The KtrAB ion transporter is a complex of two proteins, KtrB and KtrA. The integral membrane protein KtrB is expected to adopt the structural architecture typified by the pore domain of potassium channels. Here we show that homo-dimerization of KtrB proteins is most likely a general property of this family of transporters. Using cysteine mutants and bifunctional cross-linkers we define regions of the Bacillus subtilis KtrB molecule that are close to the molecular 2-fold axis and to the dimer interface. Fitting of the cross-linking data to a potassium channel-like model suggests structural similarities between potassium channels and KtrB proteins in the extracellular half of the molecule and differences in the cytoplasmic regions.

Regulation of intracellular concentrations of sodium and potassium ions is a characteristic of all living organisms and is achieved by a battery of macromolecules that include ion transporter membrane proteins. The KtrAB transporter family plays a role in these processes by mediating Na⁺/H⁺ symport activity. At the genetic level this transporter is usually arranged as a two gene operon encoding the KtrB membrane protein and the cytoplasmic KtrA protein. KtrB proteins are 400–600 residues long and predicted to have 8 transmembrane helices (TMs) (1–3). KtrA proteins are ~220 amino acids long and contain RCK domains that form octameric rings and bind adenine-ribose-phosphate-containing compounds (4, 5). RCK domains are commonly found associated with membrane proteins, including potassium channels, and function as activity regulators.

The KtrAB transporters are part of a large superfamily of ion transport membrane proteins that also includes the plant HKT, members of the superfamily, including KtrAB, are thought to be either Na⁺/K⁺ or H⁺/K⁺ symporters (1, 3, 6). Sequence homology and hydrophobicity profiles have led to the proposal that all membrane proteins in this superfamily adopt the architecture of the ion pore of potassium channels (1, 2). One KtrB polypeptide is thought to contain four repeats of the TM-Loop-TM structural motif observed in potassium channels, such as KcsA (7, 8). The repeats, labeled A to D in Fig. 1A, do not have the same sequence, except for a few conserved positions (Fig. 1B), and are connected by relatively large cytoplasmic loops (15–36 residues), which show no similarity to one another. The experimentally determined transmembrane topology of HKT1 from Arabidopsis thaliana revealed 8 TMs that agree well with a KcsA-like architecture (9). Moreover, members of the superfamily tend to have one or more conserved glycine residues in the regions equivalent to the potassium channel selectivity filter sequence GYG (glycine-tyrosine-glycine) (1, 2) (Fig. 1B). Mutation of these conserved glycines in either the KtrB or HKT proteins has been shown to cause changes in the transport properties as expected if these residues form part of the transport pathway (2, 10, 11). Although the evidence described above is compelling, to our knowledge there have been no published biochemical or biophysical studies that directly probe the three-dimensional properties of these proteins, a gap that has limited our understanding of this superfamily.

Recently we provided evidence that the KtrB membrane protein from Bacillus subtilis is a dimer in the membrane and in detergent, that the KtrA regulatory protein is an octamer able to adopt different shapes, and that the transporter complex consists of a KtrB dimer bound to a KtrA octamer (4). In that work, light scattering studies were used to determine the dimeric state of detergent-solubilized KtrB and one amino group cross-linker DSP (dithiobis(succinimidyld propionate)) was utilized to confirm that KtrB was a dimer in the membrane. Here we report substantially expanded cross-linking studies on KtrB with the aim of determining whether the structure is consistent, as has been hypothesized, with the architecture observed in potassium channels. We use several different cross-linkers to confirm the dimer state in B. subtilis and test whether the homo-dimeric organization appears to be a general property of membrane proteins in the KtrAB transporter family. In addition, bifunctional cross-linkers of various lengths are used in conjunction with a series of single-site cysteine mutants to evaluate the relative distances across the dimer interface.

We conclude that the observed cross-linking patterns for the extracellular protein regions fit well with a model of two side-by-side KcsA potassium channel molecules; however, data...
from the cytoplasmic regions reveal structural differences between KtrB and potassium channels.

