C-terminal Movement during Gating in Cyclic Nucleotide-modulated Channels*

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Activation of cyclic nucleotide-modulated channels such as CNG and HCN channels is promoted by ligand-induced conformational changes in their C-terminal regions. The primary intersubunit interface of these C termini includes two salt bridges per subunit, formed between three residues (one positively charged and two negatively charged amino acids) that we term the SB triad. We previously hypothesized that the SB triad is formed in the closed channel and breaks when the channel opens. Here we tested this hypothesis by dynamically manipulating the SB triad in functioning CNGA1 channels. Reversing the charge at positions Arg-431 and Glu-462, two of the SB triad residues, by either mutation or application of charged reagents increased the favorability of channel opening. To determine how a charge reversal mutation in the SB triad structurally affects the channel, we solved the crystal structure of the HCN2 C-terminal region with the equivalent E462R mutation. The backbone structure of this mutant was very similar to that of wild type, but the SB triad was rearranged such that both salt bridges did not always form simultaneously, suggesting a mechanism for the increased ease of opening of the mutant channels. To prevent movement in the SB triad, we tethered two components of the SB triad region together with cysteine-reactive cross-linkers. Preventing normal movement of the SB triad region with short cross-linkers inhibited channel opening, whereas longer cross-linkers did not. These results support our hypothesis that the SB triad forms in the closed channel and indicate that this region expands as the channel opens.

Cyclic nucleotide-modulated channels include two channel families: cyclic nucleotide-gated (CNG) channels and hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels. These two families have disparate physiological roles: CNG channels mediate sensory transduction such as the primary electrical signal in photoreceptors and olfactory sensory neurons, whereas HCN channels act as pacemakers in cardiac cells and neurons as well as control cellular resting potential and membrane resistance (1–3). CNG channels are nonselective cation channels that are very weakly activated by depolarizing membrane voltage and HCN channels are weakly K+-selective cation channels that are activated by hyperpolarizing membrane voltage. The similarities between these two channel families, however, become apparent when examining their functional and structural characteristics. Both CNG and HCN channels are gated by direct binding of cyclic nucleotides to the channel C-terminal cytoplasmic region, which induces a large increase in the probability of opening. Furthermore, these channels both belong to the voltage-gated potassium channel superfamily. CNG and HCN channels are comprised of four subunits, each subunit consisting of six transmembrane segments and intracellular N and C termini. The C terminus consists of a cyclic nucleotide-binding domain (CNBD), the region that connects the CNBD to the sixth transmembrane segment in the pore region (C-linker), and the distal C terminus. The architecture of the CNBD and C-linker regions was revealed by the x-ray crystallographic structures of the C-terminal regions of the HCN2 channel (4) and of another channel in the HCN family, the SpIH channel (5). This C-terminal fragment assembles as a 4-fold symmetric tetramer directly below the transmembrane portion of the channel (Fig. 1). The primary intersubunit interaction occurs between neighboring C-linkers and is comprised of hydrogen bonds, hydrophobic interactions, and salt bridges. In this interface, the first two helices (A' and B') of one subunit form an antiparallel helix-turn-helix motif that interacts with the second two helices (C' and D') of the neighboring subunit. This interacting region has been likened to an “elbow on a shoulder,” in which the “elbow” of one subunit is the A’ and B’ helix-turn-helix motif that rests on the “shoulder,” the C’ and D’ helices of its neighbor (4). There are two salt bridges in this intersubunit interface, formed between one positively charged “elbow” residue in the B’ helix of the C-linker and two negatively charged residues, one “shoulder” residue in the A’ helix of its neighbor.

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‡ The abbreviations used are: CNG, cyclic nucleotide-gated; HCN, hyperpolarization-activated cyclic nucleotide-modulated; MTS-Butyl, (L)-butyl methanethiosulfonate; MTS-1-Butyl, 1,1-methanediyl bismethanethiosulfonate; MTS-3-Butyl, 1,3-propanediyl bismethanethiosulfonate; MTS-6-Butyl, 1,6-hexanediyl bismethanethiosulfonate; NEM, N-ethylmaleimide; DTT, dithiothreitol; r.m.s.d., root mean-square deviation; CNBD, cyclic nucleotide-binding domain; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; SB, salt bridge.

