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Role of Extracellular Disulfide-Bonded Cysteines in the Ligand Binding Function of the β_2-Adrenergic Receptor†

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ABSTRACT: Evidence is presented for a role of disulfide bridging in forming the ligand binding site of the β_2-adrenergic receptor (βAR). The presence of disulfide bonds at the ligand binding site is indicated by "competitive" inhibition by dithiothreitol (DTT) in radioligand binding assays, by specific protection by β-adrenergic ligands of these effects, and by the requirement of disulfide reduction for limit proteolysis of affinity ligand labeled receptor. The kinetics of binding inhibition by DTT suggest at least two pairs of disulfide-bonded cysteines essential for normal binding. Through site-directed mutagenesis, we indeed were able to identify four cysteines which are critical for normal ligand binding affinities and for the proper expression of functional βAR at the cell surface. Unexpectedly, the four cysteines required for normal ligand binding are not those located within the hydrophobic transmembrane domains of the receptor (where ligand binding is presumed to occur) but lie in the extracellular hydrophilic loops connecting these transmembrane segments. These findings indicate that, in addition to the well-documented involvement of the membrane-spanning domains of the receptor in ligand binding, there is an important and previously unsuspected role of the hydrophilic extracellular domains in forming the ligand binding site.

The β_2-adrenergic receptor (βAR)[1] is an integral membrane glycoprotein coupled, through the guanine nucleotide binding regulatory protein G_s, to adenylyl cyclase and the production of the second-messenger cAMP. The βAR is among the best characterized of the hormone or agonist-responsive G-protein-coupled receptors. On the basis of mechanistic and structural similarities between βAR and the visual pigment rhodopsin, we proposed a topological model of the βAR as having seven membrane-spanning domains, an extracellular glycosylated amino terminus, and cytoplasmic domains which are phosphorylated by the regulatory enzymes β-adrenergic receptor kinase and cAMP-dependent protein kinase (Dixon et al., 1986a). This proposed structure has been partially validated through the use of limited proteolysis to localize these and other functional and structural landmarks (Dohlman et al., 1987a). The physical and regulatory properties of this member of the family of G-protein-coupled receptors have recently been reviewed (Dohlman et al., 1987b; Sibley et al., 1987).

One outcome of the alignment and comparison of the deduced amino acid sequences of the various members of this receptor family has been the identification of a number of highly conserved cysteine residues ($Cys^{106,184,341}$) which may play important structural or functional roles, possibly by forming intramolecular disulfide bonds (Dohlman et al., 1987b). Indeed, the effects of disulfide and sulfhydryl reactive reagents on the adenylyl cyclase coupled βAR system have been the object of extensive study. Pedersen and Ross (1985) have demonstrated the ability of DTT-treated turkey β_1-ad-

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[1] Abbreviations: DTT, dithiothreitol; βAR, β_2-adrenergic receptor; ^{125}I-CYP, 3-[^{125}I]iodocyanopindolol; ^{125}I-pBABC, *p*-(bromoacetamido)-benzyl-1-[^{125}I]iodocarazolol; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; G_s, stimulatory guanine nucleotide binding regulatory protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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renergic receptor to stimulate the GTP binding and hydrolyzing activity of G_s. Malbon and co-workers have reported a reduction-dependent mobility shift of reduced vs nonreduced βAR on SDS–PAGE (Moxham & Malbon, 1985; Moxham et al., 1988). Since denaturation by SDS is opposed when disulfide bridges are intact, such an alteration in the electrophoretic properties of a protein in the absence of a disulfide reducing agent can provide indirect evidence for intramolecular disulfide bonds. Moreover, treatment of membranes or partially purified βAR with the disulfide bond reducing agent DTT has variously been reported to result in a loss of βAR ligand binding sites, and/or result in a reduction in binding affinity, or have no effect (Mukherjee & Lefkowitz, 1977; Lucas et al., 1978; Vauquelin et al., 1979; Wright & Drummond, 1983). One possible explanation for such discrepant findings is that these studies were performed using crude receptor preparations in which effects on G_s, adenylyl cyclase, and other cellular components often could not be ruled out (Lucas et al., 1978; Bottari et al., 1979; Vauquelin et al., 1979, 1980a,b; Vauquelin & Maguire, 1980; Heidenreich et al., 1982). Similarly, the sulfhydryl-specific reagent *N*-ethylmaleimide was shown to inhibit ligand binding to the receptor, due not to modification of the receptor itself but to an allosteric effect of modification of G_s (Korner et al., 1982).

