**β**2 Adrenergic Receptor Activation

**MODULATION OF THE PROLINE KINK IN TRANSMEMBRANE 6 BY A ROTAMER TOGGLE SWITCH***

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In many rhodopsin-like G-protein-coupled receptors, agonist binding to a cluster of aromatic residues in TM6 may promote receptor activation by altering the configuration of the TM6 Pro-kink and by the subsequent movement of the cytoplasmic end of TM6 away from TM3. We hypothesized that the highly conserved Cys6.47, in the vicinity of the conserved Pro6.50, modulates the configuration of the aromatic cluster and the TM6 Pro-kink through specific interactions in its different rotamer configurations. In the β2 adrenergic receptor, mutation of Cys6.47 to Thr, which in an α-helix has a different rotamer distribution from Cys and Ser, produced a constitutively active receptor, whereas the Ser mutant was similar to wild-type receptor. Use of the biased Monte Carlo technique of Conformational Memories showed that the rotamer changes among Cys/Ser/Thr6.47, Trp6.48, and Phe6.52 are highly correlated, representing a rotamer “toggle switch” that may modulate the TM6 Pro-kink. Differential modulation of the accessibility of Cys6.47 and an engineered Cys6.52 in wild type and a constitutively active background provides experimental support for the association of this rotamer switch with receptor activation.

G-protein-coupled receptors (GPCRs) are comprised of an extracellular N terminus, seven transmembrane α-helical segments (TMs) connected by intracellular and extracellular loops, and a cytoplasmic C terminus. In the β2 adrenergic receptor (β2AR) and related rhodopsin-like GPCRs that bind small molecules, the binding site is formed among their seven TMs in a water-accessible binding site crevice (1). The transmembrane domain of these receptors, therefore, transduces the binding of ligands, such as biogenic amines, to the activation of intracellular G-proteins, which in turn mediate downstream signal transduction pathways. Even in peptide and glycoprotein hormone receptors, in which the binding site is formed, at least in part, by extracellular loops or by large N-terminal domains, respectively, agonist binding must still be transduced via the transmembrane segments to the intracellular G-protein-interacting regions of these receptors.

Understanding the function of GPCRs at a molecular level requires an understanding of how agonist binding to the receptor is converted into receptor activation (2). Studies based on electron paramagnetic resonance spectroscopy, fluorescence spectroscopy, alterations in cysteine accessibility, and engineering of metal-binding sites have altogether pointed to a key role for conformational changes of TM3 and TM6 in receptor activation (3–8). It has been suggested that protonation of the Asp in the highly conserved D/E/R/Y motif at the cytoplasmic side of TM3 leads to release of constraining intramolecular interactions, thereby resulting in movements of TM3 and TM6 and conversion of the receptor to the active state. This hypothesis has been supported by the observation that charge-neutralizing mutations of the AspGlu*4,49 in TM3 lead to increased agonist-independent activation of a number of GPCRs (7, 9–12).

Experiments using cysteine cross-linking and engineered metal ion-binding sites (3, 4, 13) suggest that residues at the cytoplasmic ends of TM3 and TM6 face each other. We proposed that in the inactive state Arg6.50, in addition to interacting with Asp3.49, also interacts with the conserved Glu6.30 at the cytoplasmic end of TM6, and that this interaction contributes to maintaining the receptor in the inactive state by holding together the cytoplasmic ends of TM3 and TM6. Our experimental data (14) together with the high-resolution structure of rhodopsin (15) suggest that ionic interactions between AspGlu*4,49, Arg6.50, and Glu6.30 may constitute a common switch governing the activation of many rhodopsin-like receptors. Disruption of the interaction between TM3 and TM6 produces constitutive receptor activation and the extent of constitutive activation is highly correlated with the extent of conformational rearrangement in TM6 (14).

Experimental and computational simulation studies indicate that conformational switches in transmembrane α-helices can be generated via Pro-containing motifs that form flexible molecular hinges (16). Although Farrens et al. (3) predicted that a rigid body motion of TM6 relative to TM3 was associated with the activation of rhodopsin, the movements crucial to activation may involve flexibility about the hinge formed by the highly conserved Pro in TM6 (Pro288*5.50 in β2AR) (17). In rhodopsin, and presumably in the β2AR, TM6 exists in a highly bent configuration, with its cytoplasmic end near to the cytoplasmic end of TM3 (15).

