Bimane Fluorescence Scanning Suggests Secondary Structure near the S3-S4 Linker of BK Channels*

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Nina P. Semenova, Karin Abarca-Heidemann, Eva Loranc, and Brad S. Rothberg1

From the Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229

Gating of large conductance Ca2+-activated K+ channels (BK or maxi-K channels) is controlled by a Ca2+-sensor, formed by the channel cytoplasmic C-terminal domain, and a voltage sensor, formed by its S0-S4 transmembrane helices. Here we analyze structural properties of a portion of the BK channel voltage sensing domain, the S3-S4 linker, using fluorescence lifetime spectroscopy. Single residues in the S3-S4 linker region were substituted with cysteine, and the cysteine-substituted mutants were expressed in CHO cells and covalently labeled with the sulfhydryl-reactive fluorophore monobromo-trimethylammonio-bimane (qBBr). qBBr fluorescence is quenched by tryptophan and, to a lesser extent, tyrosine side chains. We found that qBBr fluorescence in several of the labeled cysteine-substituted channels shows position-specific quenching, as indicated by increase of the brief lifetime component of the qBBr fluorescence decay. Quenching was reduced with the mutation W203F (in the S4 segment), suggesting that Trp-203 acts as a quenching group. Our results suggest a working hypothesis for the secondary structure of the BK channel S3-S4 region, and places residues Leu-204, Gly-205, and Leu-206 within the extracellular end of the S4 helix.

Large conductance Ca2+-activated K+ channels (BK or maxi-K channels) are opened by depolarization and raised intracellular Ca2+, and the current through these channels can contribute to membrane repolarization or hyperpolarization, thus regulating electrical excitability across a variety of tissues (1–5). BK channels are related to depolarization-activated K+ channels (Kv channels), which have a tetrameric structure with each subunit containing six transmembrane regions (6, 7). Kv channel gating is controlled by a voltage sensor domain (VSD),2 which is formed primarily by the first four transmembrane helices of each subunit, S1–S4 (8). Likewise, BK gating is controlled by a VSD formed by these segments, as well as an additional N-terminal transmembrane segment, S0, that contributes to voltage-dependent gating (1, 9–11). Mutations of several charged residues in the BK channel VSD can result in changes in the equilibrium constant for voltage sensor movement, while Arg-213 (located in the S4 segment), along with Asp-153 and Arg-167 (both in S2) and Asp-186 (in S3) potentially correspond to voltage-sensing charges that move through some portion of the electric field during voltage activation (9). These studies have provided a working hypothesis for BK channel voltage sensor activation, in terms of the relative positions of these residues with respect to the transmembrane electric field. But despite advances in our understanding of the structure of mammalian Kv channels provided by x-ray data (12), we do not yet have a clear picture of the structural correlates of these functional measures for BK channels. Further identification of structural elements in the BK VSD calls for biophysical measurements of BK channels that may provide insight toward intramolecular distances.

In cases where x-ray data is unavailable, site-specific fluorescence measurements have provided insight toward ion channel structure and activation mechanism. Fluorescent probes have provided numerous insights toward gating motions in Kv and other channels (13–21); in addition, fluorescence measurements can be used to estimate intramolecular distances to gain insight toward channel structure (22, 23). The fluorophore monobromobimane (mBBr) has recently been applied to gain structural insight toward cyclic nucleotide-gated channels and other proteins (13–21); MBBr can be covalently attached to specific positions in a protein using cysteine chemistry, and its fluorescence can be strongly quenched by Trp side chains (13, 26, 27). This property yields the possibility of using a native Trp side chain in a protein as a landmark for estimating its distance from a bimane probe (26, 28). By sampling bimane fluorescence over several labeled positions in a protein, one might gain insight toward protein structure.

Here we address the structure of the putative S3-S4 linker region of the BK channel VSD using site-specific labeling of channels, overexpressed in intact CHO cells, with the sulfhydryl-reactive fluorophore monobromo-trimethylammonio-bimane (qBBr). The quaternary amine group on qBBr carries a positive charge, rendering the qBBr molecule membrane-impermeant. We observe that qBBr fluorescence in labeled BK channels is quenched as a function of its apparent proximity to Trp-203. qBBr-quenching is reduced in labeled channels containing the mutation W203F, suggesting that Trp-203 may provide the primary quenching group. We use these fluorescence data to distinguish among several candidate structures, and conclude that the data are consistent with a local secondary structure in the S3-S4 linker that places residues Leu-204, Gly-205, and Leu-206 within the extracellular end of the S4 helix.

