The High Affinity State of the β2-Adrenergic Receptor Requires Unique Interaction between Conserved and Non-conserved Extracellular Loop Cysteines*

(Received for publication, July 28, 1993, and in revised form, October 11, 1993)

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Vol. 269, No. 9, Issue of March 4, pp. 6743-6752, 1994
Prepared in U.S.A.

A disulfide bond between two extracellular cysteines, conserved in all G-protein-coupled receptors, is believed to be critical for stabilization of the ligand-binding pocket. The β2-adrenergic receptor (β2-AR) contains two conserved cysteines (Cys106 and Cys184) as well as two other extracellular cysteines (Cys190 and Cys191). The specificity of the interactions between these four cysteines has not yet been clearly established. Mutants encoding alanines for specific extracellular cysteines in the β2-AR gene were constructed and expressed in COS-1 and Chinese hamster ovary cells. Ala106, Ala184, and Ala106,184,190,191 mutants displayed low affinity for the β-antagonist, 125I-cyanopindolol and insensitivity to dithiothreitol (DTT). The Ala106,184,190,191 mutant displayed an intermediate affinity and DTT sensitivity. Mutants Ala184, Ala106,184, and Ala106,184,190,191 displayed high affinity and DTT sensitivity, indicating that a solvent-accessible disulfide bond(s) is present in these mutant receptors as in the wild-type β2-AR. Additionally, thermal stability studies provided evidence that the extracellular disulfide bonds are essential for stabilization of the high affinity state of the receptor. These studies indicate that the covalent linkage between loops 1 and 2 of the β2-AR extracellular domains involves the formation of disulfide bonds, uniquely between Cys106 and Cys191 and Cys184 and Cys190, and is, thus, distinct from that of other G-protein-coupled receptors.

Adrenergic receptors are cell-surface glycoproteins that bind catecholamines and synthetic ligands to generate intracellular responses through the activation of specific guanine nucleotide-binding proteins (G-proteins). The β2-adrenergic receptor (β2-AR) couples to adenylate cyclase through the stimulatory G-protein, Gs (1). The proposed secondary structure of the β2-AR (Fig. 1) is similar to that of the prototypical G-protein-coupled photoreceptor, rhodopsin. Therefore, these receptors are presumed to be governed by similar structure-function relationships (2). A large number of G-protein-coupled receptors are believed to share the basic structural motif of seven transmembrane α-helical segments that form the binding pocket for the ligand. Several other structural features are conserved in the entire family of G-protein-coupled receptors, prompting the basis for a fundamental relationship in their functioning. Among these conserved structural features, the covalent linking of the first and second extracellular loops (loops B-C and D-E) by a disulfide bond is thought to be important for the generation and stabilization of receptor structure in the entire family of G-protein-coupled receptors.

There are 15 cysteine residues in the hamster lung β2-AR (Fig. 1, Table I). Four of these cysteines (106, 184, 190, and 191) are in the putative extracellular domain; five cysteines (77, 116, 125, 285, and 327) are in the putative transmembrane domain; and six cysteines (265, 341, 371, 383, 395, and 411) are in the putative cytoplasmic domain. Cys191 undergoes palmitoylation, which is thought to be important for anchoring the COOH-terminal tail to the lipid bilayer (3). The remaining 14 cysteine residues are not accessible to alkylation unless the protein is reduced under denaturing conditions, suggesting their involvement in a disulfide bond(s) (4). The compact structure thus produced is important because thiol reducing agents inactivate the β2-AR, presumably by cleaving critical disulfide bonds. Thiol reduction of the β2-AR can be blocked by agonists and antagonists demonstrating that the structurally critical disulfide bond(s) is masked by ligand occupancy (5) and, therefore, may be in proximity to the ligand-binding pocket. Furthermore, the reconstitution work of Pederson and Ross (6) demonstrated that the reduction of one or more disulfides functionally activates the β2-AR and consequently renders lability to the receptor structure. Site-directed mutational analyses showed that four cysteines critical for producing a β2-AR with high affinity ligand binding are localized to the putative extracellular domain (4, 7, 8). However, the specificity of their interaction has not been clearly established by systematic analysis of mutants and their thiol sensitivity or disulfide linkage. Such studies have been carried out, for example, with the m1 muscarinic receptor and with bovine rhodopsin (9–12).

We have prepared 15 β2-AR mutants by replacing the putative extracellular domain cysteine residues, in various combinations with alanine (Table I and Fig. 1). We have transfected COS-1 cells with either the wild-type or mutant β2-AR genes to study the effects of the mutations on the ligand-binding properties, thiol sensitivity, and effector activation of the expressed protein. High affinity antagonist-binding receptors were expressed by transfection with the wild-type gene. A significant decrease in antagonist binding was observed when only Cys106 in the loop B-C, or when all of the three cysteines, Cys184, Cys190, and Cys191 in loop D-E, were replaced. Furthermore, a
"wild-type-like" receptor was obtained by individual replacement of Cys184, by replacement of Cys184 and Cys190, or by replacement of Cys184 and Cys191. Antagonist affinity of receptor mutants retaining Cys106 and Cys190, or Cys106 and Cys191, but not of the highly conserved cysteine pair, Cys106 and Cys184, was decreased by a low concentration of DTT. These results are consistent with a promiscuous interaction between Cys106 and Cys191 and the production of the high affinity state of the \( \beta_2 \)-AR. The conserved Cys184 might be important for discrimination between Cys190 and Cys191. A disulfide interaction between Cys184 and Cys190 could be responsible for providing a unique partner to the conserved Cys106, and, in addition, affording stability to the receptor. Thus, the role of Cys184 in the folding and stabilization of the \( \beta_2 \)-AR is fundamentally different from the role suggested for the analogous residue in bovine rhodopsin or in m1 muscarinic receptor (9–12).