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the D’ helix of the neighboring subunit’s C-linker and one “β roll” residue in the eight-stranded antiparallel β roll of the CNBD (Fig. 1, inset). When this salt bridge triad (SB triad) is disrupted through mutagenesis in either CNG or HCN channels, the favorability of channel opening increases (6). Based on these findings, we hypothesized that the SB triad is formed when the channel is closed and is disrupted when the channel opens.

In this study we tested this SB triad gating movement hypothesis on the CNGA1 channel. Using modification of introduced cysteine residues, we found that manipulating charges in the SB triad of functioning channels dynamically changed the free energy of channel opening. Using x-ray crystallography on HCN channels, we show that changing the charge on the SB triad did not change the global protein configuration, but did disrupt the SB triad and hindered the simultaneous formation of both salt bridges. We then prevented normal CNGA1 SB triad movement by tethering the shoulder to the β roll and found that tethering with short MTS cross-linkers hindered channel opening whereas tethering with longer cross-linkers did not. These results suggest that the SB triad stabilizes the closed state of the channel and then is disrupted and expands as part of the conformational change coupled to channel opening.

EXPERIMENTAL PROCEDURES

Mutagenesis—In this article, we used the cysteine-less version of the bovine CNGA1 channel, CNGA1cys-free (7). All CNGA1 mutant channels were made in the CNGA1cys-free background. Mutant cDNAs were constructed by PCR methods as previously described (8), verified by sequencing the PCR-amplified regions completely, and subcloned into the pGEMHE vector (a gift from E. R. Liman, Ref. 9). cDNAs were linearized, and cRNA was transcribed in vitro using the mMessage mMACHINE kit (Ambion, Austin, TX).

Electrophysiology—Xenopus laevis oocytes were defolliculated and injected with cRNA as described (10). After manual removal of the vitelline membrane, recordings were made in the excised, inside-out patch configuration (11) using an Axopatch 200A patch-clamp amplifier (Axon Instruments) and a RSC-100 rapid solution changer (Biologic) for internal solution application. Data were acquired with PULSE acquisition software (HEKA Elektronik). Patch pipettes were pulled from borosilicate glass and had resistances of 0.25–1 MΩ after fire polishing. The solutions for CNGA1 recordings were as follows: pipette (external) solution: 130 mM NaCl, 3 mM HEPES, 0.2 mM EDTA, pH 7.2, 500 μM niflumic acid; bath (internal) solution: 130 mM NaCl, 3 mM HEPES, 0.2 mM EDTA, pH 7.2, with no cyclic nucleotides or cGMP, cIMP, or cAMP added. CNGA1 mutant channels were made in the CNGA1cys-free vector (a gift from E. R. Liman, Ref. 9). cDNAs were linearized, and cRNA was transcribed in vitro using the mMessage mMachine kit (Ambion, Austin, TX).

Cysteine-reactive Reagents—All cysteine-reactive reagents used are MTS reagents, which react with thiols via a sulphydryl substitution chemical reaction. 100 mM stock solutions of all reagents (MTSET and MTSES were dissolved in water, MTS-Butyl was dissolved in ethanol and MTS-1-MTS, MTS-3-MTS, and MTS-6-MTS were dissolved in dimethyl sulfoxide) were frozen at −80 °C in aliquots, then thawed, and diluted to final concentration just prior to application.

Calculation of L and ΔG Values—For CNGA1 channels, the opening transition for a channel that is fully bound by ligand can be approximated by a simple closed-to-open equilibrium with equilibrium constant L (12–14). The behavior for the different agonists can each be explained by ligand-specific L values shown in Reaction Scheme 1.

\[
\text{REACTION 1}
\]

We determined \( L_{\text{cGMP}} \) for each of the mutant or modified channels by fitting the currents with Equation 1.

\[
L_{\text{cGMP}} = \frac{I_{\text{max}}}{[L_{\text{cGMP}}]} \times \frac{[L_{\text{cNMP}}]}{[L_{\text{cGMP}}]} = \frac{1}{1 + \frac{[L_{\text{cNMP}}]}{[L_{\text{cGMP}}]}}
\]

\( \text{Eq. 1} \)

