Tb^{3+} peptide chelates in the presence of a sensitizing tyrosine or tryptophan residue provides a convenient handle for screening lanthanide-binding peptides. However, preliminary experiments indicated that screening of peptides covalently attached to the solid support yielded false-positive luminescent signals caused by interference from the polyethylene glycol resin matrix. In order to circumvent this problem, a

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powerful method for screening the combinatorial peptide libraries away from the solid support was developed.

The selection method was engineered to be compatible with split-and-pool peptide library synthesis,[9] and to enable interrogation of peptides dissociated from the solid support while maintaining the identity of the resin bead from which the peptide originated. A secondary evaluation of the "winning" peptides was conducted to determine the relative Tb^{3+} affinity and luminescence of the initial hits, and thereby limit the number of peptides to be resynthesized for detailed analysis. Additionally, deconvolution of the peptide library by MALDI mass spectrometry resulted in a more efficient and economical process compared with Edman sequencing.

Figure 1 summarizes the general strategy for synthesis and screening of the lanthanide-binding peptide libraries. TentaGel macrobeads (280 – 320 μm) were chosen as the solid support because of their swelling properties in both aqueous and organic solvents, as well as their high loading capacity (2 – 3 nmol/bead).[10] The resin was derivatized with 4-nitrophenylalanine, a moiety that reduces background fluorescence by quenching the eventual photoproduct of the 3-amino-3-(2-nitrophenyl)propionic acid (ANP) linker left on the solid phase during the library screening. A mixture of 80% base-labile (4-hydroxymethylbenzoic acid, HMBA) and 20% photochemically labile (ANP) linker was attached to the resin by proportional mixing of the linkers during coupling. Spectrophotometric determination of the Fmoc protecting group removed in the subsequent round of peptide synthesis was then used to quantify the ratio of ANP:HMBA incorporation.

A peptide spacer, βAla-Arg-Pro-Pro-Gly, was added to the resin to increase the mass of the shortest capped peptides above the background noise level of MALDI mass spectra (< 650 Da) and provide an ionized arginine residue to increase the signal of the peptides observable during MALDI analysis. The peptide library was assembled by a standard split-and-pool synthesis procedure that included use of encoded peptide caps during peptide elongation.[11] This method, introduced by Griesinger et al., uses a computer algorithm to calculate the minimum number of capping steps required during the library synthesis to give a nondegenerate mass ladder for each peptide. A minor modification of the literature method was employed to limit the capping reagents to those commercially available. This encoding approach is superior to other peptide laddering approaches, which cap at every randomized position during the synthesis, as it minimizes the number of caps and in turn maximizes the amount of full-length sequence produced for the screen.[12]

After cleavage of the protecting groups from the peptides, the solid-supported library was distributed in a buffered 2% agarose matrix that included Tb^{3+} ions (50 μM). Upon photolysis on a long-wavelength transilluminator (> 320 nm, 6 min), the portion of peptides linked to the solid support by the photocleavable ANP linker was released into the surrounding agarose. To sensitize the Tb^{3+} emission, the agarose gel was illuminated on a short-wavelength transilluminator. Beads carrying terbium binding peptides manifested a luminescent halo (see Figure 1).[13] In contrast to recently reported in-gel screening methods, which look for products of a reaction produced by a solid-supported catalyst, this procedure looks directly at the peptide released from the solid support.[14] Thus, this methodology avoids interference from the solid support during the screening procedure and is analogous to the bacterial lawn approach introduced by Oldenburg et al. for screening antibiotic compounds.[15]

Competing ligands such as citrate or phosphate moieties were added to the agarose to further increase the selective pressure on the library for the second, third, and fourth generations of terbium-binding peptides. The winning beads were isolated from the agarose and treated with aqueous ammonium hydroxide to liberate the remaining peptide.

