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Ligand Stabilization of the β_2 Adrenergic Receptor: Effect of DTT on Receptor Conformation Monitored by Circular Dichroism and Fluorescence Spectroscopy†

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ABSTRACT: Treatment of the β_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 β_2 adrenergic receptor in response to DTT treatment. Analysis of CD spectra of purified β_2 receptor in the detergent micelle indicated that the receptor has an α-helix content of 60%, which is substantially more than what would be attributed to the seven transmembrane domains. The α-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 β_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 β_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 β_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 β_2 adrenergic receptors (Fraser, 1989; Dohlman et al., 1990; Karnik & Khorana, 1990; Kurtenbach

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et al., 1990). Of interest, bound ligand can protect the receptor from the reducing effects of dithiothreitol (DTT)[1] (Vauquelin 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 β_2 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 β_2 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 β_2 receptor (Figure 1), the tryptophan fluorescence can be used to probe conformational changes of the receptor. Far-UV CD analysis showed that purified β_2 receptor solubilized in *n*-dodecyl β-D-maltoside has an α-helix content of around 60%, which is substantially more than what would be attributed to the seven putative membrane-spanning domains. The α-helix content of the receptor was unaffected by ligand binding or DTT treatment. However, DTT induced a reversible change in the tertiary structure of the β_2 receptor as assessed by a change in the intrinsic tryptophan fluorescence. Antagonists were able to protect DTT-induced conformational change by stabilizing the structure of the reduced receptor, rather than preventing access of DTT to the disulfide bond(s).

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 ($C_{12}M$) 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). [^{3}H]Dihydroalprenolol ([^{3}H]DHA, 95 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). Antagonists ICI 118551 and alprenolol were from RBI (Natick, MA). [^{14}C]Iodoacetamide (21.5 mCi/mmol) was from Dupont NEN (Wilmington, DE). DTT, guanidine hydrochloride, and other chemicals were from Sigma (St. Louis, MO).

Expression and Purification of the β_2 Adrenergic Receptor. DNA sequences encoding the human β_2 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) $\times$ 10^6 cells/mL and harvested after 48 h. The receptor was purified to homogeneity using a three-step purification procedure as described (Kobilka, 1995). Briefly, lysed cell membranes were solubilized in 0.8% $C_{12}M$ buffer followed by Ni-column chromatography using Chelating Sepharose. The Ni eluate was further purified on an M1 anti-FLAG antibody column and finally by alprenolol affinity chromatography. Approximately 3−5 nmol of protein with a specific activity of 12−15 nmol/mg could be obtained from 1 L of cell culture. The specific activity was determined by radioligand binding (see below) and protein determination using a Bio-Rad DC protein assay kit.

Radioligand Binding Assay. Purified β_2 receptor ($\sim$1 nM) was incubated in triplicate with a saturating concentration of [^{3}H]DHA (10 nM) in a total volume of 100 μL of $C_{12}M$−Tris buffer (100 mM NaCl, 0.05% $C_{12}M$, and 20 mM Tris, pH 7.4) for 1 h at room temperature. The binding was stopped and free [^{3}H]DHA separated from bound by desalting on a Sephadex G50 Medium column (4 cm $\times$ 0.5 cm) using ice-cold $C_{12}M$−Tris buffer. Nonspecific binding was determined in the presence of 10 μM alprenolol.

DTT and Guanidine Treatment of the β_2 Receptor. Purified receptor (1−3 μM) or L-tryptophan (12 μM) was incubated in $C_{12}M$−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 $C_{12}M$−Tris buffer (3 $\times$ 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 β_2 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 [^{14}C]iodoacetamide (4.9 mCi/mmol) was added to label DTT-reduced cysteines for 1 h at

[1] Abbreviations: CD, circular dichroism; $C_{12}M$, *n*-dodecyl β-D-maltoside; DTT, dithiothreitol; [^{3}H]DHA, [^{3}H]dihydroalprenolol; [^{14}C]-IA, [1-^{14}C]iodoacetamide; Tris, tris(hydroxymethyl)aminomethane.

