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Distance measurements reveal a common topology of prokaryotic voltage-gated ion channels in the lipid bilayer

Jessica Richardson*, Rikard Blunck**, Pinghua Ge†, Paul R. Selvin†, Francisco Bezanilla*§¶, Diane M. Papazian*, and Ana M. Correa§¶

Departments of §Anesthesiology and *Physiology, David Geffen School of Medicine, University of California, Los Angeles, CA 90095; and †Department of Physics and Biophysics Center, University of Illinois at Urbana-Champaign, Urbana, IL 61801

Contributed by Francisco Bezanilla, August 29, 2006

Voltage-dependent ion channels are fundamental to the physiology of excitable cells because they underlie the generation and propagation of the action potential and excitation-contraction coupling. To understand how ion channels work, it is important to determine their structures in different conformations in a membrane environment. The validity of the crystal structure for the prokaryotic K+ channel, KvAP, has been questioned based on discrepancies with biophysical data from functional eukaryotic channels, underlining the need for independent structural data under native conditions. We investigated the structural organization of two prokaryotic voltage-gated channels, NaChBac and KvAP, in liposomes by using luminescence resonance energy transfer. We describe here a transmembrane packing representation of the voltage sensor and pore domains of the prokaryotic Na channel, NaChBac. We find that NaChBac and KvAP share a common arrangement in which the structures of the Na and K selective pores and voltage-sensor domains are conserved. The packing arrangement of the voltage-sensing region as determined by luminescence resonance energy transfer differs significantly from that of the KvAP crystal structure, but resembles that of the eukaryotic Kv1.2 crystal structure. However, the voltage-sensor domain in prokaryotic channels is closer to the pore domain than in the Kv1.2 structure. Our results indicate that prokaryotic and eukaryotic channels that share similar functional properties have similar helix arrangements, with differences arising likely from the later introduction of additional structural elements.

High-resolution x-ray structures have been solved for two voltage-gated K+ channels, the prokaryotic KvAP (1) and the eukaryotic Kv1.2 (2). These structures differ from each other and from predictions based on a variety of experimental approaches. The KvAP structure in particular is hard to reconcile with functional and biochemical data obtained from eukaryotic channels in a native membrane environment (3, 4). Likewise, EPR scanning of purified reconstituted KvAP (9) has shown a structural arrangement that is more in accordance with predictions from data obtained from eukaryotic channels. Thus, there is general agreement that the original KvAP crystal structure represents a nonnative conformation, although the degree of

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The authors declare no conflict of interest.

Abbreviation: LRET, luminescence resonance energy transfer.

1Present address: Département de Physique, Université de Montréal, Montréal, QC, Canada H3C 3J7.

‡Present address: Département de Physique, Université de Montréal, Montréal, QC, Canada H3C 3J7.

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distortion is unknown (ref. 9, but see ref. 10). The Kv1.2 structure, on the other hand, is more compatible with most of the available data, although two independent lines of evidence obtained on functional channels suggest that the voltage sensor domains have fallen away from the pore in the crystal structure, perhaps due to the absence of lateral pressure from the lipid bilayer (5, 9–13) or due to crystal packing contacts between tetramerization (T1) domains (14–16). Subsequently, modeling of the KvAP structure, based on analogy to Kv1.2 structure (2), oxygen accessibility data (9) and biochemical cross-linking data, led Lee et al. (10) to suggest that KvAP ought to resemble Kv1.2 because manipulation of the KvAP helical arrangement renders a very similar structure to that of Kv1.2, that is diametrically different from that presented in the original crystal (1).

