Ligand Stabilization of the $\beta_2$ Adrenergic Receptor: Effect of DTT on Receptor Conformation Monitored by Circular Dichroism and Fluorescence Spectroscopy†

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ABSTRACT: Treatment of the $\beta_2$ adrenergic receptor with the reducing agent dithiothreitol (DTT) is known to abolish ligand binding to the receptor. Interestingly, the loss of binding can be prevented by preoccupation of the receptor with ligand. It is unclear, however, whether the ligand blocks access of DTT to the receptor, or the ligand stabilizes the receptor structure. In the present study, we have utilized circular dichroism (CD) and intrinsic tryptophan fluorescence to directly probe structural changes in the $\beta_2$ adrenergic receptor in response to DTT treatment. Analysis of CD spectra of purified $\beta_2$ receptor in the detergent micelle indicated that the receptor has an $\alpha$-helix content of 60%, which is substantially more than what would be attributed to the seven transmembrane domains. The $\alpha$-helix content was unchanged in the presence of DTT, suggesting that DTT treatment does not alter the secondary structure of the receptor. In contrast, the tryptophan fluorescence spectra demonstrated that DTT induces a reversible conformational change of the $\beta_2$ receptor. Thus, DTT caused a red-shift in the maximum emission wavelength of the intrinsic tryptophan fluorescence. The change in emission spectrum correlated with a loss in the ability of the receptor to bind antagonist. Both changes in receptor binding and fluorescence emission were reversible, as removal of DTT allowed the receptor to restore 70% of ligand binding and return to the initial emission spectrum. Furthermore, we found adrenergic antagonists were able to slow the rate of the conformational change induced by DTT but not the rate of disulfide reduction, suggesting that the antagonists stabilize the structure of the reduced receptor.

The $\beta_2$ adrenergic receptor belongs to a large family of G-protein-coupled receptors (Strader et al., 1994; Hein & Kobilka, 1995). Like other G-protein-coupled receptors, the $\beta_2$ receptor consists of seven hydrophobic membrane-spanning domains connected by hydrophilic loops, an extracellular amino terminus, and an intracellular carboxy terminus (Figure 1). Mutagenesis studies have revealed that several residues, which are highly conserved among members of the G-protein-coupled receptor family, possess important structural and functional roles (Kobilka, 1992; Savarese & Fraser, 1992; Strader et al., 1994). Noticeably, a pair of cysteines in the first and second extracellular loop is highly conserved and is believed to form a structurally important disulfide bridge(s) (Karnik et al., 1988; Fraser, 1989; Dohlman et al., 1990; Savarese et al., 1992). Thus, mutation of the cysteines and/or exposure of the receptor to reducing agents markedly impair the function of rhodopsin, muscarinic acetylcholine, and $\beta_2$ adrenergic receptors (Fraser, 1989; Dohlman et al., 1990; Karnik & Khorana, 1990; Kurtenbach...
et al., 1990). Of interest, bound ligand can protect the receptor from the reducing effects of dithiothreitol (DTT) (Vaquelin et al., 1979; Wright & Drummond, 1983; Dohlman et al., 1990). It is unclear, however, whether the ligand blocks access of DTT to the disulfide(s), or it stabilizes the structure of the reduced receptor.

Although mutagenesis studies have provided valuable information about the role of specific amino acids in ligand binding and G-protein coupling (Suryanarayana & Kobilka, 1993; Strader et al., 1989, 1994; Hein & Kobilka, 1995), biochemical and biophysical approaches are needed to directly probe the structure of G-protein-coupled receptors and structural changes involved in receptor activation. Recently we demonstrated the potential for using fluorescent techniques to study conformational changes in a G-protein-coupled receptor by labeling the purified β2 receptor with an environmentally sensitive, cysteine-reactive fluorophore (Gether et al., 1995). In the present study, we utilized circular dichroism (CD) spectroscopy and intrinsic tryptophan fluorescence to assess the conformational changes of the purified β2 receptor. CD and intrinsic fluorescence spectroscopy have the advantage that they do not require chemical modification of the protein. CD spectroscopy is a useful tool for evaluating the secondary structure of proteins whereas the fluorescence of endogenous tryptophan residues is sensitive to their local molecular environment. Because there are 8 tryptophan molecules throughout the 397 amino acid sequence of the β2 receptor (Figure 1), the tryptophan fluorescence can be used to probe conformational changes of the purified β2 receptor. CD and intrinsic fluorescence spectroscopy have the advantage that they do not require chemical modification of the protein. CD spectroscopy is a useful tool for evaluating the secondary structure of proteins whereas the fluorescence of endogenous tryptophan residues is sensitive to their local molecular environment.