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1 To whom correspondence should be addressed: Dept. of Biochemistry, Temple University School of Medicine, 3440 N. Broad St., Philadelphia, PA 19140. Tel.: 215-707-0855; Fax: 215-707-7436; E-mail: rothberg@temple.edu.
2 The abbreviations used are: VSD, voltage sensor domain; qBBr, monobromo-trimethylammonio-bimane; GFP, green fluorescent protein.
EXPERIMENTAL PROCEDURES

Mutagenesis, Channel Expression, and Labeling—Point mutations were introduced into the mouse BK channel gene in the pcDNA3 expression vector using QuikChange mutagenesis (Stratagene) and confirmed by DNA sequencing. Cysteine-substituted mutants were generated on a channel template in which the native cysteines at positions 14, 141, and 277 were substituted with valine ("−3C"). Channels were transiently overexpressed in CHO cells transfected using Lipofectamine (Invitrogen). CHO cells were used in these studies because this cell line seems to yield relatively low levels of endogenous background labeling, possibly due to low levels of free extracellularly accessible thiol groups (29). Cells were co-transfected with pEFYF (Clontech Laboratories, Inc.) to facilitate identification of transfected cells using fluorescence microscopy.

Labeling of mutant channels with qBBr was performed in the dark. Cells were washed three times in phosphate-buffered saline with Ca2+ and Mg2+ (PBSCM) composed of (in mM) 150 NaCl, 8.1 Na2HPO4, 1.9 NaH2PO4, 0.1 CaCl2,1 MgCl2, pH 7.4, followed by incubation with 1 mM qBBr (Molecular Probes) in PBSCM for 10 min on ice, two washes with PBSCM, and one wash in standard extracellular solution composed of (in mM) 160 NaCl, 4.5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 8 glucose, pH 7.4. Labeled cells were protected from light by covering with aluminum foil and fluorescence recordings were obtained within 2 h of labeling.

Cell Surface Biotinylation—Surface expression of mutant channel constructs was confirmed through a surface biotinylation assay (Pierce Biotechnology). Briefly, transfected cells were washed three times with PBSCM, and then incubated with sulfo-NHS-S-S-biotin for 45 min at 4 °C. Excess reagent was quenched by addition of glycine, and cells were harvested and washed twice with TBS. Cells were lysed in radioimmune precipitation assay buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholic acid, 0.1% SDS), and biotinylated proteins were precipitated by incubation with immobilized Neutravidin for ~20 h (i.e. overnight) at 4 °C. Biotinylated proteins were eluted from the beads by incubation with 50 mM dithiothreitol for 2 h. Samples from 1) lysate, 2) flow-through from Neutravidin beads, and 3) protein eluted from beads were loaded onto a 7.5% SDS gel and separated by electrophoresis. BK channels were detected by immunoblotting using an anti-BK channel antibody (gift of Dr. J. Trimmer, University of California at Davis). Each blot was reprobed with anti-extracellular signal-related kinase 1/2 (Anti-ERK 1/2 pAb, Rabbit; Promega) to detect the cytoplasmic protein ERK 1/2 in the samples, and thus verify that only membrane proteins were biotinylated during the assay. Each biotinylation experiment was repeated three times to confirm results.

Fluorescence Lifetime Measurement—Fluorescence decays were measured using a strroboscopic microscope-based fluorescence lifetime system (TM-5/2005, Photon Technology International). QBBr-labeled cells bathed in ES were placed in an imaging chamber on the stage of a Nikon TE2000 inverted microscope equipped with a 40 × 1.3 NA oil immersion objective and a photometer housing attached to the microscope side port. Cells were viewed via epifluorescence microscopy through a viewport attached to photometer housing, and an adjustable iris was used to optically isolate the light from individual cells for measurement. Only cells which displayed both apparent membrane labeling by qBBr and cytoplasmic YFP fluorescence were selected for study.