**EXPERIMENTAL PROCEDURES**

**KtrB Cloning**—KtrB genes from *B. subtilis* (GI:2635594), *Bacillus halodurans* (GI:15613161), and *Streptococcus pneumoniae* (GI:66876889) and KtrA from *B. subtilis* (GI:2635593) were defined by homology to *Vibrio alginolyticus* KtrB and KtrA (3). The genes were synthesized from genomic DNA using PCR and cloned into the pET24d expression vector (kanamycin resistance), which adds a C-terminal His tag. KtrB from *B. subtilis* was also cloned into pET24d with a stop codon before the His tag sequence. C-terminally truncated KtrBs (ending at 435 residue 10 and 15 residue truncations, respectively) were cloned into pET15b (ampicillin resistance) with an N-terminal His tag sequence. C-terminally truncated KtrBs (ending at 435 residue) were defined by homology to *B. subtilis* and the KcsA potassium channel; the loops connecting the KtrB repeats were omitted from the alignment, as were the termini. The transmembrane helices, pore helix, and selectivity filter of KcsA are marked beneath the alignment. Shading indicates positions where side-chain chemical character or volume is conserved.

The protein was concentrated and loaded into a Superdex 200 size-exclusion chromatography column equilibrated with 50 mM Tris, pH 7.5, 120 mM NaCl, 30 mM KCl, 1 mM dodecyl maltoside, 1 mM dithiothreitol.

**Radioactivity Uptake Assay**—1.1 mg of *E. coli* polar lipids (Avanti) solubilized in chloroform were dried under a nitrogen stream, resuspended in pentane, and dried again. The lipids were then resuspended in 110 μl of high potassium buffer (450 mM KCl, 10 mM Hepes, 4 mM N-methylglucamine, pH 7.4) by bath sonication. The detergent CHAPS was added to a final concentration of 34 mM, and the bath was sonicated and left to stand for 2 h for complete solubilization. Protein (5–10 μg) solubilized in dodecyl maltoside was added, and the mixture was incubated for 5 min at room temperature. To form liposomes, the mixture was passed through a spin column with 1.5 ml of G-50 beads swollen in high potassium buffer. Liposomes were subsequently passed through a spin column with 1.5-ml G-50 beads swollen with a low potassium buffer (450 mM KCl, 10 mM Hepes, 4 mM N-methylglucamine, pH 7.4) by bath sonication. The detergent CHAPS was added to a final concentration of 34 mM, and the bath was sonicated and left to stand for 2 h for complete solubilization. Protein (5–10 μg) solubilized in dodecyl maltoside was added, and the mixture was incubated for 5 min at room temperature. To form liposomes, the mixture was passed through a spin column with 1.5 ml of G-50 beads swollen in high potassium buffer. Liposomes were subsequently passed through a spin column with 1.5-ml G-50 beads swollen with a low potassium buffer (450 mM sorbitol, 10 mM Hepes, 4 mM N-methylglucamine, pH 7.4). The assay was initiated by mixing 80 μl of liposome preparation to 100 μl of radioactive tracer (50 μM KCl in low potassium buffer plus $^{86}$Rb$^+$ (at ~2000 counts per microliter)). Time points were taken by loading 70 μl of reaction mixture into a 1.5-ml Dowex cation exchange column, pre-washed with 2 ml of a solution containing 6% sucrose and 5 mg/ml bovine serum albumin, and equilibrated with 2 ml of 6% sucrose solution; finally, the Dowex matrix was washed with 1.5 ml of 6% sucrose solution. The eluate was collected in a scintillation vial and mixed with 15 ml of scintillation fluid. At the end of each experiment, valinomycin (a potassium and rubidium ionophore) was added to the remainder of liposome preparations, and the mixture was incubated for a couple of minutes; 70 μl were then loaded into the Dowex matrix as described above. Normalization of rubidium uptake was done by dividing protein-mediated uptake by total uptake from valinomycin treatment.
Probing the KcsA-like Fold of KtrB

Polyclonal Antibodies—Polyclonal antibodies against B. subtilis KtrB were obtained by injecting rabbits with purified protein. Serum was collected and pre-purified by incubation with an acetone E. coli powder for 30 min at 4 °C; this procedure removes some of the contaminating antibodies that target E. coli proteins. Antibodies were affinity-purified in the presence of detergent on a KtrB-coupled AminoLink Plus agarose gel (Pierce), prepared according to manufacturer instructions.