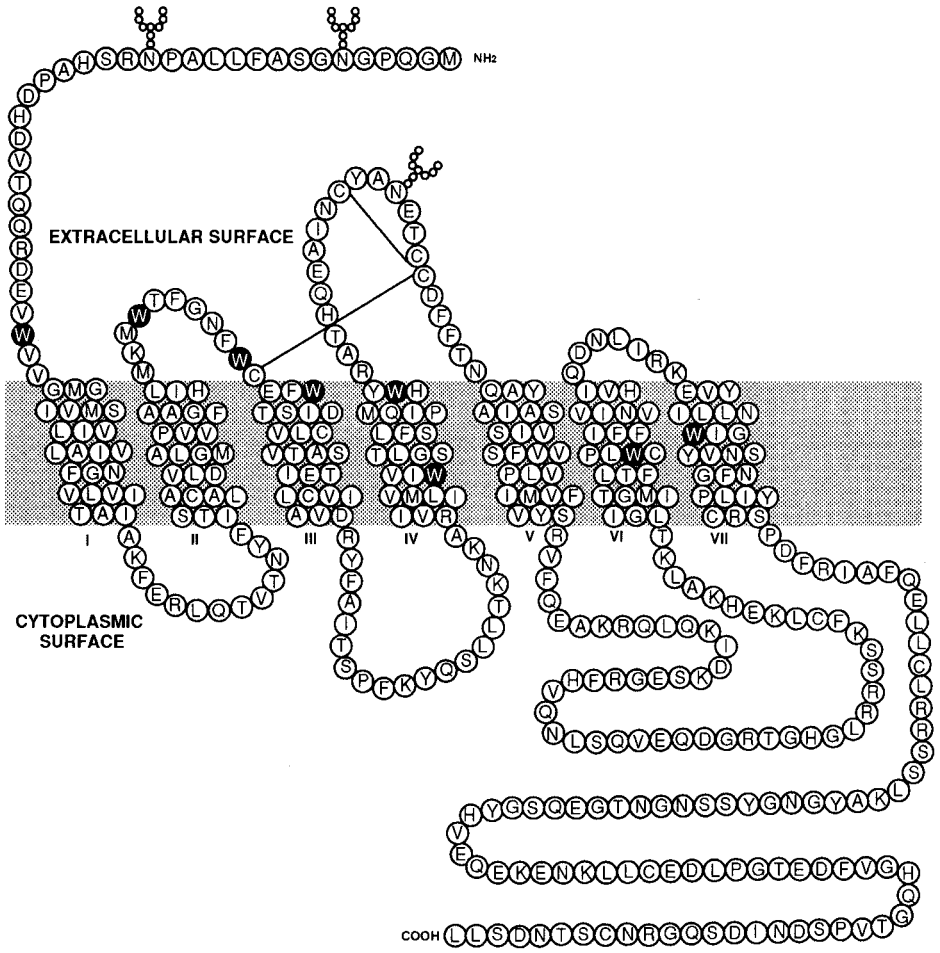

FIGURE 1: Secondary structure model of the human β_2 adrenergic receptor. The single amino acid code is used with tryptophans (W) highlighted and potential disulfide bridges of cystines (C) indicated (Noda et al., 1994).

room temperature. The bound [^{14}C]iodoacetamide was separated from unreacted free [^{14}C]iodoacetamide by gel filtration as above. Calculations of the stoichiometry of iodoacetamide binding were based on the known specific activity of the [^{14}C]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 β_2 receptor samples (1−1.5 μM in 400−500 μL of C_{12}M−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 β_2 receptor was prepared for CD analysis by dialyzing the receptor against CD buffer (0.05% C_{12}M, 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 β_2 Adrenergic Receptor. The primary amino acid sequence and proposed membrane topology for the human β_2 adrenergic receptor are shown in Figure 1. We expressed the human β_2 adrenergic receptor in Sf-9 insect cells, and were able to purify sufficient quantity of receptor in the C_{12}M micelle for CD spectroscopy studies (200−300 pmol/CD spectrum). The CD spectra of β_2 receptors are shown in Figure 2. The