We have used luminescence resonance energy transfer (LRET) (17, 18) to investigate the structural organization of KvAP and of a prokaryotic voltage-gated Na+ channel (19), NaChBac, in reconstituted lipid vesicles. LRET is a variant of fluorescence resonance energy transfer (FRET) (20), which has been widely used to estimate distances in proteins and to study protein interactions and conformational changes (11, 13, 17, 18, 20, 21). Whereas in FRET, energy transfer occurs between two organic fluorophores, in LRET, energy transfer occurs between a Tb3+-chelate (or Eu3+-chelate) donor and an organic fluorophore acceptor (Fig. 2a). The advantages of LRET over traditional FRET methods have been discussed (17, 18). Previously, LRET was used to estimate distances and describe conformational changes in Shaker K+ channels expressed in oocytes (11, 12). Because KvAP and NaChBac do not express well in oocytes, channel proteins containing single cysteine residues, introduced by site-directed mutagenesis, were expressed in Escherichia coli, purified, labeled with the Tb3+-chelate and fluorophore in detergent, and then reconstituted in functional form into liposomes for distance measurements (see Methods). Distances between analogously positioned residues on different subunits of the tetrameric channel were estimated by LRET. The results obtained with several different acceptor fluorophores with different optimal distances for energy transfer were compared to assign the measured distances as occurring between adjacent subunits or between subunits situated diagonally from each other across the pore (Fig. 2).

Results and Discussion

Supporting Information. For further details, see Figs. 6–10, which are published as supporting information on the PNAS web site.

Pore Measurements and Confirmation of Technique. The structure of K+ selective pores has been well established by x-ray crystallography of KcsA, KvAP, and Kv1.2 (1–3). To confirm the accuracy of our technique, LRET distances measured across the KvAP...
Table 1. LRET distance measurements

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>ABDMTS 15Å</th>
<th>ATTO 27Å</th>
<th>MTSF 45Å</th>
<th>FL-Mal 45Å</th>
<th>MTSR 60Å</th>
<th>TMRM 60Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>KvAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>42</td>
<td>28.7(3)±0.1</td>
<td></td>
<td>48.5(6)±1.7</td>
<td>47.2(6)±1.6</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>100</td>
<td>24.1(3)±2.6</td>
<td></td>
<td>49.8(2)±7.9</td>
<td>57.5(1)</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>122</td>
<td>19.8(2)±1.9</td>
<td>52.2(2)±1.9</td>
<td>45.3(3)±0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5</td>
<td>128</td>
<td>19.4(3)±1.0</td>
<td></td>
<td>49.4(3)±1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pore</td>
<td>173</td>
<td>52.2(2)±2.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>208</td>
<td>29.1(4)±0.9</td>
<td>27.3(3)±0.1</td>
<td>50.2(2)±0.4</td>
<td></td>
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<tr>
<td>NaChBac</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>25</td>
<td>30.0(2)±0.2</td>
<td></td>
<td>46.0(2)±2.4</td>
<td>45.8(2)±3.4</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>88</td>
<td>49.4(2)±0.7</td>
<td>46.9(2)±1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>98</td>
<td>47.8(2)±3.3</td>
<td></td>
<td>49.0(2)±2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>112</td>
<td>54.0(2)±4.7</td>
<td>45.9(6)±3.9</td>
<td>55.6(2)±0.6</td>
<td>60.5(2)±2.12</td>
<td></td>
</tr>
<tr>
<td>Pore</td>
<td>192</td>
<td>17.7(3)±0.9</td>
<td></td>
<td>49.1(3)±1.8</td>
<td>46.7(2)±0.6</td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>231</td>
<td>27.3(3)±0.1</td>
<td></td>
<td>54.9(1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Distances were calculated using the slowest component of the energy transfer for KvAP and NaChBac. Bold indicates adjacent distances (dA), and italic indicates diagonal distances (dD). Data are given as the average (number of experiments) = SEM. ABDMTS, N-[4-(aminosulfonyl)-2,1,3-benzoxadiazol-7-yl]-2-aminomethyl methanethiosulfonate; ATTO, ATTO-465 maleimide; MTSF, 2-(5-fluoresceinyl)aminocarbonyl]ethyl methanethiosulfonate; MTSR, 2-(5(6)-tetracylphthalamine)carboxylicamino)ethyl methanethiosulfonate; FL-Mal, fluorescein-5-maleimide; TMRM, tetracetylphthalamine-5-maleimide.