\( L_{\text{cGMP}} \) and \( I_{\text{max}} \) the current if the maximum open probability was equal to 1, were allowed to vary to minimize the sum of the squared difference between the calculated and the actual currents in NMP at saturating concentrations of cGMP, cIMP, and cAMP for all of these channels, we assumed that the ratio values of \( L_{\text{cAMP}}/L_{\text{cGMP}} = 0.00081 \) and \( L_{\text{cIMP}}/L_{\text{cGMP}} = 0.042 \) were held constant. Similar ratios have been found to apply for a number of CNG channel manipulations outside the ligand binding site (6, 8, 12, 15, 16). \( \Delta G_{\text{cGMP}} \) of the mutant or modified channels was calculated using Equation 2.

\[
\Delta G_{\text{cGMP}} = -RT \ln(L_{\text{cGMP}})
\]

\( \text{Eq. 2} \)

\( \Delta G_{\text{cGMP}} \) values are shown for all of the CNGA1 channels studied here, with and/or without cysteine modification.

CNG C-terminal Gating Movements

**Calculation of L and ΔG Values**—For CNGA1 channels, the opening transition for a channel that is fully bound by ligand can be approximated by a simple closed-to-open equilibrium with equilibrium constant L (12–14). The behavior for the different agonists can each be explained by ligand-specific L values shown in Reaction Scheme 1.

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\( \Delta G_{\text{cGMP}} \) values are shown for all of the CNGA1 channels studied here, with and/or without cysteine modification.

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The soluble fraction was centrifuged two additional times at 14,730 × g for 10 min at 4 °C, the resulting cell suspension was lysed with an Avestin ice-cold buffer composed of 20 mM HEPES, 1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl chloride, and 2.5 μg/ml DNase at pH 7.5. Working at 0–4 °C, the resulting cell suspension was lysed with an Avestin Emulsiflex-C5, and the lysate was cleared by centrifugation in a Ti45 rotor at 40,000 rpm. Imidazole (10 mM) was added to the supernatant, the constructs were purified by Ni-nitrilotriacetic acid chromatography, and the octahistidine tag was removed by cleavage with thrombin. Subsequently, the thrombin reaction was diluted 10-fold with a buffer composed of 20 mM HEPES, 1 mM dithiothreitol, 5 μM cAMP, pH 7. The solution was then loaded onto an S-Sepharose ion exchange column, and the protein was eluted with a linear NaCl gradient. Fractions were pooled and dialyzed against the following crystallization buffer: 20 mM HEPES, 150 mM NaCl, 1 mM dithiothreitol, 5 μM cAMP, pH 7. Following dialysis, the protein solution was supplemented with 5 mM cAMP and protein concentrated to 5–7 mg/ml.

**Crystallization, Structure Determination, and Refinement**—The HCN2I-E502K+cAMP construct was crystallized at 4 °C by the hanging drop, vapor diffusion method. In general, 1 μl of protein solution was mixed with 1 μl of a reservoir solution composed of 200 mM NaCl, 100 mM citrate (pH 4.6), and 15% PEG 400. Crystals grew within a few days. Crystals were cryoprotected in a reservoir solution supplemented with 20% glycerol and cAMP. Intensity data were collected at 110K on beamline X26C (National Synchrotron Light Source, Brookhaven...
National Laboratory in Upton, NY during a RapiData crystallography course) as summarized in Table 1. Integration, scaling, and merging of the diffraction data were carried out with the HKL2000 (21). Scaled intensities were converted to structure factors using the program TRUNCATE (22). The structure was solved by molecular replacement using the program MOLREP (23). The selenomethionine derivative structure of HCN2I (PDB code 1Q43) served as the search model. Five percent of the reflections were used to calculate the $R_{free}$ factor for cross-validation of the refinement process (24). Refinement and model building were performed using the packages Refmac (25), COOT (26), and O (27) to reach a final $R_{work}$ of 19.0% and $R_{free}$ of 21.6%. Water molecules were added gradually using COOT and ARP/wARP (28). Side-chain alternate conformations were added to the model during the final stages of refinement. Additional crystallographic calculations, including LSQMAN and PROCHECK, were employed from the CCP4 suite (29), and the final refinement statistics are presented in Table 2. Refinement and model building were performed using $2F_o - F_c$ and $F_c - F_o$ maps in addition to simulated annealing $F_c - F_o$ omit maps calculated with CNS (30).

