G protein-activated inwardly rectifying potassium channel (GIRK) plays crucial roles in regulating heart rate and neuronal excitability in eukaryotic cells. GIRK is activated by the direct binding of heterotrimeric G protein βγ subunits (Gβγ) upon stimulation of G protein-coupled receptors, such as M2 acetylcholine receptor. The binding of Gβγ to the cytoplasmic pore (CP) region of GIRK causes structural rearrangements, which are assumed to open the transmembrane ion gate. However, the crucial residues involved in the Gβγ binding and the structural mechanism of GIRK gating have not been fully elucidated. Here, we have characterized the interaction between the CP region of GIRK and Gβγ, by ITC and NMR. The ITC analyses indicated that four Gβγ molecules bind to a tetramer of the CP region of GIRK with a dissociation constant of 250 μM. The NMR analyses revealed that the Gβγ binding site spans two neighboring subunits of the GIRK tetramer, which causes conformational rearrangements between subunits. A possible binding mode and mechanism of GIRK gating are proposed.

G protein-activated inwardly rectifying potassium channel (GIRK) is a member of the inwardly rectifying potassium family, which regulates heart rate and neuronal excitability (1, 2). The Kir proteins function as tetramers, consisting of a transmembrane (TM) region and a cytoplasmic pore (CP) region. The helix bundle at the cytoplasmic side of the TM region is assumed to be a K⁺ ion gate. The opening and closing in the gate (gating) of Kirs are regulated by a variety of cytoplasmic factors. The gating of GIRK is triggered by the binding of its CP region with the heterotrimeric G-protein βγ subunits (Gβγ), which are released from the pertussis toxin-sensitive G protein α subunit (Gαツx), subsequent to the stimulation of a G protein-coupled receptor, such as M2 acetylcholine receptor.

Extensive mutational analyses to identify the GIRK residues that are critical for the Gβγ-induced activation revealed several critical residues such as His57, Leu262, Leu333, and Gly336 of GIRK1 (3–5). However, when these residues were mapped on the recently reported crystal structures of Kirs, they did not form a cluster on the protein surface (6–9). Therefore, no clear consensus has been obtained regarding the region of GIRK that is essential for Gβγ binding and/or GIRK activation. One of the reasons might be a structural alteration introduced by the mutagenesis (10), which could change the gating property of the channel.

Although the various crystal structures have provided little information about the conformational change involved in the gating of the channel, FRET analyses have clearly demonstrated the conformational rearrangements in the CP region of GIRK upon Gβγ binding (11). However, the resolution of the structural information obtained by the FRET analyses is low, primarily due to the large size of the fluorescent probe proteins.

To reveal the structural mechanism by which Gβγ binding activates GIRK, we have investigated the direct interaction between the CP region of GIRK and Gβγ by isothermal titration calorimetry (ITC) and NMR spectroscopy. Our ITC results indicated that four Gβγ molecules bind to a tetramer of the CP region of GIRK, with a dissociation constant (Kd) value of 250 μM. NMR analyses revealed that the binding of Gβγ occurs at two adjacent contiguous surfaces, clustered between the two neighboring subunits of the CP region of GIRK, and causes the conformational rearrangements of the tetramer. Based on our analysis, we propose a possible binding mode and structural mechanism of GIRK gating.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of the Cytoplasmic Regions of Mouse GIRK1—The N- and C-terminal cytoplasmic regions of mouse GIRK1 (residues 41–63 and 190–371) were fused into a single polypeptide, which is hereafter referred to as
GIRK<sub>CP</sub> (cytoplasmic pore). The GIRK<sub>CP</sub> sample for the ITC analyses was prepared by growing the *Escherichia coli* host in LB medium. The uniformly <sup>2</sup>H, <sup>15</sup>N-labeled GIRK<sub>CP</sub> samples for NMR analyses were prepared by growing the *E. coli* host in M9 minimal medium, containing <sup>2</sup>HNH<sub>4</sub>Cl, [U-<sup>13</sup>CH<sub>3</sub>]glucose, and [<sup>2</sup>H/]<sup>15</sup>N-Celtone<sup>®</sup> base powder in 99% <sup>2</sup>H<sub>2</sub>O. GIRK<sub>CP</sub> was purified as described (12). In brief, GIRK<sub>CP</sub> was purified to homogeneity by fractionating on HIS-Select<sup>®</sup> Nickel Affinity Gel (Sigma), followed by His tag cleavage by PreScission<sup>TM</sup> Protease (GE Healthcare), and then size exclusion chromatography on a HiLoad 26/60 Superdex 200 pg column (GE Healthcare). GIRK<sub>CP</sub> mainly eluted as a tetramer from the size exclusion chromatography column, which was confirmed by static light scattering (Wyatt Technology), as previously reported (10). The uniformly <sup>2</sup>H, <sup>15</sup>N-labeled GIRK<sub>CP</sub> for the transferred cross-saturation (TCS) experiments was prepared without the denaturing and refolding procedure, to leave the amide hydrogen atoms as <sup>2</sup>H in the core of the protein. The GIRK<sub>CP</sub> samples for the TCS experiments were incubated at 303 K for over 48 h to avoid <sup>2</sup>H/<sup>15</sup>N exchange during the measurements. For the chemical shift perturbation (CSP) experiments, we prepared uniformly <sup>2</sup>H, <sup>15</sup>N-labeled GIRK<sub>CP</sub>, in which the amide hydrogen atoms were fully exchanged to <sup>1</sup>H by unfolding with 6 M guanidine and subsequent refolding.