Membrane Preparations and Cross-linking Experiments—A cell pellet from 100 ml of E. coli culture expressing KtrB was resuspended in buffer and lysed by two passages through an Emulsiflex-C5 cell-cracker (Avestin). Membranes were prepared after a low speed 10-min spin at 10,000 rpm (rotor SS34), 4 °C followed by a high speed spin at 50,000 rpm (rotor Ti-70) for 1.5 h at 4 °C. Pellets were resuspended in 1 ml of buffer, and total protein concentration was determined by a colorimetric assay (Bio-Rad). For non-directed cross-linking experiments the proteins were expressed with a C-terminal His tag. The buffer used for membrane preparation was phosphate-buffered saline, pH 7.5. The suspension was diluted to 0.5 mg/ml total protein concentration, cross-linkers (stock at 25 mM in Me2SO) were added to membrane aliquots, and the mixture was incubated at room temperature for 30 min. The amino group-reactive cross-linkers used were: disuccinimidyl glutarate (DSG), DSP, 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP), and ethyleneglycol bis(succinimidyl sulinate) (EGS) (Pierce). Reactions were stopped by addition of 2% trichloroacetic acid (TCA) to a final concentration of 100 mM and incubated for 15 min. Samples were run in a SDS-PAGE, blotted onto polyvinylidene difluoride membrane, and probed with an anti-His tag monoclonal antibody (Qiagen).

For directed cross-linking experiments KtrB cysteine mutants were expressed without a His tag. The buffer used in these experiments was 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA. Membrane preparations were resuspended at 2 mg/ml total protein concentration. Methanethiosulfonate (MTS) bifunctional cross-linkers: 1,3-propanediyl bismethanethiosulfonate (M3M), 1,5-pentanediyl bismethanethiosulfonate (M5M), and 3,6,9,12,15-pentaaoxahexadecane-1,17-diyl bis(succinimidylsulfinate) (EGS) (Pierce). Reactions were stopped by addition of Tris buffer solution (pH 7.5) to a final concentration of 100 mM and incubated for 15 min. Samples were run in a SDS-PAGE, blotted onto polyvinylidene difluoride membrane, and probed with an anti-His tag monoclonal antibody (Qiagen).

RESULTS

Dimerization Is a General Property of KtrB Membrane Proteins—The predicted solvent-exposed loops of the B. subtilis KtrB membrane protein have a total of 17 lysine residues (14 cytoplasmic and 3 extracellular), all of which are potential reacting sites for amino-group cross-linkers. Amino group cross-linkers have been successfully applied in the determination of the oligomeric state of membrane proteins such as the trimeric glutamate transporters and copper uptake transporters (17, 19). In these cases where proteins exist in higher oligomeric states, increasing concentrations of the cross-linking reagent resulted in the unambiguous emergence of a strong final-state (trimer) band well before the monomer band disappeared. If any weak transient dimer band was observed, it was only in the presence of a substantial trimer band and faded before the monomer band disappeared. We have previously shown (4) that addition of the bifunctional amino-group cross-linker DSP (spacer arm, 12 Å) to E. coli membranes containing the B. subtilis KtrB membrane protein results in the appearance of a band on a Western blot that corresponds to a KtrB dimer.
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We now demonstrate that other bifunctional amino-group cross-linkers with different spanning lengths and chemical properties: disuccinimidyl glutarate (spacer arm, 7.7 Å), 3,3′-dithiobis(sulfosuccinimidyl propionate) (spacer arm, 12 Å), and ethyleneglycol bis(succinimidylsuccinate) (spacer arm, 16 Å), have the same effect. Western blots (Fig. 2A) show that increasing concentrations of these compounds result in a reduction in the intensity of a band migrating between 30 and 40 kDa, consistent with a KtrB monomer (expected molecular mass, 49 kDa), and a corresponding increase of a band that migrates between 60 and 80 kDa, equivalent to a dimer. Importantly, even though there are numerous reactive sites on each KtrB molecule, no other discrete bands are detected that may correspond to higher order oligomers. A further increase cross-linker concentration only results in a rise of nonspecific links with a variety of other proteins in the membrane.

The KtrB protein was overexpressed in E. coli, extracted with dodecyl maltoside and purified by affinity chromatography followed by size-exclusion chromatography, where it migrates as a single peak (Fig. 2B). We have recently shown by light scattering coupled to refractive index and UV absorbance measurements that the protein in this peak forms a homo-dimer (4), corroborating the cross-linking results. Our previous observations that solubilized KtrB is able to specifically bind KtrA (4) demonstrates that the presence of detergent does not cause a loss of the protein native properties.