**MATERIALS AND METHODS**

Cloning of the Hamster Lung \( \beta_2 \)-Adrenergic Receptor Genes—We constructed \( \beta_2 \)-AR mutant genes by restriction fragment replacement (15). Oligonucleotides substituted with alanine codon GCA at positions 106, 184, 190, or 191 were synthesized with a Milligen/Biosearch Cyclone™ Plus DNA Synthesizer. A 58-base pair NarI-AatII fragment was replaced in the construction of Cys106 mutants, and a 115-base pair BapM11-Ndel fragment was replaced in the construction of all other single mutants. The correctness of the mutated genes was confirmed by DNA sequence determination using the dyeoxy chain termination method (16).

Transfection of COS-1 Cells and Preparation of Membrane—COS-1 cells (American Type Culture Collection) were transfected with CaCl₂-purified plasmid DNA using the DEAE-dextran method (17, 18). Three days after transfection, membranes were prepared by Parr Bomb disruption (19) in Hanks' balanced salt solution containing protease inhibitors. The membrane pellets were resuspended in 50 mM Hepes (pH 7.2), 12.5 mM MgCl₂, and 1.5 mM EOTA (HME buffer) containing 10% glycerol and stored at -70 °C. Protein concentration was determined by the Bradford method (20).

**Immunoblotting of Receptor Protein Expressed in COS-1 Cells—**Membranes (50 µg of protein) were mixed with SDS-polyacrylamide gel electrophoresis (PAGE) buffer, allowed to remain at room temperature for 3 h, and then were analyzed by SDS-PAGE. The proteins in the gel were transferred to Immobilon-P membrane (Millipore) for 2.5 h at 450 mA. The Immobilon-P membrane was blocked with 4% bovine serum albumin and then incubated with 2.5 µg/ml of 1D4 monoclonal antibody. After washing, the membrane was incubated with 1 µCi/ml 125I-anti-mouse Ig (Amersham Corp.). The membrane was washed, dried, and exposed to x-ray film (Kodak) at -70 °C. The relative intensities of the bands were estimated by PhosphorImager analysis.

**Photolabeling of Expressed Receptor Protein in COS-1 Cell Membranes—**Membranes (30 µg of protein) and 5 nM of 125I-CYP-diazirine (Amerham Corp.) were incubated for 3 h at 25 °C in the dark. Nonspecific labeling was determined in the presence of 10⁻⁴ M propranolol (Sigma). Photolysis was performed by irradiation at 366 nm for 20 min at 0 °C (21, 22). After photolysis, ice-cold 1 mM glutathione (Sigma) was added and the membranes were centrifuged for 10 min at 30,000 × g at 4 °C. The membrane pellets were resuspended in 6 µl of 10% deoxycholic acid (Sigma) and added to SDS-PAGE sample buffer. Covalent incorporation of radioactivity was determined from the autoradiogram.

**Deglycosylation of Wild-type \( \beta_2 \)-AR—**A membrane (50 µg of protein) expressing the wild-type receptor was incubated in the presence or absence of 82.1 units/ml N-glycosidase F (Boehringer Mannheim) at room temperature overnight.

**Equilibrium Binding Studies—**Membranes expressing wild-type or mutant receptors were incubated with 0.01–10 nM 125I-CYP (Amerham Corp.) in HME buffer at 37 °C for 60 min. Nonspecific binding to the membranes was determined from 125I-CYP binding in the presence of 10⁻⁴ M propranolol. The binding reaction was stopped by filtering under vacuum (Brandel type M-24R) on FP-200 GFC filters (Whatman). Filter-bound 125I-CYP was quantitated in a γ-counter (Packard). Equilibrium binding kinetics were determined using the computer program LIGAND (23).

**Competition Binding Studies—**COS-1 cell membranes expressing the wild-type \( \beta_2 \)-AR or the mutants, Ala-184, ECCA4, and ECCA5 (Table II) were incubated at room temperature for 2 h with 60 pM 125I-CYP and various concentrations of the agonists: (10⁻²–10⁻⁶ M) (−)-isoproterenol.