There were compared with distances measured from the original KvAP crystal structure (Fig. 3 and Table 1) (Protein Data Bank accession code 1ORQ). Single cysteine mutations were introduced at positions Y173 (top of S5), V192 (pore reentrant loop), L208 (top of S6), and V231 (middle to bottom of S6). Each construct was labeled with the Tb3⁺-chelate and a specific fluorophore that would be most sensitive for the relatively short distances expected across the pore and, therefore, provide the most accurate results (Fig. 3 and Table 1). Distances estimated by LRET were very similar to those observed in the KvAP crystal structure (1). To illustrate this point, Fig. 3 shows the positions of residues 173 in S5, 192, 208 (in segment S6), and 231 in the KvAP crystal structure. In the drawing, solid orbits were drawn to connect the positions of the α-carbons of each residue in the crystal. Neighboring residues to 173 and 208 are shown in ribbon display to represent the S5 and S6 α-helices. The Tb3⁺-chelate (yellow spark, Fig. 3) was modeled to project from the α-carbon toward the orbits described by the LRET measured diagonal distances, represented as dashed lines in Fig. 3. For residues 173 and 208, the chelate was drawn to follow the same direction as the original side chains in the crystal structure. For residues 192 and 231, the LRET and crystal orbits overlapped.

There was good agreement between the positions of the Tb3⁺-chelate, modeled from the x-ray structure (1, 10), and the positions measured by LRET (dashed orbits, Fig. 3). The LRET distances were equal or larger than the crystallographic distances by no more than 10 Å (Fig. 3), about the length of the chelate (10.5 Å). At position 173 in S5, the original tyrosine side chain from the pore, placing the 10.5 Å long Tb3⁺-chelate outside the crystallographic orbit. The modeled location of the chelate and the measured LRET distance match well with the chelate projecting up and out. These results demonstrate that LRET reliably reports distances in the K⁺ channel structure.

Because a crystal structure is not yet available for NaChBac, we compared the dimensions of the Na⁺-selective NaChBac pore with the K⁺-selective KvAP pore. The agreement found in the distances between residues in the same relative positions in the top of S5, the reentrant loop and the top of S6 (Table 1), give strong support to a common architecture of the conducting pore in prokaryotic ion channels notwithstanding a lack of sequence homology in the respective pore regions (Fig. 6 and refs. 19 and...
to model the transmembrane structural organization of KVAP the topological arrangement exhibited by the KVAP crystal (1)

Structural Organization of the Voltage Sensor in KvAP and NaChBac. To investigate the structural organization of the voltage sensing domain (S1–S4) and its relationship to the pore (S5–pore loop–S6), additional single cysteine mutations were incorporated at a variety of locations in S1, S3, S4, and S6 in NaChBac and in KvAP for LRET analysis. Distances measured at the beginning and end of each individual segment were similar, indicating that S1, S3, and S4 span the membrane (Table 1) with a tilt. In the voltage sensor, diagonal distances measured across the pore were shorter for residues in S1, positioning S1 closest to the pore. In contrast, S3 and S4 were further away, positioning them at the periphery of the protein. Significantly, similar results were obtained for both KvAP and NaChBac, indicating that these two prokaryotic voltage-gated channels share a similar structural organization. These results differ significantly from the topological arrangement exhibited by the KvAP crystal (1) where S3 and S4 form a paddle that slants away from the pore and where S1 and S2 lay almost parallel to the plane of the bilayer. Our results agree better with the modeled packing of KvAP proposed recently by Lee et al. (10) to reconcile the differences between the original crystal structure, the crystal structure of the Kv1.2, and among other experimental data, the EPR results of Cuello et al. (9). The authors concluded that the lipid bilayer is necessary to maintain the correct relative orientation of channel domains in KvAP (10), precisely the case of the reconstituted channels in the present study.