In the β2AR and related aminergic receptors, a cluster of
highly conserved aromatic residues surrounding the Pro-kink, including Phe6.44, Trp6.48, Phe6.51, and Phe6.52 in catecholamine receptors, face the binding-site crevice (reviewed in Ref. 1). Binding of agonist to a residue or residues in this “aromatic cluster” has been hypothesized to induce or stabilize an altered configuration of the side chains within this cluster that promotes receptor activation (17). Such a conformational rearrangement of the aromatic cluster may be associated with an alteration in the configuration of the TM6 Pro-kink and the subsequent movement of the cytoplasmic end of TM6 away from TM3.

In the β2AR the conserved endogenous Cys-2856.47, positioned i–3 from the conserved Pro-2856.50, becomes accessible to methanethiosulfonate ethylammonium (MTSEA) in the binding-site crevice in constitutively active mutants (CAMs) (6, 7, 14). Cys, Ser, and Thr can hydrogen bond (H-bond) to the backbone in an α-helix, and this interaction may impact significantly not only the local conformation but may also lead to long range conformational changes through bends and distortions generated in the helix backbone (18–20). The highly conserved Cys-2856.47 near the Pro-kink at 6.50 can H-bond to different backbone carbonyls in different rotamer positions, and its rotamer position may modulate the TM6 kink.

We hypothesized that the configuration of the TM6 Pro-kink in a GPCR would be correlated with constitutive activity and that both Cys6.47 and the aromatic cluster may impact this configuration. We tested these hypotheses in the β2AR by accessing the binding and activation properties of Cys6.47 mutants. The biased Monte Carlo technique of Conformational Memories was used to probe for correlations between the Cys6.47 rotation position and the configuration of the aromatic cluster in TM6, and the TM6 Pro-kink conformation. We carried out cysteine accessibility experiments to test a prediction that arose from the simulations, and the experimental results supported the existence of a critical rotamer “toggle switch” (17) associated with receptor activation.

**EXPERIMENTAL PROCEDURES**

**Numbering of Residue and Residue Index**

Residues are numbered according to their positions in the human β2AR sequence. We also index residues relative to the most conserved residue in the TM in which it is located (21). By definition, the most conserved residue is assigned the position index “50,” e.g. Pro2856.50 and therefore Leu-2876.48 and Phe-2896.51. This indexing simplifies the identification of aligned residues in different GPCRs. Mutants are named as WT residue, residue number, superscript index number, and mutant residue where the residues are given in single letter codes.

**Nomenclature of χ1 Rotamer**

Different nomenclatures have been used to describe the rotamers of side chain torsion angles. When the heavy atom at the γ position is at a position opposite to the backbone nitrogen, when viewed from β-carbon to α-carbon, we define the χ1 rotamer as in the trans (t) position; from the same viewing angle, when the heavy atom at the γ position is at a position opposite to the backbone carbon, we define the χ1 rotamer as in the gauche+ (g+) position; when the γ-carbon is opposite to the α-hydrogen, we define the χ1 rotamer as in the gauche− (g−) position.

**β2-Plasmids and Site-directed Mutagenesis**

The mutations were generated by the polymerase chain reaction-mediated mutagenesis using Pfu polymerase (Stratagene). The polymerase chain reaction-generated DNA fragments containing the mutations were subcloned into the appropriate plasmid. The mutations were identified initially through the presence of diagnostic restriction sites introduced via the mutated oligonucleotides and subsequently verified by DNA sequencing analysis of the polymerase chain reaction-generated fragments. For stable expression in HEK 293 cells, this cDNA was subcloned into the bicistronic expression vector pCIN4-SFβ2AR6His (22).

**Cell Culture and Transfection**

HEK 293 cells were maintained at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium/F-12 (1:1) containing 3.15 μg/ml of glucose in 10% bovine calf serum. The HEK 293 cells were transfected with 2 μg of the pCI4 constructs using the LipofectAMINE/Opti-MEM (Invitrogen) transfection system, and a stably transfected pool was selected with Geneticin (700 μg/ml) (6).

