Lanthanide-Binding Tags as Versatile Protein Coexpression Probes

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Comprehensive proteomic analyses require new methodologies to accelerate the correlation of gene sequence with protein function. Key tools for such efforts include biophysical probes that integrate into the covalent architecture of proteins. Lanthanide-binding tags (LBTs) are expressible, multitasking fusion partners that are optimized to bind lanthanide ions and have several desirable attributes, which include long-lived luminescence, excellent X-ray scattering power for phase determination, and magnetic properties to facilitate NMR spectroscopic structure elucidation. Herein, we present peptide sequences with a 40-fold higher affinity for Tb^{3+} ions and significantly brighter luminescence intensity compared with existing peptides. Incorporation of an LBT onto ubiquitin as a prototype fusion protein allows the use of powerful protein-visualization techniques, which include rapid luminescence detection of LBT-tagged proteins in SDS-PAGE gels, as well as determination of protein concentrations in complex mixtures. The LBT strategy is a new alternative for expressing fluorescent fusion proteins by routine molecular biological techniques.

KEYWORDS:

fluorescent probes · lanthanides · peptides · protein engineering · protein modifications

Introduction

With the vast amount of genetic information continually being revealed by genome projects comes the task of assigning structure and function to the encoded protein products. The challenges of proteomics generally encompass three broad areas: analysis of protein expression levels, assignment of protein function, and determination of protein structure. Each area has its unique methodological approaches, which can be enhanced by various chemical or biochemical probes. Although numerous probes are currently available, separate protein constructs are required for each application; for example, a selenomethione-labeled protein for phase determination in X-ray crystallography,[1] and a green fluorescent protein fusion or a chemically-derivatized protein for fluorescence assays.[2] A single, versatile probe that can address challenges in multiple areas of proteomics would be an invaluable tool for biotechnology.

Existing methods for protein derivatization with reporter functionality include chemical modification of a reactive amino acid side chain,[3] native chemical ligation,[4] and transfer RNA suppression mutagenesis.[5, 6] These semisynthetic approaches often require mutagenesis and considerable optimization, and yield small quantities of the desired construct. The now common use of green fluorescent proteins[7] as fusion partners emphasizes the power of incorporating reporters at the DNA level. A facile extension of fluorescent fusion partners are fluorophores that bind to specific peptide sequences; for example, the diarsenic fluorescein derivative FlAsH binds to a tetracysteine motif,[8] whereas fluorophore conjugates of Ni^{2+}-nitrilotriacetic acid bind to polyhistidine regions.[9]

The engineering of a lanthanide (Ln) binding site into a protein provides an attractive strategy for appending a probe of versatile usage. Lanthanides possess several attractive properties for applications in biotechnology.[10–12] Given the appropriate coordination environment and the presence of a sensitizing chromophore, Tb^{3+} and Eu^{3+} ions display long-lived luminescence emissions that are ideal for time-resolved experiments and lanthanide-based resonance energy transfer.[2] Lanthanides also provide excellent X-ray scattering power and should therefore accelerate the determination of protein X-ray structures by greatly accelerating the phase determination of lanthanide-tagged proteins.[13] Finally, the paramagnetic lanthanides can be used as NMR probes of protein structure in solution.

The utility of Ln ions for solving important biological problems has been demonstrated by appending synthetic Ln chelates to biomolecules,[10, 14] as well as by exploiting calcium binding sites to bind Ln ions in proteins for both NMR spectroscopy[15] and fluorescence spectroscopy applications.[16] Modification of the metal-binding region of calcium-binding proteins with Tb^{3+}-sensitizing chromophores like tyrosine and tryptophan residues is a proven strategy for obtaining mutant proteins and fusion constructs with enhanced Tb^{3+} luminescence.[17, 18] We reasoned that considerable scope exists for further optimization of short peptide sequences (20 amino acids or fewer) specifically for intense Tb^{3+} luminescence and high binding affinity, and that such constructs could have significant impact as natively

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expressible, multitasking probes of protein structure and function. We therefore commenced work on a program combining combinatorial peptide synthesis and screening, together with explicit tactics of peptide design to develop lanthanide binding tags (LBTs). Herein we present the initial development of LBT peptides, their incorporation into fusion proteins, and the luminescence properties that allow for powerful protein visualization and quantitation techniques.

