Structures of the MthK RCK Domain and the Effect of Ca\textsuperscript{2+} on Gating Ring Stability*

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MthK is a Ca\textsuperscript{2+}-gated K\textsuperscript{+} channel from Methanobacterium autotrophicum. The crystal structure of the MthK channel in a Ca\textsuperscript{2+}-bound open state was previously determined at 3.3 Å and revealed an octameric gating ring composed of eight intracellular ligand-binding RCK (regulate the conductance of K\textsuperscript{+}) domains. It was suggested that Ca\textsuperscript{2+} binding regulates the gating ring conformation, which in turn leads to the opening and closing of the channel. However, at 3.3 Å resolution, the molecular details of the structure are not well defined, and many of the conclusions drawn from that structure were hypothetical. Here we have presented high resolution structures of the MthK RCK domain with and without Ca\textsuperscript{2+} bound from three different crystals. These structures revealed a dimeric architecture of the RCK domain and allowed us to visualize the Ca\textsuperscript{2+} binding and protein-protein contacts at atomic detail. The dimerization of RCK domains is also conserved in other RCK-regulated K\textsuperscript{+} channels and transporters, suggesting that the RCK dimer serves as a basic unit in the gating ring assembly. A comparison of these dimer structures confirmed that the dimer interface is indeed flexible as suggested previously. However, the conformational change at the flexible interface is of an extent smaller than the previously hypothesized gating ring movement, and a reconstruction of these dimers into octamers by applying protein-protein contacts at the fixed interface did not generate enclosed gating rings. This indicated that there is a high probability that the previously defined fixed interface may not be fixed during channel gating. In addition to the structural studies, we have also carried out biochemical analyses and have shown that near physiological pH, isolated RCK domains form a stable octamer in solution, supporting the notion that the formation of octameric gating ring is a functionally relevant event in MthK gating. Additionally, our stability studies indicated that Ca\textsuperscript{2+} binding stabilizes the RCK domains in this octameric state.

Potassium channels control the flow of K\textsuperscript{+} across the cell membrane and are ubiquitously expressed in nearly all organisms (1). One of the basic properties of K\textsuperscript{+} channels is gating, the opening and closing of the channel in response to external stimuli. These stimuli may be changes in membrane potential (voltage gating) or ligand binding (ligand gating).

Each kind of ligand gives rise to a ligand-gated K\textsuperscript{+} channel with a specific structure for ligand binding. Ligands can be small ions such as Ca\textsuperscript{2+} in high conductance Ca\textsuperscript{2+}-gated K\textsuperscript{+} channels (2), small molecules such as ATP in K\textsubscript{ATP} channels (3), or large proteins such as the G protein βγ subunits in G-protein-coupled inwardly rectifying K\textsuperscript{+} channels (4). Despite the structural diversity required for different ligands, most ligand-binding domains in K\textsuperscript{+} channels are located at the C terminus of the pore, close to the end of the pore-lining inner helix. This common position suggests a general mechanism used to convert the chemical energy of ligand binding to the mechanical work of opening the channel.

The majority of prokaryotic ligand-gated K\textsuperscript{+} channels are regulated by a conserved, C-terminal ligand-binding domain known as the RCK domain. This domain was first characterized based on the crystal structure of the cytoplasmic domain of an Escherichia coli K\textsuperscript{+} channel (5). The structure revealed the RCK domain to be an α/β protein with a structure similar to many dehydrogenase enzymes. The isolated RCK domain forms a homodimer with a bilobed architecture. The lobe of each RCK domain has a Rossmann fold, and dimerization produces a deep cleft between two lobes with potential ligand-binding sites within the cleft. RCK domains are also found in some bacterial K\textsuperscript{+} uptake and efflux machinery such as the Trk system (6–9), in which this domain is referred to as a KTN (K\textsuperscript{+} transport, nucleotide binding) domain (10). Some RCK domains have a conserved sequence motif (GXGXXG . . . (D/E)) for NAD\textsuperscript{+} binding (11), but others do not, reflecting the diversity of ligands for RCK domains. A structure-based sequence alignment and mutagenesis studies have shown that RCK domains also exist at the intracellular C-terminal side of the eukaryotic high conductance Ca\textsuperscript{2+}-gated K\textsuperscript{+} channels (BK or maxiK) (5, 12). The wide distribution of RCK domains in K\textsuperscript{+} channels and transporters indicates their importance in regulating the flow of K\textsuperscript{+} ions across the cell membrane.