Materials and Methods

Materials. The baculovirus expression vector pVL1392 was obtained from Invitrogen (San Diego, CA). SF-9 insect cells, SF 900 II medium, and gentamicin were purchased from Gibco (Grand Island, NY). Fetal calf serum was from Gemini Bio-Products (Calabasas, CA). n-Dodecyl β-d-maltoside (C₁₂M) was purchased from CalBiochem (La Jolla, CA). 

Chelating Sepharose fast flow and Sephadex G-50 Medium were from Pharmacia (Uppsala, Sweden). Anti-FLAG M1 affinity gel was purchased from IBI (New Haven, CT). Bio-Rad DC protein assay kit was purchased from Bio-Rad (Hercules, CA). [²H]Dihydroalprenolol ([²H]DHA, 95 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). Antagonists ICI 118551 and alprenolol were from RBI (Natick, MA). [¹⁴C]Iodoacetamide (21.5 mCi/mmol) was from Du Pont NEN (Wilmington, DE). DTT, guanidine hydrochloride, and other chemicals were from Sigma (St. Louis, MO).

Expression and Purification of the β2 Adrenergic Receptor. DNA sequences encoding the human β2 adrenergic receptor, epitope-tagged at the amino terminus with the cleavable influenza–hemagglutinin signal sequence followed by the “FLAG”-epitope, and tagged at the carboxy terminus with six histidines, were cloned into the baculovirus expression vector pVL1392, and expressed in SF-9 insect cells according to previously described methods (Guan et al., 1992). The cells were routinely grown at 27°C in SF 900 II medium containing 5% fetal calf serum and 0.1 mg/mL gentamicin. For receptor purification, 1 L cell cultures were infected with a 1:(30–40) dilution of a high titer virus stock at a density of (5–6) × 10⁶ cells/mL and harvested after 48 h. The receptor was purified to homogeneity using a three-step purification procedure as described (Kobilka, 1995).

Radioligand Binding Assay. Purified β2 receptor (~1 nM) was incubated in triplicate with a saturating concentration of [¹⁴C]DHA (10 nM) in a total volume of 100 µL of C₁₂M-Tris buffer (100 mM NaCl, 0.05% C₁₂M, and 20 mM Tris pH 7.4) for 1 h at room temperature. The binding was stopped and free [¹⁴C]DHA separated from bound by desalting on a Sephadex G-50 Medium column (4 cm × 0.5 cm) using ice-cold C₁₂M-Tris buffer. Nonspecific binding was determined by radioligand binding (see below) and protein determination using a Bio-Rad DC protein assay kit.

DTT and Guanidine Treatment of the β2 Receptor. Purified receptor (1–3 µM) or t-tryptophan (12 µM) was incubated in C₁₂M-Tris buffer containing 0–10 mM DTT for 0–2 h at room temperature before spectra were recorded. In some experiments, excess DTT was removed from DTT-treated receptors by dialyzing against C₁₂M-Tris buffer (3 × 1 L) for 24 h at 4°C. The reversibility of DTT effects was examined by analyzing the emission spectra of DTT-treated receptors and radioligand binding before and after dialysis. The purified β2 receptor was also denatured by incubation with 6 M guanidine hydrochloride and 10 mM DTT for 2 h at 37°C. The effect of ligand on the DTT-induced conformational change was examined by preincubation of 1 µM receptor with 2 µM antagonists ICI 118551 or alprenolol for 2 h at room temperature prior to DTT treatment.

To investigate whether antagonist blocks access of DTT to disulfide(s), 16 µM purified receptor was treated with 100 molar excess of iodoacetamide for 1 h at room temperature to block free cysteines. After removal of excess iodoacetamide by two sequential gel filtrations on a 1 mL Sephadex G50 spin column, alkylated receptors were pretreated with or without 2 molar excess of ICI 118551 for 2 h at room temperature before incubation with 10 mM DTT for 0–1 h. At the indicated time intervals, aliquots of samples (0.1 mL) were separated from unreacted DTT by gel filtration as above, and 100 molar excess of [¹⁴C]Iodoacetamide (4.9 mCi/mmol) was added to label DTT-reduced cysteines for 1 h at
room temperature. The bound [14C]iodoacetamide was separated from unreacted free [14C]iodoacetamide by gel filtration as above. Calculations of the stoichiometry of iodoacetamide binding were based on the known specific activity of the [14C]iodoacetamide and the concentrations of the receptor determined by ligand binding.