1 The synthetic gene encoding the hamster lung polypeptide \( \beta_2 \)-AR was designed, characterized, and expressed in COS-1 cells by the group of Daniel Oprian, the Graduate Dept. of Biochemistry of Brandeis University, Waltham, MA, prior to our work.
Novel Disulfide Linkage in β2-Adrenergic Receptor

Table II

| Construct | 125I-CYP | (-)-Iso | (+)-Epi | (-)-Nor | (+)-Alp | (-)-Pro |
|-----------|---------|--------|--------|--------|--------|--------|
|           | Bmax | Kd | IC50 | Kd | IC50 | Kd |
| Endogenous | pmol/mg | pm | μM | μM | μM | nm |
| Wild type | 42.7 ± 4.5 | 35.6 ± 4.6 | 0.78 ± 0.77 | 0.80 ± 0.22 | 3.2 ± 1.95 | 19.4 ± 18.10 | 0.43 ± 0.22 | 0.62 ± 0.19 |
| Ala-184 | 18.2 ± 2.0 | 47.3 ± 2.3 | 1.11 ± 0.62 | 5.20 ± 1.68 | 18.7 ± 4.21 | 15.2 ± 6.35 | 1.07 ± 0.31 | 1.36 ± 0.19 |
| ACCA4 | 15.0 ± 2.4 | 44.2 ± 5.2 | 1.59 ± 0.89 | 6.4 ± 2.71 | 24.3 ± 3.44 | 29.4 ± 22.50 | 0.99 ± 0.31 | 1.00 ± 0.17 |
| ECCA5 | 9.7 ± 1.3 | 52.4 ± 5.0 | 1.66 ± 0.49 | 12.3 ± 5.25 | 22.2 ± 4.21 | 56.3 ± 4.81 | 2.24 ± 0.23 | 4.04 ± 0.97 |
| Group 1 (high affinity receptors): | | | | | | |
| Wild type | 42.7 ± 4.5 | 35.6 ± 4.6 | 0.78 ± 0.77 | 0.80 ± 0.22 | 3.2 ± 1.95 | 19.4 ± 18.10 | 0.43 ± 0.22 | 0.62 ± 0.19 |
| Ala-184 | 18.2 ± 2.0 | 47.3 ± 2.3 | 1.11 ± 0.62 | 5.20 ± 1.68 | 18.7 ± 4.21 | 15.2 ± 6.35 | 1.07 ± 0.31 | 1.36 ± 0.19 |
| Group 2 (intermediate affinity receptors): | | | | | | |
| Ala-106 | 4.2 ± 0.3 | 276.8 ± 44.4 | 1178 ± 133.2 | |
| ECCA3 | 3.3 ± 0.6 | 258.9 ± 38.7 | 281.0 ± 61.5 | |
| ECCA5 | 8.2 ± 1.6 | 258.9 ± 38.7 | 281.0 ± 61.5 | |
| Group 3 (low affinity receptors with low expression): | | | | | | |
| Ala-106 | 5.5 ± 0.6 | 1178 ± 133.2 | 1932 ± 153.3 | |
| ECCA1 | 7.1 ± 1.9 | 1932 ± 153.3 | 1678 ± 223.3 | |
| ACCA2 | 4.6 ± 0.2 | 712.6 ± 92.6 | 1678 ± 223.3 | |
| ECCA7 | 12.3 ± 0.4 | 728.9 ± 13.7 | 1678 ± 223.3 | |
| ECCA8 | 7.7 ± 1.0 | 1932 ± 153.3 | 1678 ± 223.3 | |
| ECCA9 | 5.8 ± 1.1 | 1678 ± 223.3 | 1678 ± 223.3 | |
| ECCA10 | 4.2 ± 0.2 | 1467.7 ± 77.4 | 1678 ± 223.3 | |
| ECCA11 | 5.4 ± 0.6 | 924.6 ± 104.3 | 1678 ± 223.3 | |
| Group 4 (low affinity receptor with high expression): | | | | | | |
| ECCA6 | 32.1 ± 4.2 | 1838.6 ± 90.9 | 1838.6 ± 90.9 | |

Results

Expression of Wild-type β2-AR Gene in COS-1 Cells

Expression of the native β2-AR cDNA, the synthetic β2-AR gene, or its mutants in COS-1 cells was examined by immunoblotting, photoaffinity labeling, and by antagonist-binding studies (Table II, Figs. 2 and 3). Immunoblotting revealed heterogeneous expression with predominant molecular weight forms of ~40–55 kDa (Fig. 2A). A ~65–70-kDa band corresponding to about 20–25% of the expressed wild-type β2-AR was also observed. The heterogeneity of the expressed β2-AR species resulted from differences in Asn-linked glycosylation, since treatment of the membranes with N-glycosidase F produced a single band of approximately 45 kDa, which corresponds to the calculated molecular mass of the unglycosylated β2-AR polypeptide (Fig. 2B). A 24–28-kDa band was also detected by immunoblot analysis with the 1D4 antibody. The mobility of this band was not altered by N-glycosidase F treatment. Thus, this species probably represents a proteolytic COOH-terminal fragment of the β2-AR.

Of all the bands, only the 65–70-kDa band could be photolabeled with the antagonist, 125I-CYP-diazirine (Fig. 2C). Photo labeling of this band was specific because it could be blocked by 10−4 M (-)-propranolol (Fig. 2C). A 65–70-kDa photolabeled species was also observed in membranes prepared from COS cells transfected with a native β2-AR cDNA that lacks the 1D4 epitope tag. The abundance of this species was similar to that observed with the wild-type synthetic gene. In both membrane preparations, the efficiency of photolabeling was proportional to the Bmax values estimated from antagonist equilibrium binding studies (discussed later). Furthermore, it correlated with the intensity of the 65–70-kDa band as determined by immunoblotting. Since the 40–55-kDa receptor forms, as determined by immunoblotting, were expressed at higher levels than the 67–70-kDa forms, these species should have been detectable by photoaffinity labeling as well. This suggests that nascent, folding intermediates are present in the cell at a steady state due to a high level of expression. Moxham and Molbon (28) have
shown that rat fat cell β1-AR undergoes apparent changes in conformation upon treatment with β-mercaptoethanol and DTT. However, these intermediates do not interfere with antagonist binding analysis, because they do not contain an intact ligand-binding pocket. On the other hand, the 24–28-kDa band cannot be detected in photocross-linking, suggesting that it represents a non-functional proteolytic fragment of the receptor.