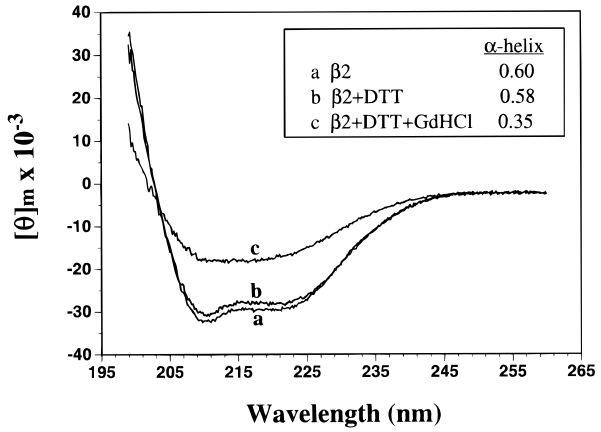

FIGURE 2: CD spectra of native and denatured β_2 receptor. Far-UV CD spectra of affinity-purified β_2 receptor (trace a), β_2 receptor treated with 10 mM DTT for 2 h at room temperature (trace b), and β_2 receptor treated with 10 mM DTT and 6 M guanidine hydrochloride for 2 h at 37 °C (trace c). The CD spectra were collected as described under Materials and Methods. Spectra represent the average of five scans expressed as molar ellipticity ($[\theta]_m$) in units of deg cm^2 dmol^{-1}. Insert: α-helix contents of the β_2 receptor estimated from the corresponding CD spectra.

spectrum of native β_2 is typical of a protein with a high degree of α-helical secondary structure, showing minima at both 209 and 222 nm (Figure 2, trace a). The α-helix content of the β_2 receptor was estimated to be 60% (Figure 2, insert), assuming the secondary structure of the β_2 receptor, like rhodopsin and m2 muscarinic acetylcholine receptor, is composed of mainly α-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% α-helix content (Figure 2, trace c, insert).

Effects of DTT on the Tertiary Structure of the β_2 Receptor. To examine the effect of DTT on the β_2 receptor conformation, we monitored the intrinsic tryptophan fluorescence. The human β_2 adrenergic receptor having eight tryptophan residues (Figure 1) showed an intrinsic fluorescence spectrum with a maximum emission wavelength (λ_{max}) centered around 329 nm when excited at 295 nm (Figure 3, trace a). The λ_{max} of a comparable concentration of L-Trp solution was at 355 nm (Figure 3, trace d). The blue-shifted λ_{max} in the β_2 receptor indicates that tryptophan residues are on average in a nonpolar (hydrophobic) environment. Treatment with 10 mM DTT caused a shift in the λ_{max} of the receptor to 332 nm (Figure 3, trace b). In comparison, addition of DTT to L-Trp solution under the same condition did not change the λ_{max} (Figure 3, trace e). Although the λ_{max} of purified receptors varies from 328 to 330 nm depending on the particular preparation of the protein, a consistent red-shift in the λ_{max} of 3−4 nm was observed after DTT treatment (Table 1). This red-shift in the λ_{max} of the β_2 receptor indicates that DTT treatment causes a conformational change which exposes some of the tryptophan residues to a more hydrophilic environment. The red-shift in the λ_{max} reached maximal at DTT concentrations above 7.5 mM (Figure 3, insert). 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 β_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 λ_{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.