To determine whether the purified protein retains its functional properties, we reconstituted purified KtrB into liposomes and performed a radioactivity uptake assay. The assay is equivalent to the one used in the characterization of the KcsA potassium channel; briefly, proteoliposomes loaded with 450 mM potassium are exchanged into a solution with a low potassium concentration (50 μM). If the protein is a potassium transporter, it will mediate the flow of K⁺ ions, but not their counterions, down the concentration gradient, thereby establishing a difference potential across the membrane. When ⁸⁶Rb⁺, a good substitute for K⁺, is added to the external solution, it is driven into the proteoliposomes by the electrical gradient. Fig. 2C shows that uptake of rubidium is protein concentration-dependent. A time course (Fig. 2D) shows that uptake by KtrB is slow and that even after 20-min saturation has not been reached. These data were fitted with a double exponential with time constants of ~2 min (amplitude, 0.03) and ~120 min (amplitude, 0.45). We believe that the apparent initial burst of activity (corresponding to the short time constant exponential) is a systematic feature of the assay and that the activity of KtrB is expressed in the slow rising phase of the data. In equivalent experiments with the KcsA potassium channel, performed by Heginbotham and colleagues (20), saturation is attained within ~3 min, and uptake is fitted with single exponential with a time constant of ~30 s. Our experiments show that KtrB can behave like a channel; by this we mean that, albeit not very efficiently (slow uptake), K⁺ and Rb⁺ flow through the protein driven solely by their electrochemical gradients. This agrees well with the report by Bakker and colleagues (11), which showed in vivo that the KtrB protein alone (without KtrA) can transport potassium ions independently of sodium. Importantly these functional results demonstrate that detergent solubilization of KtrB preserves the protein's basic functional properties.

To verify that other KtrB proteins are also organized as dimers, we repeated the cross-linking experiments with KtrB proteins from B. halodurans and S. pneumoniae. These membrane proteins were expressed in E. coli, and membrane preparations were exposed to the bifunctional amino group cross-linker DSP. After the reaction, just like with B. subtilis KtrB, a
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**FIGURE 3. Experimental setup.** A, schematic depicting the use of bifunctional cross-linkers in our experiments with the two polypeptide chains of a KtrB dimer represented by continuous and dashed lines; single cysteine mutations are represented by the circles on the lines. B, KtrB schematic roughly showing the positions of cysteine mutations. The star marks the position of the C407A mutation. TM s are labeled according to repeat (A–D) and helix position in repeat (1 or 2).

strong band consistent with a dimer appeared in Western blots (Fig. 2E), suggesting that the dimeric organization is a general feature of all KtrB proteins.

**Probing KtrB with Cysteine-reactive Cross-linkers**—The discovery of the dimeric nature of KtrB opened up the possibility of using biochemical approaches to gain structural insights about the protein. We used cysteine-reactive bifunctional compounds and a series of single cysteine mutants to identify protein regions close to the dimer interface and to investigate the structure of KtrB. As a constraint, we have assumed that the subunits of the homo-dimeric membrane protein are related by a 2-fold axis of symmetry perpendicular to the plane of the bilayer. Introduction of a single cysteine mutation in a cysteine-less KtrB will result in the dyad axis being positioned midway between this cysteine and its counterpart in the other molecule.

Bifunctional MTS reagents of different lengths can be used to estimate the relative separation between the single cysteine mutations in each of the two subunits (Ref. 21, and references within). Under oxidizing conditions the reaction between the MTS compounds and a cysteine is irreversible, and, given enough time, the macromolecule samples many conformations and cross-linking occurs even at positions that seem too far apart. Therefore, distance restraints are usually defined from the rate of formation of the cross-linked protein, or efficiency of cross-link. For practical reasons we have chosen not to measure the rates of dimer cross-linking but to expose each cysteine mutant to a long (M17M, Ca to Ca maximum span of ~31 Å) and a short (M3M, Ca to Ca maximum span of ~13 Å) bifunctional reagent for a fixed amount of time. Reaction with the short compound will occur only at a restricted set of mutant sites (Fig. 3A), identifying positions close to the dyad axis (interface). Conversely, the long reagent will react with most or all mutant positions. Local protein structure will also affect the reaction, so it is important to look for trends across several positions.

We obtained a cysteine-less KtrB by mutating the only natural cysteine (Cys-407) to alanine; in this background we prepared 13 single cysteine mutants in the cytoplasmic and extracellular loops (Fig. 3B) guided by sequence analysis algorithms and the experimentally determined topology of the related protein HKT1 from *A. thaliana* (9). The selected positions are occupied by non-conserved amino acids with polar side chains and are therefore probably non-essential and exposed to the aqueous solution. A mutation that completely wipes out function may have only minimal structural effects and therefore still provide useful information for our purposes. For this reason, we did not test the functional impact of the mutations, but assessed only whether the mutant proteins are still dimeric.