The ligand for and physiological function of the E. coli K\textsuperscript{+} channel are still unclear, and the structure of the E. coli RCK domain provides only limited information on its role in regulating channel function. The structure of the MthK channel, another RCK-regulated K\textsuperscript{+} channel, provides some important insights into understanding the RCK-regulated gating mechanism (13). MthK is a tetrameric K\textsuperscript{+} channel from Methanobacterium thermoautotrophicum that can be activated by Ca\textsuperscript{2+}. Each MthK subunit has two membrane-spanning segments that together form the ion conduction pore and an RCK domain at the C terminus. The x-ray structure of the MthK channel in a liganded (Ca\textsuperscript{2+})-bound open state suggested that the functional channel requires eight RCK domains to form an octameric gating ring on the intracellular side of the pore. Four RCK domains are covalently linked to the pore, and four additional RCK domains, co-expressed from the MthK gene using an alternative internal start site (Met-107), are co-assembled in the cytosol. Ca\textsuperscript{2+} binding is thought to cause conformational changes in the gating ring that then lead to the opening of the pore.

Two distinct protein interfaces between the MthK RCK domains hold the octameric gating ring together. Based on a structural compar-
ison between RCK domains from the MthK channel and the E. coli K+ channel, one dimer interface was suggested to be flexible, and the other was suggested to be fixed (13). The protein-protein contacts at the putative fixed interface are formed by three hydrophobic residues on the external face of helix αD. These three residues are either hydrophobic or aromatic among all RCK domains but not among other Rossmann folded proteins. The protein contacts at the putative flexible interface are more extensive, involving a helix-turn-helix (αF-turn-αG) interlock and a C-terminal subdomain that protrudes out from the gating ring.

Although the structure of the open MthK channel provides some crucial insights into RCK-regulated gating in K+ channels, many fundamental questions remain unanswered. Most importantly, the structure of the MthK channel in a ligand-free closed conformation, which could facilitate a much better understanding of the ligand-induced conformational change in RCK domains, is yet to be solved. A simple approach to this problem is to study the structure of the RCK domain alone, with or without bound ligand. In this study, we have presented high resolution structures of the MthK RCK domain determined from three different crystals. One of the structures is in a Ca2+-bound state and was refined to 1.7 Å. The high resolution structure provided a much more accurate model, allowing us to visualize critical protein-protein interactions such as van der Waal contacts, salt bridges, and solvent interactions at atomic detail. It also undoubtedly served as a better guide for structure-based mutagenesis studies. Unlike the RCK domains in the MthK structure, which form an octameric gating ring, the isolated RCK domains form dimers through interactions at the flexible interface, whereas the interactions at the fixed interface are absent in all three crystal structures solved. This prevented us from visualizing the gating ring movement upon Ca2+ binding. However, the structural comparisons among all these dimers along with modeling exercises provided informative insight into the conformational change at the flexible interface and raised the possibility that the fixed interface may not remain fixed during channel gating. Biochemical analyses demonstrated that at pHs above neutral (pH 7.5–8.0), the RCK domain by itself can form a stable octameric gating ring in solution. The octamer is disrupted in these crystals by the low pH crystallization conditions, which may cause a histidine residue (His-193), important for protein contacts at the fixed interface, to become protonated, destabilizing the interface. Denaturation studies of RCK octamers in urea showed that the binding of Ca2+ has a strong effect on the stability of the gating ring.