The qBBr fluorophore (excitation peak: 378 nm, emission peak: ~490 nm; Molecular Probes/Invitrogen, Carlsbad, CA) was excited by a 500-ps pulse of 375-nm light (2-nm bandwidth) using a high-resolution nitrogen/dye laser (GL3300/ GL302; Photon Technology International), containing 5 mm 2-[1,1′-biphenyl]-4-yl-5-phenyl-1,3,4-oxadiazole (PBD; Exciton, Dayton OH) dissolved in toluene/ethanol (50/50 v/v) in the dye laser cuvettes. Light was guided to the microscope epifluorescence port through a 40-m quartz optical fiber, which generates a static delay of ~50 ns between the generation of the light pulse and excitation of the sample. Excitation light was deflected to the sample via a filter cube containing a 365–395-nm bandpass excitation filter (D380/30) and 420-nm longpass dichroic (420DCLP), and sample fluorescence was filtered using a 460–500-nm bandpass emission filter (D480/40; all filters from Chroma Technology Corp.). Fluorescence was detected by a photomultiplier tube (PMT) attached to the side port of the microscope.

Data acquisition was controlled by Felix32 software (Photon Technology International). Fluorescence decays were generated from 200 data points, in which the interval between data points over the course of the decay was increased by a constant increment. Thus for the decays reported below, the interpoint interval was ~5 ps at the early points in the decay, and increased arithmetically to ~500 ps by the last points in the decay. This data collection strategy enabled us to acquire both brief and long lifetime components with the appropriate time resolution. Each decay was averaged over three acquisitions for each labeled cell. Instrument response functions (IRFs) were measured using identical data acquisition protocols by replacing the D380/30–420DCLP–D480/40 filter cube with a 50/50 beamsplitter (Chroma), with attenuation of the scattered light by neutral density filters.

Data Analysis—Fluorescence decays from individual cells were fitted with sums of exponential components, using a χ2 statistic. Fits were limited to the portion of the decay where the IRF decayed to less than 5% of its maximal amplitude (~1.5 ns after the peak of the fluorescence signal), and deconvolution of the IRF was not used. The minimum number of significant exponential components required to describe a given decay was determined by comparing the ratio of the reduced χ2 (χ2R) values for two fits with the F-statistic; a (χ2R) ratio >1.35 warrants an additional exponential component at the p < 0.05 level (30). Kinetic analysis was based on fluorescence decays from a total of 119 cells for these studies; data are reported as mean ± S.E.

Electrophysiology—Wild-type and C14V/C14V/C277V ("−3C") BK channels were transiently overexpressed in CHO cells, and currents were recorded from excised inside-out patches as described previously (31), at 22–24 °C. Solutions bathing both sides of the membrane contained 160 mM KCl and 10 mM HEPES, pH 7.4. The solutions at the cytoplasmic face of the patch additionally contained 2 mM N-(2-hydroxyethyl)-ethylenediamine-triacetic acid (HEDTA), with CaCl2 added to
raise the free $[\text{Ca}^{2+}]$ in the solution to 10 $\mu\text{M}$, estimated using MaxChelator. Free $[\text{Ca}^{2+}]$ in the solution was measured using a $\text{Ca}^{2+}$-sensitive electrode (Orion). Currents were recorded using a Dagan PC-ONE amplifier, low-pass filtered at 5 kHz, and digitized at 20 kHz using pClamp 9.0.

RESULTS

Bimane derivatives (i.e. bromobimanes) are small, thiol-reactive fluorescent molecules that are useful probes of short range molecular interactions (13, 26, 32, 33). It was previously established that bimane fluorescence can be quenched by Trp or Tyr side chains in proteins (13, 26, 27). This quenching occurs through an excited-state electron transfer mechanism (Fig. 1A) that requires close proximity between the bimane and the Trp side chain, on the order of $\sim 10$ Å (26). Thus bimane fluorescence shows the potential for use as a point-to-point proximity detector either within or between proteins, between a specific position in the protein to which the bimane can be covalently attached and a native (or substituted) Trp side chain.