Membrane preparations of each mutant were exposed for 1 h to the reagents M3M and M17M, with a Ca to Ca span of ~13 Å and ~31 Å (22–25), respectively. The reactions were terminated with the addition of NEM and analyzed by Western blot. As a control, the efficiency of the stop reagent was demonstrated by simultaneously adding NEM and M17M to the samples, incubating 1 h then Western blotting (supplemental data). Exposure to the M3M reagent (Fig. 4A) resulted in the formation of strong dimer bands at only a few positions: S159C, N218C, E291C, E335C, and G440C. The apparent weak dimer band at position 252 was not reproduced in other equivalent blots, therefore M3M cross-linking at position 252 was not considered further.

The level of protein expression for the mutants was monitored by Western blot analysis of samples not exposed to cross-linkers (Fig. 4B); in this case samples were incubated as before but without reagents. NEM was added after 1 h. Surprisingly, mutants S159C and G440C each showed intense dimer bands that must result from the spontaneous formation of disulfide bonds at those positions between the two subunits. These dimer bands disappear if the samples are incubated with reducing reagent prior to running the SDS-PAGE (data not shown). The band at N119C was disregarded due to its very weak intensity. This blot also shows that, with the exception of T73C, mutants expressed at similar levels. Despite its relatively low expression, the T73C mutant still cross-links well with the long compounds (Fig. 4C, M17M blot), and results obtained at this position are included in our analysis below.

Exposure to the longest reagent M17M (Fig. 4C) resulted in strong dimer cross-linking for almost all mutants (except for T15C), demonstrating that the large majority of cysteines introduced are accessible and reactive to the MTS reagents. No reaction was observed at T15C, probably because the side chain is not accessible, and we did not consider this position further. As expected, the cysteineless KtrB control shows no dimer cross-linked band after exposure to M17M. There are a large number of higher molecular weight bands in the cysteine mutant lanes relative to the cysteineless protein. Because all of the KtrB variants used here contain just one cysteine residue per monomer then the higher molecular weight bands must result from cross-linking of KtrB with other protein components in the membrane.
To account for the possibility that we are observing interdimer and not intra-dimer cross-linking, we also compared the effects of two different temperatures, room temperature and 0 °C, on the M17M reaction pattern (Fig. 4D). The low temperature was set below a phase transition of the lipid bilayer so that lateral diffusion within the membrane was decreased, resulting in a reduction of collisions between dimers (22, 26). It has been reported that cross-linking easily occurs in the monomeric LacY, giving rise to an apparent dimer. Equivalent temperatures have been reported that cross-linking easily occurs in the monomeric LacY, giving rise to an apparent dimer. Equivalent temperatures have been reported that cross-linking easily occurs in the monomeric LacY, 

**FIGURE 4. Directed cross-linking experiments.** Experiments were performed with single cysteine mutants of _B. subtilis_ KtrB without a tag. All mutants were made in the cysteine-less (C407A) background. Blots were probed with polyclonal antibodies against KtrB protein. For all blots the lane with molecular mass markers (kDa) is labeled MW. A, blot for M3M cross-linker. B, control (no cross-linker). C, blot for M17M cross-linker. Each lane corresponds to a cysteine mutant as labeled. “Cyst” lane corresponds to the cysteine-less KtrB incubated with M17M. D, temperature dependence of M17M cross-linking reaction. Tested mutants are indicated above each pair of lanes. “0” and “rt” labels correspond to reactions performed at 0 °C or room temperature, respectively. “+” and “-” symbols mark the mutants where reaction is temperature-dependent or independent, respectively. “++” and “+-” indicates the large difference between the two reaction conditions at position 403. E, spontaneous disulfide and M3M cross-linking results are summarized on the topology plot of KtrB. “S-S” indicates spontaneously formed disulfide bond. Reaction with the short cross-linker is indicated by “M3M.” “Tdep” marks mutant positions where M17M temperature dependence was observed. F, Western blot of cross-linking by M3M or disulfide bond at the new positions: 125, 164, 225, and 346. The lane labeled 159 is the positive control; M3M cross-link at position 159. G, blot of M17M cross-linking reactions at 0 °C and room temperature. Labels are as in D.

