In Vivo Measurement of Intramolecular Distances Using Genetically Encoded Reporters

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ABSTRACT The function of membrane proteins occurs in the context of the cell membrane in living cells acting in concert with various cell components such as other proteins, cofactors, etc. The understanding of the function at the molecular level requires structural techniques, but high resolution structural studies are normally obtained in vitro and in artificial membranes or detergent. Ideally the correlation of structure and function should be carried out in the native environment but most of the techniques applicable in vivo lack the high resolution necessary to track conformational changes on a molecular level. Here we report on the successful application of an improved variant of lanthanide-based resonance energy transfer a fluorescent based technique, to *Shaker* potassium channels expressed in live *Xenopus* oocytes. Lanthanide-based resonance energy transfer is particularly suitable to measure intramolecular distances with high resolution. The improvements reported in this work are mainly based on the use of two different small genetically encoded tags (the Lanthanide Binding Tag and the hexa-histidine tag), which due to their small size can be encoded at will in many positions of interest without distorting the protein's function. The technique reported here has the additional improvement that the two tags can be placed independently in contrast to previously described techniques that rely on chemical labeling procedures of thiols.

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To conduct fluorescence studies on membrane proteins in the native environment of a living cell, it is either required to chemically react a fluorophore to an amino-acid side group, (e.g., the thiol group of a cysteine) or to create a fusion construct of the protein of interest together with a fluorescent tag such as *Aequorea victoria* fluorescent proteins (1). Chemical labeling procedures usually demand an excess of fluorescent label, which frequently will result in unspecific labeling of endogenous proteins or unspecific incorporation of label into the lipid membrane. Genetically encoded tags, on the other hand, have the disadvantage of their large size, which for many applications outweighs their advantageous qualities. Here we show that it is feasible to use much smaller genetically encoded tags—the lanthanide binding tag (LBT) (2)—in proteins in vivo, as exemplified with tagged *Shaker* potassium (K^+) channels expressed in the membrane of *Xenopus laevis* oocytes. We also show that it is possible to perform lanthanide-based resonance energy transfer (LRET) (3) distance measurements employing two fully genetically encoded tags: the LBT and the hexa-histidine (6-His) tag.

LRET, a spin-off of fluorescence energy transfer, has gained wide acceptance as a powerful technique to measure distances at a molecular level. It is based on the use of chelating molecules that bind lanthanide ions with high affinity and protect them from collisional quenching from the aqueous media. The lanthanide is used as a donor element to transfer energy upon excitation to an acceptor element. Because lanthanides have a low extinction coefficient, additional modifications have been introduced into these chelating molecules or motifs that serve as sensitizer antennae. The widely used terbium (Tb^{3+})-chelate (4) has a carbostyril group as sensitizer. It is bound to the protein by labeling cysteine residues using cysteine-specific reagents. Acceptor molecules in the proximity of the donor lanthanide receive the energy and quench the donor luminescence according to the distance between the donor-acceptor pair following Förster energy transfer theory (Supplementary Materials). Acceptor molecules are usually fluorophores that react with cysteine residues in the protein. As both donor and acceptor molecules require cysteine modification, limitations emerge in terms of the labeling procedure and stoichiometry, and also with regard to the distances measured, as donor and acceptor compete for the same pool of cysteines (5,6). Thus, we hypothesized that by using the LBTs introduced by Imperiali and collaborators (2,7) as high affinity lanthanide binding motifs we would be able to overcome most of the above-mentioned shortcomings. LBTs have tryptophan (Trp) and tyrosine (Tyr) residues at specific and well-defined positions in their sequence, close to the bound lanthanide, such that when illuminated by ultraviolet (UV) light these residues are excited and transfer energy to the trapped lanthanide, which then luminesces. LBTs are introduced in the open reading frame of a protein and hence can be used in combination with reporter fluorophores bound to individually introduced cysteines (Cys) or as shown here with another encoded motif as acceptor molecule: the 6-His tag.

The use of LBTs with bound Tb^{3+} as donors was validated in experiments in which the binding affinity and the Tb^{3+} luminescence time course was determined for the LBT sequence: YIDTNNDGWYEGDELLA (7) (Fig. 1 *a*, *inset*) incorporated into a protein and compared to the same parameters