RESULTS

CNGA1 channels are activated by direct binding of cyclic nucleotides. Homomeric CNGA1 cys-free channels were expressed in Xenopus oocytes, and currents were recorded using the patch-clamp technique in the inside-out configuration. Fig. 2A (left) shows CNGA1 cys-free currents in response to voltage pulses to $+60$ mV in the presence of saturating concentrations of three different cyclic nucleotides: guanosine 3',5'-cyclic monophosphate (cGMP), inosine 3',5'-cyclic monophosphate (cIMP), and adenosine 3',5'-cyclic monophosphate (cAMP). cGMP produced the most current, and cAMP the least, reflecting the observation that cyclic nucleotides stabilize the closed-to-open equilibrium of CNGA1 channels, and cGMP stabilizes this transition the most while cAMP stabilizes it the least (14). Thus cGMP is a full agonist whereas cIMP and cAMP are partial agonists. We term this differential activation by the three cyclic nucleotides the “fractional activation” of the channel, where cIMP produced 76% $\pm$ 1.5% of cGMP-induced current and cAMP produced 5.8% $\pm$ 0.6%.

Using these fractional activations we can calculate the stability of the closed-to-open transition for each of the cyclic nucleotides as the change in free energy ($\Delta G$) between the closed and open states (14, 16). Previously we have shown that the $\Delta G_{cGMP}$ for CNGA1 cys-free channels is $-2.5$ kcal/mol (6, 16). Assuming that the change in $\Delta G$ is the same for each of the cyclic nucleotides, we can then calculate $\Delta G$ for each manipulation such as an amino acid substitution or modification by a reagent (see “Experimental Procedures”). For example, a manipulation that decreases the fractional activation by cIMP and cAMP would predict an increase in the stability of the open state relative to the closed state and a more negative $\Delta G$ value. The assumption that the change in $\Delta G$ is cyclic nucleotide-independent appears valid for virtually all CNG channel manipulations outside the ligand binding site (6, 8, 12, 15, 16), like the ones studied here. This method for calculating $\Delta G$ has an advantage over using the open probability in that it is not sensitive to closed states, such as inactivated states, that are not part of the activation pathway.

Manipulation of the SB Triad Alters Channel Gating—We hypothesize that the SB triad is formed when the channel is closed and is disrupted upon channel opening. Thus, we predict that permanently breaking the SB triad will lead to a channel with a very favorable opening. Normally the elbow residue Arg-431 interacts with both negatively charged residues (Glu-462 in the shoulder and Asp-502 in the β roll) of the SB triad (Figs. 1 and 2A), but if Arg-431 is mutated to a negatively charged amino acid (R431E), it should repel Glu-462 and Asp-502 and neither of the salt bridges should form. As predicted, this R431E mutation significantly increased the fractional activation by both cIMP (to 98.9% $\pm$ 1.2%) and cAMP (to 94.1% $\pm$ 1.2%), producing a change in $\Delta G$ of $-3.6$ kcal/mol (Fig. 2A, right) (6). The increase in the favorability of CNGA1 channel opening with SB triad disruption was also evident by the leftward shift of the cGMP dose response curve with the R431E channel (Fig. 2B). With the SB triad intact in the CNGA1 cys-free channels, the $K_{50}$ for cGMP ($K_{50cGMP}$) was 87.4 μM $\pm$ 1.2 μM, but for R431E the $K_{50cGMP}$ significantly decreased about 17-fold to 510 nm $\pm$ 45 nm without significantly changing the Hill slope of the dose response curve (Fig. 2B). This shift in $K_{50cGMP}$ is consistent with
a −2.9 kcal/mol change in the free energy of opening ($\Delta G$), similar to the −3.6 kcal/mol predicted from the fractional activation of partial agonists (see “Experimental Procedures”). This result suggests that mutation of the positive charge at position 431 to a negative charge creates a channel whose opening is significantly more favorable than that of CNGA1cys-free channels, probably because the SB triad is disrupted.