**Fluorescence Spectroscopy.** Fluorescence spectroscopy was performed at room temperature on a SPEX Fluoromax spectrofluorometer (SPEX, NJ) with the photon counting mode. Both excitation and emission bandpaths were set at 4.2 nm to minimize photobleaching. Fluorescence spectra of the β2 receptor samples (1–1.5 µM in 400–500 µL of C12M—Tris buffer) were recorded from 310 to 400 nm with excitation at 295 nm to minimize the contribution of tyrosine fluorescence (Lakowicz, 1983). Spectra were typically scanned using 0.5-nm intervals with an integration time of 0.5 s/0.5 nm. Microcuvettes of 0.5 cm × 0.5 cm were used to reduce the inner filter effect. Spectra were corrected for background fluorescence from buffer, DTT, and ligand in all experiments (usually negligible). All samples had an absorbance of less than 0.05 at 295 and 330 nm; thus, correction for the inner filter effect was unnecessary.

**Circular Dichroism.** Purified β2 receptor was prepared for CD analysis by dialyzing the receptor against CD buffer (0.05% C12M, 5 mM phosphate, pH 7.4). The receptor concentration was 2 µM with a specific activity of 15 nmol/mg of protein. The total concentration of amide bonds of the receptor was determined from the product of protein concentration and total amino acid residues of 427. DTT- or guanidine-treated receptor samples were desalted to remove excess reagents just before CD spectra were recorded. To study the effect of agonist or antagonist on the secondary structure of the protein, the CD spectra of purified receptor were taken before and after incubation of the protein with 2–4 molar excess of isoproterenol or alprenolol for 3 h at room temperature. Buffer controls with or without ligand, DTT, and guanidine were prepared in parallel with the receptor preparation. Spectra were recorded at 25 °C using an AVIV 60DS spectropolarimeter (AVIV Associates, Inc., Lakewood, NJ). The CD of each sample was measured in 0.1-cm path length cells from 260 to 192 nm with a total absorbance less than 1.0 to ensure sufficient light transmission. The data were collected at 0.2-nm intervals, and five spectra for each sample were averaged and corrected by subtraction of each corresponding buffer spectra. α-Helix contents were analyzed assuming the secondary structure of the receptor was predominantly α-helices with some random coils (Chen et al., 1974; Scholtz et al., 1991).

**RESULTS**

**Secondary Structure of the Purified β2 Adrenergic Receptor.** The primary amino acid sequence and proposed membrane topology for the human β2 adrenergic receptor are shown in Figure 1. We expressed the human β2 adrenergic receptor in Sf-9 insect cells, and were able to purify sufficient quantity of receptor in the C12M micelle for CD spectroscopy studies (200–300 pmol/CD spectrum). The CD spectra of β2 receptors are shown in Figure 2. The
The spectrum of native $\beta_2$ is typical of a protein with a high degree of $\alpha$-helical secondary structure, showing minima at both 209 and 222 nm (Figure 2, trace a). The $\alpha$-helix content of the $\beta_2$ receptor was estimated to be 60% (Figure 2, insert), assuming the secondary structure of the $\beta_2$ receptor, like rhodopsin and m2 muscarinic acetylcholine receptor, is composed of mainly $\alpha$-helices and random coils (Park et al., 1992; Peterson et al., 1995). The agonist- or antagonist-bound receptor exhibits similar CD spectra (not shown), suggesting that the secondary structure of the protein is not changed upon ligand binding. When the receptor was treated with 10 mM DTT, it showed a similar spectrum (Figure 2, trace b, insert) indicating that the secondary structure of the receptor is not perturbed. In contrast, harsher denaturing conditions with guanidine and DTT treatment drastically change the CD spectrum; however, the receptor still retains about 35% $\alpha$-helix content (Figure 2, trace c, insert).