**Construction of Recombinant Baculoviruses**—The cDNA encoding the Gγ<sub>2</sub> subunit was amplified from a human heart cDNA library (BD Biosciences) by PCR and inserted into the baculovirus transfer vector pVL1393 (Invitrogen). The site-directed mutagenesis of the cysteine residue at position 68 of Gγ<sub>2</sub> to serine and the insertion of a hexahistidine tag at the N terminus (Gγ<sub>2</sub>-His-C68S) were performed by the QuikChange<sup>®</sup> method (Stratogene). The recombinant baculovirus, encoding the bovine Gβ<sub>2</sub> subunit, was kindly provided by Drs. Y. Fukada and M. Katadae (The University of Tokyo, Japan). The amino acid sequences of Gβ<sub>1</sub> are fully conserved among the bovine, human, and mouse forms.

**Expression and Purification of Gβγ**—The Gβ<sub>1</sub> and Gγ<sub>2</sub> subunits were co-expressed in expressSF<sup>®</sup>-<sup>TM</sup> insect cells (Protein Sciences) by infection with the amplified recombinant baculoviruses, encoding the Gβ<sub>1</sub> and Gγ<sub>2</sub>-His-C68S subunits at a multiplicity of infection of 2.5 for each baculovirus. After an incubation at 27 °C for 60–72 h, the cells were harvested and re-suspended in 100 ml of buffer A (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol (DTT)) containing 1× protease inhibitor mixture (EDTA free) (Nacalai Tesque) per 1 liter of culture cells. All of the following purification procedures were carried out at 4 °C. The cell suspension was homogenized with Teflon homogenizer (15 strokes) or by nitrogen cavitation (Parr bomb) at 800 p.s.i. for 30–60 min. Cell lysates were centrifuged at 750 × g for 10 min to remove intact cells and nuclei. The supernatants were further centrifuged at 100,000 × g for 30 min, and the supernatants were used for purification. The supernatant was purified using HIS-Select<sup>®</sup> Nickel Affinity Gel (Sigma), followed by further purification using His-Trap HP (GE Healthcare). The elution was applied to a HiLoad 16/60 Superdex 200 pg column (GE Healthcare). The purified proteins were concentrated by ultrafiltration and buffers were adjusted for the following experiments using an Amicon Ultra filter (Millipore).