To ensure that marked effects in channel behavior produced by mutagenesis, such as those of the R431E mutation, are not due to a global change in protein structure, we wanted to insert and remove negative and positive SB triad charges in real time. To do this, we created a construct with a cysteine at the elbow residue 431 (R431C). As expected, simply neutralizing the charge at position 431 such that the SB triad could no longer opening by changing the charge at a SB triad position other than the elbow residue Arg-431. We previously investigated the effect of charge substitution at the negatively charged residues Glu-462, the shoulder residue on the D helix of the C-linker, and Asp-502, on the $\beta$ roll (6). Reversing the charge at these positions (E462R and D502R) significantly increased the fractional activation and resulted in significantly more negative $\Delta G_{cGMP}$ as compared with R431C channels, mimicking the behavior of channels with a negatively charged amino acid at this position, for example, R431D channels. Finally, removal of both of the MTS reagents by a reducing agent (DTT) reinstated pretreatment R431C behavior (Fig. 4).

As the SB triad is comprised of three residues, we asked if we could affect the favorability of channel opening by changing the charge at a SB triad position other than the elbow residue Arg-431. We previously investigated the effect of charge substitution at the negatively charged residues Glu-462, the shoulder residue on the D helix of the C-linker, and Asp-502, on the $\beta$ roll (6). Reversing the charge at these positions (E462R and D502R) significantly increased the fractional activation and resulted in significantly more negative $\Delta G_{cGMP}$ values. Here we further investigated the effect of charge substitutions on the shoulder residue Glu-462. We made increasingly positive amino acid substitutions at this position, first conservatively mutating Glu-462 to another negatively charged residue, an aspartate. This subtle substitution did not affect channel behavior (Fig. 5). However, neutralizing the charge (E462Q) and reversing the charge (E462R) signifi-
cantly increased the fractional activation and resulted in significantly more negative \( \Delta G_{cGMP} \) values as compared with CNGA1_cys-free channels, just as with position Arg-431 (see Fig. 3). These results indicate that the effect of charge neutralization and substitution is qualitatively the same at Glu-462 and Arg-431: neutralizing the charge resulted in channels that open more favorably than CNGA1_cys-free channels and reversing the charge resulted in even more favorable channel opening. But the magnitude of the change is not the same: the charge reversal at 431 resulted in channels that open much more easily than the charge reversal at 462, as is evident from the \( \Delta G_{cGMP} \) values (Figs. 3 and 5).

Structure of an SB Triad Mutant—Why would reversing the charge at position Arg-431 favor channel opening more than reversing the charge at position Glu-462? To answer this question, we turned to the HCN2 channel for structural information. The HCN2 construct containing the C-linker and CNBD is a stable, monodispersed protein that readily forms crystals suitable for x-ray crystallography (4, 5). Our aim was to solve the crystal structure of the HCN2 C-terminal region for both the elbow and the shoulder charge reversal mutations. While we were unable to obtain crystals for the elbow mutation, the shoulder mutant HCN2-E502K, analogous to E462R in CNGA1, crystallized in the presence of 5 mM cAMP. The co-crystal structure was solved by molecular replacement in the space group C222₁, with data extending to 1.65 Å (Table 1). The asymmetric unit contains four protein molecules, each bound to one molecule of cAMP. The final work and free values for refinement were 19.0% and 21.5%, respectively (Table 2). Overall the structure of HCN2-E502K was very similar to that of wild-type HCN2, with an r.m.s.d. for C superposition of 0.23 Å, as indicated by the overlay in Fig. 6 (left). Like the wild-type HCN2 C-terminal region, E502K forms a 4-fold pseudosymmetric tetramer with the C-linkers positioned between the CNBD and the presumed location of the membrane.