**Effects of DTT on the Tertiary Structure of the $\beta_2$ Receptor.**

To examine the effect of DTT on the $\beta_2$ receptor conformation, we monitored the intrinsic tryptophan fluorescence. The human $\beta_2$ adrenergic receptor having eight tryptophan residues (Figure 1) showed an intrinsic fluorescence spectrum with a maximum emission wavelength ($\lambda_{\text{max}}$) centered around 329 nm when excited at 295 nm (Figure 3, trace a). The $\lambda_{\text{max}}$ of a comparable concentration of $L$-tryptophan ($L$-Trp) in C$_{12}$M-Tris buffer (trace a), $\beta_2$ receptor treated with 10 mM DTT and 6 M guanidine hydrochloride for 2 h at 37 °C (trace c). The CD spectra were collected as described under Materials and Methods. Spectra represent the average of five scans expressed as molar ellipticity ($\theta_{\text{m}}$) in units of deg cm$^2$ dmol$^{-1}$. Insert: $\alpha$-helix contents of the $\beta_2$ receptor estimated from the corresponding CD spectra.

At concentrations higher than 20 mM, DTT has an absorbance greater than 0.05 at 295 nm. Thus, 10 mM DTT was used in subsequent experiments to minimize the inner filter effect. If the $\beta_2$ receptor was treated by the chemical denaturant guanidine hydrochloride in the presence of DTT for 2 h at 37 °C, the emission maximum was further red-shifted to 336 nm with a significantly decreased fluorescence intensity (Figure 3, trace c). The attenuated fluorescence signal and a 6-nm red-shift in the $\lambda_{\text{max}}$ suggest that, in the guanidine-denatured receptor, tryptophan residues are in an even more hydrophilic environment compared with those in native or DTT-treated receptors.

The reversibility of the DTT-induced $\lambda_{\text{max}}$ red-shift in the $\beta_2$ receptor was examined by removing excess DTT following DTT treatment. After 24 h dialysis, DTT-treated receptor displayed a spectrum of native $\beta_2$ receptor (1 µM) in C$_{12}$M-Tris buffer (trace a), $\beta_2$ receptor treated with 10 mM DTT for 1 h at room temperature (trace b), and $\beta_2$ receptor treated with 10 mM DTT and 6 M guanidine hydrochloride for 2 h at 37 °C (trace c). Lower panel: fluorescence emission spectra of 12 µM $L$-tryptophan ($L$-Trp) in C$_{12}$M-Tris (trace d), and $L$-Trp treated with 10 mM DTT for 1 h at room temperature (trace e). Spectra were obtained at room temperature with an excitation wavelength at 295 nm. Shown are representative spectra from at least four separate experiments. The $\lambda_{\text{max}}$ values of each sample are listed. Insert: Dose response of the DTT-induced $\lambda_{\text{max}}$ shift of the $\beta_2$ receptor. The $\lambda_{\text{max}}$ of emission spectra of the $\beta_2$ receptor (1 µM) was recorded after the receptor was incubated with various concentrations of DTT for 45 min at room temperature. $\Delta\lambda_{\text{max}}$ is calculated from the difference between the $\lambda_{\text{max}}$ of DTT-treated samples and control. $\Delta\lambda_{\text{max}}$ is shown as mean ± SD of three separate experiments.
Spectroscopy Studies of the $\beta_2$ Adrenergic Receptor

Table 1: DTT-Induced Reversible Shift in the $\lambda_{\text{max}}$ of Tryptophan Fluorescence and Loss of Antagonist Binding in the Purified $\beta_2$ Adrenergic Receptor

<table>
<thead>
<tr>
<th></th>
<th>$\Delta \lambda_{\text{max}}$ (nm) $^a$</th>
<th>$[^3]$H[DHA] (%) $^b$</th>
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<tr>
<td></td>
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<tr>
<td>pre-dialysis</td>
<td></td>
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</tr>
<tr>
<td>$\beta_2$</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>$\beta_2 + \text{DTT}$</td>
<td>3.5 $\pm$ 0.3</td>
<td>12.5 $\pm$ 4.1</td>
</tr>
<tr>
<td>$\beta_2 + \text{DTT} + \text{GdnHCl}$</td>
<td>5.7 $\pm$ 0.5</td>
<td>$&lt;1$</td>
</tr>
<tr>
<td>post-dialysis$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>DTT-treated $\beta_2$</td>
<td>1.2 $\pm$ 0.2</td>
<td>70.2 $\pm$ 9.8</td>
</tr>
<tr>
<td>DTT-treated $\beta_2 + \text{DTT}^*$</td>
<td>3.2 $\pm$ 0.5</td>
<td>15.5 $\pm$ 5.1</td>
</tr>
</tbody>
</table>