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obtained from the same LBT as a synthetic peptide in solution. As reported (7), upon excitation of the LBT with UV light (266 nm), the Tb^{3+} luminescence of the LBT peptide decayed monoexponentially with a time constant (τ_D) of 2.6 ms (not shown). Such a slow time course indicates that the Tb^{3+} is well protected from collisional quenching. The same experiment was done with the LBT encoded in the open reading frame of the *Shaker* potassium channel, a member of the six-transmembrane segment family of tetrameric voltage-gated ion channels. The LBT was introduced in the loop between trans-membrane segments S3 and S4 (Fig. 1 *a*). The LBT mutant channels were expressed in *Xenopus laevis* oocytes (Supplementary Materials) and recordings were done from channels on the oocyte surface. In the absence of an acceptor, upon excitation, Tb^{3+} luminescence decayed with a bi-exponential time course with a main component, with time constant of 2.45 ± 0.05 ms that was identified as the luminescence originating from the LBT-bound Tb^{3+} (Fig. 1 *c* and LBT only plot in Fig. 2, *b* and *d*). The optimal Tb^{3+} concentration (2–3 μM) was determined based on the balance of emission intensity and prevalence of the 2.4 ms decay component (Fig. 1 *b* and 1 *c*). Nonsaturable Tb^{3+} emission was seen at higher concentrations suggesting unspecific binding of Tb^{3+} by other proteins or the lipids. The results indicated that, when incorporated in the protein sequence, the LBT had similar properties to those of the LBT peptide. When positioned in the S3-S4 loop, the LBT was kept intact and functional. In fact, LBTs placed in the intersegmental loops S1-S2 and S3-S4 of the *Shaker* channel displayed very similar decay properties (data of the S1-S2 LBT not shown). Measurements of gating currents of the *Shaker* potassium channel constructs showed that neither the insertion of the LBTs nor of the 6-His tag distorted functionality (Fig. S1, Supplementary Material).

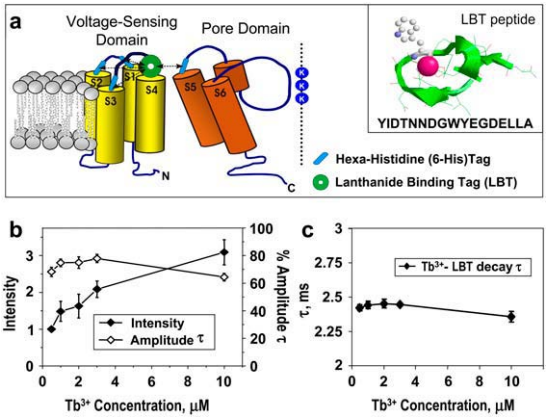


FIGURE 1 (*a*) Location of donor and acceptor groups. Schematic representation of the *Shaker* K^+ channel monomer illustrating the positions of the LBT (between transmembrane, TM, segments S3 and S4) and of the four 6-His tags tested. Displayed is the voltage sensing domain, comprised of TM segments S1-S4, and the pore region, segments S5-pore loop-S6, of the adjacent subunit. (*Inset*) Structural representation of the LBT used with a Tb^{3+} ion bound. (*b*) Concentration dependence of time resolved Tb^{3+} emission. Tb^{3+} concentration dependence of the phosphorescence intensity normalized to the lowest Tb^{3+} concentration, 0.5 μM (*left axis*) and the fractional amplitude (%) of the main component of Tb^{3+} luminescence decay (*right axis*). While the luminescence intensity increased with increasing Tb^{3+}, the fractional contribution of the main component decreased at higher Tb^{3+} concentrations. The optimal Tb^{3+} concentration range was that at which the signal was mostly carried by the 2.4 ms component. (*c*) Time constant of Tb^{3+} luminescence decay versus Tb^{3+} concentration. The time constant was fairly insensitive to the Tb^{3+} concentration up to 10 μM (note expanded scale). Data in *a* and *b* are mean ± SE.

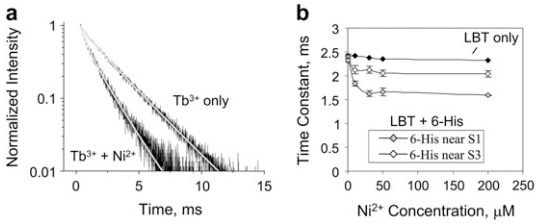
6-His tags are known to bind transition metals such as Ni^{2+}, Co^{2+}, and Cu^{2+} with high affinity (8). As these metal ions absorb in the region in which Tb^{3+} emits (Fig. S3, Supplementary Material), we considered employing them as energy transfer reporters. Distance measurements between Tb^{3+} and Co^{2+} have been introduced in an earlier study in which the authors took advantage of the presence of multiple Ca^{2+}-binding sites within thermolysin (9).

Several LBT and 6-His tag combinations were tested in the *Shaker* K^+ channel with the LBT in the S3-S4 loop and the 6-His in the S1-S2 loop (close to S1 and close to S2), S3-S4 loop (close to S3), and S5-S6 loop (close to S5) (Fig. 1 *a* and Supplementary Materials).

Addition of nickel caused no significant decrease in lifetime of the Tb^{3+} emission in the absence of a 6-His tag (Fig. 2 *a* and *b*) up to a concentration of 200 μM. The graph in Fig. 2 *b* shows the concentration dependence of the time constant of Tb^{3+} emission in a construct with a 6-His tag close to S1 and S3. The acceleration of the time constant of Tb^{3+} was clearly saturated at 50 μM Ni^{2+}. Distance estimates obtained with Ni^{2+} (R_o = 12 Å) for the different constructs are shown in the table presented in Fig. 2 *e* along with the corresponding transfer efficiencies.