The steady-state luminescence spectra of the solution derived from winning beads was recorded at low terbium concentration (200 nM – 1 μM). The brightest of these winners were then titrated

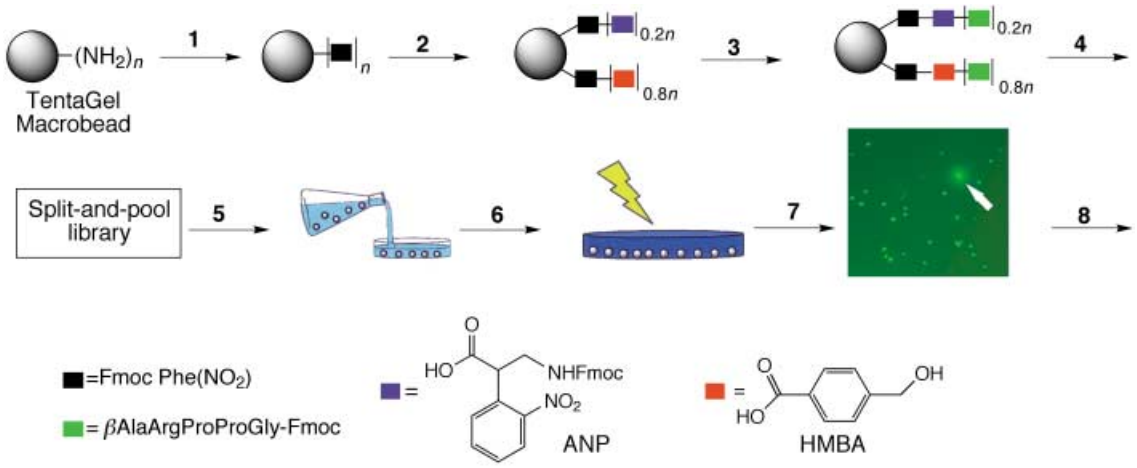
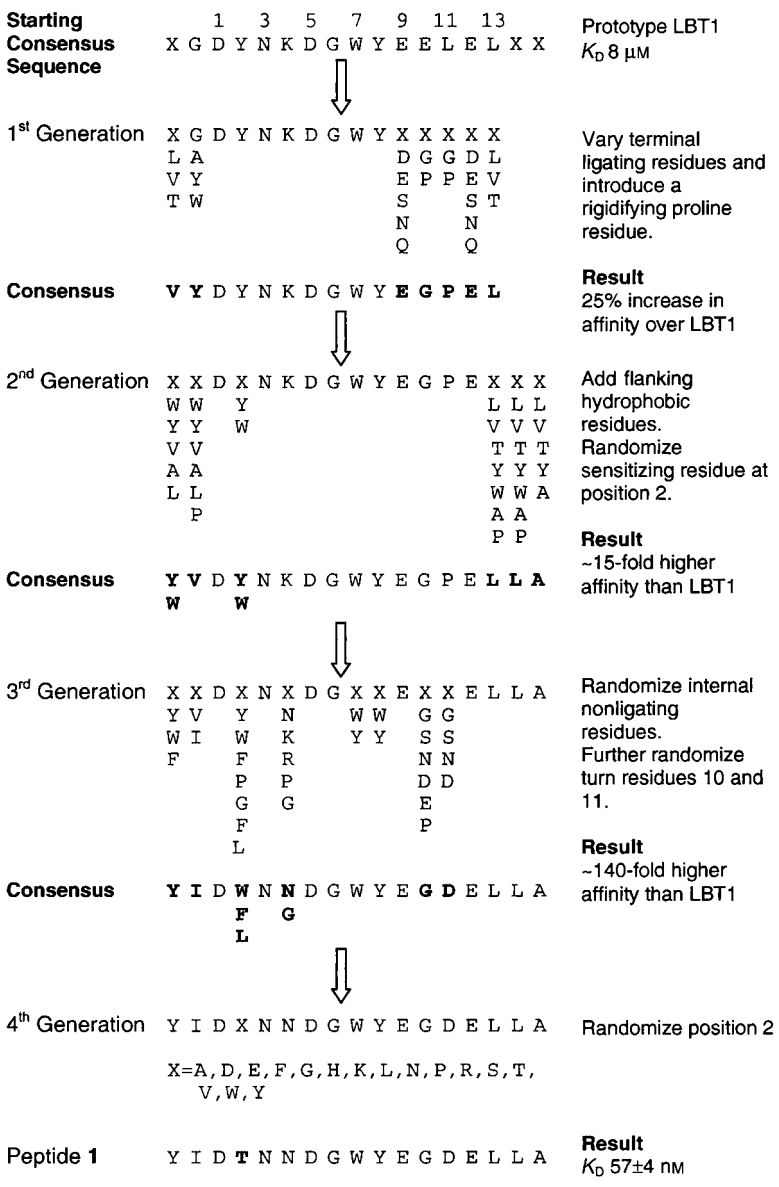