**EXPERIMENTAL PROCEDURES**

*Protein Purification and Crystallization*—Taking advantage of the fact that the MthK gene expressed in E. coli also produces the soluble RCK domain in excess to the channel, we were able to obtain the purified domain from the soluble fraction of E. coli cell lysates in which the MthK gene was overexpressed. The gene was cloned into a pQE70 vector (Qiagen) with a thrombin cleavage site between the channel and the C-terminal hexahistidine tag. The channel was overexpressed in E. coli SG13009 cultures by induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside at A660 ∼0.8, and cells were harvested and lysed in 50 mM Tris-HCl, pH 8.0, 250 mM NaCl containing leupeptin, pepstatin, aprotonin, and phenylmethylsulfonyl fluoride (Sigma) to inhibit proteases. The supernatant of the cell lysate containing an excess of the RCK domain was purified on a Talon Co2+ column (Clontech) and eluted with 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 300 mM imidazole. Protein eluted from the Co2+ column was incubated for 3 h at room temperature (∼21 °C) in the presence of 1.0 unit of thrombin (Roche Applied Science) per 2.0 mg of protein to remove the hexahistidine tag and further purified on a Superdex 200 (10/30) size-exclusion column (Amersham Biosciences) in 20 mM Tris-HCl, pH 8.0, 250 mM NaCl.

The purified protein was concentrated to about 6 mg/ml using a 10-KDa cutoff Amicon Ultra (Millipore) for crystallization. All crystals were grown by sitting-drop vapor diffusion at 20 °C by mixing equal volumes of protein and reservoir solutions. The crystal of space group R32 was grown over a reservoir solution of 200 mM NH4SO4, 100 mM cacydate, pH 5.5, and 25–30% polyethylene glycol 4000 and has a unit cell of a = b = 164.88 Å, c = 82.852 Å, α = β = 90°, γ = 120° with one subunit per asymmetric unit. The two P21 crystals were both grown over a reservoir solution of 1 M NH4 formate, 100 mM sodium acetate, pH 4.0–4.5, 10% polyethylene glycol 4000. One of the protein solutions used for crystallization contained 20 mM CaCl2. The P21 crystal with Ca2+ has a unit cell of a = 57.979 Å, b = 37.908 Å, c = 96.652 Å, α = γ = 90°, β = 95.486°, and that without Ca2+ has a unit cell of a = 57.900 Å, b = 38.167 Å, c = 96.700 Å, α = γ = 90°, β = 95.946°. Both crystals contain two subunits in an asymmetric unit.

**Structure Determination**—All data were collected at the Argonne National Laboratory Structural Biology Center (19 ID or 19 BM beamlines) at the Advanced Photon Source at −180 °C under a nitrogen stream. The data sets were processed and scaled with HKL2000 (14), and the structures were determined by molecular replacement methods with CNS (15) using the RCK domain from the MthK structure as a search model. The model was corrected using the program O (16), and the refinement was performed using CNS (15) by iterative cycles of simulated annealing and model rebuilding. The model for the R32 crystal form was refined to 2.8 Å with an Rwork of 22.4% and Rfree of 26.2% and contains residues 113–336, two extra amino acids from the thrombin cleavage site, and five water molecules. The model for the P21 crystal with Ca2+ was refined to 1.7 Å with an Rwork of 22.8% and Rfree of 26.8%. It contains residues 116–336 plus three extra amino acids from the thrombin cleavage site in one subunit, residues 116–334 in the other subunit, two Ca2+ ions, and 542 water molecules. The model for the P21 crystal without Ca2+ was refined to 2.1 Å with an Rwork of 21.1% and Rfree of 24.5%. It contains residues 116–336 plus three extra amino acids from the thrombin cleavage site in one subunit, residues 116–335 in the other subunit, and 290 water molecules. The numbering in the models is based on the sequence of the MthK channel.