Antagonist Binding—A binding affinity (Kd) of 30–50 pm was displayed by the receptor expressed by transfection of either the β2-AR cDNA or the 1D4 epitope-tagged synthetic β2-AR gene (Table I). These two receptor forms were considered as “wild type.” Their affinity for the ligand was comparable to that of the endogenous β2-AR of COS cells, which is expressed at 200-fold lower levels. This suggests that expression of heterogeneous forms of the β2-AR in COS-1 cells does not interfere with formation of a functional receptor. Two different approaches were taken to further strengthen this observation. (i) Antagonist binding kinetics were evaluated for the presence of more than one affinity state. As shown in Fig. 3, only a single affinity state was observed for the wild-type receptor expressed in COS-1 cells. (ii) Agonist competition binding experiments (Table II) were performed. These demonstrated that the expressed receptor exhibited the expected relative potencies for agonists at β2-ARs, i.e. (-)-isoproterenol > (-)-epinephrine > (-)-norepinephrine. Thus, the observed heterogeneity of β2-ARs is presumably due to the production of the mature protein, as well as nascent species, which do not bind ligand, are partially glycosylated, and have not yet folded completely, rather than due to the end products of proteolysis within the cells.

We, therefore, conclude that high level expression of β2-AR is achieved in COS-1 cells by pMT3 vector, and that the level and nature of the expressed receptor is essentially the same for either the wild-type synthetic gene or the native cDNA.

Expression of the β2-AR Cysteine Mutants in COS-1 Cells

The pattern of protein expression of all of the mutants was similar to that of the wild type (Fig. 2A). The 40–55-kDa bands were expressed at the same level for all of the mutants, suggesting that there are no defects in the transcription/translation of the mutant genes. A 65–70-kDa form of the receptor was observed with all of the mutants, although the amount of these species was variable. For example, with the mutants Ala-106, ECCA7, and ECCA11 (Table I), the 65–70-kDa band was less abundant than that observed for the wild-type receptor. In the mutant ECCA6, the expression of the 65–70-kDa band was almost the same as that of the wild type. With all of the mutants, only the 65–70-kDa receptor could be detected by 125I-CYP-diazirine photocross-linking (Fig. 2C). However, the extent of labeling differed significantly among the mutants. For example, the photocross-linked band intensities with the mutants Ala-184, ECCA4, and ECCA5 were similar to that of the wild type. It was considerably reduced with the mutant ECCA6, although by immunoblotting the protein expressed by this mutant gene was the same as that of wild type. As indicated below, antagonist binding with some of the mutants was not directly related to the level of the 65–70-kDa receptor. Therefore, it appears that the mutant receptors are appropriately glycosylated in COS-1 cells, but unlike the wild-type receptor, are not homogeneously folded.

Antagonist Binding to Mutants—The mutants could be classified into four groups based on their affinity for the antagonist radioligand 125I-CYP (Table II). Group I mutants, which in-
This mutant presented some interesting additional phenotypic fe...nts 11). The 65-70-kDa receptor form for these mutants is produced at the same abundance...osition 184 is not essential for producing the wild-type-like group I phenotype. Among the mutants displayed a single class of high affinity antagonist reduced ability to form a 65-70-kDa receptor (Fig. 2A). The presence of complex, multiple affinity binding sites for the antagonist is apparent in this group of mutants (Fig. 3).

The group IV phenotype is represented by only one mutant, ECCA6, which retains the conserved Cys90 and Cys106 residues but lacks the vicinal Cys190 and Cys191 (ECCA6). Expression of the 65-70-kDa mutant receptor is enhanced (as also indicated by its B_max) but the affinity of the receptor for 125I-CYP is greatly decreased (K_d > 1500) (Table II, Fig. 2A). Also, antagonist-binding studies demonstrated the presence of multiple affinity forms in this mutant.

**Effect of DTT on Antagonist Affinity**

Wild-type—Incubation of the wild-type β2-AR with 1–10 mM DTT leads to the loss of high affinity antagonist binding (Fig. 4). This is thought to be due to reduction of disulfide bonds. Preincubation of the wild-type receptor with 1 mM DTT led to a decrease in the affinity of the receptor for 125I-CYP (Table III, Fig. 4). However, incubation with 5–10 mM DTT results in a substantial decrease in affinity combined with loss of binding sites. The DTT inactivation follows a biphasic time course (Fig. 4). The initial decrease in binding represents the fast reduction of solvent accessible disulfide bonds, by DTT. In contrast, the slower decrease in binding presumably represents the ensemble reduction of other accessible disulfide bonds. We assumed that the initial phase involved the reduction of the extracellular disulfide bonds. Therefore, we chose 1 mM DTT to investigate the disulfide interaction of extracellular cysteines in mutants. Inactivation was complete in 30 min at 37 °C (Fig. 4). The receptor could be protected from inactivation by preincubation with 125I-CYP. Inactivation of β2-adrenergic receptors by treatment with DTT or β-mercaptoethanol also leads to loss of antagonist binding with similar kinetics (28).