Figure 1. *Library synthesis and screening strategy for Tb^{3+}-binding peptides. Peptide coupling procedures can be found in the Experimental Section. 1) Coupling of* N-α-*Fmoc-4-nitrophenylalanine (Fmoc = 9-fluorenylmethoxycarbonyl); 2) coupling of ANP:HMBA; 3) introduction of spacer sequence; 4) split-and-pool synthesis and introduction of a mass spectral ladder; 5) amino acid side chain deprotection and casting in 2% agarose (50 μM Tb^{3+} in 100 mM NaCl, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7.0; 15 cm × 18 cm plate; 6) photolysis (> 320 nm) and visualization (280 nm); 7) selection of beads with luminescent halos, removal of agarose and incubation of beads in 28% NH_4OH (12 – 16 hrs); 8) single-bead Tb^{3+} titrations and sequence deconvolution by MALDI MS.*

with Tb^{3+} ions to determine the relative affinities of the peptides for terbium (III) species. The resulting titration curves were fit on the assumption that the peptide concentration was considerably lower (> 10 times lower) than the K_D value, to give the relative affinities of the winning sequences for Tb^{3+} ions. The sequences of winning peptides obtained from this final assay were then deconvoluted by interpretation of the mass ladder, as revealed by MALDI mass spectrometry. Selected peptides from each library were then resynthesized without the spacer sequence and evaluated in a purified form to confirm the results of the library screen.

Library Design

Prototype LBT sequences were based on the calcium-binding motifs of EF-hand proteins, which provide seven coordinating residues at positions 1, 3, 5, 7, 9, and 12 in pentagonal bipyramidyl geometry.[5] Studies of EF hands have shown that the loops with the highest affinity for the lanthanides have the following primary sequence of coordinating ligands: Asp-Xxx-Asn-Xxx-Asp-Xxx-Xxx-Glu-Xxx-Xxx-Glu (Xxx = any amino acid).[16] A systematic study by MacManus et al. established that a tryptophan residue at position seven of the loop, which donates a backbone carbonyl group to the metal ion, is optimal for sensitizing Tb^{3+} luminescence.[17] Further studies in our laboratory showed that tyrosine residues at positions 8 and 2 further increase the Tb^{3+} luminescence of the loop.[1] These factors were taken into account for the iterative and targeted synthesis of four peptide libraries each of fewer than 15 000 members, with each library improving on the last, to identify the peptide sequence with optimal terbium binding and sensitized luminescence (Scheme 1).



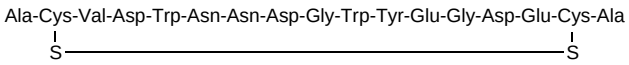
Scheme 1. *Description of peptide libraries. For details of LBT1 construction, see ref. [1].*

Each library addressed a specific concern in the design of the lanthanide-binding peptide, as outlined in the right-hand column of Scheme 1. Strong consensus sequences and improvements in luminescence and/or Tb^{3+} binding affinity were observed after each library iteration. After each round of screening, the winning peptide sequences were resynthesized and evaluated in a purified form to confirm the results of the screen.