**Fluorescence and Circular Dichroism Spectroscopy**—The RCK domain was purified in 20 mM Tris-HCl, pH 8.0, and 250 mM NaCl as described above. The urea stock solutions at various concentrations were prepared fresh in the same buffer, and their pH was adjusted to 8.0. The RCK samples were mixed with urea stocks at a ratio of 1:3 (v/v) and left at room temperature for half an hour before subjecting them to fluorescence or CD spectroscopy measurements. CaCl2 was added to the protein samples before mixing with urea. Samples were excited at 280 nm, and the fluorescence emission from 300 to 380 nm was scanned using a PTI model A1010 fluorometer (South Brunswick, NJ). The CD spectra in a wavelength range of 260–200 nm was recorded on an AVIV Model 62DS spectropolarimeter (Lakewood, NJ) using a 1-mm path length cuvette.

**RESULTS**

**Overall Structure of MthK RCK Domain**—All crystals used for the structural analysis were grown in sitting drops by vapor diffusion, and their structures were determined by molecular replacement using the RCK domain from the open MthK channel structure (13). The RCK domains crystallized in two distinct crystal forms. One crystal has an
The MthK RCK domain forms a dimer in all three crystal structures and has a bilobed architecture with a deep cleft between the lobes, where two Ca\(^{2+}\)/H\(_{11001}\) ions can bind (Fig. 1B). Although the R32 crystal contains only one subunit in an asymmetric unit, the molecular dyad of the dimer coincides with one of the crystallographic two-fold axes. Each RCK subunit can be divided into three subdomains (Fig. 1A): the N-terminal subdomain, which forms a Rossmann folded lobe; the intermediate subdomain with a helix-turn-helix (αG-turn-αF) structure that crosses over to the neighboring subunit to form an interlocking dimerization interaction with its counterpart; and the C-terminal subdomain, which forms an extension of the dimer interface and further stabilizes the RCK homodimer. The C-terminal subdomain is less conserved among RCK domains, being absent in some, such as the one from E. coli (5).

The dimer interface between the RCK domains is extremely extensive and buries about 3200 Å\(^2\) of otherwise solvent-accessible area. The interactions within the interface are composed of van der Waals contact between nonpolar surface and hydrogen bonds, involving almost all the residues from the intermediate subdomain and some interfacial residues from the C-terminal subdomain. The extensiveness of this dimer interface and the fact that monomeric RCK is never detected under nondenaturating conditions suggest that the dimerization of RCK...
domain through this interface is conserved in the assembly of the MthK channel.

In addition to the MthK RCK, structures of RCK homologs from other systems are also available. These are the RCK domain from the six-transmembrane E. coli K+/H11001 channel (5) and the KTN domains of the Ktr K+/H11001 import systems from Methanococcus jannaschii and Bacillus subtilis (10). They all form homodimers with the same bilobed architecture and use the same helix crossover motif to hold the two Rossmann folded lobes together with a deep cleft in between (Fig. 1, B–D). However, the dimerization interface of the MthK RCK domain is distinct from that of the E. coli RCK domain. The E. coli RCK does not have the C-terminal subdomain (Fig. 1C), and its dimer interface has a helix-strand-helix (F-H9251-F-H9252-F-H9251) structure. This helix-strand-helix is equivalent to the helix-turn-helix intermediate domain in the MthK RCK but forms a handshake-like motif for dimerization instead of an interlock.

The KTN domain shares high sequence similarity to the MthK RCK despite the fact that the ligand for the KTN domain is NAD/NADH. The KTN structure was solved using a truncated construct in which the C-terminal region right after helix F was removed (Fig. 1D). Considering the sequence similarity, we expect the full-length KTN domain to have a similar structure and the same dimer interface (a helix interlock with the C-terminal subdomain) as the MthK RCK.

**Ca2+ Binding Site**—The high resolution structure from the P21 crystal of the MthK RCK in the presence of Ca2+ allowed us to accurately define the coordination of the Ca2+ ions. The RCK dimer has two Ca2+-binding sites located at the bottom of the cleft between the two Rossmann folded lobes. The two sites are 11 Å apart and are separated by two phenylalanine residues (Phe-232), one from each subunit (Fig. 2A).