**Membrane Preparation and [3H]CGP-12177 Binding**

To increase the expression of the constitutively active mutant, a 10 μM concentration of the inverse agonist sotalol was added to the growth medium of HEK 293 cells stably expressing WT or mutant β2AR for 1–2 days. Membranes were prepared as described previously (14). Aliquots of diluted membrane suspension (200 μl) were incubated in binding buffer either with six different concentrations of the antagonist [3H]CGP-12177 between 40 and 1100 pM (in saturation binding experiments) or with increasing concentrations of the agonists in the presence of 100 pM [3H]CGP-12177 (in competition binding experiments). The total volume was adjusted to 1.0 ml, and the binding experiments and data analysis were performed as described previously (22, 23).

**cAMP Accumulation Assay**

HEK 293 cells stably expressing the WT β2AR or the mutants were plated in 96-well cell culture plates (pretreated with poly-l-lysine, 0.1 mg/ml) at a density of 40,000–60,000 cells/well. After incubation overnight at 37 °C in 5% CO2, the cells were 95–100% confluent. The medium was removed, and 100 μl of 0–60 μM N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in Opti-MEM (Invitrogen) was added in order to normalize the concentration of receptor (22). The concentration of EEDQ used for a particular experiment was adjusted based on the level of cell surface expression of the particular mutant, as higher expressing receptors such as WT required higher concentrations of EEDQ for appropriate normalization. The level of cell surface expression of WT and the mutants was determined by specific binding of the hydrophilic radioligand [3H]CGP-12177 at a concentration 10-fold higher than its Kd (0.9 nM). cAMP accumulation assay was performed as described previously (22).

**Reactions of F2906.52C Mutants with MTS Reagents**

Whole cells from a 35-mm dish were resuspended in 400 μl of buffer A. Aliquots (50 μl) of cell suspension were incubated with freshly prepared MTSEA (Toronto Research Biochemicals) at the stated concentrations at room temperature for 2 min. Cell suspensions were then diluted 20-fold, and 300-μl aliquots were used to assay for [3H]CGP-12177 (1.2 nM) binding in triplicate as described above. The fractional inhibition was calculated as I (specific binding after MTS reagent/ (specific binding without reagent).

**Rotamer Statistics of Cys/Ser/Thr of Membrane Proteins**

The distributions of the χ1 rotamer of Cys/Ser/Thr were calculated from all the high resolution (≤3 Å) membrane protein structures currently available. The structures were downloaded from the Protein Data Bank. The list and definitions of transmembrane α-helical segments were updated and modified using the Membrane Protein Topology data base (24). Cys/Ser/Thr in transmembrane α-helical segments were only included if at least 5 residues N-terminal to the identified position were within the α-helix. Only regions with a B-value ≤40 (25) and structures with a resolution ≤2 Å were included in the analysis. Our rationale was that a resolution ≤3 Å can differentiate whether or not a region is α-helical and B-values ≤40 of the Cys/Ser/Thr side chain atoms ensure that coordinate information was reliable.

**Conformational Memories**

The approach of Conformational Memories, which is a two-phase biased Monte Carlo simulation, was described previously (26). We used this algorithm, which was implemented in CHARMM (27), to probe the conformational space of TM6 of human β2AR.

**Initial Structures—**The template was TM6 (6.40–6.56) of chain A of the bovine rhodopsin structure (Protein Data Bank number 1H2X). The side chains of the residues were mutated to those of the aligned positions in the human β2AR. Four Ala residues in a standard α-helical conformation were added at the N-terminal and C-terminal. Thus, there are totally 25 residues in the system. The α-helix was capped by aceticamide at the N terminus and N-methylamide at the C terminus. χ1 of 6.47 (Cys/Ser/Thr) was manually placed in gauche+ (g+), gauche− (g−), or trans (t). χ1 of 6.48 and 6.52 were manually placed in g+ or t, and in
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Results

Potential Conformations and Backbone Interactions of Cys6.47—Cysteine residues in an α-helical context are restricted to the gauche+ (+g+) and trans (t) rotamer conformations (Table I), because the gauche− (−g−) configuration induces a sterically clash between the sulfur atom and the backbone carbonyl of the i−3 position (20). The rotamer distribution of these side chains in an α-helical context is quite similar in soluble and membrane proteins (Table I) and is consistent with the theoretical free energy calculated for different rotamers (29).