Results and Discussion

LBT peptide design and optimization

Lanthanides have long been used as probes of calcium-binding proteins because of the similarities in ionic radii and coordination preferences between Ca^{2+} and Ln^{3+} ions.[19, 20] Numerous studies have investigated the metal-binding properties of synthetic loops composed of 12- to 33-residue segments of calcium-binding proteins.[21, 22] Although 12-residue loops bind Ln^{3+} more tightly than Ca^{2+} ions, the affinity even for Ln^{3+} ions decreases significantly for these isolated loops (dissociation constant, K_D, values around 10 – 20 μM) compared with full-length proteins (K_D values can be low nanomolar for high-affinity sites). Whereas the goal of many of these studies was to investigate how variations in sequence affect the function of calcium-binding proteins, our approach is to optimize peptides of similar size specifically for Tb^{3+} affinity and sensitized luminescence. By varying the position and type of aromatic side chain in a series of 14-residue loops based on the calmodulin protein family, Szabo and co-workers established the presence of a tryptophan residue at loop position 7 as optimal for enhancing Tb^{3+} luminescence.[22] We reasoned that further refinements to 14-mer peptides would provide tighter and brighter LBTs and constructed a combinatorial library of peptides based upon the results of Szabo.

The prototype LBT sequence was discovered in a split-and-pool library in which potential metal-binding residues Xaa were varied between Asp, Asn, Ser or Glu, and Zaa residues could be a variety of hydrophobic amino acids, including potential energy donors Tyr and Trp, which gives potentially 500 000 peptides of the general sequence: Ac-Gly-Xaa-Zaa-Xaa-Zaa-Xaa-Gly-Trp-Zaa-Glu-Zaa-Zaa-Glu-Leu. The peptide library was evaluated on resin by UV illumination of Tb^{3+}-soaked beads. Luminescent beads were isolated and subjected to Edman degradation for primary sequence identification. Five peptides were resynthesized after the on-bead screen to study their properties in solution. One was insoluble, three showed similar Tb^{3+} luminescence intensity compared with REF (Ac-GDYNADGWIEFEEL),[22] and one was at least twice as bright.[23] This first-generation LBT peptide, designated LBT1, has the sequence Ac-GDYNKDGWYEELEL. A mutant peptide of LBT1 in which the principal energy-sensitizing residue Trp is replaced by Phe is only slightly less intense than REF (data not shown). This observation suggests that the brighter luminescence of LBT1 is due in part to the additional energy-sensitizing Tyr residue found in LBT1 but absent in REF.

It is important to note that our initial screening technique was designed specifically to select for brightness, and it did not have an intrinsic preference for peptides with improved Tb^{3+}-ion affinity. We have developed a more sophisticated screening protocol, however, that can discriminate not only bright Tb^{3+}-ion binders, but also tight Tb^{3+}-ion binders. Details of this later-generation library synthesis and screening procedure, along with a description of the resulting LBT peptides of even further refinement, can be found in the following paper of this issue.[23]

The sequence similarity among LBT1, REF, and native loops like site III of Troponin C (DKNADGIDIEE) suggests that the metal-binding ligands themselves are already optimal for Ca^{2+} and Tb^{3+} binding and that improvements elsewhere in the sequence are necessary to enhance the affinity of LBTs for Tb^{3+} ions. A single disulfide linkage was therefore established to constrain the LBT1 loop in order to provide a more preorganized, macrocyclic array of ligands for metal binding. A series of nine peptides was made, in which the position of cysteine residues flanking the core LBT1 sequence was systematically shuffled to optimize the macrocycle. The sequences are listed in Table 1.

Table 1. *Amino acid sequences of REF and LBT peptides, together with apparent K_D values for Tb^{3+} ions.*[a]

Peptide	Sequence	K_D [μM]
REF	Ac-GDYNADGWIEFEEL	9 ± 1
LBT1	Ac-G(LBT)L	8 ± 1
LBTC1	**C**(LBT)**C**	0.60 ± 0.1
LBTC2	A**C**A(LBT)**C**AA	0.220 ± 0.03
LBTC3	A**C**AA(LBT)**C**AA	6.0 ± 0.9
LBTC4	AA**C**(LBT)A**C**A	9.3 ± 0.4
LBTC5	A**C**A(LBT)A**C**A	6.4 ± 0.8
LBTC6	A**C**AA(LBT)A**C**A	5.3 ± 0.9
LBTC7	AA**C**(LBT)AA**C**A	5.0 ± 0.3
LBTC8	A**C**A(LBT)AA**C**A	5.9 ± 0.5
LBTC9	A**C**AA(LBT)AA**C**A	(insoluble)

[a] REF and LBT1 sequences have N-terminal acetyl capping groups (Ac), the others are N-terminal amines. Peptides LBTC1 – LBTC9 are oxidized to form disulfide-bonded macrocycles. (LBT) = DYNKDGWYEELE.