Ca2+ binding is coordinated by three acidic residues (Asp-184, Glu-210, and Glu-212) and three water molecules. As shown in Fig. 2B, the Ca2+ ion in each RCK subunit is chelated by seven oxygen atoms as indicated by the dotted lines, four from carboxylate groups of Asp-184, Glu-210, and Glu-212 and three from water molecules (red sphere).

**The RCK Dimer Interface Is Flexible**—The dimer interface of the MthK RCK domain was previously suggested to be flexible based on a structural comparison between RCK domains from the MthK channel and the E. coli K+ channel, two different proteins that are closely related but have distinct differences. With an intact flexible interface in all these MthK RCK dimer structures, we are now able to evaluate the intermolecular conformation across the interface in the same protein in different states.

As the RCK dimers from both the Ca2+-bound and the unbound P21 crystals have almost identical structures and can be superimposed with minimal differences (Fig. 3A), only the Ca2+-bound structure was used in the comparison with the gating ring-forming RCK dimer from the MthK structure. The individual Rossmann folded lobes superimpose...
quite well with each other in these RCK structures, but the relative positions of the two lobes across the dimer interface are different. To visualize this difference, we positioned the gating ring-forming RCK dimer about its molecular dyad (Fig. 3, B and C, vertical arrows) and superimposed the other RCK dimer against the lobe on the left. The P21 RCK dimer adopts a more open conformation with the right lobe swinging clockwise (Fig. 3B). To accommodate such a change, the C-terminal subdomains from both subunits swing clockwise accordingly. The movement is as if the molecular dyad rotates clockwise around the flexible hinge point located at the bottom of the cleft while keeping the left lobe stationary. The R32 RCK dimer, on the other hand, adopts a more closed conformation, and the right side lobe and the C-terminal subdomains have a counterclockwise rotation, leading to a narrower and deeper cleft (Fig. 3C). It is likely that the RCK structure from the R32 crystal is more closely related to a closed conformation.

The observation that two RCK subunits adopt different relative positions across the flexible interface in different crystals clearly demonstrated that this interface is indeed flexible as previously proposed from the MthK structure. In the context of the gating ring, we expect that each RCK subunit will undergo a similar kind of conformational change at the flexible interface during channel gating as we observed in those dimer structures. A similar interface in the KTN domain was also proposed to be flexible from structural studies (10).

It is interesting to note that the two Ca$^{2+}$-binding sites, located within the interdomain cleft, are in the vicinity of the flexible hinge point between βF and αF. The two ligand (NAD/NADH)-binding sites in the KTN dimer, although not the same as the Ca$^{2+}$-binding sites in the
MthK RCK, are also within the cleft and close to the hinge point (Fig. 1D). The common positions of the ligand-binding sites in different RCK dimers indicate a similar conformational change across the flexible interface upon ligand binding.

It is worth noting that the conformational differences between the flexible interfaces of the isolated RCK dimers and the gating ring-forming RCK dimer are not large enough to account for the previously hypothesized gating ring movement. Furthermore, an attempt to reconstruct these dimers into octamers by applying protein-protein contacts at the fixed interface failed to generate the enclosed, four-fold symmetrical gating ring. This suggested that to form a gating ring, the protein contacts at the fixed interface can no longer stay fixed. These observations pointed toward a high probability that the fixed interface is actually not fixed during channel gating. With the movement of the fixed interface, a larger conformational change at the flexible interface is no longer necessary to achieve a substantial gating ring movement.