The cross-linking results are summarized in Fig. 4E. All cysteine residues introduced in the short extracellular loops are within a few residues of the nearest TM and therefore serve as positional restraints for the nearby transmembrane segment. In the cytoplasmic loops, with the exception of the mutant in the loop B2-C1 (N218C), cysteine mutants were introduced in the center of the loops. Based on the observed cross-linking patterns, we propose that the regions close to the molecular dyad axis, positioned at or near the dimer interface, are as follows: on the extracellular side, the ends of TM-B1 (disulfide bond at the external position S159C) and TM-C2 (M3M cross-link at the external site E291C); on the cytoplasmic side, the end of TM-B2 (M3M cross-link at N218C), the loop between TM-C2 and TM-D1 (M3M cross-link at E335C) and the C terminus (disulfide bond at G440C). Regions determined to be close to the periphery of the molecule, as determined from the temperature where almost no dimer band is present at 0 °C, indicating that only inter-dimer cross-linking is occurring. Smaller temperature effects were observed at positions 43, 73, 190, and 252, where at 0 °C dimer bands are slightly weaker and the monomer band is slightly stronger than at room temperature. It cannot be ruled out that temperature has an impact either on the rate of the chemical reactions or on the flexibility of the protein and therefore on the efficiency of cross-linking. However, if either one of these effects played a significant role in the temperature dependence of the cross-links, we would expect to observe a decrease in the intensity of the dimer bands relative to the monomer bands for most of the mutants at the lower temperature. Because this is not the case, we favor the explanation that the weakly temperature-dependent dimer bands result from a mix of both inter- and intra-dimer cross-linking. Based on the relative intensities of the dimer bands at the two different temperatures, we estimate that roughly half of the observed cross-linking at these positions is intra-molecular. These temperature dependence experiments also aid in the analysis of observed cross-linking by identifying regions sensitive to dimer collisions and therefore probably close to the periphery of the dimer. In addition, the presence of dimer bands at 0 °C for almost all mutant positions (Cys-430 is the exception) demonstrates that the mutations do not drastically alter the dimeric nature of KtrB.

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dependence of M17M cross-linking (Fig. 4D), are also marked in Fig. 4E. Strikingly, none of the cytoplasmic positions tested were temperature-dependent.

To increase the number of restraints for the ends of TM segments in the cytoplasmic regions we generated four more mutants (G125C just before TM-B1, S225C before TM-C1, P346C just before TM-D1, and T430C just after TM-D2) and performed M3M and M17M exposures, as well as spontaneous cross-linking. In the last TM-Ploop-TM repeat, both Cys-346 and Cys-430 formed M3M-mediated cross-links, with Cys-346 proving more efficient at forming a disulfide bond. The M17M blot demonstrates that the cross-links at Cys-346 and Cys-430 are not temperature-dependent and, therefore, occur within the dimer molecule (Fig. 4G). The absence of cross-linking at cytoplasmic positions Cys-125 and Cys-225 is clarified by a lack of reactivity even after exposure to M17M, indicating that these positions are not accessible to the reagents. The same was found for a new mutation made on the extracellular side of the molecule, A164C, located just after Cys-159 and before the pore helix of repeat B (Fig. 4, F and G).

Properties of the C Terminus—We have interpreted the spontaneous formation of direct disulfide bonds, or of cross-links using the short M3M molecule, as identifying regions that are close to the molecular dyad axis. Because these residues are presumably located at or near the dimer interface, sequence perturbations in these regions could possibly alter the oligomeric state of the membrane protein and impact the ability of the transporter to function.

To test this interpretation we created several mutations in the C-terminal end of KtrB, a region that contains one of the sites of spontaneous disulfide bond formation, at G440C, just five residues before the end of the protein. This region displays a substantial degree of sequence conservation (Fig. 5A). A truncation mutant (residues 1–435) of KtrB with the last 10 residues removed yields two peaks in a size-exclusion chromatography elution profile (Fig. 5B), clearly different from the single peak profile of the wild-type protein (residues 1–445) (Fig. 2B).

Three-detector light scattering analysis reveals the first peak to be a dimer (105.1-kDa molecular mass) like that observed for the wild-type protein, whereas the new second peak is a monomer (47.9 kDa). The expected molecular mass is 48.6 kDa. A similar result was also obtained using a mutant with the last 15 residues truncated (data not shown). Hence, removal of the final 2% of the protein sequence, a region able to form spontaneous disulfide bonds with its counterpart in the dimer and presumably located near the interface, results in significant disruption of the dimer. These data demonstrate that the C-terminal end of KtrB is important for dimer stability, probably by establishing contacts across the interface, and reinforce our molecular interpretation of the cross-linking data in general.