Packing model of KvAP and NaChBac. The LRET results were used to model the transmembrane structural organization of KvAP and NaChBac (Fig. 4). Because the distances measured at equivalent positions in KvAP and NaChBac were comparable and because the crystal structure of the pore is consistent among Kv channels, the S5 and S6 helices were positioned according to the KvAP x-ray structure. S1, S3, and S4 were then positioned based on the LRET distances measured from the tops and bottoms of each helix. To assist in the modeling, orbits of the measured distances were drawn first, and 10-Å circles, representing the span of the α-helical segments, were centered on top of them. From the extracellular perspective, S1 was positioned next to the S5 and S6 helices because of the close proximity found between S1 and the pore. The S1–S2 loops in KvAP and NaChBac are short, only 3 aa in NaChBac, constraining S2 to be very close to S1. The S3–S4 linker is also extremely short, constraining S3 and S4 to be in close proximity to each other on the extracellular side. The packing model is shown with a counterclockwise orientation (Fig. 4a). For comparison, an equivalent packing model based on the Kv1.2 crystal structure is shown as an inset below the model (Fig. 4a Inset). Following the arrangement seen in the Kv1.2 crystal structure, the voltage sensor (S1–S4) was positioned next to the S5 and S6 of the neighboring monomer. However, LRET measurements do not allow discriminating between monomers. A counterclockwise arrangement of the transmembrane segments in the voltage sensor is the only organization compatible with structural con-
strains derived from functional eukaryotic channels in a native membrane environment and is also seen in the x-ray structures of the isolated KvAP voltage sensor and Kv1.2 (2, 23). Although the LRET data alone do not exclude a clockwise orientation (shown in Fig. 1b), this arrangement is unlikely given the weight of the other evidence obtained with both prokaryotic and eukaryotic channels.

The packing arrangement as seen from an intracellular perspective is shown in Fig. 4b. Again, the packing of the pore is taken directly from the KvAP crystal structure. Although the S5 and S6 segments of the same subunit are separate from the voltage sensor on the extracellular side, these helices cross in the membrane. As a result, S5 and S6 are adjacent to the voltage sensor on the intracellular side. S1 was placed between neighboring subunits in proximity to S5 and S6. The resulting segment placement as seen from the inside reveals not only tighter packing than that seen in the Kv1.2 structure (2, 24) (Fig. 4 inset) but also a more peripheral position for S3 and S4.

The Kink in the S4 Helix. To investigate the secondary structure of the S4 segment in NaChBac, single cysteine mutations were introduced throughout S4 at noncharged positions. The adjacent distances exhibited periodicity when plotted versus residue number, and therefore were fitted to a sinusoidal function with a period of 3.6 residues per cycle, the periodicity of an α-helix (Fig. 5a). A simple sinusoidal function could not fit the data for the entire S4 segment, but by allowing a break and change in phase after residue 123, a good fit was obtained (Fig. 5a). A break in the S4 helix is consistent with EPR and crystallographic data of KvAP, suggesting that it may be a common feature in voltage-gated channels (9). Interestingly, the four charged residues that are expected to carry the bulk of the gating charge are located above the break in the S4 secondary structure. It is tempting to speculate that the interruption in the S4 helix allows rotation of S4 during activation, as proposed in some models for the voltage-dependent conformational changes of the voltage sensor (7, 13, 24).

To illustrate the orientation of the S4 segment, residues 112–130 were plotted on a helical wheel. The corresponding adjacent distances measured by LRET are also noted (Fig. 5b). Above the break in secondary structure at residue 123, the shorter distances cluster on one face of the helix, with the longer distances on the opposite face. The face with the longer distances is likely to be located at the periphery of the channel protein, facing the lipid bilayer. Interestingly, the four positively charged arginines involved in voltage sensing are located on the face where the shorter distances cluster. These data suggest that the arginine residues face into the protein in the open/inactivated conformation of the NaChBac channel, not out into the lipid. A similar conclusion was drawn for charged residues in the KvAP S4 segment on the basis of EPR analysis (9). The differential exposure of EPR probes in positions throughout the S4 segment suggested an interrupted α-helical arrangement along the segment’s axis in KvAP, supporting the idea that there is a slight rotation of the segment, so that most of the S4 charged residues involved in voltage sensing face away from the bilayer in the open-inactivated state, even though S3–S4 are peripheral (9). In contrast, the model proposed by Lee et al. (10) places the arginines in contact with the bilayer.