In the + rotamer, Cys6.47 can H-bond the backbone carbonyl of the i−4 residue, and this is the most common rotamer configuration for a Cys residue in an α-helix. In an α-helix, the cyclic side chain of proline prevents its H-bonding to the i−4 backbone carbonyl and also induces steric clash, thereby bending the helix. The resulting Pro-kink conformation also prevents H-bonding of the i+1 backbone amide to the i−3 backbone carbonyl (16, 30). Cys6.47 in i−3 from Pro6.50, and in the t rotamer, the backbone carbonyl of 6.47 can act as an H-bond acceptor for its own side chain. Thus, Cys6.47 can adopt two different rotamers, and each rotamer can interact with the TM6 backbone differently, with the potential to induce different conformations of the adjacent Pro-kink. Unlike Cys and Ser, which can populate the t rotamer, the β-branched Thr does not exist in t, because of steric clash between the side chain methyl and the backbone carbonyls at i−3 and i−4. We therefore mutated Cys-2856.47T to Thr to assess the effect of eliminating the t rotamer at this position, and to Ser as a control.

Pharmacological Characterization of Mutant Receptors—The affinity of the antagonist [3H]CGP-12177 was not significantly different in the mutants (C2856.47T and C2856.47S) and WT (Table II). C2856.47T had a 5-fold higher affinity for the agonist isoproterenol than did WT, whereas the affinity of C2856.47S was reduced 2-fold (Table II). In intact cells, the maximum level of [3H]CGP-12177 binding to C2856.47T was −20% of that of WT, whereas that of C2856.47S was −50% of WT (data not shown). After normalization of receptor number with the alkylation reagent EEDQ, the mean and standard error values for the EC50 of the agonist epinephrine in the accumulation of cAMP were 6.0 ± 1.2 (n = 3), 9.4 ± 1.2 (n = 3), and 1.6 ± 0.43 nm (n = 3) for WT, C2856.47S, and C2856.47T, respectively (Fig. 1). Thus, the potency of epinephrine was increased ~4-fold in C2856.47T. A higher potency for agonist activation and increased agonist affinity in competition experiments are features commonly associated with CAMs (31).

The Increased Basal cAMP Accumulation of C2856.47T Can Be Reduced by Inverse Agonist—We measured cAMP accumulation after attempting to equalize the number of receptors by inactivating WT and C2856.47S by pretreatment with EEDQ. Under these conditions, basal cAMP accumulation in C2856.47T was about 2-fold higher than in WT and 6-fold higher than in C2856.47S (Fig. 2A). In the presence of an appropriate concentration of forskolin, which directly activates adenylyl cyclase, we still observe an effect of receptor activation on cAMP accumulation because of the synergistic effects of forskolin and Gαs on adenylyl cyclase. The increase in cAMP produced by forskolin, however, raises "basal" levels to a level where our assay is more sensitive. Therefore, we also examined the effects of the mutations of 6.47 on cAMP accumulation in the presence of 10 μM forskolin. In the presence of forskolin the 2-fold elevation in cAMP in C2856.47T relative to WT and 6-fold relative to C2856.47S was also observed (Fig. 2B), consistent with the basal

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**Table I. Rotamer statistics of Cys/Ser/Thr within an α-helical context**

| Residue | All proteins (32) | Membrane proteinsa |
|---------|------------------|--------------------|
| Cys     |                  |                    |
| g+      | 75               | 69                 |
| g−      | 5                | 8                  |
| t       | 20               | 25                 |
| Ser     |                  |                    |
| g+      | 44               | 43                 |
| g−      | 33               | 36                 |
| t       | 22               | 21                 |
| Thr     |                  |                    |
| g+      | 75               | 71                 |
| g−      | 25               | 27                 |
| t       | 0                | 2                  |

a From 23 membrane protein structures, filtered by resolution <3 Å, B-value <40, and residue position at least 5 from the N terminus.
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Table II
Antagonist and agonist binding to WT βAR and the mutants

| Construct | [3H]CGP-12177 | Isoproterenol |
|-----------|---------------|---------------|
|           | $K_a$ | $n$ | $K_i$ | $n$ |
| WT        | 0.073 ± 0.012 | 4 | 71.3 ± 10.3 | 10 |
| C285T     | 0.081 ± 0.011 | 5 | 13.8 ± 1.8 | 6 |
| C285S     | 0.090 ± 0.012 | 4 | 146.0 ± 11.1 | 5 |

In contrast, this combination of rotamer conformations is tolerated without clash in a standard α-helix in a number of known proteins (data not shown). The clash between Trp6.48 and Phe6.52+ implies the following correlated motions: if Trp6.48→Phe6.52+ and if Phe6.52+→Trp6.48g+. Thus, as proposed by Visiers et al. (17), these two aromatic residues act as a toggle switch.