To investigate the functional importance of the C-terminal tail of KtrB we tested deletion mutants, point mutants, and wild-type KtrB using an in vivo complementation assay. We made use of the TK2420 E. coli strain in which a series of proteins involved in potassium ion uptake have been disabled and therefore cells require additional K+ in the medium for optimal growth. The presence of a functional KtrAB transporter aids K+ uptake and allows these cells to grow in low K+ medium.

Results from the complementation assay are presented in Fig. 5C. Others have shown that complementation is observed only when KtrB and KtrA are expressed together in cells (2, 3). Under the conditions of this assay, co-expression of wild-type KtrA and wild-type KtrB allowed the cells to achieve saturated growth in the presence of 1 mM K+ (Fig. 5C). As a negative control, cells containing no KtrAB transporter show no detectable growth at 10 mM K+ or below, only slight growth at 30 mM K+, but hearty growth indistinguishable from cells containing wild-type KtrAB at 115 mM K+. Cells co-expressing either of the C-tail deletion mutants of KtrB (missing the last 10 or 15
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To further test the dimer model shown in Fig. 6 (B and C), we exposed single cysteine mutants of KtrB to M5M, a bifuncational cysteine cross-linker that is slightly longer (Cα to Cα maximum span, ~16 Å) than M3M. If the KcsA-based model is correct, the extracellular cross-linking pattern would now be expected to expand outward to include transmembrane helices located further away from the dyad axis, such as TM-B2, C1, D1, or A2. Exposure to M5M was performed with all mutants except for 159C and 440C, which spontaneously form a disulfide bond. As with M3M, cross-linking was detected at mutant positions Cys-218, Cys-291, Cys-335, Cys-346, and Cys-430 (Fig. 6D). As anticipated, the reaction pattern now expands to the extracellular ends of TM-C1 and TM-D1 with new dimer bands detected at positions Cys-252 and Cys-374, respectively. We have shown that Cys-374 is not temperature-dependent (Fig. 4D), indicating that the M5M band at this position corresponds to intra-
FIGURE 6. Modeling the data. A, schematic representation of some of the possible arrangements of repeats within a single KtrB monomer as viewed from the extracellular side. Each circle represents an individual TM (transmembrane helix) labeled as in Figs. 3B and 4E. Each TM-Ploop-TM repeat (A–D) is shown as a pair of circles of the same color. The four possible arrangements (I to IV) in which the B and C repeats are adjacent within a KtrB monomer are shown. Two other possible arrangements (where repeats B and C are in opposite corners) are not shown. B, extracellular view of dimer model used to analyze KtrB cross-linking data. Two KcsA channels placed side by side and related by 2-fold symmetry serve as the structural scaffolding onto which KtrB cross-linking data is mapped. TMs are labeled as in Figs. 3B and 4E. TM-Ploop-TM repeats within each monomer are colored and arranged as in disposition III of Fig. 6A. Unlabeled short "pore" helices are positioned between the labeled TM helices of each repeat. Solid lines connecting select helices are imaginary representations of loop regions. Thick-dashed lines connect the cysteines (filled circles) found to form a disulfide bond; finely dashed lines connect regions cross-linked by M3M. Open circles correspond to the extra positions defined by M5M cross-linker. "Tdep" marks positions at which cross-linking shows some temperature dependence. C, same dimer model as in B but viewed from the cytoplasmic side. Labeling is the same as in Fig. 6B except that open-dashed circles positioned at ends of TM-D1 and TM-D2 correspond to disulfide and M3M cross-links established between Cys-346 and Cys-430, respectively. D, blot of M5M cross-linking reactions with mutants indicated. M3M reaction with Cys-159 is included as a positive control. MW, molecular mass markers lane (kDa). E, schematic representation of all cross-linking data showing residue positions and type of cross-links observed (S-S is a disulfide bond; M3M and M5M are cross-links defined by those compounds).

dimer cross-linking. Cross-linking at Cys-252 was shown to be weakly temperature-dependent, probably resulting from a mix of inter- and intra-dimer reactions. If, as previously discussed, half of the intensity of a dimer band formed at Cys-252 is considered to result from intra-dimer cross-linking, then this position can be considered for our model. A much weaker band was also observed at Cys-403, a position shown to be highly temperature-dependent, indicating a location on the periphery of the KtrB dimer involved in inter-dimer reac-
ture seen in the pore domain of potassium channels. It implies that two ion transport pathways exist side by side and raises the question of whether the pores are structurally independent, that is, formed by one polypeptide chain wrapped around a central pore axis or created out of different regions of two polypeptides. Our C-terminal tail truncation data shed some light on this question. The observation that the removal of as little as 10 residues from the C terminus of KtrB causes the dimer to break down into stable monomers suggests that there