**ITC Analyses**—The binding of Gβγ to GIRK<sub>CP</sub> was measured by ITC, using a MicroCal ITC200 calorimeter (GE Healthcare), with stirring at 1000 rpm at 25 °C. The protein samples were dialyzed against a buffer containing 10 mM HEPES-NaOH (pH 7.5), 50 mM KCl, and 1 mM tris-(2-carboxyethyl)phosphine. The titration of the GIRK<sub>CP</sub> tetramer with Gβγ involved 25 injections of 1.5 μl of the GIRK<sub>CP</sub> solution (1.8 mM as the tetramer) at intervals of 180 s into a sample cell containing 200 μl of Gβγ (380 μM). The heat of dilution of the titrant (the GIRK<sub>CP</sub> tetramer) was subtracted from the titration data for the GIRK<sub>CP</sub> titration into Gβγ. The data were analyzed with the MicroCal Origin<sup>TM</sup> 5.0 software to determine the enthalpy (ΔH), dissociation constant (K<sub>d</sub>), stoichiometry of binding (N). Thermal titration data were fit to a single site binding model and thermodynamic parameters ΔH, K<sub>d</sub>, and entropy change (ΔS) were obtained by fitting to the model. The error of each parameter shows the fitting error.

**NMR Analyses**—All NMR experiments were performed at 303 K on a Bruker Avance 600 spectrometer equipped with a cryogenic probe. All spectra were processed by the Bruker TopSpin 2.1 software and the data were analyzed by Sparky (T. D. Goddard and D. G. Kneller, Sparky 3, University of California, San Francisco). The error bars were presented based on the signal to noise ratio calculated by the Sparky software. The assignments of the backbone NMR signals from GIRK<sub>CP</sub> were reported (BioMagResBank accession number 11067 (12)).

The TCS experiments were carried out as described with minor modifications (13). A solution containing the uniformly <sup>2</sup>H, <sup>15</sup>N-labeled GIRK<sub>CP</sub> (300 μM as a tetramer) and the non-labeled Gβγ (200 μM) was prepared in buffer (10 mM HEPES-NaOH (pH 7.5), 50 mM KCl, 2 mM DTT, 1 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate, 20% <sup>2</sup>H<sub>2</sub>O, 80% <sup>1</sup>H<sub>2</sub>O). The selective saturation for the aliphatic protons of Gβγ was performed with a 15-ms IBURP2 pulse centered at 1.0 ppm. The saturation duration and the relaxation delay were set at 2.5 and 3.5 s, respectively. To evaluate the effect of the residual aliphatic protons within the GIRK<sub>CP</sub> protein, a TCS experiment was also carried out for the control sample without Gβγ, containing the uniformly <sup>2</sup>H, <sup>15</sup>N-labeled GIRK<sub>CP</sub> (250 μM as a tetramer) alone. It should be noted that the residues with overlapping resonances were excluded from the analyses. In the CSP experiments, samples containing the uniformly <sup>2</sup>H, <sup>15</sup>N-labeled GIRK<sub>CP</sub> (50 μM as a tetramer) mixed with Gβγ at molar ratios (GIRK tetramer:Gβγ) of 1:0, 1:1, 1:2, 1:4, and 1:8, in buffer (10 mM HEPES-NaOH (pH 7.5), 50 mM KCl, 5 mM DTT, 1 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate, 10% <sup>2</sup>H<sub>2</sub>O, 90% <sup>1</sup>H<sub>2</sub>O), were individually prepared, and an <sup>1</sup>H<sub>2</sub>/<sup>15</sup>N transverse relaxation-optimized spectroscopy (TROSY) spectrum of each sample was recorded. The signal intensities among distinct samples were normalized by the intensity of the sodium 2,2-dimethyl-2-silapentane-5-sulfonate signal. The residues with overlapping resonances and small signal intensities in the absence of Gβγ (the signal-to-noise ratios<15) were omitted from the analyses.
RESULTS

The Binding Affinity of the Cytoplasmic Pore Region of GIRK for Gβγ—In this study, we used the cytoplasmic pore regions of mouse GIRK1, composed of residues 41–63 and 190–371 as a single polypeptide (hereafter referred to as GIRKCP), and the prenylation-deficient mutant of Gβγ, composed of the wild-type Gβ and the C68S mutant of Gγ, which has a hexahistidine tag at the N terminus (hereafter referred to as Gβγ).