In our initial conformational searches, we found that particular starting conformation/rotamer positions significantly impacted upon the final distribution of distinct conformations. For example, if we started the conformational search from Trp6.48g+/Phe6.52t, >90% of the resulting structures contain Trp6.48g+/Phe6.52t (data not shown). This likely resulted from the favorable interaction between the aromatic side chains, the propensity for aromatic residues to be in t in α-helices (32), and the need for complex coordinated changes of multiple side chains and/or backbone angles for movement of these side chains. In contrast, if the search started from Trp6.48g+/Phe6.52g+, the position of these two side chains in the resulting structures was highly correlated, with either g+/g+ (59 and 79%) or t/t (34 and 9%), depending on the rotamer at 6.47 (g+ or t, respectively; see below).

In an attempt to minimize the impact of the starting structure, we started simulations with Trp6.48g+ and Phe6.52t in t, thereby breaking the correlation between the rotamers at these two positions. If we started from Trp6.48g+/Phe6.52t, a high percentage of the resulting structures leave the starting uncorrelated state and become correlated, either in t/t or g+/g+.

2 Although the lack of effect of timolol on the level of basal activity in WT and C285S was not surprising given their lack of substantial constitutive activity (see Ref. 14), it is somewhat surprising that the basal activity in the presence of timolol was higher in WT than in C285T or C285S. This might conceivably relate in part to the 2-fold higher expression level of WT after EEDQ treatment and/or to differences in cAMP levels in the different stable pools that were independent of β3 receptor expression.

We analyzed the relationship in the final structures between the 6.47 rotamer and the different rotamer combinations of 6.48 and 6.52 (Table III). There was a clear correlation between the rotamer position of Cys6.47 and the resulting rotamer conformation of 6.48. Specifically, when Cys6.47 was in $g^+$, 73% of 6.48 moved from the initial $g^+$ to $t$. In contrast, when 6.47 was in $t$, 87% of 6.48 remained in $g^+$ (Table III). Similarly, with Thr6.47 in $g^+$ or $g^-$, 73 and 67% of 6.48, respectively, moved from the initial $g^+$ to $t$. With Ser6.47 in $g^+$ or $g^-$, 61 and 68% of 6.48, respectively, moved from the initial $g^+$ to $t$, whereas with Ser6.47 in $t$, 70% of 6.48 remained in $g^+$. Because Thr does not exist in $t$, it is likely, therefore, that in the C285S6.47 mutant the Trp$S^{6.48}$ is significantly overrepresented in $t$ compared with its rotamer distribution with Cys or Ser at 6.47.

Modulation of the Pro-kink in TM6 by the $\chi_1$ Rotamer of 6.47, 6.48, and 6.52—The resulting simulation structures can be clustered into two major groups based on the rotamer conformations of 6.47, 6.48, and 6.52, one with Cys$S^{6.47}$, Trp$S^{6.48}$, and Phe$S^{6.52}$, and the other with Cys$S^{6.47}$, Trp$S^{6.48}$, and Phe$S^{6.52}$. These two clusters have significant differences in the parameters representing the global characteristics of the Pro-kink, which results primarily from the significant alteration in the backbone torsion angles of 6.47 and 6.48 (Table IV). Even a small local change in backbone resulting from the different rotamer combinations could have a large impact on the position of the cytoplasmic end of TM6.

Thr at 6.47 exists predominantly in the $g^+$ rotamer, and it does not exist in the $t$ rotamer. Because the Thr$S^{6.47}$ mutant shows an activated phenotype, the 6.47 $g^+$ conformation seems to favor and/ or mimic the active state. In contrast, Ser, which can populate the $t$ rotamer, showed a distribution of states similar to that of WT. Thus, we hypothesize that Cys$S^{6.47}$, Trp$S^{6.48}$, and Phe$S^{6.52}$ represents the inactive conformation of the receptor and that Cys$S^{6.47}$, Trp$S^{6.48}$, and Phe$S^{6.52}$ represents the active state (see “Discussion”).