In this report, biochemical techniques using pure βAR preparations reveal a functional role of disulfide bonds in ligand binding. Through the use of site-directed mutagenesis, the location of the cysteines sensitive to DTT treatment has been identified. Their location in extracellular domains of the receptor has important implications for our understanding of how multiple membrane-spanning domains might assemble to form the ligand binding pocket.

Experimental Procedures

Materials. All buffers and hydrogen peroxide were purchased from Mallinckrodt. [α-^{35}S]dATP, [^{3}H]dihydroalprenolol, ^{125}I Bolton–Hunter reagent, and Enlightning were from New England Nuclear. [^{14}C]Iodoacetamide and 3-[^{125}I]iodocyanopindolol (^{125}I-CYP) were from Amersham. V8 protease from *Staphylococcus aureus* was purchased from ICN. All reagents for electrophoresis were purchased from Bethesda Research Laboratories, except for the protein standards which were obtained from Pharmacia. Photographic supplies were from Kodak. Phosphate-buffered saline and all other reagents used for tissue culture were from Gibco. Dithiothreitol was from Bachem. Chloramine T, β-mercaptoethanol, iodoacetamide, all adrenergic ligands, bovine serum albumin (BSA), and protease inhibitors were purchased from Sigma. All reagents for molecular biology were purchased from Promega, except pTZ vector (Pharmacia LKB Biotechnology). Reagents for the purification of βAR, and the preparation of ^{125}I-pBABC, are given in the original citations below.

Purification and Reconstitution of βAR. Hamster lung βAR was purified to >95% purity by sequential alprenolol–Sepharose affinity chromatography and steric-exclusion high-performance liquid chromatography, as previously described (Benovic et al., 1984). βAR was quantified by saturation radioligand binding according to Caron and Lefkowitz (1976), as detailed below. Specific activity for the receptor (~15000 pmol/mg of protein) was determined by [^{3}H]dihydroalprenolol binding and protein determination using the Amido Shwarz assay (Caron & Lefkowitz, 1976; Schaffner & Weissmann, 1975). Receptor purity was assessed by radioiodination using the Chloramine T method (Greenwood et al., 1963) or with ^{125}I Bolton–Hunter reagent, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (10% acrylamide) and autoradiography (Laemmli, 1970). All samples were solubilized prior to electrophoresis by diluting 1:1 with SDS–PAGE sample buffer (8% SDS, 10% glycerol, 25 mM Tris-HCl, pH 6.5, and 0.003% bromphenol blue) and 5% β-mercaptoethanol (where indicated). Gels were dried and subjected to autoradiography on Kodak XAR-5 film at −80 °C.

^{14}C-Alkylation. Pure soluble βAR samples were diluted 5-fold with denaturation buffer (7 M guanidine hydrochloride, 500 mM Tris-HCl, pH 8.5, and 2 mM EDTA), in the absence or presence of 5 mM DTT. Samples were incubated for 20 min at 60 °C, then cooled to 23 °C, and transferred to a tube containing 20 μCi of dried [^{14}C]iodoacetamide (8 mM final concentration), as described (O'Dowd et al., 1984). After 1 h at 23 °C, an equal volume of 200 mM iodoacetamide in the absence or presence of 125 mM DTT in water was added and incubated for an additional hour at 23 °C to complete the reaction. Alkylation was stopped by chromatography over Sephadex G-25 in 25 mM Tris-HCl, pH 7.4. The samples were lyophilized and resuspended for electrophoresis in 50% SDS–PAGE sample buffer. SDS–PAGE and autoradiography were carried out as described above, except that prior to being dried, the gel was fixed and treated with Enlightning autoradiography enhancer according to the manufacturer's instructions. To validate the methodology, protein standards, for which the number of disulfide bonds are known, were similarly treated in parallel with pure βAR. These are bovine serum albumin (M_r ~67000) (Brown, 1974), ovalbumin (M_r ~43000) (Thompson & Fisher, 1978), carbonic anhydrase (M_r ~30000) (Sciaky et al., 1976), soybean trypsin inhibitor (M_r ~20100) (Brown et al., 1966), and α-lactalbumin (M_r ~14400) (Vanaman et al., 1970). Phosphorylase *b* (M_r ~94000) was also used, although information on disulfide bonding was not found.