Conclusion

Our LRET data and packing model are compatible with previous EPR analysis of KvAP (9). The results contrast with models of membrane-bound eukaryotic K+ channels, in which S4 is closer to the pore (23) and mostly surrounded by S1–S3 in particular in the internal face of the channel. By comparison, the extracellular face of NaChBac and KvAP compares well with that of the Kv1.2 crystal, whereas the intracellular face differs significantly (Fig. 4) (2, 24). Taken together, the data indicate that there are intrinsic differences in the relative orientation of the voltage sensor and pore domains in prokaryotic and eukaryotic voltage-gated channels, but that the main differences rest on the tightness of helical packing within the voltage sensor and relative to the pore as well as in the tilt of the transmembrane helices.

The LRET data indicate that the structure of S1–S4 in NaChBac strongly resembles that in KvAP as the distances from tops and bottoms of the segments are within the same range. Furthermore, the results indicate that LRET is a sensitive probe of the secondary structure and orientation of transmembrane segments. The packing model generated by our data corresponds to the structural organization of these prokaryotic channels in a bilayer environment and in the absence of a membrane potential. Therefore, the distances measured correspond to those found in an open/slow inactivated conformation, which is also true for the available EPR and crystallographic data. It is conceivable, however, that these segments move and reorient slightly as the channels transition between states (7, 13, 24). In future studies, imposition of a membrane potential should make it feasible to gain insights into the structure of other channel conformations, particularly the closed conformation.

We conclude from our LRET distance measurements that KvAP and NaChBac share similar topologies but differ from eukaryotic K channels in that the voltage sensor is closer to the pore domain and more tightly packed than what is seen in the x-ray crystal structure. In prokaryotic channels, the S3, S4 and the S1, S2 helices are not parallel, but they do traverse the membrane, although with a slight tilt. It is likely that the voltage-sensing domain is reoriented relative to the pore in eukaryotic channels due to the presence of the large cytoplasmic T1 assembly domain present in Kv channels (2, 13–16). We might speculate that, with the expansion in the number of Kv genes in eukaryotes, acquisition of the T1 domain was likely advantageous to regulate subunit assembly. This structure would impose a different topology in the transmembrane segments that did not affect the function of the channel.

Methods

Purification. His-6-tagged constructs of NaChBac and KvAP in the pQE-60 vector (Qiagen, Valencia, CA) were used for bacterial expression. The single endogenous cysteine in KvAP was mutated to serine (C247S). Single cysteine mutations were introduced by using the QuikChange mutagenesis method (Stratagene, La Jolla, CA). Constructs were expressed in the XL-10 Gold (Stratagene) strain of Escherichia coli, grown to an OD = 1.0 and then induced with 0.5 mM IPTG for 5 h (NaChBac) or 3 h with 10% glycerol (KvAP). Bacteria were resuspended in 50 mM Na-Pi, 240 mM NaCl, and 50 mM KCl (150 mM NaCl and 140 mM KCl for KvAP), pH 7.0, with a mixture of noncysteine modifying protease inhibitors. Bacteria were disrupted with temperature-controlled sonication (Misonix, Farmingdale, NY, Sonicator-3000). The protein was solubilized overnight in 40 mM dodecyl-maltopyranoside (NaChBac) or 40 mM dodecyl-maltospyranoside (KvAP) with 10% glycerol and noncysteine modifying protease inhibitors. The solubilized proteins were centrifuged at 50,000 × g. NaChBac and KvAP were bound to affinity His-6 binding Co2+ resin (BD Biosciences, Franklin Lakes, NJ), and washed with 10 volumes of the resuspension buffer plus 40 mM detergent, 40 mM imidazole, and 10 mM TCEP (Pierce, Rockford, IL) to remove nonspecific binding. Because the chelate is sensitive to phosphates, the solutions were exchanged while the protein was bound to the resin with 50 mM Pipes and 100 mM NaCl (NaChBac) or 100 mM KCl (KvAP) pH 7.0. The protein was eluted in buffer containing 0.5 mM detergent with 200 mM imidazole and concentrated to 5 mg/ml (NaChBac) and 3 mg/ml (KvAP) with Millipore (Eugene, OR) 50-kDa concentrators.
Labeling. Purified protein was labeled overnight with the Tb$_{3}{^+}$-chelate alone or a 5:1 ratio of Tb$_{3}{^+}$-chelate to fluorophore. MTS or maleimide linkers were used to modify the protein. In a given labeling experiment, the linkers of the chelate and fluorophore were matched. Labeling was done in detergent to label the entire protein, not only extracellular residues accessible to dyes when in the native membrane. Protein aggregates and excess dye were removed in PD-10 columns (Amersham, Piscataway, NJ). N-[4-(amino-sulfonyl)]-2,1,3-benzoazadiazol-7-yl]-2-aminoethyl methanethiosulfonate (ABD-MTS), 2-(5-fluoresceinyiamino)carbonyl]ethyl methanethiosulfonate (MTSF), and 2-((4(6)-N-0xime)benzyl)ethyl methanethiosulfonate (MTSR) were purchased from Toronto Research Chemicals (North York, ON, Canada); ATTO-465 maleimide was purchased from Atto-Tec (Siegen, Germany); and fluorescein-5-maleimide (MTSR) were purchased from Molecular Probes.