The peptides were evaluated by steady-state luminescence measurements, examples of which are shown in Figure 1. The improvements made to the LBT loop compared with the REF peptide clearly result in Tb^{3+}-binding peptides with enhanced luminescence intensities. Apparent dissociation constants were obtained spectrophotometrically from Tb^{3+} titrations in pH 7.0 buffer containing 100 mM NaCl. The REF and LBT1 peptides bind Tb^{3+} ions with K_D values of 9 μM and 8 μM, respectively. These affinities are similar to values obtained for other synthetic loops of similar size.[21] In contrast, the LBTC2 loop has a greatly improved K_D value of 220 nM, a dissociation constant more aligned with full-length proteins than short peptide loops. Optimization of the cysteine positions is clearly important, as is most dramatically demonstrated by the increase in K_D values from 220 nM to 9.3 μM when the flanking cysteine residues are shifted from their positions in LBTC2 (ACADYNKDGWYEELECAA) to their positions in LBTC4 (AACDYNKDGWYEELEACA). A recent study also reported the use of a disulfide bridge across a metal-binding loop of an EF-hand peptide to stabilize a native-like conformation.[24] However, the K_D value for Tb^{3+} ions was only

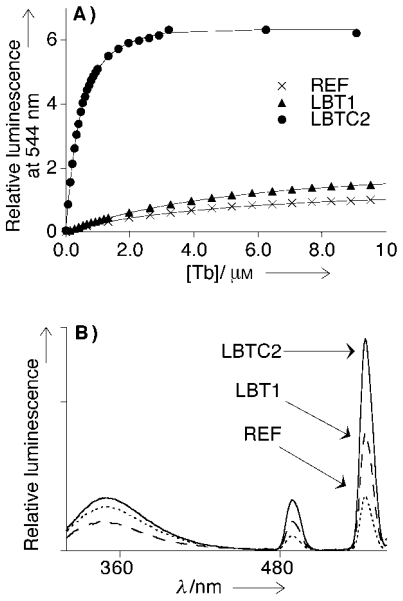

Figure 1. *A) Examples of Tb^{3+} titration data used to calculate K_D values for 1:1 complexes are listed in Table 1. Solid lines represent the best fits calculated with the Specfit program. Peptide concentrations are 250 nM in HEPES buffer (10 mM; pH 7.0), NaCl (100 mM). B) Luminescence spectra of peptides REF, LBT1, and LBTC2 (250 nM) in the same buffer as in (A), with saturating Tb^{3+} ion concentration ($\lambda_{ex} = 280$ nm).*

3.5 μM for a 33 amino acid peptide, which indicates that the cysteine residues were perhaps not optimally positioned for metal binding.

LBT fusion proteins

To test the utility of the LBT tags, both LBT1 and LBTC2 sequences were appended to ubiquitin by standard molecular biological techniques. Polyhistidine tags were also incorporated into the constructs to establish the compatibility of LBTs with this common metal-affinity purification tag. Figure 2 shows the

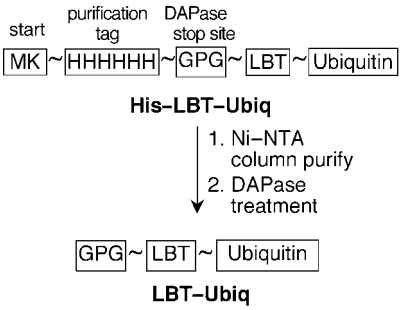

Figure 2. *The general strategy for construction of LBT-tagged proteins, where LBT represents either of the LBT1 or LBTC2 sequences.*

general strategy, in which an intervening Gly-Pro-Gly sequence between the N-terminal His Tag and the LBT tag was introduced as a stop site to allow subsequent removal of the His tag, if desired, by the TAGZyme protocol. Protein expression was not diminished by the presence of the LBT, and the fusion proteins were easily purified by Ni^{2+} – nitrilotriacetate (NTA) affinity chromatography.

Metal-ion competition experiments established that the LBTs demonstrate good selectivity for Tb^{3+} ions. Figure 3 compares the relative intensity of Tb^{3+} emission at 544 nm of His-LBT1-Ubiq and its counterpart LBT1-Ubiq, in which the His tag has

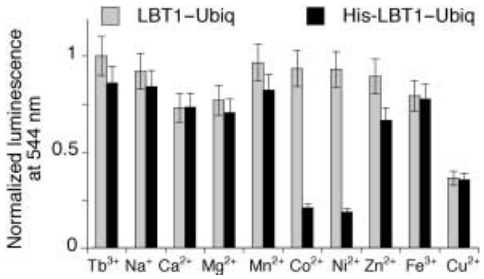

Figure 3. *Normalized luminescence intensity of the Tb emission at 544 nm of His – LBT1 – Ubiq or LBT1 – Ubiq protein (5 μM) loaded with Tb^{3+} ions (10 μM) in the presence of competing metal ions (5 mM Na^+, Ca^{2+}, Mg^{2+} and 5 μM Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}). Buffer = HEPES (10 mM; pH 7.0), $\lambda_{ex} = 280$ nm.*