Cys → Ala Mutants—The mutants fall into two different
FIG. 4. Influence of DTT concentration on $^{125}$I-CYP binding. $^{125}$I-CYP binding to membranes prepared from transfected COS-1 cells treated with varying concentrations of DTT was examined as described under "Materials and Methods." $^{125}$I-CYP binding in the absence of DTT was expressed as 100%.

Table III

| Construct | $B_{max}$ | $K_d$ | $K_r$ | Retained cysteine(s) |
|-----------|-----------|-------|-------|----------------------|
|           | DTT (-) (+) | (+) | (+) | 106 | 184 | 190 | 191 |
| DTT sensitive | | | | |
| Wild type | 41.9 | 30.7 | 43.5 | 361.6 | 8.3 | + | + | + |
| Ala-106 | 23.3 | 18.0 | 46.6 | 235.5 | 5.1 | + | + | + |
| Ala-184 | 3.2 | 4.7 | 192.3 | 434.2 | 2.3 | + | + | + |
| ECCA4 | 6.7 | 6.3 | 216.4 | 846.2 | 4.0 | + | + | + |
| Ecca5 | 16.4 | 13.1 | 40.6 | 89.2 | 2.2 | + | + | + |
| DTT insensitive | | | | |
| Ala-106 | 11.1 | 8.5 | 51.0 | 203.6 | 4.0 | + | + | + |
| Ala-190 | 2.9 | 3.4 | 922.2 | 809.9 | 0.9 | + | + | + |
| ECCA1 | 3.4 | 3.3 | 744.5 | 864.2 | 1.2 | + | + | + |
| ECCA2 | 4.5 | 4.1 | 826.8 | 981.4 | 1.2 | + | + | + |
| ECCA5 | 20.1 | 15.1 | 1894.1 | 1879.3 | 1.0 | + | + | + |
| ECCA7 | 10.1 | 8.8 | 737.8 | 754.1 | 1.0 | + | + | + |
| ECCA11 | 17.4 | 18.6 | 1336.5 | 1560.0 | 1.2 | + | + | + |

categories based on the concentration of DTT required to inactivate their antagonist binding sites (Table III and Fig. 4). The insensitive mutants show little change (0.9-1.4-fold) in their $K_d$ values upon incubation with 1 mM DTT (compare Tables II and III). These results suggest that reduction of putative disulfide bond(s) in other domains was not taking place under our experimental conditions. Furthermore, they indicate that these mutants do not contain a DTT-accessible disulfide bond(s). The mutants lacking all four extracellular cysteines (ECCA11), mutants lacking loop D-E cysteines (ECCA7), and the mutant Ala-106 fall into this category. Some of the mutants retaining a single pair of extracellular cysteines also showed resistance to 1 mM DTT. For example, DTT treatment did not influence antagonist binding of mutants retaining Cys150 and Cys151 (ECCA1), Cys184 and Cys193 (ECCA2), or Cys106 and Cys184 (ECCA6) (Table III). These mutant receptors, like mutants ECCA7 and ECCA11, however, were inactivated at elevated temperatures in the absence of DTT (data not shown). Therefore, it seems unlikely that cysteine pairs, Cys150 and Cys151, Cys106 and Cys184, or Cys184 and Cys193 are linked by a disulfide bridge.
Cys

mutants produced a single antagonist-affinity class of receptors with moderate affinity for 125I CYP. Thus, the Cys184-Cys190 disulfide bond most likely represents a genuine interaction required for efficient production of high affinity receptors. Therefore, a second DTT-sensitive disulfide bond might also exist between Cys184 and Cys190 in the wild-type receptor.

Temperature Dependence of Antagonist Affinity

DTT inactivation of the mutants did not distinguish the stability of two putative disulfide bonds, presumably due to similar roles they play in receptor structure-function. Thermal stability of the wild-type or mutant receptors was compared to distinguish the role of the two putative disulfide bonds (Fig. 5B).

The wild-type receptor was stable for 3 h at 37 °C, whereas mutants Ala-184, ECCA3, ECCA4, and ECCA5 showed about a 30-40% loss in total binding. Thus, differences between the stabilities of different mutants could not be readily discerned at 37 °C. At 42 °C, however, thermal-stability differences between the mutants were apparent. The thermal stability of the wild-type receptor at 42 °C was similar to that observed at 37 °C.

The mutants Ala-184 and ECCA4, which lack the putative Cys184-Cys190 disulfide bond, showed similar kinetics of inactivation (Fig 5B). The mutant ECCA3 that retains the putative Cys184-Cys190 disulfide bond and ECCA5 that retains the putative Cys106-Cys191 disulfide bond were slowly but gradually inactive. Therefore, it appears that the contribution of the Cys106-Cys191 bond to receptor stability is greater than that of the Cys184-Cys190 bond. However, with the wild-type receptor, a concerted effect of both of these bonds on the receptor stability is evident.