Reconstitution. The protein was concentrated to 3 mg/ml and reconstituted in 10 mg/ml 3:1 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoethanolamine/1-palmitoyl-2-oleyl-sn-glycero-3-phospho-rac-(1-glycerol)] (POPE/POPG) (Avanti Polar Lipids, Alabaster, AL) in a 10:1 protein/lipid ratio. Detergent and remaining excess dye was removed with four rounds of BioBeads (Bio-Rad, Hercules, CA) overnight.

Data Acquisition. Tb$_{3}{^+}$ luminescence was induced by excitation with pulses of a nitrogen laser at 337 nm and sensitized emission was measured at 510–530 nm (ABD, ATTO-465, or fluorescein) or 560–575 nm (rhodamine) from samples labeled with Tb$_{3}{^+}$-chelate to fluorophore. MTS or maleimide-chelate and dye, the donor-acceptor pair. MTS (or maleimide)-chelate alone at wavelengths longer than 515 nm. Distances between various positions across subunits were determined from the energy transferred between donor and acceptor groups at defined positions in single-Cys constructs. Cys-less wild-type NaChBac and C247S-KvAP were used as negative controls (Fig. 7).

Identification of Distances. Tb$_{3}{^+}$-chelate has two time constants, a slow ($\tau_{slow}$) and a fast ($\tau_{fast}$) in its emission decay (donor only) (12). In the present experiments, the $\tau_{fast}$ made up 34–50% of the total decay; therefore, both time constants were considered in the final analysis (Fig. 2). In addition to the two components of the Tb$_{3}{^+}$-chelate, the present experiments measure two distances: the adjacent distance ($D_{a}$) between subunits and the diagonal distance ($D_{d}$) across the pore (Fig. 2). These distances are detected as the emission of the organic fluorophore that is excited from the emission of the Tb$_{3}{^+}$-chelate (sensitized emission, SE). Thus, with the initial two components of the donor and the two distances of the protein, four different components are theoretically present in the SE (Fig. 2b). Although the slowest component can be detected with confidence, the second and third components are not well resolved, and the fourth component is so fast that it is partially lost in the fast transient that follows the laser pulse (Fig. 2b). For these reasons, only the slowest component (SE $\tau_{slow}$) can be used confidently for distance determinations. Using only the SE $\tau_{slow}$ and ignoring the other components means that only one distance can be calculated from each experiment. Because there are two possible distances measured, $D_{a}$ or $D_{d}$, the distance obtained will be the one optimally measured by the $R_{o}$ of the Tb$_{3}{^+}$-chelate-fluorophore combination. Thus, to determine whether the measured distance is $D_{a}$ or $D_{d}$, multiple experiments with various fluorophore acceptors of different $R_{o}$ values were used (Table 1 and Fig. 9).

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