We previously reported that the purified GIRKCP protein forms a tetramer determined by size exclusion chromatography combined with multiple angle light scattering (10). The 1H-15N TROSY spectrum of GIRKCP was well dispersed and provided a number of signals somewhat consistent with the number of the residues within a single subunit of GIRKCP, indicating that the four subunits within the tetramer are in an equivalent environment in solution (12). The validity of this construct was confirmed by its crystal structures (7, 8), which were essentially identical to the structures of the CP region in the full-length Kir channels, such as KirBac1.1 (14) and a chimeric channel of KirBac1.3 and GIRK1 (6). The purified Gβγ contains approximately equimolar amounts of Gβ and Gγ, and its binding activity to Ga was confirmed by size exclusion chromatography (supplemental Fig. S1).

ITC experiments were carried out to determine the binding affinity and stoichiometry between GIRKCP and Gβγ. Fig. 1 shows the isotherm upon the titration of GIRKCP into the Gβγ solution (upper panel), and the amounts of the heat exchange obtained by the integration of each peak, after the subtraction of the heat of GIRKCP dilution (lower panel). The best fit of the integrated isotherm was obtained, by using a single-site model, which generated an apparent dissociation constant (K_d) value of 250 μM (Fig. 1, lower panel). Although the concentration of Gβγ in the ITC cell (380 μM) was not sufficient for the determination of the precise binding stoichiometry when the K_d value is 250 μM, the fitting ended with a binding stoichiometry of 0.25 eq of GIRKCP tetramer per one Gβγ molecule. Because the 0.25 eq of GIRKCP tetramer corresponds to the concentration of the GIRKCP monomer, the ITC result suggests that four Gβγ molecules bind to one GIRKCP tetramer, where the K_d value for the binding of GIRKCP monomer to Gβγ is 250 μM.

The Gβγ Binding Site on GIRKCP, Revealed by Transferred Cross-saturation Experiments—To identify the Gβγ binding site on GIRKCP, TCS experiments were performed. The radio frequency irradiation to the sample containing GIRKCP tetramer, where the frequency irradiation to the sample containing GIRKCP tetramer, where the cross-saturation of the proton signals of the CP regions in GIRKCP, a control experiment in the absence of GIRKCP tetramer was also performed (Fig. 2B). We evaluated the cross-saturation from Gβγ, by subtracting the intensity reduction ratios of this control experiment, which were scaled up considering the slower tumbling time of GIRKCP caused by the binding to Gβγ (supplemental Fig. S2, gray; see the legend of supplemental Fig. S2 for details) from the ratios in the presence of Gβγ (supplemental Fig. S2, beige) (16). The differences in the reduction ratios, ΔRR, are shown in Fig. 2C with error bars calculated based on the signal-to-noise ratios. The minimum values of ΔRR within the error ranges (ΔRR_min) were utilized for the evaluation.

The residues with large intensity reductions (ΔRR_min > 0.20) are Arg236, Gly241, Phe243, and Leu333, and those with small but significant intensity reductions (0.083 < ΔRR_min ≤ 0.20) are Glu237, Thr238, Glu240, Leu244, Met308, Glu320, Glu334, Phe337, Lys339, Asp341, and Glu350. These residues mostly belong to one of two regions: the region from the βD to βE strands (Arg236–Thr238 and Glu240–Leu244) and the region from the βL to βM strands (Leu333, Glu334, Phe337, Lys339, and Asp341) (Fig. 2C). The other three residues, principle, would reduce the signal intensity of the proximal amide groups, because of the enhanced dipole-dipole interactions due to the high molecular weight of GIRKCP (nearly 100,000). To exclude the effects of the direct saturation of the GIRKCP resonances, a control experiment in the absence of Gβγ was also performed (Fig. 2B). We evaluated the cross-saturation from Gβγ, by subtracting the intensity reduction ratios of this control experiment, which were scaled up considering the slower tumbling time of GIRKCP caused by the binding to Gβγ (supplemental Fig. S2, gray; see the legend of supplemental Fig. S2 for details) from the ratios in the presence of Gβγ (supplemental Fig. S2, beige) (16). The differences in the reduction ratios, ΔRR, are shown in Fig. 2C with error bars calculated based on the signal-to-noise ratios. The minimum values of ΔRR within the error ranges (ΔRR_min) were utilized for the evaluation.