The findings were extended to copper ions, which exhibited very efficient acceleration of the Tb^{3+} decay at about 10-fold lower concentration (Fig. 2 *c* and *d*). The R_o value for the Tb^{3+}-Cu^{2+} is longer, 20 Å (Supplementary Materials). Relative to Ni^{2+}, a higher transfer-efficiency (80% vs. 14%; Fig. 2 *e*) was observed for the same donor-acceptor pair. The calculated *r*-value was 15.8 ± 0.3 Å, which coincides with that estimated with Ni^{2+}. Results were obtained for the 6-His tag placed at various positions on extracellular loops of the *Shaker* channel (Fig. 2 *e*). For Cu^{2+} only, 6-His positions close to S2 and S3 gave *r* values within the range $R_o/2 < r < 2R_o$ both coinciding well with those obtained from the Ni^{2+} data (Fig.2 *e*). Even though it was tested to determine its energy transfer properties, Co^{2+} (R_o = 10 Å) was not considered further for distance determinations (see Supplementary Materials). Lacking specific information on the orientation of the reporter probes, a fair comparison with intersubunit distances obtained from the crystal structure of $K_V1.2$ (10), a *Shaker* channel relative, is too ambitious at present (but see Supplementary Materials).

As shown in Fig. 2 *f*, we also tested whether shorter genetically encoded tags such as a 2-His tag and a 4-His tag can be used to bind transition metals. We used truncated 6-His motifs inserted close to S1. We found 2-His to be too short, but 4-His was sufficient to form a high affinity His-transition metal complex.



e

	Ni2+		Cu2+	
6-His Position	Transfer Efficiency	Distance Å	Transfer Efficiency	Distance Å
S1	31%	13.7	-/-	-
S2	14%	16.3	80%	15.8
S3	11%	16.8	76%	16.4
S5	22%	14.9	-/-	-

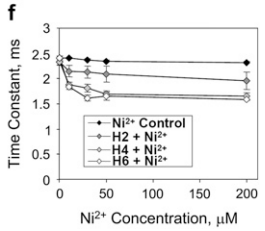


FIGURE 2 Energy transfer and distance determinations. (*a* and *b*) Energy transfer data obtained with Ni^{2+}. (*c* and *d*) Data obtained with Cu^{2+}. (*a* and *c*) Logarithmic representation of Tb^{3+} luminescence decay of *Shaker* channel constructs with the LBT in the S3-S4 loop with and without a 6-His tag close to S1 and S3 for Ni^{2+} and Cu^{2+}, respectively. In the presence of the acceptor 6-His tag-ion complex, Tb^{3+} luminescence decayed with a faster time course. (*b* and *d*) Time constant as a function of transition metal concentration. The main time constant of Tb^{3+} decay is shown for a construct without a 6-His tag (LBT only) and for constructs with 6-His close to (*b*) S1 and S3 and (*d*) S2 and S3. (*e*) Energy transfer efficiency and distance determinations for constructs with the donor LBT in the S3-S4 loop (close to S4) and with the 6-His close to the segments denoted in the first column. 6-His tags in segments S1 and S5 were too close to resolve when Cu^{2+} ions were used as acceptors. (*f*) Effect of the length of the His tag on transfer efficiency. A histidine tag containing four consecutive histidine residues was as effective as the 6-His tag used in this study for Tb^{3+} luminescence quenching. A 2-His motif was less efficient presumably because of its shorter length. The truncated 6-His tags were positioned close to S1. The Ni^{2+} control was obtained in the absence of a His tag. Data are mean ± SD.

Successful application of this technique depends on the following: 1), As for many spectroscopic studies, a large expression of the subject protein is required for good signal/noise ratios; 2), The background signals have to be minimized for unequivocal interpretation of the results; 3), The structures of the reporters, donors, and acceptors have to be intact and accessible to the ions and/or the fluorophores; and 4), Terbium and metal ion binding has to be specific.

There are several advantages of this method over other energy transfer configurations. The reporter groups are genetically encoded, which means that protein manipulation is reduced by avoiding prolonged labeling procedures that can be deleterious to protein function. The unspecific background is also reduced. Because LBTs have very high affinity for Tb^{3+} and because the Tb^{3+} emission depends on the presence of the sensitizer Trp and its time constant of decay is ~2 ms, the donor signal is very specific and distinguishable from background. By encoding the tags at will, it is possible to perform distance measurements while breaking symmetry in homomeric proteins. The measurements can be done within a subunit or across subunits with the appropriate choice of donor-acceptor pairs with different R_o values. Furthermore, the recordings can be done in the native environment of the protein, be it in solution, or, as in this case, in the membrane of a living cell. In addition, precise molecular modeling of the complexes can be achieved because the sequence is known and the atomic distances within the complexes are also known, in particular for the Tb^{3+} in the LBT. Thus, one can precisely predict the location, orientation, and effect of the motifs for each mutant protein. The information becomes either an excellent source of relative structural data or a complement to the static picture obtained from solved crystal structures.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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