been removed, in the presence of potentially competing metal ions. A decrease in signal indicates competitive binding, since only Tb^{3+} ions afford a luminescent complex. This analysis demonstrates that the Tb^{3+} – LBT complex itself is relatively unaffected by most metal ions, with the exception of Cu^{2+} ions. Importantly, the presence of oxophilic and biologically prevalent cations such as Ca^{2+} and Mg^{2+} has little consequence for Tb^{3+} binding, even at concentrations three orders of magnitude higher than that of the lanthanide. Both Co^{2+} and Ni^{2+}, metal ions with high affinity for histidine ligands, significantly compete with Tb^{3+} ions in full-length His – LBT1 – Ubiq, but not in LBT1 – Ubiq. Metal binding within the His tag likely disrupts the Tb^{3+} binding site in the LBT, but in the absence of histidine residues, the LBT loop itself is selective for Tb^{3+} ions. Since the His tag has negligible effect on Tb^{3+} binding and luminescence intensity, and because the presence of Co^{2+} and Ni^{2+} ions is not a concern for most applications, the full-length His – LBT – Ubiq protein was left intact for most experiments.

The Tb^{3+}-binding properties of the purified LBT-protein fusions were assessed by luminescence titration experiments, as shown in Figure 4 A, and found to be comparable to the model peptides, with the LBTC2 protein construct significantly brighter than the LBT1 construct. The binding studies reveal dissociation constants for Tb^{3+} ions that indicate slightly tighter binding for the protein conjugates than for the independent peptides, with K_D values for His – LBT1 – Ubiq and His – LBTC2 – Ubiq of 6 μM and 150 nM, respectively. The intensity of the Tb^{3+} emission confirms that appending a His tag as well as the ubiquitin protein to the LBT sequences does not affect the luminescence properties of the LBT.

Counterions with negatively charged oxygen atoms are potential ligands for lanthanides and therefore may represent challenges for some biological LBT applications. These limitations, however, can be overcome by improving the binding affinity of LBTs. Figure 4 B compares the normalized lumines-

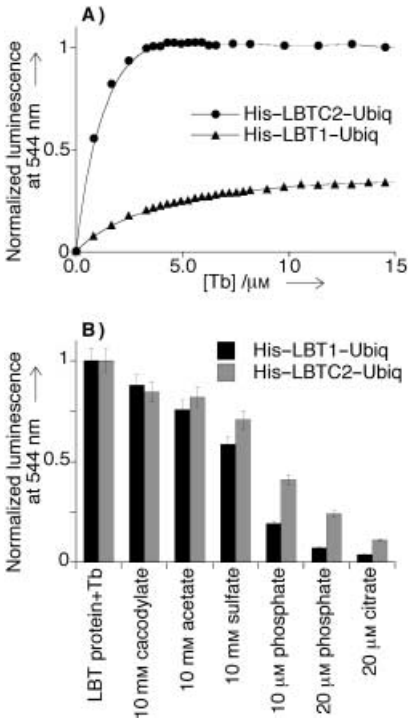

Figure 4. *A) Tb^{3+} titrations of His – LBTC2 – Ubiq and His – LBT1 – Ubiq proteins monitored by luminescence intensity at 544 nm, $\lambda_{ex} = 280$ nm. The protein concentration was 2 μM in HEPES buffer (10 mM; pH 7.0), NaCl (100 mM). Solid lines represent the fit calculated with the Specfit program to obtain Tb binding affinity. B) Comparison of the relative Tb luminescence response of His – LBT1 – Ubiq and His – LBTC2 – Ubiq proteins (10 μM) in the presence of Tb^{3+} (10 μM) and various competing ions (buffer = 10 mM HEPES; pH 7.0). The data are normalized so that the maximum emission at 544 nm for both proteins = 1 before addition of competing anions.*

cence intensities of Tb^{3+}-loaded His – LBT1 – Ubiq and His – LBTC2 – Ubiq fusion proteins in the presence of various anions. Cacodylate, acetate, and sulfate diminish Tb^{3+} emission, but only at elevated concentrations. Phosphate and citrate however, are much stronger competitors. The improvements made with respect to the Tb^{3+} binding affinity of LBTC2 over the prototype LBT1 are quite noticeable here, as the phosphate and citrate effect is less pronounced for the tighter-binding LBTC2 protein construct. Future improvements to the LBT loop will further minimize this counterion limitation.[23]