Affinity Labeling of βAR. Pure βAR was affinity labeled using *p*-(bromoacetamido)benzyl-1-[^{125}I]iodocarazolol (^{125}I-pBABC) as previously described (Dickinson et al., 1985). Briefly, 10 nM pure βAR was incubated in binding buffer B (20 mM NaH_2PO_4, pH 7.2, 100 mM NaCl, and 0.1% digitonin) and 100 nM ^{125}I-pBABC for 1 h at 25 °C, either in the absence or in the presence of 10 μM alprenolol. The ^{125}I-pBABC alkylation reaction was stopped by addition of cysteine (1 mM final concentration) and alprenolol (10 μM final concentration). The samples were lyophilized and resuspended for electrophoresis in 50% SDS–PAGE sample buffer. SDS–PAGE and autoradiography were carried out as described above.

Assessment of the Effects of DTT Treatment of Pure βAR on Radioligand Binding. All binding experiments for Figures 2–4 were performed in a final volume of 0.5 mL of binding buffer A, 3–10 pM βAR, and 600 pM ^{125}I-CYP (unless otherwise indicated), in triplicate. After equilibrating for 3 h at 23 °C, samples were chromatographed over Sephadex G-50 columns in binding buffer A, to separate bound from free ligand. To measure the time course of DTT inhibition of ^{125}I-CYP binding (Figure 2), pure βAR was treated with or without 10 mM DTT in 50 μL of binding buffer A. At the indicated times, the samples were effectively stopped by dilution with 450 μL of binding buffer A, 600 pM ^{125}I-CYP, and 20 mM hydrogen peroxide (where indicated), and equilibrated for 3 h at 23 °C before chromatography on Sephadex G-50, as before. The effect of 10-fold-diluted DTT alone (1 mM final concentration) on binding is minimal (cf. the 0-min time point in Figure 2, in which the final concen-

tration of DTT is 1 mM throughout the experiment). To examine the ability of β-adrenergic ligands to block the DTT inhibition of ^{125}I-CYP binding (Figure 3), pure βAR was added to binding buffer A with varying concentrations of either (−)-isoproterenol, (+)-isoproterenol, (−)-norepinephrine, or (−)-alprenolol and incubated for 1 h at 23 °C. Fifty microliters of 100 mM DTT in buffer A, or buffer A alone, was added to a 450-μL sample (10 mM final DTT concentration) and incubated for another 30 min at 23 °C. These were then chromatographed on Sephadex G-50 in binding buffer A, and an aliquot was used to determine ^{125}I-CYP binding as before. To determine the mechanism by which DTT inhibits ligand binding (Figure 4), pure βAR was added to binding buffer A with varying concentrations of ^{125}I-CYP and final concentrations of 10, 3, 1, or 0 mM added DTT and equilibrated for 1 h at 23 °C before chromatography on Sephadex G-50.

V8 Proteolysis. ^{125}I-pBABC-labeled pure βAR was incubated at 37 °C in 75 μL of phosphate-buffered saline, 0.1% digitonin, 0.07% SDS, and (where indicated) 25 mM DTT. After 1 h, 135 μL of *Staphylococcus aureus* V8 protease in water was added to a final concentration of 500 μg/mL. The digestions were allowed to proceed for 24 h at 37 °C and stopped by a 1:1 dilution with SDS–urea–PAGE sample buffer (1% SDS, 8 M urea, and 10 mM phosphoric acid, pH 6.8, with Tris-HCl, 1% β-mercaptoethanol, and 0.003% bromphenol blue). SDS–PAGE was carried out as before, except using 6.5 M urea, 0.1% SDS, and 100 mM H_3PO_4, pH 6.8 with Tris-HCl and 9% acrylamide in the separating gel and 5% acrylamide in the loading gel (SDS–urea–PAGE) (Swank & Munkres, 1971).