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Met\textsuperscript{308}, Glu\textsuperscript{320}, and Glu\textsuperscript{350}, are located in the loop between the βH and βI strands, the loop between the βI and βJ strands, and in the βN strand, respectively.

These residues were mapped on a single subunit of GIRK\textsubscript{CP} (PDB code 1N9P) (Fig. 2D). Although the mapped residues are dispersed on a single subunit of GIRK\textsubscript{CP}, the residues except for Met\textsuperscript{308} Glu\textsuperscript{320} and Glu\textsuperscript{350} formed two adjacent contiguous surfaces in the two neighboring subunits of the GIRK\textsubscript{CP} tetramer (Fig. 2E). The two surfaces are separated by two proline residues (Pro\textsuperscript{245} and Pro\textsuperscript{329}), which lack amide protons and thus were excluded from the analysis, suggesting that the two surfaces could form a single Gβγ binding site (supplemental Fig. 3A).

The intensity reduction of Met\textsuperscript{308} would be induced by the direct saturation of the hydrogen atoms in the hydroxyl groups of the three proximal threonine residues (Thr\textsuperscript{305}, Thr\textsuperscript{306}, and Thr\textsuperscript{309}). The distances between the nitrogen atom of Met\textsuperscript{308} and the γ1 oxygen atoms of Thr\textsuperscript{305}, Thr\textsuperscript{306}, and Thr\textsuperscript{309} are 7.4, 3.1, and 7.0 Å, respectively, in the crystal structure. Thus, the direct saturation of the threonine hydroxyl groups would result in the large ∆RR value of Met\textsuperscript{308}, by decreasing the distances due to the conformational change (see below) and/or enhancing the dipole–dipole interactions upon Gβγ binding, even though significant intensity reductions were not observed in the control experiment.

Glu\textsuperscript{320} and Glu\textsuperscript{350} might be included in the Gβγ binding site. Although these residues are separated from the contiguous surfaces, the intervening residues are difficult to detect by cross-saturation. Glu\textsuperscript{320} is separated from the clustered surface by Arg\textsuperscript{43} and Gln\textsuperscript{44} of the N-terminal region (supplemental Fig. S3A). Because the exchange rates for the amide protons of several residues in the N-terminal and loop regions under the TCS experimental conditions seem to be faster than those of the other regions (supplemental Fig. S3B), the intensity reductions of the signals for these regions might be underestimated. Glu\textsuperscript{350} is separated by two phenylalanine residues (Phe\textsuperscript{328} and Phe\textsuperscript{349}), and their side chain aromatic rings are oriented toward the protein surface (supplemental Fig. S3A). The amide groups of these two phenylalanine residues are buried in the protein and thus are more than 6 Å away from the contiguous surface for Gβγ binding. Therefore, Phe\textsuperscript{328} and Phe\textsuperscript{349} are unlikely to be detected as binding residues by the TCS experiment, even when they are involved in the Gβγ binding interface. Altogether, we concluded that the Gβγ binding site on GIRK\textsubscript{CP} is composed of the following residues clustered between the two neighboring surfaces of the adjacent subunits: Arg\textsuperscript{36} Gln\textsuperscript{37} Thr\textsuperscript{38} Gln\textsuperscript{40} Gln\textsuperscript{41} Glu\textsuperscript{242} Phe\textsuperscript{243} Leu\textsuperscript{244} Glu\textsuperscript{320} Leu\textsuperscript{333} Glu\textsuperscript{344} Phe\textsuperscript{337} Lys\textsuperscript{339} Asp\textsuperscript{341} and Glu\textsuperscript{350}.

**Chemical Shift Perturbation of GIRK\textsubscript{CP} upon Binding to Gβγ**—The Gβγ binding effects on GIRK\textsubscript{CP} were also investigated by CSP experiments, which reflect the effects of the direct binding of Gβγ as well as the conformational change of GIRK\textsubscript{CP} induced by the Gβγ binding. Upon the addition of 50, 100, 200, and 400 μM Gβγ to 50 μM uniformly \textsuperscript{3}H,\textsuperscript{15}N-labeled GIRK\textsubscript{CP} tetramer, no signals exhibited chemical shift changes larger than 0.05 ppm, but significant intensity reductions were observed for a number of signals (Fig. 3A).