Protein concentration determination and expression profiling

A desirable attribute of a fluorescently tagged protein is the ability to detect it selectively within a complex mixture. A fluorescent tag that is also natively expressed allows detection immediately upon expression and can therefore be used as a marker of expression efficiency. Initial attempts to detect the first-generation His – LBT1 – Ubiq protein in complex mixtures were unsuccessful because the Tb^{3+} ion concentration required to observe a strong luminescence signal also elicited a significant response from nonselective cellular components, such as other proteins and DNA fragments. The improvements in Tb^{3+}-ion affinity made in the second-generation His – LBTC2 – Ubiq construct, however, permit assays at Tb^{3+} concentrations below the threshold of nonspecific binders. Aliquots from a cell culture can therefore be harvested, resuspended in a suitable lysis buffer, and the cleared cell lysate then assayed by luminescence spectroscopy in the presence of Tb^{3+} ions. Under the optimized luminescence assay conditions (6 M guanidine hydrochloride, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer, and 2 μM $TbCl_3$ at pH 7.0), cell lysates that do not contain LBTC2-tagged protein do not sensitize Tb^{3+} emission, as shown in Figure 5 A. The concentration of expressed protein can be estimated by comparing its luminescence

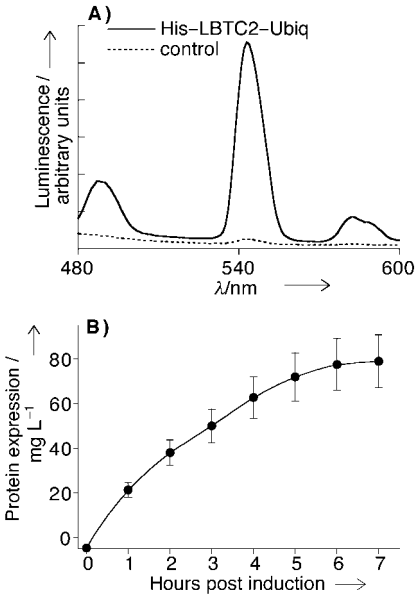

Figure 5. *A) Luminescence spectra of crude cell lysates from a culture expressing His – LBTC2 – Ubiq (blue) or a control culture (black), in guanidine hydrochloride (6 M), HEPES (10 mM), $TbCl_3$ (2 μM), pH 7.0, $\lambda_{ex} = 280$ nm. B) Plot of the Tb luminescence response (converted into mg protein per liter culture by comparison with a standard curve) obtained at various times after inducing expression of His – LBTC2 – Ubiq by treatment with isopropyl-β-D-galactopyranoside (IPTG). Buffer conditions are as described in A. Error bars represent 15% standard deviation, based on duplicate measurements.*

response to a standard curve generated with the LBTC2 peptide. Comparable amounts of a control lysate that does not contain any LBT protein are added to the buffer of the standards in order to duplicate the conditions of the samples. Figure 5 B shows an example of an expression profile of His – LBTC2 – Ubiq. The analysis predicts a protein yield of 80 mg after 6 hour of induction by treatment with IPTG. An independent, 1-L expression yielded 60 mg isolated and purified protein, which establishes a reasonable correlation between the luminescence assay and final isolated yield.

In-gel visualization

Another powerful new application made possible by the improved binding and brightness of the LBTC2 tag is the ability to readily and selectively visualize LBT-tagged proteins directly in sodium dodecyl sulfate (SDS) polyacrylamide gels. The 20-

minute, 2-step procedure involves a 10-minute wash followed by a 10-minute soak in buffered, 1-μM $TbCl_3$ solution. Gels are illuminated with a UV source, such as a hand-held lamp or a UV transilluminator, and bands corresponding to His – LBTC2 – Ubiq are easily detected by eye and imaged with a digital camera. Figure 6 A shows two images of a gel that was initially visualized