There are several advantages of using bimane as a probe of protein structure. Specifically, the small size of the bimane molecule (Fig. 1A) and the ability to attach it at essentially any position on a protein via cysteine substitution and covalent labeling make it both versatile and presumably minimally invasive toward protein structure, compared with larger fluorophores such as GFP. In addition, the property of quenching by Trp side chains can preclude the requirement of attaching an exogenous quenching "partner," which can sometimes be problematic in FRET-based assays.

We have exploited the bimane quenching assay in the present study by using site-specific labeling of cysteines in BK channels with the bimane derivative qBBr, which carries a positive charge and is membrane-impermeant (34–38). The use of qBBr thus enables the targeted labeling of thiols that are accessible only from the extracellular side of the cell.

Our goal here was to use relative distance constraints derived from bimane fluorescence data to distinguish among models for local secondary and tertiary structure near the extracellular S3-S4 linker of the BK channel. For example, Fig. 1B shows two plausible partial sequence alignments between the BK channel and the prokaryotic voltage-gated K$^+$ channel KvAP (39–43), with a schematic of the corresponding secondary structure of KvAP in the region of the alignment (known from crystallographic studies) shown underneath. In one alignment ("BK"), the extracellular end of the S4 transmembrane helix is bounded by Trp-203; residues C-terminal to this would be located within the S4 helix. In an alternative alignment

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FIGURE 1. Fluorescent labeling of the BK channel S3-S4 region with bimane. A, schematic of qBBr after covalent attachment to a cysteine side chain, and the proposed mechanism of bimane fluorescence quenching by electron transfer from nearby tryptophan. The size of qBBr is comparable to that of the Trp side chain. B, two plausible partial sequence alignments of the S3-S4 region of the BK channel with KvAP. The position of Trp-203 is indicated by asterisk; positions of arginines in the BK sequence are indicated by plus signs. Residues targeted for cysteine-substitution and qBBr labeling (Leu-199 through Leu-206) is indicated by green shading. Secondary structure corresponding to KvAP (PDB ID 1ORS) is indicated below the aligned sequences. C, schematic of the BK channel subunit showing hypothetical positions of basic residues, corresponding to the alignment "BK" (in B). Positions of native cysteines on extracellular loops (C14, C141, C277) are indicated by yellow circle; these were substituted with valines in our experiments to generate the -3C BK channel. Position of Trp-203 is indicated by the cyan hexagon.
("BK*") the extracellular end of S4 is, in contrast, bounded by Leu-206. Distinguishing between these two models could provide insight toward the structure of the VSD in BK channels. VSDs in K<sup>+</sup> channels are known to be targets for highly selective peptide components of spider venoms (44–47). Thus insight toward the structure of the BK channel VSD could lead to a better understanding of mechanisms underlying toxin specificity. In addition, these experiments might place structural constraints on the location of voltage-sensing charges in the BK VSD.

**QBBr Fluorescence Can Be Quenched by Tryptophan or Tyrosine Side Chains**—Although it was previously observed that monobromobimane (mBBr) fluorescence is quenched by Trp (13, 26, 27), we used the charged bimane derivative qBBr in our studies to prevent bimane-labeling of cytoplasmic thiols. To determine whether qBBr fluorescence could also be quenched by Trp, we measured fluorescence decays using qBBr-labeled bovine serum albumin in aqueous solution. We tested individual solutions containing Trp, Phe, Tyr, Glu, Lys, and Gly, each at a concentration of 160 mM to yield a theoretical mean distance of ~13 Å from the bimane group.

Fig. 2 illustrates the robust effect of Trp (open circles) on qBBr fluorescence decay. Under the conditions of these experiments, the decay in the absence of added free amino acids was described by a single exponential, as was the decay with Phe ($\tau_{control} = 6.3$ ns; $\tau_{Phe} = 6.6$ ns). Decays observed with 160 mM Glu, Lys, and Gly were also similar to control (not shown). In contrast, the decay with Trp is described by the sum of two exponential components, with a fast time constant of 1.4 ns and fractional amplitude of 0.69 (slow time constant of 7.8 ns). Similarly, the decay with Tyr is described by a fast time constant of 3.1 ns and fractional amplitude of 0.60 (slow time constant of 8.8 ns). Previous work has illustrated that the presence of Trp does not alter the peak bimane emission wavelength, but acts effectively as a collisional quencher, increasing the rate of fluorescence decay, consistent with the present observations (13).