Electron density for the amino acid side-chains of the SB triad residues was well resolved for two of the four subunit interfaces in the asymmetric unit (Fig. 6A). Two different SB triad configurations were seen. In one, both the elbow residue Lys-472 and shoulder residue E502K point toward the roll residue Asp-542, forming two salt bridge interactions via water molecules (Fig. 6A, middle). In this configuration, the wild-type salt bridge between the elbow and the shoulder is replaced by a new salt bridge between the shoulder and the roll (Fig. 6, B and C). In the other configuration, E502K points away from the other two SB triad residues (Fig. 6A, right), and only the salt bridge between the elbow and the roll is present (Fig. 6, B and C). Electron density representing the side-chain atoms of E502K was resolved for only two of four molecules in the asymmetric unit (Fig. 6A). Two different SB triad configurations were seen. In one, both the elbow residue Lys-472 and shoulder residue E502K point toward the roll residue Asp-542, forming two salt bridge interactions via water molecules (Fig. 6A, middle). In this configuration, the wild-type salt bridge between the elbow and the shoulder is replaced by a new salt bridge between the shoulder and the roll (Fig. 6, B and C). In the other configuration, E502K points away from the other two SB triad residues (Fig. 6A, right), and only the salt bridge between the elbow and the roll is present (Fig. 6, B and C).
Constraining the SB Triad Movement—As perturbing the charge-charge interactions in the SB triad facilitates channel opening, we imagine that the SB triad reversibly changes conformation as the channel opens and closes. More specifically, because SB triad disruption results in channels that open more easily than CNGA1_{cys-free} channels, we hypothesize that the SB triad residues move apart upon channel opening. Thus, we predict that if we inhibit SB triad movement, we will inhibit channel opening. In order to inhibit SB triad movement, we made a double mutant CNGA1 channel construct, L459C+S499C. L459C is in the shoulder of the C-linker and S499C is in the β roll of the CNBD. These introduced cysteines are near the SB triad residues, but because the SB triad itself is not mutated, the channel should be able to form and break its SB triad interactions as in the CNGA1_{cys-free} channel. We then modified L459C+S499C channels with homobifunctional cysteine-reactive cross-linkers of different lengths (Fig. 7) to estimate how far these regions move during channel opening (31, 32). We assumed that the residues most likely to be cross-linked would be L459C and S499C residues in the shoulder and β roll of neighboring subunits, as these cysteines (the only cysteines in the channel) are ~8 Å apart in the HCN2 C-terminal region crystal structure and any other combination of L459C and S499C are more than 30 Å apart. Moreover, because the reagents we chose are similar in length to the SB triad interaction to stabilize the protein in the R431E construct may render it very flexible, accounting for our inability to crystallize it.

Tethering residues L459C to S499C via MTS cross-linker reagents alters channel gating. Application of the homobifunctional cross-linker MTS-3-MTS to L459C+S499C channels

FIGURE 5. Charge reversal at position Glu-462 favors channel opening. A, behavior of Glu-462 (E462), E462D, E462Q, and E462R channels. Currents in response to voltage pulses to +60 mV are shown in the presence of saturating cAMP (red), cGMP (green), and cGMP (green). The schemes show which SB triad residue charge is changed for each panel. B, box plots of ΔG_{cAMP} values for Glu-462 (CNGA1_{cys-free}) channels with different charges at this position. Negatively charged: Glu-462 channels (CNGA1_{cys-free}, n = 14) and E462D (n = 3); neutral: E462Q (n = 5); positively charged: E462R (n = 2). The dashed line indicates the median ΔG_{cAMP} value for Glu-462 (CNGA1_{cys-free}) channels.

TABLE 1
HCN2-E502K crystallographic data collection statistics

| Statistic                  | Value                          |
|----------------------------|--------------------------------|
| Wavelength (Å)             | 0.97                           |
| Resolution (Å)             | 50-1.65 (1.71-1.65)            |
| Space group                | C22                           |
| Unit-cell dimensions (Å)   | 123.9, 134.3, 134.4            |
| Total reflections          | 1,844,008                      |
| Total unique reflections   | 132,486                        |
| Completeness (%)           | 99.0 (91.5)                    |
| Redundancy (%)             | 40.0 (4.2)                     |
| Beaminle (%)               | 139.3 (8.7)                    |

TABLE 2
HCN2-E502K refinement statistics

| Statistic                  | Value                          |
|----------------------------|--------------------------------|
| Resolution (Å)             | 30.1-65                        |
| R_{work} (%)               | 19.0                           |
| R_{free} (%)               | 21.6                           |
| R.m.s.d. bonds (Å)         | 0.011                          |
| R.m.s.d. angles (°)        | 1.32                           |
| Residues in most favored region of ϕ (°) | 95.0                            |
| Residues in allowed region of ϕ (°) | 4.9                            |
| B_{iso} (protein) (Å²)     | 15.8                           |
| B_{iso} (cAMP) (Å²)        | 12.4                           |
| No. of protein atoms       | 6123                           |
| No. of protein residues    | 775                            |
| No. of non-hydrogen ligand atoms | 88                             |
| No. of water molecules     | 1044                           |

a Values in parentheses are for the highest resolution shell.
b R_{work} = \frac{\sum_{i} F_{i}-\sum_{j} F_{i}}{\sum_{i} F_{i}}, where F_{o} and F_{c} are the observed and calculated structure factors, respectively.