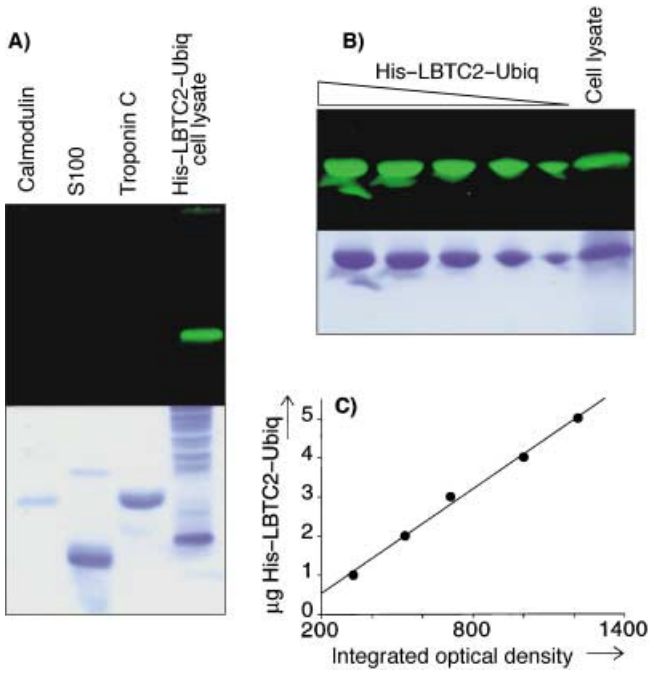

Figure 6. *Visualization and quantification of LBTC2-tagged protein directly in SDS polyacrylamide gels. A) Comparison of calcium-binding proteins (5 μg each) and crude cell lysate of His – LBTC2 – Ubiq stained with Tb and visualized by UV illumination (top) or stained with Coomassie blue (bottom). The results demonstrate the selectivity of the Tb staining method. B) Purified His – LBTC2 – Ubiq (decreasing quantity of 5 – 1 μg) is used to generate a standard curve (C) to estimate amount of expressed protein in the crude cell lysate (50 mg L^{-1} in this example).*

by the Tb method, then subsequently stained with Coomassie Blue to highlight the LBT selectivity of the new procedure. Significantly, proteins in the cell extract are not visible by the Tb stain, and neither are calmodulin, S100, or troponin C, calcium-binding proteins that have high affinity for Tb^{3+} ions. This rapid, highly selective staining procedure is analogous to a conventional Western blot, but takes only a fraction of the time. Unlike Western blot analyses, which require binding of specific antibodies and secondary antibodies of unknown concentration, this method is a direct measurement of the desired protein and can therefore be analyzed with digital imaging techniques to quantify the amount of tagged protein by comparison with standards, as shown in Figure 6 B and C.

Mutant calcium-binding proteins containing the REF peptide sequence have been visualized on SDS-PAGE gels previously,[25] but weak binding and dim fluorescence necessitated an extensive staining procedure to reduce nonspecific binding, and a specialized camera to acquire time-resolved images. The improvement in Tb^{3+}-binding affinity of LBTC2 greatly reduces nonspecific binding, which allows gels to be stained at low Tb^{3+} ion concentrations. The improvements in luminescence also make the proteins detectable by eye without the need for nonstandard equipment. The current detection limit for visualizing His – LBTC2 – Ubiq by our method is 50 – 100 ng per protein band, approximately the limit of detection for Coomassie Blue staining. This detection limit is likely to improve with upgraded technology for image acquisition and background reduction.

Conclusions

Lanthanide-binding tags represent an alternative approach for conveniently generating noninvasive, fluorescent protein tags of minimal dimensions. These short peptide sequences are compatible with polyhistidine tags for facile protein purification. The presence of the tag did not interfere with expression efficiency or protein stability in this first prototype example. The luminescent properties of Tb^{3+}-loaded LBT fusion proteins allow rapid and selective visualization of a protein of interest in complex solution mixtures, as well as directly in SDS polyacrylamide gels. These characteristics mean that the tags should find broad application in proteomic studies, for example in cases where functional assays are not established and antibodies are not available. The presence of an Ln binding site also suggests the usefulness of these tags in protein structure elucidation by X-ray crystallography and NMR spectroscopy, applications that we are currently pursuing.

Experimental Section

Peptide synthesis and purification: Peptides were synthesized on an Advanced ChemTech automated synthesizer with standard 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and 1-hydroxy-1*H*-benzotriazole (HOBt)/*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate coupling reagents (Novabiochem). Peptides were synthesized on polyaniline – poly(ethylene) glycol – polystyrene resin (PerSeptive Biosystems) and deprotected in a cocktail of 90% trifluoroacetic acid (TFA), 5% dichloromethane (DCM), 2.5% triisopropyl silane, and 2.5% H_2O (2.5% ethane dithiol was included for cysteine-containing peptides) for 1 – 2 h. Peptides were purified by reverse-phase HPLC on a YMC C_{18} preparative column with a linear gradient from 7 – 70% acetonitrile in water with 0.2% TFA. Purity was confirmed by analytical HPLC and correct mass validated by ESI MS on a Mariner electrospray mass spectrometer (PerSeptive Biosystems).

Cysteine-containing peptides were air-oxidized in NH_4HCO_3 (0.1 M) with peptide concentrations of 10 mg mL^{-1}. Reactions were monitored with Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid) to completion.