Thus Trp has a pronounced effect on qBBr fluorescence decay, decreasing the lifetime by over 4-fold under these experimental conditions, while the apparent quenching efficiency of Tyr was around half that of Trp, consistent with previous observations using mBBr (27). Finally, Phe, Glu, Lys, and Gly were
essentially ineffective at quenching qBBr fluorescence under the conditions of these experiments.

**Nonspecific Component of qBBr Fluorescence, and Endogenous Cellular Fluorescence**—Because components of observed fluorescence decays in labeled, transfected CHO cells may arise from either qBBr labeling of endogenous surface membrane proteins or endogenous fluorescent molecules in the cell (such as NAD or NADH), we estimated the possible contribution of these components to our measured fluorescence signals. This was done by performing measurements using qBBr-labeled cells transfected with 1) BK channels in which native extracellular cysteines (Cys-14, Cys-141, and Cys-277) were substituted with valine ("-3C"), 2) sham-transfected cells (transfected with pcDNA3 vector with BK cDNA excised), and 3) non-transfected, non-labeled cells.

The representative fluorescence decay traces in Fig. 3A illustrate that the maximal amplitudes of fluorescence signals from -3C-transfected cells with were nearly the same as the maximal amplitudes of fluorescence signals from sham-transfected CHO cells. This suggests that qBBr-labeling of BK channels from which native extracellular cysteines were removed is nominal. However, it also indicates that there are thiols on the CHO cell surface that can be labeled by qBBr. Thus it is critical that the fluorescence signal from qBBr-labeled, single cysteine-substituted BK channels is sufficiently higher than this background fluorescence, to allow one to systematically observe differences in fluorescence due to the position of the qBBr label.

Of the background signal, we further observed a brief lifetime component that is due to endogenous fluorescent molecules in the cell, as indicated by the trace recorded from a non-labeled cell. Such background fluorescence could be due to cytoplasmic or mitochondrial NAD⁺ and NADH, which have excitation and fluorescence spectra that overlap with those of bimane (48). This endogenous cellular fluorescence decays with a single exponential time course with a time constant of 2.0 ns.

In contrast, fluorescence signals from cells transfected with BK channel cDNA were greater than the maximal fluorescence from -3C or sham-transfected CHO cells, as illustrated by the representative fluorescence decay trace from a qBBr-labeled cell expressing the N200C mutant. Fig. 3B further shows the fluorescence decay amplitudes for -3C and each of the qBBr-labeled mutants; based on these observations, we estimate that for each fluorescence measurement, ~30% of the signal could arise from "background" fluorescence components. This is not nominal, and it is therefore important to consider the effect of the background fluorescence on the interpretation of data. Because Trp acts as a collisional quencher of bimane fluorescence, we hypothesize that one might observe effects on the fluorescence decay kinetics (i.e. exponential time constants and relative component amplitudes) as a function of the qBBr label position. Thus, if the decay kinetics of the background fluorescence remain constant (on average), then it should be possible to resolve differences in channel-specific component of the fluo-
Secondary Structure in the Voltage Sensor Domain of BK Channels

We estimated the minimum numbers of exponential components required to describe the fluorescence decays by fitting individual decay curves with sums of exponentials. Consistent with previous observations (26), we found that the bimane fluorescence decays from the labeled cells were generally described by two significant exponential components, although fits from some of the mutants could be described by a single exponential (6/78 cells total).

Fig. 6 shows the mean time constants and fractional amplitudes obtained from the double exponential fits to the each single cell fluorescence decay. These data illustrate the quantitative differences among fluorescence decays for each labeled mutant. While the time constants of the long and brief lifetime components of the fluorescence decays did not vary significantly with the position of the labeled cysteine, the fractional amplitudes of the components did vary with position. In general, fractional amplitudes of the long-lifetime (slow) component (4) were decreased at positions that were proximal to Trp-203 in the linear sequence, with the exception of L206C, which showed a smaller slow component amplitude than G205C. This could be explained by secondary structure in this region, to be discussed below.