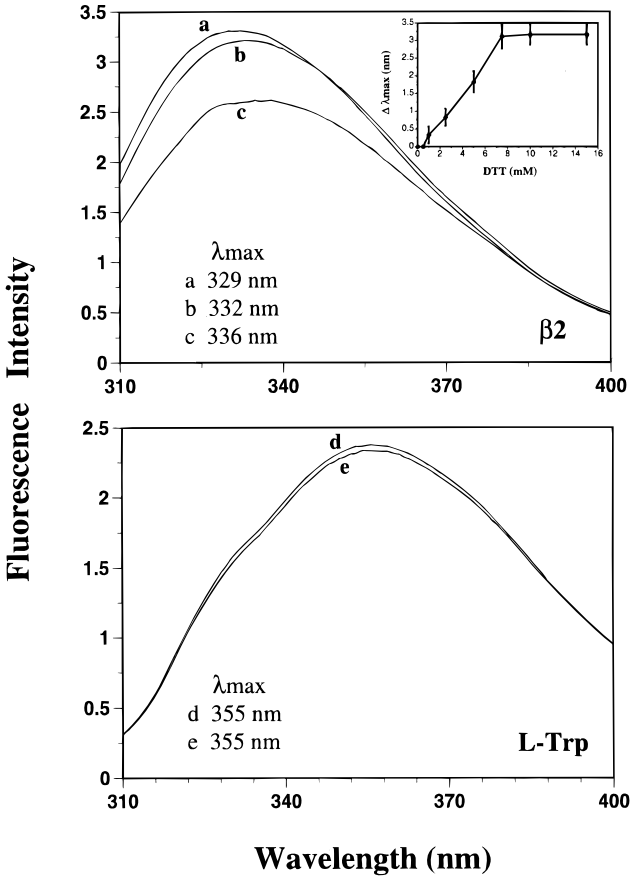

FIGURE 3: Effect of DTT on the maximum emission wavelength (λ_{max}) of the β_2 adrenergic receptor and of L-Trp. Top panel: fluorescence emission spectra of purified β_2 receptor (1 μM) in $C_{12}M$−Tris buffer (trace a), β_2 receptor treated with 10 mM DTT for 1 h at room temperature (trace b), and β_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 λ_{max} values of each sample are listed. Insert: Dose response of the DTT-induced λ_{max} shift of the β_2 receptor. The λ_{max} of emission spectra of the β_2 receptor (1 μM) was recorded after the receptor was incubated with various concentrations of DTT for 45 min at room temperature. $\Delta\lambda_{max}$ was calculated from the difference between the λ_{max} of DTT-treated samples and control. $\Delta\lambda_{max}$ is shown as mean ± SD of three separate experiments.

The Effects of DTT Are Reversible. The reversibility of the DTT-induced λ_{max} red-shift in the β_2 receptor was examined by removing excess DTT following DTT treatment. After 24 h dialysis, DTT-treated receptor displayed an emission spectrum with a λ_{max} at 331 nm (Figure 4A, dotted line), which is very close to the native receptor (Figure

Table 1: DTT-Induced Reversible Shift in the λ_{max} of Tryptophan Fluorescence and Loss of Antagonist Binding in the Purified β_2 Adrenergic Receptor

	$\Delta\lambda_{max}$ (nm)[a]	[^{3}H]DHA (%)[b]
pre-dialysis		
β_2	0	100
β_2 + DTT	3.5 ± 0.3	12.5 ± 4.1
β_2 + DTT + GdnHCl	5.7 ± 0.5	<1
post-dialysis[c]		
β_2	0	100
DTT-treated β_2	1.2 ± 0.2	70.2 ± 9.8
DTT-treated β_2 + DTT*	3.2 ± 0.5	15.5 ± 5.1

[a] DTT- or guanidine-induced λ_{max} shift of tryptophan fluorescence in the purified β_2 receptor was expressed as $\Delta\lambda_{max}$, the difference of λ_{max} between treated receptor samples and controls. Values are mean ± SD of $\Delta\lambda_{max}$ from 2−6 separate experiments. [b] Binding activity of the purified β_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 β_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.

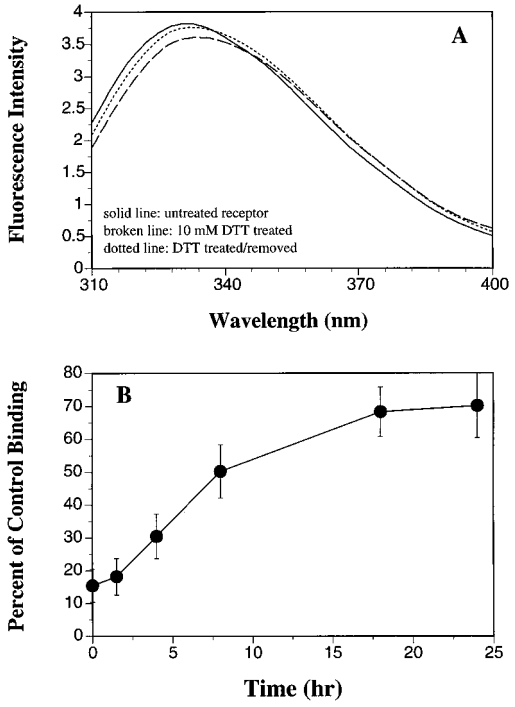

FIGURE 4: DTT-induced red-shift in λ_{max} and inhibition of antagonist binding in the β_2 receptor are reversible. **(**A) Fluorescence emission spectra of 1 μM purified, untreated β_2 receptor after 24 h dialysis against C_{12}M−Tris buffer at 4 °C (*solid line*), and DTT-treated β_2 dialyzed against 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 β_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 ± SD of four separate experiments.