$^a$ DTT- or guanidine-induced $\lambda_{\text{max}}$ shift of tryptophan fluorescence in the purified $\beta_2$ receptor was expressed as $\Delta \lambda_{\text{max}}$, the difference of $\lambda_{\text{max}}$ between treated receptor samples and controls. Values are mean $\pm$ SD of $\Delta \lambda_{\text{max}}$ from 2–6 separate experiments. $^b$ Binding activity of the purified $\beta_2$ receptor was measured by incubating receptor with a saturating concentration of $[^3]$H[DHA] as described under Materials and Methods, and expressed as percent of control binding. $^c$ Control or DTT-treated $\beta_2$ receptor was dialyzed against C$_{12}$M–Tris buffer for 24 h at 4 °C, except $^*$, which was dialyzed against the buffer containing 10 mM DTT.

Figure 4: DTT-induced red-shift in $\lambda_{\text{max}}$ and inhibition of antagonist binding in the $\beta_2$ receptor are reversible. (A) Fluorescence emission spectra of 1 nM purified, untreated $\beta_2$ receptor after 24 h dialysis against C$_{12}$M–Tris buffer at 4 °C (solid line), and DTT-treated $\beta_2$ dialyzed against C$_{12}$M–Tris buffer for 24 h at 4 °C (dotted line) or against C$_{12}$M–Tris buffer containing 10 mM DTT (broken line). The conditions for recording spectra were the same as described in Figure 3. The spectra are representative of three separate experiments. (B) Time course of recovery of binding activity of DTT-treated $\beta_2$ receptor. The purified receptor was treated with 10 mM DTT for 2 h at room temperature followed by removal of excess DTT through dialysis. Binding activity of the receptor was measured at the indicated time intervals by incubating receptor samples with saturating concentrations of $[^3]$H[DHA], and expressed as a percent of control, untreated receptor binding. The values shown are the mean $\pm$ SD of four separate experiments.

Figure 5: Effect of antagonist on stabilizing the DTT-induced $\lambda_{\text{max}}$ shift of the $\beta_2$ receptor. The $\lambda_{\text{max}}$ of emission spectra of $\beta_2$ receptor was recorded after the receptor (1 µM) was treated with 10 mM DTT (open circles) or preincubated with 2 µM antagonist ICI 118551 for 1 h prior to DTT treatment (closed circles). Experiments were carried out as described under Materials and Methods and in Figure 3. $\Delta \lambda_{\text{max}}$ was calculated from the difference between the $\lambda_{\text{max}}$ of DTT-treated samples and control. Data are the mean $\pm$ SD of three separate experiments.

Ligand Stabilization of the DTT-Induced Conformational Change. The effect of ligand on the DTT-induced conformational changes of the $\beta_2$ receptor was investigated by monitoring the emission of the DTT-treated receptors in the presence or absence of an antagonist. The purified $\beta_2$ receptor was first incubated with antagonist before it was exposed to DTT. As shown in Figure 5, the rate of $\lambda_{\text{max}}$ red-shift induced by DTT was significantly slower when the receptor was preincubated with the antagonist ICI 118551 (Figure 5). The antagonist by itself did not induce a detectable change of $\lambda_{\text{max}}$. A similar effect was observed with the antagonist alprenolol (data not shown). Thus, antagonists stabilized the receptor from DTT-induced conformational changes. The effect of agonists could not be tested because agonists, which all share the catechol ring, emit considerably at the range of tryptophan fluorescence.