In EF-hand motifs the ligating residues at positions 9 and 12 are constrained in the N-terminal end of a helix. The first library randomized the ligating residues at positions 9 and 12 and introduced a rigid proline residue between these residues in an effort to optimize the geometry and flexibility of this region of the LBT. The second library incorporated hydrophobic residues at both termini of the loop to constrain the loop by replacing the hydrophobic interactions between the two flanking helices of the EF-hand in the complete calcium-binding protein or the disulfide bond in previous LBT sequences.[1] The third generation presented a greater number of possible turn-forming sequences by randomization of residues 10 and 11 and randomized N-terminal non-metal-ligating residues. Finally, a consensus at position 2 was found by complete randomization at this position, to arrive at the optimized peptide sequence, Peptide **1**.

Although cysteine residues may reduce the utility of the LBT under some redox-sensitive conditions, there are numerous applications for which a disulfide bond would be benign and would provide significant improvements in lanthanide binding affinity. Based on previous research, the optimal position for a disulfide bond is between positions −1 and 12.[1] Introduction of a disulfide bond between these positions led to Peptide **2** (Scheme 2), with an impressive 30-fold increase in Tb^{3+} affinity compared to the best linear sequence ($K_D = 2 \pm 1$ nM; see Figure 2).

Numerous screening techniques have been developed for combinatorial peptide libraries but the majority have either limited throughput in the absence of robotic liquid handling and



Scheme 2. *Sequence of disulfide-containing Peptide* ***2****.*

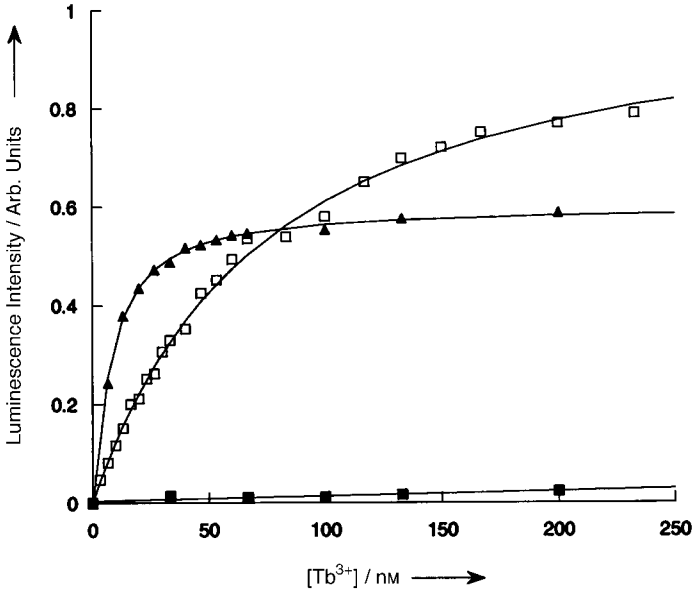


Figure 2. *Terbium titrations of peptides. All peptides at 10 nM in NaCl (100 mM), HEPES (10 mM; pH 7.0).* □*, Peptide* ***1*** *(*$K_D = 57 \pm 4$ *nM);* ■*, LBT1*[1] *(*$K_D = 8.0 \pm 0.5$ *μM);* ▲*, Peptide* ***2*** *(*$K_D = 2 \pm 1$ *nM). Binding curves were fit based on a 1:1 binding model by using the SPECFIT/32 software. Only a portion of the curve is shown for the lower affinity peptides.*

microarrays, or they are performed on the solid phase.[18] We have introduced a method for screening luminescent peptides off the solid support by using a technique that requires no special equipment. Outside the context of a protein, 12 – 20-residue loops bind Tb^{3+} ions with a reported affinity of approximately 5 – 10 μM.[7, 8] The iterative approach described herein, which relies on focused peptide libraries, an efficient screening technique, and specific elements of the peptide design, has identified a cysteine-free peptide that binds Tb^{3+} ions with a K_D value of 57 nM. Constraint of this peptide with a disulfide linkage further improves the affinity for terbium ions to an impressive K_D value of 2 nM. These vastly improved affinities for lanthanides should circumvent most nonspecific lanthanide binding problems since the LBT affinity is now of the same order of magnitude as the ion affinities of the majority of naturally occurring calcium-binding proteins ($K_D = 1 - 10$ nM). Thus, peptides **1** and **2** should prove exceptionally useful in the generation of LBT fusion proteins for use in biotechnological applications.