4A, solid line). In comparison, the spectrum of DTT-treated receptor dialyzed in the presence of DTT remained red-shifted (λ_{max} = 334 nm) (Figure 4A, broken line). Recovery of the apparent original conformational state was confirmed by regaining the function of the receptor. Thus, DTT

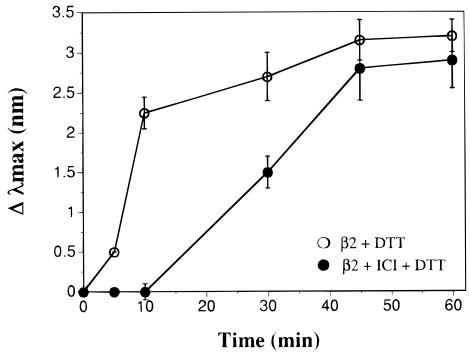

FIGURE 5: Effect of antagonist on stabilizing the DTT-induced λ_{max} shift of the β_2 receptor. The λ_{max} of emission spectra of β_2 receptor was recorded after the receptor (1 μM) was treated with 10 mM DTT (open circles) or preincubated with 2 μM antagonist ICI 118551 for 1 h prior to DTT treatment (closed circles). Experiments were carried out as described under Materials and Methods and in Figure 3. $\Delta\lambda_{max}$ was calculated from the difference between the λ_{max} of DTT-treated samples and control. Data are the mean ± SD of three separate experiments.

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_2O_2. Table 1 summarizes the reversible effects of DTT on the structure and function of the receptor.

Ligand Stabilization of the DTT-Induced Conformational Change. The effect of ligand on the DTT-induced conformational changes of the β_2 receptor was investigated by monitoring the emission of the DTT-treated receptors in the presence or absence of an antagonist. The purified β_2 receptor was first incubated with antagonist before it was exposed to DTT. As shown in Figure 5, the rate of λ_{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 λ_{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 β_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

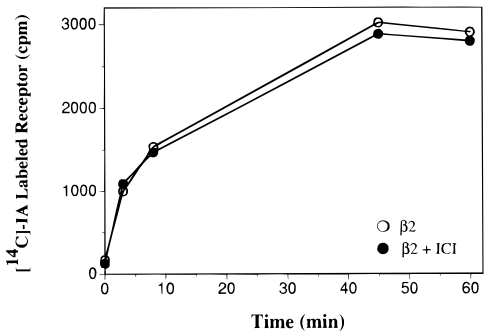

FIGURE 6: [^{14}C]Iodoacetamide labeling of DTT-reduced cysteines of the β_2 receptor. Purified and iodoacetamide-treated β_2 receptors were incubated with antagonist ICI 118551 (closed circles) or without (open circles) before they were treated with 10 mM DTT for 0−60 min. At the indicated time intervals, DTT was removed, and [^{14}C]iodoacetamide was added to label DTT-reduced cysteines. [^{14}C]Iodoacetamide incorporation into receptors was determined by scintillation counting following removal of unreacted [^{14}C]iodoacetamide by gel filtration. Data are the mean of duplicate determinations. Similar data were obtained in another separate experiment.

118551 before they were exposed to DTT. After being exposed to DTT for different periods of times, aliquots of receptor were removed, separated from excess DTT, and incubated with [^{14}C]iodoacetamide to label DTT-reduced cysteines. Approximately 63% of DTT-reduced sulfhydryls (average of two determinations of 61% and 65%) were labeled by [^{14}C]iodoacetamide, assuming all four sulfhydryls per receptor molecule can be alkylated. As shown in Figure 6, the rate of labeling DTT-reduced cysteines in antagonist-pretreated receptors (Figure 6, closed symbols) was similar to that without the antagonist pretreatment (Figure 6, open symbols). Thus, the accessibility of DTT to disulfide-bonded cysteines is not affected by antagonist treatment, suggesting that ligand does not block access of DTT to disulfide(s).