Modeling the Structure of Extracellular Loop D-E

The presence of a disulfide bond between 2 of the 3 cysteine residues in the D-E loop raises the question of which pair is sterically favored. The choice of partners in the formation of the two putative disulfide bonds will depend on the tertiary structure of the D-E loop. To address this issue, we carried out modeling studies to predict the potential, interacting cysteine pairs. A pool of 27,000 disulfide-bonded protein sequences in the Swiss protein database was searched using the computer program Geneworks™ by providing a sequence motif C-X(>4)<6)-CC. The pattern match picked 84 sequences. Structural information was available in the protein data base for six of the proteins in this pool. There is no sequence homology in the segments separating the cysteines in any of these six proteins. However, there is a remarkable similarity in the backbone structures of these segments. Thus, the structure might represent a motif that is frequently used in several proteins unrelated to the β2-AR. These segments are all present in the solvent-exposed regions of their respective proteins. We reasoned that the D-E loop of the β2-AR might assume a similar structure, and, therefore, performed comparative modeling studies with this segment of the β2-AR. The packing of residues depicts a β-strand conformation. The side chains of cysteines 184 and 190 face inward. We estimated the range of distances between their sulfur atoms through rotation of the C6 bond. The minimal distance estimated was 1.87 Å, and the maximal distance was 8.32 Å, implying a potential Cys184-Cys190 disulfide interaction bond based on their proximity (29). Similar estimates for the interaction between Cys184 and Cys191 gave a range of distances of 6.66-11.85 Å and for the interaction between Cys190 and Cys191 a distance of 5.81-9.29 Å. This analysis strongly suggests that the Cys184-Cys190 interaction is favored in this model. The structure obtained for the D-E loop suggests that within this region a disulfide bond interaction

![Fig. 5. Time-course effect of DTT reduction and temperature on 125I-CYP binding. A, inactivation of 125I-CYP binding due to pre-incubation with 1 mM DTT of wild-type (●); Ala-184 (○); ECCA3 (△); ECCA4 (□), or ECCA5 (▲) receptors for various times. 125I-CYP binding in the presence of DTT at each preincubation time was calculated as a percentage of 125I-CYP binding in the absence of DTT at the same preincubation time. Arrow indicates percent binding without DTT addition and with 60-min incubation of all samples. Therefore, it is the same point for all inactivation studies. B, inactivation at 37 °C of 125I-CYP binding due to temperature-induced destabilization of wild-type (●); Ala-184 (○); ECCA3 (△); ECCA4 (□), or ECCA5 (▲) receptors for the times indicated.](image)
Figure 6: A model of the second extracellular loop of hamster lung \( \beta_2 \)-adrenergic receptor. The peptide segment from residues Cys\(^{184} \) to Cys\(^{191} \) was modeled based on the assumptions described under “Results.” The thick line depicts the backbone, thin lines the side chains, and the dotted lines depict the sulfur atoms of the cysteines. The broken line triangle depicts minimal distances for interactions between the three cysteines. The minimal distance estimated was 1.87 Å between Cys\(^{184} \) and Cys\(^{190} \), 5.81Å between Cys\(^{190} \) and Cys\(^{191} \), and 6.66Å between Cys\(^{184} \) and Cys\(^{192} \). Two views of the model are shown between Cys\(^{184} \) and Cys\(^{190} \) is more likely than between Cys\(^{184} \) and Cys\(^{191} \) (Fig. 6). Additionally, an interaction between Cys\(^{190} \) and Cys\(^{191} \) (8) seems unlikely.

Antagonist Binding and Agonist-induced Function in CHO Cells

Clonal CHO cell lines expressing some of the mutants (Table IV) were established, as described earlier, for examining antagonist affinity profiles and receptor-coupled adenylylate cyclase activation. The expression level of the wild-type \( \beta_2 \)-AR was 0.74 pmol/mg and of the mutants between 0.11 to 2.27 pmol/mg. In CHO membranes, the wild-type receptor and the mutants, Ala-184, ECCA4, or ECCA5 exhibited a single affinity state for agonists. Two mutants, Ala-106 and ECCA5, exhibited a mixture of high and low affinity receptors as in COS-1 membranes. The heterogeneity of receptor-affinity states, therefore, seems to be an intrinsic property of the mutants rather than arising from the high level expression observed in COS-1 cells, and binding of \( ^{125}\text{I}-\text{CYP} \) by the wild type and by each mutant receptor was similar to that observed for these receptors when expressed in COS-1 cells. The mutants Ala-184, ECCA4, and ECCA5, which showed a similar affinity for agonists, were functionally active (Table IV). The mutant Ala-106 also retained the ability to stimulate adenylylate cyclase, but the mutant ECCA6 did not show agonist-induced stimulation of adenylylate cyclase. The level of expression of each of the proteins could not be estimated independently by immunoblotting, and, therefore, the fold stimulation of cyclase by each of the mutants could not be used to interpret defects in coupling ability. These studies are currently underway.

DISCUSSION

Alteration in the properties of hormone receptors by thiol-reducing agents has been known for some time (30). \( \beta_2 \)-AR alteration by DTT was first suggested by the observation that DTT modifies beating of guinea pig atria (31). In all native systems examined, DTT alters the affinity of the receptors for their ligands without affecting the total number of receptors, due to the reduction of one or more disulfide bonds present in \( \beta_2 \)-receptors (32). Protection of the receptor from thiol reduction by \( \beta_2 \)-adrenergic agonists and antagonists implied that the bonds reduced by DTT are shielded in the ligand-occupied receptor (5). The reconstitution work of Pederson and Ross (6) and the in situ experiments of Moxham et al. (28, 32) clearly demonstrated the presence of disulfide bridges, reduction of which causes functional activation of the receptor.