A total of 5% of all intensities was randomly selected and excluded from the refinement to follow R_{work} for cross-validation.
rapidly inhibited current in response to a half-saturating concentration of cGMP (Fig. 8A), suggesting that MTS-3-MTS modification favors channel closing. Consistent with this, modification of L459C/H11001S499C channels by MTS-3-MTS significantly decreased the fractional activation by cIMP and cAMP (Fig. 8B) and resulted in significantly less negative $\Delta G_{cGMP}$ values (Fig. 9A) of the channels. Similar results were seen with the shorter linker MTS-1-MTS (Fig. 9A). Application of the longer homobifunctional cross-linker MTS-6-MTS potentiated current in response to a half-saturating concentration of cGMP (Fig. 8A), suggesting that MTS-6-MTS modification favors channel opening. Modification by MTS-6-MTS significantly increased the fractional activation (Fig. 8B) and made the $\Delta G_{cGMP}$ significantly more negative (Fig. 9A) for L459C+S499C channels. These results suggest that the cross-linkers whose lengths are similar to or smaller than the distance between L459C and S499C, MTS-1-MTS and MTS-3-MTS, inhibit channel opening, while the cross-linker that is longer than the inter-residue distance, MTS-6-MTS, facilitates channel opening.

To control for the effect of modification of each cysteine without the possibility of cross-linking, we made single cysteine constructs, L459C and S499C. These constructs have only one cysteine per subunit, each separated by at least 30 Å from neigh-
boring subunits, a distance that should prohibit cross-linking by these reagents. Modification of L459C alone by MTS-1-MTS, MTS-3-MTS, or MTS-6-MTS significantly increased the fractional activation (Fig. 8B) and resulted in significantly more negative ΔH9004G values (Fig. 9B) compared with L459C. In contrast, modification of S499C alone by MTS-1-MTS, MTS-3-MTS, or MTS-6-MTS had no statistically significant effect on the fractional activation (Fig. 8B) or ΔH9004G values (Fig. 9B). Because modification by MTS-1-MTS and MTS-3-MTS is different for L459C+S499C channels than for either L459C or S499C, it is likely that MTS-1-MTS and MTS-3-MTS are tethering L459C to the neighboring S499C, suggesting that the shorter linkers constrain movement of the SB triad during gating.

But why does application of MTS-6-MTS favor channel opening in L459C and L459C+S499C channels? To determine the effect of modification by a reagent without the possibility of cross-linking, we used a reagent that is approximately the same size as the MTS cross-linkers but that does not have the capability of cross-linking two cysteine residues: MTS-Butyl (Fig. 7). Modification by MTS-Butyl had no significant effect on the ΔH9004G values of L459C+S499C or the S499C channels and resulted in significantly more negative ΔH9004G values for L459C channels (Fig. 9). These results indicate that modification of L459C by any bulky reagent favors channel opening, perhaps due to steric interference. Thus, it is possible that the effect of MTS-6-MTS on L459C+S499C channels is dominated by the effect of modification of L459C.

Modification of the double cysteine channels, L459C+S499C, with the MTS cross-linkers affected the energetics of channel gating differently from modification of the single cysteine channels. We wanted to ascertain whether the MTS cross-linkers are tethering the subunits at residues L459C and S499C, especially in the case of MTS-6-MTS where modification of the L459C+S499C channels produces qualitatively similar effects to modification of L459C channels. We expressed CNGA1 L459C+S499C channels in Xenopus oocytes and homogenized the oocytes with MTS-Butyl, MTS-3-MTS, or MTS-6-MTS. Total protein was solubilized and run out on SDS-PAGE gels either in nonreducing or reducing conditions, and channel protein was visualized using Western blotting (Fig. 10). In the presence of MTS-3-MTS and MTS-6-MTS, under nonreducing conditions, portions of L459C+S499C subunits ran at approximately the size of a dimer and the size of a tetramer. But when these channels were modified by MTS-Butyl, the L459C+S499C channels ran at the size of a monomer only. Under reducing conditions, the percent of channel subunits that ran at the size of a multimer decreased and the percent that ran at the size of a monomer increased. Thus, L459C+S499C subunits can multimerize either upon modification by MTS-3-MTS or MTS-6-MTS, but not upon modification by MTS-Butyl, indicating that these reagents do tether one subunit to another.
DISCUSSION