MTSEA Reaction Rate of F290C in WT and CAM Background—Cys-285$S^{6.47}$ in $\beta_2$AR is responsible for the inhibitory effect of MTSEA on ligand binding in several $\beta_2$AR CAMs (6, 7, 14). We inferred that this increased accessibility of Cys6.47 to MTSEA resulted from a rotation and/or tilting of TM6 associated with receptor activation. Although a substantial backbone movement of TM6 around the Pro-kink might contribute to such a conformational rearrangement, it is also possible that a local change in the relative orientations of Cys6.47 and Trp6.48 may contribute to the difference in the accessibility of Cys6.47 in the inactive and active states. In the simulations we noted an interaction between the side chain of Cys6.47 in $g^+$ and that of Trp6.48 in $g^+$ (Fig. 3B). In the putative inactive state, this interaction may impact on the dielectric environment of Cys6.47, making it more hydrophobic and likely decreasing the ionization and therefore the reactivity of the thiol. Furthermore, the bulky Trp6.48 side chain may also sterically block MTSEA from accessing Cys6.47. In the putative active state, when Trp6.48 is in the $t$ position, the Trp side chain would be much farther from Cys6.47, and therefore Cys6.47 would be more reactive with MTSEA because of the removal of the factors described above (Fig. 3B).
In considering ways to test this hypothesis, we noted that a Cys residue at position 6.52 would be able to have a similar stabilizing interaction with Trp$^{6.48}$ in the t conformation (Fig. 3C). Thus, the F290$^{6.52}C$ mutant would have an activation-dependent MTSEA accessibility phenotype opposite to that of WT Cys$^{6.47}$. That is, in the active state, Trp$^{6.48}$ in t would be expected to interact with Cys$^{6.52}$, thereby decreasing its reactivity with MTSEA. Thus, we predicted that F290$^{6.52}C$ would react with MTSEA more rapidly in the WT background than in a CAM background. To test this prediction, we made the mutants F290$^{6.52}C$ in WT and F290$^{6.52}C$ in a well characterized CAM background. To test this prediction, we made the mutants F290$^{6.52}C$ in WT and F290$^{6.52}C$ in a well characterized CAM background that contains several mutations at the cytoplasmic end of TM6 (31). The binding affinity of these mutants for $[^3H]$CGP12177 was significantly lower than that of WT (31). The aromatic stacking interactions between Trp$^{6.48}$ and Phe$^{6.52}$ may stabilize the g$^+/g^+$ and t/t rotamer configurations. These favorable interactions between 6.48 and 6.52 are facilitated by the presence of the Pro-kink, which brings these two residues closer to each other, altering the relationship between these two side chains from what would be much less favorable interactions in a standard a-helix (data not shown).

We also found that the rotamer conformation of 6.47 was significantly correlated with the rotamer of 6.48, making 6.47 a critical part of the rotamer toggle switch. Specifically, 6.47 in g$^+$ was associated with 6.48 in t, and 6.47 in t was associated with 6.48 in g$^+$ (Table III). We propose that the impact of mutation of 6.47 to Thr on activation results from both altered H-bonding as well as modulation by the 6.47 side chain of the rotamer toggle switch. Thus, we propose that Cys$^{6.47}C$/Trp$^{6.48}C/Ph$^{6.52}C$ represents the active state and that Cys$^{6.47}T/Trp^{6.48}g^+/Phe^{6.52}C$ represents the inactive state. With Trp$^{6.48}$ in t in the active state, Phe$^{6.52}$ must also be in t to avoid steric clash. In contrast, as discussed above, with Trp$^{6.48}$ in g$^+$ in the inactive state, although there is an increased propensity for 6.52 to be in g$^+$, 6.52 may also exist in t, suggesting the possibility of a diversity of inactive states, with the rotamer positions of 6.47 and 6.48 being more critical to its phenotype.