Cloning, expression, and the mutagenesis of the \( \beta_2 \)-AR gene provided new insight into the role played by disulfide bonds in \( \beta_2 \)-AR structure and function. Dixon et al. (7) demonstrated that mutagenesis of cysteines in the intracellular domain, and the cysteines in the transmembrane domain, did not critically influence the ligand-binding properties of the receptor. This conclusion seems to be borne out in other studies as well (4, 8). The four cysteines in the extracellular domain have been examined in several studies (4, 7, 8, 33). Substitution of Cys\(^{184} \) or Cys\(^{185} \) of the hamster lung \( \beta_2 \)-AR by valine resulted in altered agonist-binding properties, with no apparent alteration of \( ^{125}\text{I}-\text{CYP} \) binding. The possibility that these two residues might be involved in a disulfide bond was suggested by the finding that substitution of either cysteine caused a loss of affinity for ligands (7). By analogy to disulfide interaction of the conserved cysteines in other members of this receptor superfamily, the conclusion that these cysteines, Cys\(^{100} \) and Cys\(^{184} \), are similarly involved in \( \beta \)-AR integrity seemed obvious (7). However, direct evidence for an interaction between Cys\(^{106} \) and Cys\(^{184} \) in a disulfide bond is lacking so far. With the human \( \beta_2 \)-AR three mutants involving Cys\(^{100} \) and Cys\(^{191} \) demonstrated a loss of affinity for both antagonists and agonists. Involvement of Cys\(^{184} \) and Cys\(^{191} \) in ligand binding was proposed (8). Dohlman and colleagues (4) created eight different cysteine-substitution mutants. The properties of receptors with mutations of the cysteines in the transmembrane domain were unaffected. The replacement of cysteines in the putative extracellular domain resulted in profound changes in the normal ligand-binding affinities. They concluded that extracellular disulfide-bonded cysteines are critically important for forming or stabilizing the ligand binding site and are likely to be sensitive to DTT treatment (4).

Although the critical nature of the cysteines in extracellular domain is clearly established in the studies mentioned above, several questions remained. If residues Cys\(^{100} \) and Cys\(^{184} \) are connected by a disulfide bond, then their contribution to receptor structure and function is expected to be similar. This expectation seems clearly satisfied in the mutagenesis studies of bovine opsin and the m1 muscarinic receptor. The cysteine residues thus identified in these receptors were shown to be disulfide-bonded (9–12). However, the effects of Cys\(^{100} \) muta- tion in the \( \beta_2 \)-AR are very different from those of Cys\(^{184} \) mutation. In two independent studies, Cys\(^{184} \) substitution showed near wild-type affinity toward the antagonist, \( ^{125}\text{I}-\text{CYP} \), but showed a greater decrease in affinity toward agonists. The Cys\(^{100} \) mutation had a more severe effect on both properties (4, 7). Agonist binding is dependent on the folding of the receptor, while agonist binding affinity is also modulated by cognate G-protein coupling (or precoupling). It seems possible that these properties are achieved by independent disulfide bond formation in the wild-type receptor. The differential effects of Cys\(^{184} \) or Cys\(^{100} \) mutations on these properties then might suggest that these cysteines are not linked by a disulfide bond. Rather, DTT inactivation of the \( \beta_2 \)-AR might actually reflect reduction of two different disulfide bonds. The work presented in this paper attempts to address these questions.
Our studies began with the observation that a single mutant, Ala-184 displays high affinity antagonist binding (Tables I and II). This property differed from that of the Ala-106 mutant, the partner proposed to be involved in a critical disulfide bond. The Ala-106 mutant produced low affinity receptors, and was expressed at reduced levels. A quadruple mutant, ECCA11, and a triple mutant, ECCA7, also expressed low affinity receptors like the Ala-106 mutant, suggesting that removal of either Cys106 or removal of all three cysteines in the loop D-E, results in the low affinity receptor phenotype. Therefore, it is likely that Cys184 is not essential for the formation of a high affinity β2-AR. In addition, the two double mutants (ECCA4 and ECCA5), both lacking Cys184, and either Cys106 or Cys191, produce high affinity receptors. A double mutant lacking both Cys190 and Cys191 produces low affinity receptors, as observed in earlier studies (8). Furthermore, inactivation of high affinity antagonist binding by DTT suggests that these residues are involved in disulfide bond formation. It seems possible, therefore, that the two cysteines, Cys106 or Cys191, could potentially interact with Cys190. A promiscuous interaction between Cys106, and either Cys106 or Cys191, appeared to produce the high affinity state of the β2-AR in mutants lacking Cys184. Which one of these is the partner for Cys150 in the native receptor?