The ligand protection of the DTT-induced conformational changes of the receptor was further investigated. One possible mechanism is that a ligand blocks access of DTT to the putative disulfide(s) either directly or through an induced change in the structure of the receptor (Dohlman et al., 1990). If this mechanism were true, ligand binding would block the access of DTT to disulfide bond(s) such that the rate of DTT reduction would be slower in the presence of an antagonist. The following experiment was designed to assess the rate of DTT reduction of cysteines. The purified $\beta_2$ receptor was first treated with iodoacetamide to block free sulfhydryls. The alkylation of the receptor did not affect ligand binding (data not shown), agreeing with previous results (Dohlman et al., 1990; Gether et al., 1995). The alkylated receptor was then treated with or without ICI treatment decreased the binding of the receptor to antagonist $[^3]$H[DHA] to about 15% of the control (Figure 4B, 0 h). When DTT was removed, the ability of the receptor to bind antagonist was gradually restored to about 70% after incubation for 24 h at 4 °C (Figure 4B). In agreement, previous experiments have shown that addition of hydrogen peroxide, an oxidizing agent, to DTT-treated receptor partially restored ligand binding (30%) (Wright & Drummond, 1983; Dohlman et al., 1990). Removal of DTT slowly by dialysis may be more effective at restoring the correct disulfide bonds than rapid oxidation by H$_2$O$_2$. Table 1 summarizes the reversible effects of DTT on the structure and function of the receptor.
### DISCUSSION

The $\beta_2$ adrenergic receptor is one of the best characterized G-protein-coupled receptors. Many studies employing genetic and molecular biology techniques have advanced our understanding about specific amino acids involved in ligand binding and the orientation of the transmembrane domains (Strader et al., 1994; Hein & Kobilka, 1995; Mizobe et al., 1996). In comparison, only a few biophysical or structural studies have been conducted to directly assess the receptor structure and ligand binding sites. Strader and co-workers studied the quenching of the receptor-bound fluorescent antagonist carazolol, and provided direct biophysical evidence that the antagonist resides in a constrained, hydrophobic pocket that is deeply buried into the core of the $\beta_2$ adrenergic receptor (Tota & Strader, 1990). Recently we described ligand-specific conformational changes of the $\beta_2$ adrenergic receptor following ligand binding by labeling the purified $\beta_2$ receptor with a cysteine-reactive fluorophore (Gether et al., 1995). In this study, we used CD and intrinsic tryptophan fluorescence to monitor structural changes following removal of guanidine prior to or during CD analysis. By constructing mutant receptors with a limited number of tryptophan residues, it should be possible to determine which tryptophan(s) is (are) responsible for the observed spectral change. However, Trp residues (Trp$^{105}$, Trp$^{109}$, and Trp$^{175}$) that are at the boundary of the putative membrane domains and in proximity to the postulated disulfide bridges (Figure 1) may be most sensitive to the local environmental changes caused by DTT treatment. The reversible effects of DTT on the receptor suggest that DTT changes the conformation of the $\beta_2$ receptor by reducing crucial disulfide bond(s). Sulfhydryls are readily oxidized under nonreducing conditions, and removal of DTT leads to a gradual recovery of receptor function, probably as a result of re-forming the original disulfide bond(s) (Figure 4, Table 1). Consistent with our finding that antagonists stabilize the receptor conformation, previous reports found that pretreatment of the receptor with adrenergic ligands blocks DTT-induced inhibition of ligand binding (Wright & Drummond, 1983; Dohlman et al., 1990). Binding of adrenergic antagonists slowed the rate of DTT-induced
conformational change (Figure 5) but not the rate of DTT reduction of disulfide(s) (Figure 6). Therefore, antagonists most likely do not change the accessibility of DTT to disulfide bridges, but rather stabilize the structure of the receptor following reduction.

The results described above are consistent with the model illustrated in Figure 7. There are at least a pair of disulfide-bonded cysteines that are highly susceptible to DTT reduction. Mutagenesis studies indicated that these cysteines are likely located on the extracellular surface (Fraser, 1989; Dohlman et al., 1990; Noda et al., 1994). If Cys$^{106}$ on the first extracellular loop forms a disulfide bond with Cys$^{191}$ on the second extracellular loop as proposed by Noda and co-workers (Noda et al., 1994), the transmembrane helices 3 and 5 will be held in close proximity, a conformation implicated for agonist-bound receptor (Strader et al., 1989, 1991; Hein & Kobilka, 1995). Reduction of disulfide bond(s) by DTT may relax an important conformational constraint in the receptor structure causing the receptor to become nonfunctional (Figure 7). Removal of DTT, however, allows the receptor to return to its apparent original conformation and to restore ligand binding (Figure 7). A bound ligand does not affect the DTT reduction of the receptor; instead, the ligand stabilizes the conformation of the reduced receptor through its high-affinity binding (Figure 7). The ligand-bound reduced receptor, however, is thermodynamically unstable: it undergoes much slower confor

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