The observed intensity reductions of the NMR signals are caused by slower tumbling, due to the increased average molecular weight of the complex of GIRK\textsubscript{CP} and Gβγ. Furthermore, the intensity reductions are induced by the chemical shift differences of GIRK\textsubscript{CP} between the free and bound states (18). The differences reflect the changes of the microenvironments on the Gβγ binding site on GIRK\textsubscript{CP}, including the disruption of the 4-fold symmetry of the GIRK\textsubscript{CP} tetramer, as well as the concomitant conformational changes of the remote region from the binding site.

The intensity reduction ratios of the TROSY signals are plotted in Fig. 3B for the sample containing 50 μM of the \textsuperscript{2}H,\textsuperscript{15}N-labeled GIRK\textsubscript{CP} tetramer mixed with 100 μM Gβγ, in which the GIRK\textsubscript{CP} tetramers in the free form and 1:1 complex with Gβγ are predominantly present, assuming the K\textsubscript{d} value of 250 μM obtained by the ITC analysis (supplemental Fig. S4). In the analysis, the signal intensity of free GIRK\textsubscript{CP} was divided by a factor of 1.36, considering the increase in the averaged molecular weight of GIRK\textsubscript{CP} in complex with Gβγ. Several residues such as Gly\textsuperscript{61}–Glu\textsuperscript{63} Ser\textsuperscript{368} Ser\textsuperscript{369} and Leu\textsuperscript{371} exhibited the negative RR values lower than −0.20, suggesting that these residues tumble faster than other regions of GIRK\textsubscript{CP}, due to their local flexibility.
The residues with intensity reduction ratios larger than 0.32 are Arg45, Phe46, Arg236, Gln237, Glu242, Phe243, Leu244, Asp235, Asp319, Leu333, Glu334, Gln344, and Thr348, and those with smaller but significant intensity reduction ratios within the 0.18–0.32 range are Val253, Asn50, Gly51, Ala226, Ser235, Thr238, Glu240, Gly241, Gly242, Phe243, Leu244, Thr290, Thr317, Glu18, Asp319, Glu320, Leu333, Glu334, Gly336, Phe337, Phe338, Tyr342, Ser343, Gln344, Ala347, and Thr348, which reside in the Gβγ binding site (Fig. 2E) and its proximity. The other residues are located on the inside of the pore (Ala226, Val233, Gly237, Thr238, Glu240, Gly241, Gly242, Phe243, Leu244, Thr290, Thr317, Glu18, Asp319, Glu320, Leu333, Glu334, Gly336, Phe337, Phe338, Tyr342, Ser343, Gln344, Ala347, and Thr348), at the membrane side (Gly237 and Met308), and at other distal sites (Ser78 and Val558) (Fig. 3D, right). In particular, the CSPs for the residues outside of the Gβγ binding interface (see Fig. 2E for comparison) reflect their conformational changes, as allosteric effects of the Gβγ binding.

**DISCUSSION**

The Binding Affinity between Gβγ and GIRK—The ITC results indicated a dissociation constant (K_d) value of 250 μM for the interaction between the GIRK_{CP} monomer and Gβγ.
The GIRK<sub>cp</sub> tetramer seems to bind to four G<sub>βγ</sub> molecules, and no significant affinity difference was observed among the multiple G<sub>βγ</sub> binding sites. The binding stoichiometry is consistent with the results of the cross-linking experiments indicating that 1 – 4 G<sub>βγ</sub> molecules bind to a tetramer of GIRK (19). The binding of 1 – 4 G<sub>βγ</sub> molecules to a GIRK tetramer might be relevant to the G<sub>βγ</sub>-dependent multiple modes of GIRK activation, where increased G<sub>βγ</sub> binding seems to result in increased current frequencies (open probability) of GIRK, observed by the single-channel analyses (20).