This hypothesis is consistent with a number of experimental results in rhodopsin. Movement of the side chain of Trp$^{6.48}$ from g$^+$ to t upon activation is consistent with the inference from UV absorption spectrometry in rhodopsin that the indole side chain of Trp-265$^{6.48}$ changes orientation during the inactive to active conformational transition (34) as well as with data suggesting that a single Trp tilts toward the membrane during activation (35). Although 6.52 is an alanine in rhodopsin, in the inactive rhodopsin structure the $\beta$-ionone ring and carbon chain of retinal interact with 6.48 in g$^+$ in a configuration very similar to that of the interaction of 6.48 in g$^+$ with Phe$^{6.52}$ in g$^+$ in the $\beta$AR (Fig. 5). Photoisomerization of retinal from cis to trans moves the $\beta$-ionone ring toward TM4 (36) and thus away from Trp$^{6.48}$, whose enhanced freedom and subsequent rearrangement is involved in the activation mechanism of rhodopsin. The

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**Table IV**

**Structural effects of different rotamer combinations of 6.47, 6.48, and 6.52**

| x1 of 6.47/6.48/6.52 | $t^g$/$g$ (inactive) | $g^+$/$t^+$ (active) |
|----------------------|----------------------|----------------------|
| Mean $\phi$          | -74.1                | -80.1                |
| Mean $\psi$          | -53.8                | -45.8                |
| Bend angle           | 18.3                 | 24.8                 |
| Wobble angle         | -100.1               | -84.0                |
| Face shift           | -57.8                | -94.7                |

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**Fig. 4.** Rate of reaction of F$^{6.52}C$ in the WT background (open circle) and F$^{6.52}C$ in a CAM background (filled circle) with MTSEA. Specific binding was assayed as described under "Experimental Procedures" after a 2-min incubation with the indicated concentration of MTSEA. The mean ± S.E. are shown (n = 4 for WT-F290$^{6.52}C$; n = 5 for CAM-C285$^{6.47}S$/F290$^{6.52}C$).
exquisite lack of constitutive activity of rhodopsin may result from the inability of 6.48 to move to the t rotamer when con-strained by bound 11-cis-retinal. For GPCRs, such as the β2AR, which are activated by diffusible ligands, a ligand that stabi-lizes Trp6.48 in the g+ conformation would, therefore, behave as an inverse agonist. One such mechanism of inverse agonism may involve forcing or stabilizing Phe6.52 into the g+ rotamer conformation, which would in turn force Trp6.48 into the g+ conformation, and thereby promote the inactive state. Con-versely, an agonist that stabilizes Phe6.52 in the t rotamer conformation would free Trp6.48 to adopt the t conformation, and thereby promote the active state.

FTIR difference spectroscopy has been used to study the role of cysteine residues in the photoactivation of rhodopsin (37). At least one cysteine sulfhydryl was inferred to be structurally active during rhodopsin photoactivation and to form a stronger hydrogen bond in the activated state. The transmembrane cysteines proposed as candidates are Cys-1674.56, Cys-2225.57, and Cys-2646.47. Movement of Cys6.47 into the g+ rotamer and the associated formation of a strong H-bond with the i+4 car-bonyl could be responsible for the spectroscopic observations. In the high-resolution structure of rhodopsin, which represents the average of its conformations, the χ1 of 6.48 is in g+ and the χ1 of Cys-2646.47 is in a nonstandard rotamer position between g+ and t. The modification of Cys-2646.47 by mercury may have affected its rotamer position. It is also possible that the Cys6.47 side chain may be dynamic, with its coordinates representing the average of its g+ and t rotameric conformations.

Based on the proximity of the side chain of Cys6.47 in t and Trp6.48 in g+ in the β2AR simulations, we propose the existence of an interaction between Sy of Cys6.47 and the Ne of Trp6.48 (Fig. 3B). This configuration is consistent with the favored orientation of interaction between Cys and Trp in the Residue Contact Atlas (39). The sulfur-aromatic interaction has been characterized as an intermediate interaction between a purely van der Waals interaction and a H-bond (40). In the inactive state of rhodopsin, the side chain of Trp2655.48 forms a H-bond with another residue (34). In the bovine rhodopsin structure (15), which represents the inactive state of receptor, Cys-2646.47 appears to be the only residue that could form a H-bond-like interaction with the indole side chain of Trp-2655.48. Receptor activation alters tertiary interactions and weakens this H-bond (34). We propose that the interaction between Cys6.47t and Trp6.48g+ in the inactive state accounts for the H-bond feature of Trp-2655.48 that is lost during the activation process.