The RCK Domain Forms a Stable Octameric Gating Ring in Solution—The structure of the MthK channel suggested that a functional MthK channel requires the formation of an octameric gating ring on the intra-cellular side. However, under the conditions used in this study, the RCK domain forms a dimer. To determine the identity of the stable form of the RCK domain in solution, we used size-exclusion chromatography to estimate its oligomeric state (Fig. 4). At a pH above neutral (i.e. pH 7.5–8.0), with or without Ca$^{2+}$, the purified RCK domain elutes at a position equivalent to the size of a 200-kDa protein, consistent with the formation of an octameric complex. At lower pH (i.e., pH ~4.5–5.5), however, the RCK domain elutes as a dimer. This dimer in the low pH solution is likely to be the same as the crystallographic dimers, in which the flexible interface is maintained, whereas the fixed interface, associated with higher-order RCK assembly, is absent. The disruption of the RCK octamer at low pH can be attributed to the protonation of His-193, a residue important for hydrophobic protein contacts at the fixed interface. Mutations at the fixed interface can also lead to the disruption of the octamer at high pH (data not shown), confirming the importance of the fixed interface in octamer formation. The RCK octamer in solution is most likely to have the same gating ring architecture as in the MthK structure, using fixed and flexible interfaces to hold eight subunits together in a ring. The observation that isolated RCK domains form a stable octamer in solution near physiological pH in the presence or absence of ligand indicated that the formation of the gating ring is an independent event and does not require help from the tetramerization of the channel pore. It provided compelling evidence to support the conclusion that the octameric gating ring observed in the MthK structure is not a crystallographic artifact but a functionally relevant assembly, and the ring architecture is maintained during Ca$^{2+}$-regulated channel gating. The effect of pH on gating ring stability also hints to the possibility that both pH and Ca$^{2+}$ are involved in the physiological regulation of the channel.

Ca$^{2+}$ Stabilizes the RCK Octamer—The formation of a stable octameric gating ring by RCK itself at a pH above neutral provides a simple system to study the gating ring conformational change induced by Ca$^{2+}$ binding. While pursuing the structures of the gating ring with and without Ca$^{2+}$ bound, we also studied the effects of Ca$^{2+}$ on the stability of the gating ring by measuring protein fluorescence and circular dichroism spectra in the chemical denaturant urea. For the stability experiments, the measurements were made at pH 8.0, at which RCK exists as a stable octamer.

Each RCK domain contains one tryptophan residue. When excited at 280 nm, the RCK octamer emits strong fluorescence at 340 nm. Denaturation in urea leads to a significant decrease of fluorescence as shown in Fig. 5A. We generated a denaturation curve of the RCK domain by measuring its fluorescence at 340 nm with increasing urea concentrations. As shown in Fig. 5B, Ca$^{2+}$ stabilizes the RCK domain in a concentration-dependent manner, shifting the denaturation curve toward a much higher urea concentration with increasing [Ca$^{2+}$]. It is interesting to note that adding Ca$^{2+}$ leads to about a 15% increase in the fluorescence of the RCK octamer, which may reflect the initial conformational changes associated directly with Ca$^{2+}$ binding.

The secondary structure change of RCK during urea denaturation was monitored by using CD spectroscopy. Shown in Fig. 5, C and D, are the CD spectra of RCK in the absence and presence of 0.5 mM [Ca$^{2+}$], respectively. In the absence of Ca$^{2+}$, the RCK domain is susceptible to unfolding by low concentrations of urea, and its secondary structure content decreases with increasing urea concentrations above 1 M (Fig. 5C). In the presence of 0.5 mM Ca$^{2+}$, we see no obvious secondary structural change at 2 M urea, partial unfolding at 3 M urea, and substantial loss of secondary structure at 4 M urea (Fig. 5D). These results are consistent with the fluorescence study in which, at 0.5 mM [Ca$^{2+}$], the RCK fluorescence remains unchanged up to 2.5 M urea and then undergoes a rapid decrease between 3 and 4 M urea before reaching baseline at about 4 M urea.