The dissociation constant between GIRK<sub>cp</sub> and G<sub>βγ</sub> (K<sub>d</sub> value of 250 μM) determined in this study is considerably larger than the reported constant for the G<sub>βγ</sub>-induced activation of GIRK (K<sub>d</sub> value of 11 nM) determined by electrophysiological experiments (21). Theoretically, the affinity between membrane proteins could be enhanced by 10<sup>6</sup>-fold compared with that in solution, because of the effects of the excluded volume, the substantially higher local concentra-

tions, and the preorientation of proteins in membranes (22). Indeed, the affinities of protein-protein interactions within membranes were reported to be enhanced by 10<sup>1</sup>-10<sup>3</sup>-fold (23, 24). Because G<sub>βγ</sub> is anchored to the membrane by the lipid moiety covalently attached to the C terminus of G<sub>γ</sub>, the orientational constraint of G<sub>βγ</sub> should be smaller than those of integral membrane proteins. Therefore, the enhancement of the binding affinity in the membrane is expected to be significantly smaller than 10<sup>6</sup>-fold, suggesting that the K<sub>d</sub> value of the GIRK-G<sub>βγ</sub> interaction falls in the nanomolar to micromolar range. Considering that GIRK activation is terminated by the removal of G<sub>βγ</sub> from GIRK through the competitive binding of the GDP-bound Ga to G<sub>βγ</sub> (IC<sub>50</sub> = 10 nM (21)), the affinity between GIRK and G<sub>βγ</sub> should be lower than that between Ga and G<sub>βγ</sub> (K<sub>d</sub> = 6 – 9 nM (25)) to accomplish the rapid closure of GIRK by the GDP-bound Ga. Therefore, the K<sub>d</sub> value of 250 μM, determined in this study, would be reasonable for the interaction between GIRK and G<sub>βγ</sub> in solution.

The Possible Binding Mode between GIRK and G<sub>βγ</sub>—The TCS method identified the site of the direct G<sub>βγ</sub> binding, which is mainly located on the region from the βD to βE strands of one subunit and the region from the βL to βM strands of the adjacent subunit of GIRK<sub>cp</sub> (Fig. 2). The latter region contains Leu<sup>313</sup> and Gly<sup>336</sup>, which were previously identified by the mutational analyses as important residues for the G<sub>βγ</sub>-induced activation of GIRK (3, 5, 26). Although the other residues postulated by the mutational studies, His<sup>57</sup> and Leu<sup>262</sup>, were excluded from the present analyses, unfortunately, due to insufficient signal intensity and lack of the backbone assignment of the NMR signal, respectively.

On the other hand, the mutational analyses have suggested several residues of G<sub>βγ</sub> that are important for GIRK activation (residues Leu<sup>285</sup>, Lys<sup>279</sup>, Ile<sup>280</sup>, Lys<sup>283</sup>, Trp<sup>299</sup>, Asp<sup>328</sup>, and Trp<sup>332</sup> of G<sub>β</sub>) (27, 28). It should be noted that most of these residues are located in the Ga binding site, in the crystal structure of the heterotrimeric Ga<sub>βγ</sub> complex (Fig. 4A). The former four G<sub>β</sub> residues (Leu<sup>285</sup>, Lys<sup>279</sup>, Ile<sup>280</sup>, and Lys<sup>283</sup>) bind to the N-terminal helix of Ga<sub>α</sub>, which undergoes a large conformational change upon G<sub>βγ</sub> binding (29), suggesting that this site on Ga<sub>α</sub> is formed specifically in the complex with G<sub>βγ</sub>. Meanwhile, the latter four residues (Trp<sup>299</sup>, Asp<sup>328</sup>, Asp<sup>346</sup>, and Trp<sup>332</sup>) of G<sub>β</sub> reside on the “top” face of the G<sub>βγ</sub> torus, also known as the “hot spot” (Fig. 4A, left), which binds to the GTPase domain of Ga<sub>α</sub> (Fig. 4A, right). The center of the binding site of the G<sub>βγ</sub> torus on Ga<sub>α</sub> (Fig. 4A) contains the hydrophobic residues, Ile<sup>180</sup>, Phe<sup>195</sup>, and Phe<sup>211</sup>, which are located in the proximity of the Trp<sup>299</sup> side chain of G<sub>β</sub>. These three hydrophobic residues of Ga<sub>α</sub> are flanked by two acidic residues, Glu<sup>182</sup> and Glu<sup>212</sup>, where Glu<sup>182</sup> hydrogen bonds with the Trp<sup>299</sup> side chain of G<sub>β</sub> and Glu<sup>212</sup> makes an electrostatic interaction with Lys<sup>279</sup> of G<sub>β</sub>. Indeed, the importance of Trp<sup>299</sup> was indicated by the mutational analyses, and affected the activation of G<sub>βγ</sub> effectors besides GIRK, such as phospholipase C-β2 and adenyl cyclase 2 (30). Although G<sub>βγ</sub> binding affects the conformation of the GTPase domain of Ga<sub>α</sub>, the structural motif comprising “the hydrophobic region”
and “the two flanking acidic residues” is preserved in GDP-bound Ga, in both the presence and absence of Gβγ.