LBT library synthesis and screening: The LBT library was constructed on poly(ethylene) glycol acrylamide resin (150 – 300 μm, Polymer Laboratories) by using a split-and-mix combinatorial approach that provided one peptide sequence per bead.[26] The library contained a possible 500 000 unique sequences in fourfold redundancy. Standard side-chain and Fmoc-protected amino acids (0.8 M in dimethyl formamide (DMF)) were manually coupled with benzotriazol-1-yl-oxotripyrrolidinophosphonium hexafluorophosphate/HOBt (0.8 M in DMF) and diisopropylethylamine (1.6 M) for 1 h. A second coupling step was added in cases where a 2,4,6-

trinitrobenzenesulfonic acid test indicated an incomplete coupling reaction. After each cycle, the resin was washed with acetic anhydride/HOBt solution (0.3 M) in DMF/DCM (9:1) to acetyl-cap any remaining unreacted amines. At the completion of the synthesis, all peptides were N-terminally capped with monochloro acetic anhydride/HOBt as described previously.[27]

The chloroacetyl-capped, resin-bound peptides were loaded into 6-well plates (approximately 5000 beads per well) and incubated for 2 h at ambient temperature in HEPES buffer (3 mL, 10 mM; pH 7.0), containing $TbCl_3$ (100 μM). When an average of 50 pmol peptide per bead is assumed, one well can be calculated to contain approximately 250 nmol peptide and 300 nmol Tb^{3+} ions. The 6-well plates were illuminated with a hand-held, short-wave UV lamp in a dark room to reveal 3 – 4 beads per well that were visually brighter than average. These initial winners were then transferred to individual wells of a 96-well plate containing Tb^{3+} (100 μL, 2.5 mM) in HEPES (10 mM, pH 7.0). A digital picture was taken of each bead under UV illumination on an Olympus 1X50 fluorescence microscope coupled to a DVC-1300C RGB color CCD camera controlled by C-View software (DVC Company, Austin, TX). The pictures were captured with XCAP-Lite interactive image analysis software (EPIX, Inc. Buffalo Grove, IL) and analyzed with Image-Pro Plus software (version 4.0, Media Cybernetics, Silver Spring, MD) by counting the green pixel density contained within an outline of the bead on each picture. These values were used to rank the relative luminescence of each bead. Of the twenty highest-scoring beads that were identified after screening, a quarter of the fourfold redundant library, 5 (0.004%) scored similarly or better than the REF peptide, which was used as a benchmark.

To prepare beads for sequencing, they were first soaked in ethylenediaminetetraacetate (0.1 M, 100 μL; 3 – 4 h), rinsed with double-distilled H_2O (ddH_2O; 100 μL), then treated with $NH_{3(aq)}$ (15%, 200 μL; overnight) into convert the chloroacetyl cap to an N-terminal glycine residue.[27] After a final rinse in ddH_2O, the beads were stored individually in water/methanol (1:4, 200 μL). The peptides were sequenced by Edman degradation at the Protein Service Laboratory at the University of British Columbia.

Preparation of stock solutions: Concentrations of purified, lyophilized peptides dissolved in nanopure water were determined by measuring the absorbance at 280 nm in 6 M guanidine hydrochloride and by using calculated extinction coefficients, as described in the literature.[28] Stock solutions of $TbCl_3$ were prepared in 1 mM HCl and calibrated by a complexometric technique.[29]

Luminescence spectroscopy: Luminescence emission spectra were recorded on a Fluoromax 2 instrument (Jobin Yvon Horiba) in 1-cm pathlength quartz cells equilibrated at 25 °C by a jacketed water bath. Tryptophan-sensitized Tb^{3+} emission spectra were collected by exciting at 280 nm and by using a 315-nm longpass filter to avoid interference from harmonic doubling. The spectra were corrected for emission intensity by using manufacturer-supplied correction factors. Slit widths were 4 nm and 5 nm for excitation and emission slits, respectively. Spectra were measured at pH 7.0 in HEPES buffer (10 mM) with NaCl (100 mM) and either 1 – 2 μM peptide (or protein) for the weak Tb^{3+} sites, or 250 nM peptide for the tighter Tb^{3+} sites. Luminescence spectra obtained from Tb^{3+} titrations were analyzed with the program SPECFIT/32 (Spectrum Software Associates, version 3.0.30).[30] A Tb^{3+}:peptide binding stoichiometry of 1:1 provided the best fit of the data in all cases. Errors reported for the K_D measurements represent the standard deviation of the results from three independent trials.