We studied gating rearrangements in the C-terminal region of cyclic nucleotide-modulated channels by examining the effect of charge substitution at two key residues in the SB triad and then examining the effect of restricting normal channel movements in the SB triad region through tethering. Charge substitution at position Arg-431 in CNGA1 channels resulted in channels with an extremely favorable opening transition, no matter how the substitution was achieved: through mutation or through modification with a charged reagent. Similarly, charge substitution at position Glu-462 resulted in channels that open more favorably than wild type. The x-ray crystallographic structure for the C-terminal region of HCN2 channels with this charge substitution, E502K, showed that the global structure of the protein did not change, but that the SB triad did change configuration, decreasing the number of salt bridge interactions. We conclude that the increased ease of channel opening for E462R CNGA1 channels occurs because both salt bridges in the SB triad cannot always form simultaneously. Furthermore, we conclude that the more dramatic effects of R431E channels occur because neither of the salt bridges in the SB triad can form. Finally, as disrupting the SB triad favors channel opening, we predicted that inhibiting SB triad movement would hinder channel opening. We tethered the shoulder of one subunit to the β roll of the neighboring subunit and found that short cross-linkers hindered channel opening whereas longer tethers did not. These data suggest that rearrangements in the SB triad region occur as the channels gate open and closed.

Compiling these data from the current study with other recent discoveries about gating rearrangements in cyclic nucleotide-modulated channels (6, 33, 34), we propose a mechanism by which cyclic nucleotide binding may lead to pore opening: In the absence of ligand, the C-linkers are packed together tightly, stabilized by SB triad interactions and other intersubunit interactions, as seen in the HCN2 C-terminal crystal structure. This intact SB triad favors closed channels, as indicated by the inhibitory effects of restoring the positive charge to position Arg-431 and by tethering the SB triad region with short cross-linkers. Cyclic nucleotides bind to the β roll and C-helix of the CNBD, inducing a quaternary rearrangement that leads to C-linker conformational changes. The proposed C-linker rearrangement would result in the SB triad region expanding and disrupting the salt bridge interactions. From the cross-linker results that the short cross-linkers inhibit channel opening, we propose that the shoulder moves away from the β roll further than MTS-1-MTS or MTS-3-MTS can stretch but not as far as...
MTS-6-MTS can stretch. Based on the estimated lengths of the cross-linkers, ~6 Å for MTS-1-MTS, ~8 Å for MTS-3-MTS, and ~11 Å for MTS-6-MTS (Fig. 7), L459C appears to move more than 8 Å but less then 11 Å apart from S499C. In order for this SB triad expansion to take place, we propose that each subunit twists away from the central axis of the channel, such that the tip of the elbow points away from the center, much as the iris of a camera moves. As the C-linkers move centrifugally, the sixth transmembrane helices also move, widening the pore and allowing for ion flux through the open channel.

Although SB triad expansion occurs during channel opening, we suggest that the crucial function of the SB triad is to maintain channel closure. Support for the assertion comes from a previous study on HCN channels in which deletion of a portion of the C terminus produces channels that open more favorably than wild-type channels (35). These data suggest that the unliganded C termini inhibit cyclic nucleotide-modulated channel closure. Although SB triad expansion occurs during channel opening, and our data provide a mechanism for this inhibition: the SB triad. These intersubunit SB triad interactions, along with the hydrophobic and hydrogen bond interactions in the intersubunit interface, likely serve to hold the channel pore closed. Thus the binding of cyclic nucleotide and expansion of the SB triad probably does not “pull” the channel pore open, rather it relieves the inherent inhibition the C termini exert on the pore.

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