In our simulations of the β2AR, Cys6.47 in t is positioned at an appropriate distance to H-bond to its own backbone car-bonyl, i−3 from Pro6.50. H-bonding of the Cys6.47 side chain to its own carbonyl may contribute to the altered ϕ angles at 6.47 and 6.48, thereby altering the Pro-kink conformation (Table IV, Fig. 3A) and repositioning the cytoplasmic end of TM6 away from TM3 upon activation. Therefore, Cys6.47, through the interaction of its Sy with the indole Ne of Trp6.48 and the H-bond of its side chain to its own carbonyl, may link the conformation of the aromatic cluster with the conformation of the Pro-kink, thereby playing a critical role in the mechanism of receptor activation.

This local network of interactions may underlie the observed correlations between the rotamer conformations of Cys6.47 and Trp6.48. The conformational changes associated with receptor activation must involve a change in interhelical interactions. Interestingly, in the high-resolution structure of bovine rho-dopsin, there is a water molecule in the cavity surrounded by Cys6.47, Tyr6.51, Pro6.50, Pro7.41, Phe7.43, and Ala7.42 (38). Okada et al. (38) have hypothesized that electrostatic interactions between TM6 and TM7 through this bound water may stabilize the inactive state, and it is further possible that alterations of these interactions may facilitate the movements associated with activation.

The activation process of rhodopsin has also been found to produce bulk dielectric changes surrounding Trp (34). The proximity of the bulky aromatic side chain of Trp might sub-stancially decrease the accessibility of Cys6.47 in the inactive state, both by decreasing the local dielectric and decreasing the ionization of the sulfhydryl as well as through direct steric block. This is consistent with the observation from our laboratory that Cys6.47 is unreactive with MTSEA in the inactive state (6). In the proposed active state, Cys6.47 and Trp6.48 are substantially further apart, consistent with the observed reac-tivity of Cys6.47 with MTSEA in the CAM background (6, 7, 14). Based on our simulations and our clustering of the simulated structures into what we inferred to be inactive and active configurations, we hypothesized that in the active state, with 6.48 in the t rotamer, a cysteine substituted for 6.52 might be shielded from reaction with MTSEA, much like Cys6.47 is in the inactive state. We carried out this experiment and found that F2906.52C was ~5-fold less reactive in the CAM background than in the WT background (Figs. 3C and 4), consistent with our predictions. This provides experimental support for the proposed inactive and active configurations and for the existence of a rotamer toggle switch. Thus, with Trp6.48 in the inactive state (g+), Cys6.47 is less accessible and with 6.48 in the active state (t) Cys6.52 is less accessible. In addition, these data are significant because they demonstrate for the first time that in a CAM particular positions are less reactive with MTSEA, thereby arguing against a simple model in which the
CAM is simply a less stable, more dynamic molecule.

It is important to note that our simulations are performed with an isolated TM6 out of the context of the helical bundle and lipid. It is clear that studying a helix in isolation ignores known constraining interactions with other TMs, and is clearly inadequate to predict the global configuration of TM6. For this reason, we think it wise not to overinterpret the exact Pro-kink conformation of the TM6 Pro-kink are generally involved in GPCR Cys6.47, Trp6.48, and Pro 6.50 are highly conserved and 6.52 is receptor activation. Because in rhodopsin-like receptors may modulate the movement of TM6 about the Pro-kink during activation.

ulation of the TM6 Pro-kink are generally involved in GPCR above), it is likely that the rotamer toggle switch and its mod-
tations, and mutations like those presented here can be designed to test the validity of these hypotheses. In summary, both our experimental findings and the literature, it appears that our experimental findings and the literature, it appears that the hypothesis that coordinated rotamer changes of 6.47, 6.48, and 6.52 appear to be associated with receptor activation. This rotamer toggle switch may impact the α-helix backbone and may modulate the movement of TM6 about the Pro-kink during receptor activation. Because in rhodopsin-like receptors Cys6.47, Trp6.48, and Pro6.50 are highly conserved and 6.52 is conserved as a bulky residue (except in rhodopsin as discussed above), it is likely that the rotamer toggle switch and its modulation of the TM6 Pro-kink are generally involved in GPCR activation.

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