The key to answer this question comes from the DTT sensitivity and thermal stability experiments on the mutants (Table III, Figs. 4 and 5). From the data presented in Table III, some of the potential disulfide bond interactions can be ruled out, based on their temperature sensitivity and insensitivity to DTT. For example, a disulfide interaction between Cys190 and Cys191 in the mutant ECCA11, between Cys184 and Cys191 in the mutant ECCA2, or between Cys106 and Cys184 in the mutant ECCA6 is not evident. However, these mutants are sensitive to temperature inactivation, like the mutants lacking all extracellular cysteines. Therefore, resistance to DTT suggests that the remaining cysteines bear a free sulfhydryl, rather than being involved in solvent-inaccessible disulfide bonds. It is possible that the secondary structure of the polypeptide and side chain packing precludes an interaction between the pairs of cysteines mentioned above. Complementing this observation, the mutant ECCA3, retaining Cys184 and Cys190, and the mutant ECCA5, retaining Cys106 and Cys191, were found to produce a single-affinity class of receptors, and both mutants were sensitive to DTT reduction. Extending this wild-type receptor might indicate that two disulfide bonds linking Cys106 and Cys191, and Cys184 and Cys190 exist in the native receptor. Only in one mutant (ECCA5) a high affinity antagonist binding state seems to be generated by a non-native disulfide interaction between Cys106 and Cys184. The absence of Cys184 and Cys191 might position Cys190 at an interacting distance with Cys106. Based on our modeling studies, it appears that a Cys184-Cys190 disulfide bond may be induced by the proximity of their sulfhydryl groups. Since localized folding in the loop region could drive this interaction, native folding of the β2-AR might involve a multi-step process (Fig. 7). A Cys184-Cys190 interaction leads to a linking of loops 1 and 2 through disulfide bond formation between Cys106 and Cys191. The mutants suggest that the high affinity antagonist binding property correlates with the formation of a Cys106-Cys191 disulfide bond. The role of a second disulfide bond is unclear at this stage, although involvement in agonist affinity seems most likely based on previous observations on Cys184 mutants (4, 7). Our studies suggest that a disulfide linkage does exist between Cys184 and Cys190. Therefore, it is not essential for the high affinity antagonist binding but might be important to achieve high Bmax values.

Two questions still remain. First, the basis for the conservation of Cys184 is not clear at this stage. As discussed earlier, a cysteine residue analogous to Cys184 is conserved in the D-E loop of the family of the G-protein-coupled receptors, which is believed to be disulfide-bonded to a similarly conserved cysteine, analogous to Cys106, at the boundary of loop 2 and helix C (34). This linkage has been confirmed for bovine opsin and the m1 muscarinic receptor (9-12). The critical role played by this pair of cysteines in receptor folding was shown by mutagenesis for a number of other receptors in addition (35). Based on their studies Khorana (36) proposed a scheme for the putative steps involved in the folding of bovine opsin; the assembly of the receptor begins by transfer of the hydrophobic segments into the endoplasmic reticulum membrane where high mannose glycosylation then takes place. The insertion of helices into the bilayer does not ensure their alignment. Rather, the correct alignment of helices is achieved by a cooperative interaction of the extracellular loops stabilized by disulfide bond formation. A unique disulfide bond between the conserved cysteines is obligatorily required for bovine rhodopsin (11, 12). The number of disulfide bonds may differ in other receptors (35, 37). The folding of the β2-AR may follow the same general scheme (see Fig. 7). For example, Kobilka (38) observed a lag time in the acquisition of specific ligand-binding properties of the β2-AR after its incorporation into the microsomal membrane. This lag might be due to a disulfide exchange reaction involving the extracellular cysteines. We speculate that the kinetically preferred route of folding may involve the initial formation of a Cys106-Cys184 disulfide bond (Fig. 7). The basic seven transmembrane α-helical motif generated at this step does not contain the ligand-binding pocket. In subsequent steps, a disulfide-exchange reaction resulting in the formation of disulfide bonds between Cys106 and Cys191, and Cys184 and Cys190, might then lead to the final structure. Heterogeneity in glycosylation might reflect such folding steps. Intra- and posttranslational modification are independent processes thought to be coupled to the folding of proteins into complex structural motifs. In the folding of soluble proteins, it is known that disulfide bond formation is directed by other
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non-covalent, intramolecular interactions, which may proceed through distinctly non-native intermediates (39, 40). The role of disulfide exchange and isomerization of prolyl side chains have been speculated in the folding of membrane proteins, but have not been clearly established (41).

Second, although epinephrine is also a natural ligand for α1-adrenergic receptors, they do not share the structural features that characterize the β2-AR extracellular domain. For example, a vicinal cysteine pair in loop D-E is found only in β-adrenergic receptors. The concentration of DTT that inactivates the β2-AR does not inactivate the α1-adrenergic receptor, although this latter receptor clearly contains a solvent-inaccessible disulfide bond (42). Furthermore, functional activation by thiol compounds is a unique property of the β2-AR. β-Adrenergic agonists conserve redox properties, while β-AR antagonists do not share this property. Based on this observation, involvement of a redox mechanism in the activation of the receptor has been suggested (43). Direct agonist-mediated disulfide bond reduction may, therefore, be involved in receptor activation, but this has not been shown. Therefore, it is possible that disulfide exchange is a property uniquely acquired in the divergence of the folding of β-adrenergic receptors from other adrenergic receptors and G-protein-coupled receptors.

Acknowledgments—We are indebted to Dr. D. D. Oprián, Brandeis University, Waltham, MA, for providing the synthetic gene of β2-AR prior to publication of the group's work. A manuscript describing their work is also being reviewed at this time. We thank JoAnne Holl for the preparation of our manuscript, Robert J. Gavrin and Cynthia Boehm for technical assistance, Dr. Shen-Shu Sung for assistance in the modeling studies, and Dr. Robert Molday, University of British Columbia Van-couver, Canada, for the hydromab culture of 1D4.

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