There are two general physical processes occurring during the denaturation of the RCK octamer, a disruption of the octameric gating ring into a lower-order protein complex and the unfolding of the RCK domains themselves. Both processes are expected to cause significant changes in fluorescence emission. Both the fluorescence data and the CD data suggested that Ca$^{2+}$ stabilizes the RCK domain in its octameric form, which is more resistant to unfolding. The decrease in fluorescence at high urea concentration was correlated to the loss of secondary struc-
FIGURE 5. Effect of Ca\(^{2+}\) on the stability of the RCK domain. A, fluorescence emission scanning of the RCK octamer with and without urea. B, denaturation curves of RCK at various Ca\(^{2+}\) concentrations measured by its fluorescence emission at 340 nm. C, CD spectra of RCK without Ca\(^{2+}\) at various urea concentrations. D, CD spectra of RCK with 0.5 mM Ca\(^{2+}\) at various urea concentrations. E, profiles of RCK on a size-exclusion column (Superdex 200, 10/30) at various Ca\(^{2+}\) concentrations in a buffer containing 2M urea. F, denaturation curves of RCK in the presence of 0.5 mM Mg\(^{2+}\) and Ba\(^{2+}\).
ture. This suggested that under these experimental conditions, the disruption of the RCK octamer and the unfolding of the RCK domains are most likely coupled.

The conclusion that Ca$^{2+}$ stabilizes RCK as an octamer was also supported by size-exclusion chromatography monitoring the oligomeric state of RCK in urea as a function of [Ca$^{2+}$]. As shown in Fig. 5F, the formation of the RCK octamer in the presence of 2 M urea is [Ca$^{2+}$]-dependent. At high [Ca$^{2+}$], the RCK domains exist predominantly as octamers, whereas at lower [Ca$^{2+}$], the octamers are disrupted, and the RCK domains exist predominantly as dimers. The apparent sensitivity of octamerization to [Ca$^{2+}$] is lower than that seen in the spectroscopic measurements, perhaps due to the dilution effect on the column. However, the size-exclusion chromatography assay clearly demonstrated that Ca$^{2+}$ stabilizes the RCK domain as an octameric gating ring in a concentration-dependent manner.

To test whether other divalent cations have similar stabilization effects as Ca$^{2+}$, we measured the denaturation curve of the RCK octamer in the presence of MgCl$_2$ or BaCl$_2$. As shown in Fig. 5F, at 0.5 mM, whereas Ca$^{2+}$ has strong effect on RCK stability, Mg$^{2+}$ or Ba$^{2+}$ do not have any obvious effect on the spectroscopic properties of the RCK domains. This indicates that the stabilization effect on the RCK domain is Ca$^{2+}$-specific.

**DISCUSSION**

This study has provided three new crystal structures of the MthK RCK domain. Unlike the RCK domain of the open MthK structure, which forms an octameric ring, the isolated RCK domain only forms dimers in the crystallization conditions employed. Similar to the E. coli RCK domain and the KTN domain, the MthK RCK dimer has a bilobed architecture with a deep cleft between the two lobes, where two Ca$^{2+}$ ions can bind. The structure of the Ca$^{2+}$-bound state, refined to 1.7 Å, has provided the molecular details of Ca$^{2+}$ binding and the dimer interactions that hold the two RCK subunits together. A comparison of these structures with the RCK dimer from the open MthK channel indicated that the two lobes across the dimer interface can have multiple relative positions, confirming the flexible nature of this interface as proposed from the MthK structure. The extent of the conformational change at the dimer interface is smaller than the hypothetical gating ring movement proposed from the open MthK structure. These dimers are unable to form an enclosed octameric gating ring by simply applying protein contacts at the previously defined fixed interface, raising the possibility that the putative fixed interface associated with gating ring formation may not be fixed during channel gating.

Two of the RCK domain structures were determined from crystals grown in the same conditions, except that one is in a ligand-bound state due to the presence of Ca$^{2+}$ in the protein solution. The two crystals have the same space group, and the structures show no obvious conformational change caused by Ca$^{2+}$ binding. This may be because at low pH, the RCK domain forms only a dimer utilizing the flexible interface. The restraint exerted on this dimer from the protein contacts at the fixed interface in the context of the octamer are absent, leaving crystal packing as the dominant force in determining the conformation of the RCK dimer.