A closer inspection of the Gβγ binding sites on GIRKCP revealed a similar structural motif, the hydrophobic region consisting of the residues on the βE strand (Phe243, Leu244, and Pro245) of one subunit and the residues at the beginning of the βL strand (Pro229 and Ile231) of the neighboring subunit, and the two flanking acidic residues (Glu242 and Glu334) (Fig. 4B). Thus, we conclude that the Gβγ binding sites on GIRK possess surface properties similar to those on Ga. It is tempting to speculate that, upon binding, the Trp99 side chain of Gβγ is accommodated in the hydrophobic region supported by the interactions with the two flanking acidic residues of GIRK. The binding orientation would be possible for intact GIRK and Gβγ that anchors to the membrane with a lipid moiety attached to the C terminus of Gγ. Because the hydrophobic region spans two neighboring subunits, Gβγ binding to this site could cause the rearrangements of the CP regions of GIRK, as evidenced by the CSPs at distant sites far away from the Gβγ binding site (see below).

Propagation of the Gβγ-induced Rearrangements of GIRKCP to the K⁺-ion Gate—The Gβγ binding induced significant intensity reductions originating from CSPs not only for the residues at the direct binding site, but also for those in other regions (Fig. 3, C and D). In particular, the significant CSPs observed for the residues located at the subunit-subunit interface away from the Gβγ binding site (Fig. 3D, right) strongly suggest that the rearrangements of the four subunits were induced by Gβγ binding. Indeed, two distinct conformations of the CP region by the rigid body subunit rearrangements were observed in the crystal structure of a chimeric channel of KirBac1.3 and GIRK1 (PDB code 2QKS) (6). The quite recently reported structures of KirBac3.1 (31) also revealed conformational changes in the orientation of the CP region relative to the TM region (PDB codes 2WLI and 2WLJ, as the representative structures).

In the crystal structure of the chimeric channel, several residues around the K⁺-ion gate (Phe272 and Thr73 in the slide helix, Ile182 and Ser185 in the inner TM helix, and Gln196 and Pro187 in the C-linker) directly interact with the residues in the loop between the βH and βL strands (H-I loop) of the CP region of GIRK1 (Val303, Thr305, Thr306, Met308, and Cys310), as summarized in Fig. 5. In addition, Thr73 (slide helix) and Lys188 (C-linker) interact with His222 and Arg219, respectively, which are in the loop between the βC and βD strands (C-D loop) of the CP region (supplemental Fig. S5A). The interactions of the residues in the H-I loop with the TM region residues including the slide helix, inner helix, and C-linker are

![Figure 5](image-url)
also observed in the crystal structure of KirBac3.1, whereas more interactions were observed between the C-D loop and the slide helix in one form of the KirBac3.1 structures (supplementary Fig. S5, panels B and C).

As shown in Fig. 3, significant CSPs upon Gβγ binding were observed for Gly307 and Met308 on the H-I loop, suggesting that the relative positions between the loops of the four subunits change upon Gβγ binding, which could result in the opening of the K+ ion gate. It should be noted that the residue at the top of the H-I loop, Thr305, provided a very weak NMR signal, and was excluded from the analysis, and no significant CSP was observed for the adjacent residue, Thr306, due to the lack of the interacting TM residues in GIRK CP, used in this study. The residues in the C-D loop were also excluded from the analysis, because their backbone NMR signals were not assigned. Further studies are awaited to reveal the mode of the Gβγ-induced structural rearrangements of the CP region of GIRK, and their effects on the conformation of the K+ ion gate.

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