Quenching of qBBr Fluorescence Is Due in Part to Trp-203—Our results so far are consistent with quenching of bimane fluorescence in a subset of the labeled cysteine residues decay kinetics, which should typically account for \( \sim 70\% \) of the fluorescence signal. The experiments below aim to resolve observed differences and use them to distinguish among plausible structures for the S3-S4 region of the BK channel.

Cell Surface Expression of Cysteine-substituted BK Channels—We generated a series of single cysteine-substituted BK channel mutants to be probed with qBBr (residues Leu-199 through Leu-206; Fig. 1); native extracellular cysteines in these mutant channels were substituted with valine. Because qBBr fluorescence is quenched by Trp, we aimed to exploit the presence of a native tryptophan, Trp-203, as a potential landmark for use in estimating relative intermolecular distances.

Surface expression of mutant channel constructs was confirmed through membrane protein biotinylation. Substitution of native extracellular cysteines with valine yielded robust surface expression of BK channels ("−3C," Fig. 4A). These results were consistent with robust currents observed in recordings from excised patches of −3C BK channels, which were comparable in amplitude with wild-type BK currents (Fig. 4B). Further substitutions at positions on the S3-S4 linker did not significantly disrupt surface expression, as indicated by surface biotinylations (Fig. 4, C and D); these results are consistent with previous observations that mutations in this region do not disrupt functional assembly of the channels at the plasma membrane (14). While not quantitative, these results demonstrate overall that each of the constructs form channels that reach the plasma membrane and that the mutations are not likely to place a severe limitation on surface expression.

Bimane Fluorescence Shows Apparent Position-specific Quenching in Cysteine-substituted BK Channels—Bimane fluorescence decays were recorded from individual qBBr-labeled, transfected cells, and decay lifetimes were estimated using exponential fitting. We observed bimane fluorescence in each of the labeled mutants generated for these studies (from L199C to L206C). Fig. 5 shows normalized, averaged fluorescence decays obtained from the site-specifically labeled BK channels.

We estimated the minimum
mutants by the nearby Trp-203. If this were a viable mechanism, then elimination of this Trp by substitution to Phe should abolish quenching in the mutants that showed a greater fraction of brief lifetime component in qBBr fluorescence. We tested this by measuring the bimane fluorescence decays from cysteine-substituted channels with the additional mutation W203F (Fig. 7).

Fluorescence decays from labeled cells expressing the W203F-containing cysteine mutants were generally best described by two exponential components. Fig. 8, which shows the mean time constants and fractional amplitudes obtained from the double exponential fits (compared with the kinetic parameters from Trp-203-containing channels, replotted from Fig. 6), illustrates that the fraction of brief-lifetime component was significantly reduced by the W203F mutation in the mutants R201C, S202C, L204C, and L206C. These results suggest that in these mutants, quenching of qBBr fluorescence is due at least in part to Trp-203.

Mechanism of Bimane Quenching at Selected Positions in the S3–S4 Linker—We observed with exponential fitting that the bimane fluorescence decay time constants did not vary much as a function of label position, but in bimane-labeled cells expressing R201C, S202C, L204C, and L206C, bimane fluorescence was effectively quenched due to decrease in the long lifetime component and an increase in the brief lifetime component of fluorescence. The observation of two components is consistent with a minimum of two excited states of bimane in the labeled cells, with the long lifetime component corresponding to excited/unquenched bimane and the brief lifetime component corresponding to excited/quenched bimane. In addition, part of the brief-lifetime component is due to a relatively constant level of endogenous cellular fluorescence (Fig. 3A). The observation of a two-component fluorescence decay is consistent with previous measurements of site-specific bimane fluorescence decay in labeled lysozyme (26).

Our results with the W203F mutation (Fig. 7) hold Trp-203 at least partially accountable for the quenching of bimane fluorescence in our labeled mutants. However, each of the W203F mutants displays a level of apparent quenching that is intermediate between the maximal and minimal levels of apparent quenching we observed in the absence of the W203F mutation. Therefore, we must consider the possibility of additional quenching groups.