Plasmid preparation: A DNA sequence encoding LBT1 – ubiquitin was initially inserted into a *pCRT7/NT-TOPO* plasmid (Invitrogen) by a standard PCR and Topo-ligation strategy in which ubiquitin was PCR amplified with a primer containing the LBT-coding region as an N-terminal extension (the ubiquitin plasmid was a kind gift from T. M. Handel). Unfortunately, treatment of the protein product with enterokinase to remove the N-terminal His tag in the commercial vector resulted in nonspecific cleavage of the LBT sequence. Another PCR strategy was therefore used to reconfigure the LBT region to include an intervening Gly-Pro-Gly sequence as an intrinsic DAPase (Qiagen) stop point between the His and LBT tags. DNA sequences encoding Met-Lys-His_6-Gly-Pro-Gly-LBT1 or Met-Lys-His_6-Gly-Pro-LBTC2 were PCR amplified as Nde1/BglII fragments and inserted into the corresponding site of our original *ubiquitin-TOPO* vector. All constructs were verified by DNA sequencing (MIT Biopolymers Laboratory).

Protein expression and purification: The LBT-containing plasmids were transformed into BL21-Gold(DE3) competent cells (Stratagene) and grown to midlog phase in Luria-Bertani media containing carbenicillin (50 μg mL^{-1}) at 37 °C with shaking at 250 rpm. Protein expression was induced by treatment with IPTG (1 mM) for 3 – 6 h at 37 °C. High protein yields were also obtained by growing cultures overnight with no induction. Cells were harvested by centrifugation at 4 °C, 5 K for 30 min. Pellets were resuspended and lysed by sonication in buffer containing HEPES (10 mM; pH 7.5), NaCl (300 mM), lysozyme (1 mg mL^{-1}), imidazole (10 mM), 4-(2-aminoethyl)-bezenesulfonylfluoride (0.1 mM), leupeptin (0.5 μg mL^{-1}), and pepstatin (0.5 μg mL^{-1}). Soluble fractions were purified under native conditions with Ni^{2+} – NTA resin (Qiagen) by following the manufacturer's instructions, with the exception that we used the buffer described above. Protein was eluted with buffer containing imidazole (100 mM). Correct protein masses were confirmed by ESI MS. Concentrations were determined by the fluorescence method described below and verified by Pierce BCA protein assay. Yields of purified protein ranged from 20 – 65 mg L^{-1}, depending on induction times.

Removal of His tags: His – LBT1 – Ubiq and His – LBTC2 – Ubiq were treated with DAPase enzyme according to the TAGZyme protocol provided by Qiagen. 4 mg protein were successfully processed in overnight reactions at 4 °C. The purity and correct mass of the final products were verified by analytical HPLC and ESI MS.

Luminescence of cell lysates: Aliquots of cell culture (1 mL) were harvested by centrifugation on a table-top microfuge for 2 min. Cell lysis was achieved by resuspending the cell pellets in guanidine hydrochloride (1 mL, 6 M) and HEPES buffer (10 mM; pH 7) for 20 minutes. The samples were again centrifuged to remove insoluble debris. Alternatively, the pellets were resuspended in HEPES buffer (10 mM; pH 7.0) containing NaCl (100 mM) and lysed by sonication. The cell lysates were then diluted 25- to 50-fold into buffer containing $TbCl_3$ (2 μM) in guanidine hydrochloride (6 M) and HEPES (10 mM; pH 7.0). Sensitized luminescence spectra were recorded as described above. The integrated area of the Tb^{3+} emission band centered at 544 nm was then compared to a standard curve in order to estimate the concentration of LBT-tagged protein. Standard curves were calculated from the integrated luminescence spectra of varying concentrations of the LBTC2 peptide under the same conditions as the lysate samples. In order to reproduce similar background conditions for the standards as for the samples, aliquots of 25- to 50-fold dilutions of cell lysate samples from cell lines that did not express any LBT protein were added to the standards.

In-gel luminescence: Proteins were loaded onto 15% SDS-polyacrylamide gels in nondenaturing gel-loading buffer, and subjected to electrophoresis at 100 mV for 1 – 2 h, or until the blue dye from the loading buffer passed through the gel. The gels were first washed in buffer (25 mL; pH 7.0) containing HEPES (10 mM) and NaCl (100 mM)

for 10 minutes with agitation, then soaked again in the same buffer (25 mL) plus $TbCl_3$ (1 μM) for an additional 10 minutes, with agitation. The luminescent Tb^{3+} bands were visualized by placing the gel directly on a UV transilluminator (UltraLum, 115 V, 50/60 Hz), taking care not to touch the gel in order to avoid unwanted smudges that increase background fluorescence. Alternatively, the gels were placed on a dark background and illuminated with a low-wavelength UV lamp. Digital pictures were taken through a UV-blocking shield and a 475-nm longpass filter (Schott) with a DVC-1300C RGB color camera controlled by C-View software (DVC Company, Austin, TX). The digital images were processed with Image-Pro Plus software, version 4.0 (Media Cybernetics, Silver Springs, MD). The integrated optical density of each luminescent band was used to estimate the content of LBT-tagged protein by comparison with standards of known LBT-protein content.

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