Experimental Section

Library synthesis: Solid-phase peptide synthesis was performed manually by using Fmoc chemistry on TentaGel macrobeads (0.2 mmol g^{-1}, 90 μm, Rapp Polymere). Standard peptide coupling procedures were used for all single amino acid couplings, in which we treated amino acid (3 equiv/equiv resin) with benzatriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (3 equiv/equiv resin) and diisopropylethylamine (8 equiv/equiv resin) in dimethylformamide (DMF) for one hour at room temperature. Standard deprotection conditions were employed (20% piperidine in DMF for 10 minutes at room temperature). Couplings with more than one free acid, that is, mass encoding steps and orthogonal linker coupling, involved treatment with *N*,*N*′-diisopropylcarbodiimide (1 equiv/equiv acid) and *N*-hydroxybenotriazole (HOBt; 1 equiv/equiv acid) in DMF for 1 hour. Orthogonal linker coupling was achieved by coupling of HMBA and 3-N^{α}-Fmoc-amino-3-(2-nitrophenyl)propionic acid (Fmoc – ANP) linkers (10:1, total 6 equiv/equiv resin) to the peptide. The ratio of HMBA:Fmoc – ANP incorporation was estimated from spectrophotometric quantification of the released dibenzofulvene – piperidine adduct during two subsequent steps of peptide synthesis. The βAla residue directly coupled to the orthogonal linkers was introduced by treatment of the beads with the symmetrical anhydride of the residue (6 equiv/equiv resin) and 4-dimethylaminopyridine (0.1 equiv/equiv resin) in DMF for 1 hr. Encoded mass capping was achieved by coupling a mixture of the desired amino acid and mass cap (85:15, 10 equiv/equiv resin) to the peptide. Positions in the library that required capping were calculated with the Biblio software.[11] Once capping positions were revealed, manual calculations determined which commercially available capping reagent combination would lead to a nondegenerate mass ladder. Mass caps used: Boc-Ser(OBzl)-OH, Boc-Asp(OBzl)-OH, Boc-Glu-(OBzl)-OH, Boc-Pro-OH, Boc-Leu-OH, Boc-Tyr(O*t*Bu)-OH, Boc-Tyr(OBzl)-OH, Boc-Thr(O*t*Bu)-OH, Boc-Phe-OH, Boc-Ala-OH, Boc-Arg(Tos)-OH, Boc-Lys(Boc)-OH, Ac-Phe-OH, benzoyl-Leu-OH, Ac-Leu-OH, benzoyl-Leu-OH (Boc = *tert*-butoxycarbonyl, Bzl = benzyl, Tos = toluene-4-sulfonyl, Ac = acyl). Where possible, the capping agent most similar to the amino acid that it encodes was used, for example, Ac-Ala-OH codes for Ala or Boc-Phe-OH codes for Tyr. Examples of a complete capping strategy and MALDI spectrum are available in the Supporting Information.

Side chains were deprotected by treatment with 94% trifluoroacetic acid (TFA), 2.5% 1,2-ethanedithiol, 2.5% H_2O, and 1% triisopropyl silane for 2 h at room temperature. Resin was then washed sequentially with TFA (10 mL), dichloromethane (5 × 10 mL), DMF (5 × 10 mL), and finally HEPES (100 mM, 3 × 10 mL; pH 7.0).

Combinatorial screening: Approximately 300 – 500 resin beads were suspended in Tb^{3+} (5 mM), NaCl (200 mM), and HEPES (20 mM, 1 mL; pH 7.0) and allowed to equilibrate (5 min). This solution was then diluted with molten agarose (2%) in NaCl (100 mM) and HEPES (10 mM, 80 mL; pH 7.0), poured into a flat rectangular dish (18 × 15 cm, gel thickness ~ 5 mm), and allowed to cool to room temperature. To increase the selective pressure on later generations of the library, $NaHPO_4$ (10 – 75 μM) was added to the molten agarose until only 3 – 5 beads with halos were visible in each gel screened.