DISCUSSION

The β_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 β_2 adrenergic receptor (Tota & Strader, 1990). Recently we described ligand-specific conformational changes of the β_2 adrenergic receptor following ligand binding by labeling the purified β_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 of the β_2 adrenergic receptor in response to DTT treatment. While reducing agents such as DTT and 2-mercaptoethanol are documented to impair ligand binding to the β_2 adrenergic receptor, the structural and conformational changes of the receptor following DTT treatment are not known.

The Secondary Structure of the β_2 Adrenergic Receptor. Secondary structure determinations can provide information for developing protein structural models. However, methods used to probe secondary structure, such as CD and infrared spectroscopy, require micromolar concentrations of purified protein, and this has generally been difficult to obtain for G-protein-coupled receptors. We have engineered a human β_2 adrenergic receptor that preserves the pharmacological properties of the wild-type receptor and yet facilitates the production and purification of sufficient quantities to study the structure of the receptor by CD and intrinsic fluorescence. The CD spectrum of the β_2 adrenergic receptor is indicative of a high degree of α-helical structure, which is unaffected upon binding to adrenergic ligands. A similar study on the muscarinic acetylcholine receptor showed a marginal decrease in α-helical structure on binding of agonists, but not antagonists (Peterson et al., 1995). The estimated 60% α-helix content in the β_2 receptor is substantially more than what would be attributed to seven transmembrane domains, which constitute about 33% of the total protein sequence. This implies that nearly half of the α-helical structures derives from extramembranous regions of the β_2 adrenergic receptor. DTT treatment, which leads to inhibition of ligand binding (Table 1), does not perturb the secondary structure of the receptor (Figure 2). In contrast, guanidinium denaturation both abolishes ligand binding (Table 1) and significantly alters the secondary structure (Figure 2). Of interest, guanidine-denatured receptor still retains 35% α-helical content. A similar result has been reported in rhodopsin after thermal denaturation (Traxler & Dewey, 1994). It is tempting to speculate that the seven hydrophobic membrane-spanning domains remain α-helical structures in the presence of guanidine, or rapidly re-form α-helices following removal of guanidine prior to or during CD analysis.

Ligand Stabilization of the DTT-Induced Conformational Change. While DTT does not alter the secondary structure of the β_2 receptor, it does change the receptor conformation as reflected by the red-shift in the λ_{max} of tryptophan fluorescence (Figure 3, Table 1). The red-shift of 3−4 nm induced by DTT suggests that some of the tryptophans are exposed to a more hydrophilic environment. It is impossible to discern at this stage which Trp residue(s) is (are) responsible for the observed spectral change. However, Trp residues (Trp^{105}, Trp^{109}, and Trp^{173}) 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. 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 λ_{max} shift.

The reversible effects of DTT on the receptor suggest that DTT changes the conformation of the β_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

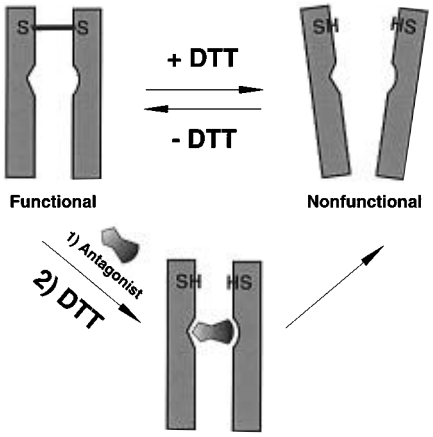

FIGURE 7: Mechanism of antagonist stabilization of the DTT-induced conformational change of the β_2 adrenergic receptor. The receptor contains a pair of disulfide-bonded cysteins that is critical for receptor function and is susceptible to DTT reduction. The reduced receptor assumes a different conformation and is nonfunctional. Antagonist occupation stabilizes the DTT-induced conformational change through its high-affinity binding, but does not change the accessibility of DTT to disulfide.

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 conformational changes initially but reaches the same conformational state at equilibrium (Figure 7).

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