The S0 segment contains two Trp residues near its extracellular end (Trp-22 and Trp-23), which may be located near the extracellular border of the lipid bilayer. The extracellular half of S0 con-
contains residues that may interact with the other transmembrane segments in the channel to modulate voltage sensor movement (31); thus it is plausible that either (or both) of these Trp side chains may be close enough to the S3-S4 linker to contribute to quenching in the labeled double mutants. This hypothesis is consistent with cross-linking studies demonstrating that the residues extracellular to S0 are close enough to the S3-S4 linker to form disulfide bonds (50) (expression of channels containing substitutions at both Trp-22 and Trp-23 was too low to enable testing of this using bimane-quenching). Alternatively, a Tyr side chain (Tyr-198) is located on the apparent extracellular end of S3, close to the labeled positions, and thus could also contribute to quenching.

FIGURE 9. Working hypothesis for secondary structure near the S3-S4 linker in BK channels. A, side chains from the KvAP S3-S4 linker were substituted with BK channel side chains using the PYMOL mutagenesis tool according to alignment “BK” in Fig. 1, consistent with fluorescence quenching data. B, rear view of the structure shown in A. C–F, correlations between $A_{\text{slow}}$ and $C_\gamma-C_\gamma$ distances between Trp-203 and indicated side chains in four different candidate models: C, model “BK” (shown in Fig. 9, A and B); D, model BK* (Fig. 1B); E, a straight $\alpha$-helix; and F, a parallel $\beta$-strand.
While other quenching groups may contribute, the identification of Trp-203 as an endogenous quenching group enables the interpretation of the bimane fluorescence data in the context of several candidate models, considered below.

Working Hypothesis for the Structure of the S3–S4 Linker of the BK Channel—Although fluorescence data of this type cannot, on their own, define a unique three-dimensional structure, they do provide constraints that can be used to distinguish among plausible candidate models. Because of the apparent length conservation between KvAP and BK channels in the S3–S4 region (Fig. 1), we used a candidate model based on the KvAP VSD (39, 41) as a working hypothesis to predict possible distances corresponding to the fluorescence data presented in Figs. 5 and 6, and made additional comparisons using predictions of model secondary structures. Quantitative predictions of the model in Fig. 9, A and B are shown in Fig. 9C, which plots distances between $C_{\beta}$ of Trp-203 and the $C_{\beta}$s of each side chain in the model, scaled, and superimposed on the fractional amplitudes determined by exponential fitting of qBBr decays. Predictions for the model described by the aligned sequence BK* (Fig. 1), as well as an $\alpha$-helix and a parallel $\beta$-strand are presented in Fig. 9, D–F, respectively.

The plot in Fig. 9C illustrates a strong correlation between the fluorescence data and distances in the model shown in Fig. 9, A and B, and suggests that the model accounts well for the position-dependence of the fluorescence decay kinetics with the assumption that Trp-203 is the primary qBBr quencher. With respect to the S4 helix, L204C showed robust quenching, consistent with this residue lying on the opposite face of an $\alpha$-helix (at the i + 2 position), distant from Trp-203. Quenching is again robust with L206C, consistent with this position lying on the same face of the helix as Trp-203 (at the i + 3 position), one helical turn away.

On the other hand, each of the model predictions in Fig. 9, D–F show clear discrepancies with the fluorescence data. Fig. 9D compares the data with the prediction of model with Trp-203 in the middle of a short turn region (BK*, Fig. 1B). The plot illustrates that in a non-helical region, residues near Trp-203 are predicted to be quenched with a nearly monotonic relation to proximity in the linear sequence, while a phasic relation emerges in the helical region (residues Leu-199 to Arg-201). The phasic relation that is observed within an $\alpha$-helix is more clearly illustrated by the predicted distances in Fig. 9E. Here we can observe that data from the more C-terminal residues are consistent with an $\alpha$-helix, while the more N-terminal residues are not. In contrast, data from the more N-terminal residues are potentially consistent with the predicted distances of a $\beta$-strand (Fig. 9F), while the data for G205 and L206 is not. Together, these comparisons support the model presented in Fig. 9, A and B for this stretch of residues addressed by these studies, and provide evidence that Trp-203 may represent the N-terminal end of the S4 helix in the BK channel.

In the context of Fig. 9, the range of distances that can be resolved using bimane quenching, on the order of 5–10 Å, supports previous studies illustrating that bimane is a sensitive reporter of protein conformation (13, 26, 28). Further experiments using fluorescence lifetime methods may provide additional structural constraints in this region of the channel.

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