DISCUSSION

In a previous study (4) focused on the structural and functional properties of the KtrA regulatory protein, we established that the KtrAB complex is composed of a KtrA octamer and a KtrB dimer. Here we have explored the structural properties of the KtrB protein, provided additional data supporting the dimeric organization of B. subtilis KtrB, and show that homodimerization is probably a general property of the KtrB membrane protein family. We have identified several regions of KtrB that react as if close to the molecular dyad axis and have bolstered our interpretation of the cross-linking data by demonstrating that removal of one of these regions promotes the breakdown of the dimer into monomers. In light of the hypothesis that KtrB may be similar in structure to potassium channels, we have mapped the cross-linking data onto a model consisting of two side-by-side KcsA channels. Interestingly, the conserved nature of the KtrB dimer organization does not seem to be reflected by sequence conservation along peripheral transmembrane regions (data not shown).

The dimeric organization of these proteins is intriguing in light of the proposal that they adopt the archi-

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is no extensive domain swapping. We believe that our data are best explained by two side-by-side KtrB monomers. Whether the two ion pores are functionally independent is unknown.

The revelation that KtrB forms homo-dimers has provided us with the opportunity to probe the structure of the protein in a simple fashion, using cysteine mutagenesis and directed cross-linking reactions. With this approach we have established that the regions close to the molecular dyad axis and therefore probably close to or participating in the dimer interface include the extracellular ends of TM-B1, TM-C2, the cytoplasmic regions of TM-B2, TM-D1, and TM-D2 as well as the loop C2-D1 and the C-terminal tail (Fig. 6E). The cross-linking results have also allowed us to probe the proposed structural relationship between the KtrB transporter and the KcsA potassium channel. The KcsA-based dimer model presented in Fig. 6B explains well the cross-links (disulfide bonds or short M3-M3-mediated links) observed in the extracellular half of KtrB by placing the ends of TM-B1 and TM-C2 near the 2-fold axis. The use of M5M, an MTS reagent slightly longer than M3M, expands the interaction radius in a manner consistent with this interface. The temperature-dependent nature of cross-linking observed at some positions is also explained by this model, because these regions lie on the extracellular periphery of the dimer where collisions between KtrB dimers are more likely to occur.

Our cross-linking data support the view that KcsA serves as a good model for the extracellular half of KtrB but that adjustments are required on the cytoplasmic half of the model. This is not surprising, because even among potassium channels the structural core that includes the selectivity filter is located on the extracellular side and tends to remain predominantly unchanged, whereas the cytoplasmic half can undergo major conformational adjustments during gating and is therefore structurally more adaptable.

Either of the following possible adjustments to the model could remedy the apparent too-long cross-links of the cytoplasmic region. First, the axes of the KtrB monomers might be tilted somewhat with respect to the membrane plane, with cytoplasmic loop interactions across the dimer interface drawing the cytoplasmic ends of the subunits closer together than in the present model while leaving the spacing of the extracellular halves unchanged. The resulting ion pathways would no longer be perpendicular to the membrane or parallel to each other. The somewhat inverted-teepee shape of KcsA, wider on the extracellular side than on the cytoplasmic side, could easily accommodate a modest inward rotation, and the effect would be to shorten all of the cytoplasmic cross-link vectors depicted in Fig. 6C. A second possible explanation is that the KtrB sequence asymmetry is manifested in structural asymmetry, especially in the cytoplasmic half. Because KtrB contains one or more glycine residues in the regions where helical bending has been shown to occur during potassium channel gating (27) (Fig. 1A), it is possible that the cytoplasmic ends of some KtrB inner-helices are bent toward the dyad axis. However, the trajectories or bending of the four inner-helices of KtrB do not need to be like one another, making this scenario difficult to model.

In conclusion, the KtrB membrane protein is a homo-dimer and we suggest the extracellular half of its constituent monomers resemble KcsA in structure. By contrast, the cytoplasmic half of the KtrB transporter appears to differ from our simple KcsA-based model and to contain features that are specific to the transporter family.

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