Biochemical assays demonstrated that Ca$^{2+}$ stabilizes the RCK domain as an octamer, consistent with the RCK complex seen in the MthK gating ring. How does this stabilization effect correlate to the gating ring conformation and channel gating? Without external force, the K$^+$ channel pore is more stable in a closed conformation (19). To open the channel, the gating ring in a Ca$^{2+}$-bound state must adopt an expanded and relatively rigid conformation to exert a lateral force onto the pore through the linkers between the gating ring and the pore lining inner helices, similar to the "passive spring" model proposed for the gating of BK channels (20). This rigid open conformation of the gating ring may have a geometry favoring protein-protein contacts at the fixed interfaces and provide extra stability to the gating ring against denaturation. In the absence of Ca$^{2+}$, the gating ring may adopt a less expanded, more flexible conformation to release the force on the pore. The flexibility of the gating ring without Ca$^{2+}$ may destabilize the dimer interactions at the fixed interfaces and cause the protein to be more susceptible to denaturation. So far, all attempts to crystallize the RCK domain at high pH in the absence of Ca$^{2+}$ (presumably as an octameric gating ring) led to poorly diffracting crystals. This may be related to the flexible nature of the gating ring in the absence of ligand.

Previous functional assays in lipid bilayers showed that the activation of the MthK channel requires [Ca$^{2+}$] in the millimolar range, with the channel open probability ($P_o$) reaching 50% at about 2.9 mM [Ca$^{2+}$].

However, our biochemical assays showed that at a submillimolar [Ca$^{2+}$] (0.5 mM), at which channel $P_o$ is still low, there is a significant effect on the stabilization of the gating ring. Although neither assay is a direct measurement of Ca$^{2+}$ affinity, they suggested that Ca$^{2+}$ has a higher apparent affinity for the isolated gating ring as compared with the gating ring with the transmembrane pore attached. Considering the ligand-gating process, this makes perfect sense. To open the MthK channel, part of the chemical energy from Ca$^{2+}$ binding has to be converted to the mechanical force required to open the pore, which will decrease the apparent affinity of Ca$^{2+}$ for the gating ring.

The formation of an octameric gating ring by RCK domains in a functional channel requires four extra copies of RCK to co-assemble from the cytosol. The MthK channel achieves this by expressing an additional copy of the RCK domain from an internal methionine of the MthK gene. This co-expression phenomenon was also observed in several other prokaryotic RCK-regulated K$^+$ channels such as the E. coli K$^+$ channel (21), MjK1, and MjK2 from M. jannaschii (22, 23). K$^+$ uptake complementation assays of MjK2 (23) and gain-of-function assays of the E. coli K$^+$ channel (21) show that these two channels can still conduct K$^+$ after the expression of the extra RCK domain is abolished by mutating the internal start methionines. However, several observations lead us to believe that the co-expression of the extra RCK domain together with the channel is a functionally relevant phenomenon that plays an important role in the regulation of channel gating. Firstly, the expression of an extra copy of the soluble RCK domain from an internal start site of the channel gene has been seen in several RCK-regulated K$^+$ channels. Secondly, the MthK RCK domain forms an octameric gating ring both in the functional channel and as the isolated domain, indicating that the octameric gating ring is the stable form of RCK domains. Also, the two distinct dimer interfaces that hold the MthK RCK domains in an octameric ring are conserved among all RCK domains. Finally, the expression of wild-type MjK1 produces a complex of channels and soluble RCK domains just like wild-type MthK.

It is also interesting to note that the overexpression of the wild-type E. coli K$^+$ channel or MthK channel in E. coli has no effect on cell growth, whereas overexpression of the mutant channels that have internal start methionines replaced (M107I for MthK or M240I for E. coli K$^+$ channel) significantly inhibits cell growth, indicating the importance of the soluble RCK domain in regulating the channel gating. It is possible that these mutant channels, which lack the co-
assembled RCK domains, become leaky and are no longer as tightly regulated as wild-type channels.

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