The agarose gel was placed on a mid-range UV transilluminator (UVP, high-performance transilluminator) for 6 minutes. The gel was then transferred to a shortwave UV transilluminator (Ultra Lum) and was observed from behind a UV shield. Beads with halos were cut from the agarose with a small section of glass tubing (3 mm internal diameter) and transferred to a 1.5-mL ependorf tube. The residual agrose surrounding the bead was melted away in distilled H_2O (1 mL) at 110 °C. The selected bead was subsequently washed with ethylenediaminetetraacetate (0.5 M; pH 7.0), then extensively with H_2O, and then covered with ammonium hydroxide (28%, 50 μL) and left to incubate for 12 – 15 hrs at room temperature. After cleavage, the ammonium hydroxide was removed under vacuum and the

peptide residue resuspended in an acetonitrile/H_2O (50%, 200 μL) stock solution.

One quarter of the peptide solution was then assayed for its ability to sensitize Tb^{3+} luminescence. A portion of the peptide stock solution (50 μL) was diluted into HEPES (pH 7.0) containing NaCl (100 mM) and a Tb^{+3} ion solution (3 mL, 200 nM – 1 μM) and the fluorescence spectrum of the solution was obtained by excitation at 280 nm and observation between 535 and 555 nm. A sample of the remaining stock solution (100 μL) was then titrated with Tb^{3+} ions in the same buffer to yield the relative affinity of the peptide for Tb^{3+} ions.

MALDI mass spectra were obtained on a PerSeptive Biosystems Voyager MALDI-TOF instrument. A portion of the peptide stock solution (1 ul) was mixed on the MALDI plate with matrix solution (1 ul, 0.15 M in 7:3 CH_3CN/H_2O) and allowed to crystallize under a gentle stream of nitrogen.

Peptide synthesis: Peptides were synthesized on an Advanced ChemTech automated synthesizer with standard Fmoc-protected amino acids and HOBt/*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate coupling reagents and piperidine deprotection on polyaniline – poly(ethylene) glycol – polystyrene (PerSeptive Biosystems) resin. Peptides were purified by reverse-phase HPLC on a YMC C_{18} preparative column with a linear gradient from 7 – 70% acetonitrile in water and 0.2% TFA. Purity was confirmed by analytical HPLC and the correct mass validated by ESI MS on a Mariner electrospray mass spectrometer.

Disulfide-containing peptides were formed by oxidation in a solution of dimethyl sulfoxide (25% v/v), β-morpholinoethanesulfonic acid (20 mM; pH 6.5), and $CaCl_2$ (5 mM) and incubated for 12 – 16 h at room temperature.

Stock solutions: Concentrations of purified, lyophilized peptides dissolved in nanopure water were determined by measuring the absorbance at 280 nm in guanidine hydrochloride (6 M) and by using calculated extinction coefficients, as described in the literature.[19]

Stock solutions of $TbCl_3$ were prepared in HCl (1 mM) and calibrated by complexometric titration.[20]

Luminescence spectroscopy: Luminescence emission spectra were recorded on a Fluoromax 3 instrument (Jobin Yvon Horiba) in a quartz cuvette with a 1-cm path length. Tryptophan-sensitized Tb^{3+} emission spectra were collected by exciting the solution at 280 nm, with a 315-nm longpass filter to avoid interference from harmonic doubling. Excitation and emission slit widths were 4 nm and 5 nm, respectively. Luminescence spectra obtained from Tb^{3+} titrations were analyzed with the program SPECFIT/32 (Spectrum Software Associates, version 3.0.30).[21] Titration data represent individually prepared solutions of peptide (10 nM) and variable Tb^{3+} ion concentrations in NaCl (100 mM), HEPES (10 mM; pH 7.0). Errors reported for K_D values represent the standard deviation calculated from the curve fit.

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