Mutagenesis Constructs and Expression of βAR Transcripts. Standard molecular biology techniques were used throughout (Maniatis et al., 1982). The *NcoI*/*SalI* fragment (−1 to 1817 bp relative to the initiator ATG) of the human βAR cDNA (Kobilka et al., 1987a) was ligated into the polylinker of vector pTZ18R (Pharmacia). Single-stranded DNA from this construction was prepared and used in the Amersham oligonucleotide-directed mutagenesis system, according to the manufacturer's instructions. Briefly, oligonucleotides encoding a Val for Cys substitution and a unique restriction enzyme recognition site were synthesized (Applied Biosystems Model 380B), annealed to single-stranded βAR in pTZ, and extended with Klenow fragment in the presence of dCTPαS. The nonmutant (non-phosphorothioate) strand was removed by nicking with *NciI* and exonuclease III digestion, and the double-stranded homoduplex mutant was generated with DNA polymerase I. Unique restriction fragments (*NcoI* and *BglII*, or *BglII* and *EcoRV*) encompassing each mutant sequence were ligated into the βAR cDNA (*NcoI*/*SalI* fragment) under the control of the Rous sarcoma virus promoter in the expression vector pBC12B1 (Cullen, 1987). Cells were maintained at 37 °C, 5% CO_2, in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). DNA for each construction was prepared and sequenced by the dideoxy chain-termination method to verify all base pair substitutions (Sanger et al., 1977) and transfected into COS-7 cells by the DEAE-dextran method (Cullen, 1987). The nine pBC12B1–βAR constructs, including wild-type βAR, are identical except for the indicated Cys (TGC) for Val (GTC) codon substitution, and it is assumed that each is comparably transcribed and translated. COS-7 cells were harvested after 60–72 h, and membranes were prepared for ligand binding. Briefly, the cells were washed with phosphate-buffered saline, then scraped in lysis buffer (5 mM Tris-HCl, pH 7.4, and 5 mM EDTA), and vigorously polytron-treated for 5 s on ice. The homogenate was then centrifuged at 2000*g*, and the resulting supernatant was centrifuged at 45000*g*. The membrane pellet was washed twice in lysis buffer, resuspended to a final concentration of 300 mg of protein/mL in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA, and frozen. ^{125}I-CYP binding in these membranes was assessed, typically using 0.06–4.8 μg of protein/1 mL (~2 pM βAR) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA. B_{max} and K_d values were determined by varying ^{125}I-CYP concentrations and competing with alprenolol (10 μM). Low-affinity (non-G-protein coupled) K_i values were determined in the presence of 100 μM GTP by displacing 20 or 130 pM ^{125}I-CYP with various concentrations of agonists. After equilibrating for 2 h at 23 °C, incubations were stopped by filter binding on GF/C (Whatman) paper, followed by 3 × 4 mL rinses with ice-cold 25 mM Tris-HCl, pH 7.4.

Results

Evidence for Disulfide Bonds within the β_2-Adrenergic Receptor. This study examines the structural and functional role of possible disulfide bridging in the βAR. As shown in Figure 1, [^{14}C]iodoacetamide labeling of free sulfhydryls was performed on pure βAR denatured by heating and the addition of 6 M guanidine (O'Dowd et al., 1984). While there is only very weak labeling of the denatured βAR migrating at M_r ~64 000 (Figure 1B, "none"), labeling of the receptor in the presence of DTT (Figure 1B, "DTT") leads to a marked (≥15-fold) increase in [^{14}C]iodoacetamide incorporation. Since the DTT-reduced form of βAR is a much better substrate for alkylation than the denatured but nonreduced form, most of the cysteines appear to be involved in disulfide bonding. This is consistent with the findings of Malbon and co-workers, showing a reduction-dependent mobility shift of reduced vs nonreduced βAR on SDS–PAGE (Moxham & Malbon, 1985; Moxham et al., 1988). However, direct alkylation of βAR sulfhydryls has an added advantage that an estimate of the number of disulfide bonds can be made. Thus, since we have deduced from the gene that there are 15 cysteine residues in the βAR, a *ratio* of labeling of ≥15:1 in reduced vs nonreduced forms of the protein indicates that there are several, and probably as many as the maximum of 7, pairs of disulfide-bonded cysteines in the βAR in vitro.

Biochemical Evidence for Disulfide Bonds Near the Ligand Binding Site. In Figures 2–4, we demonstrate that DTT inhibits binding of the β-adrenergic radioligand ^{125}I-CYP to pure βAR. The inhibition of ^{125}I-CYP binding by DTT is demonstrated to be time dependent (Figure 2, "DTT") and then partially reversible with the subsequent addition of hydrogen peroxide (Figure 2, "DTT + H_2O_2"). A strong oxidizing agent, hydrogen peroxide, has no effect on ^{125}I-CYP binding alone (Figure 2, "H_2O_2") but accelerates re-formation of disulfide bridges. Incomplete recovery of ligand binding may be due to incorrect pairing between intramolecular cysteines, protein denaturation, or modification. One hundred millimolar *N*-ethylmaleimide or iodoacetamide has no effect on ^{125}I-CYP binding; β-mercaptoethanol is ~10-fold less potent than DTT (not shown).

In Figure 3, DTT inhibition of ^{125}I-CYP binding to the βAR is shown to be blocked by pretreatment with either an agonist or an antagonist, indicating that occupancy of the binding site confers protection from reduction. Protection by a ligand or enzyme substrate is commonly used to selectively prevent the modification of binding site residues by group-specific reagents. If activity is retained following modification in the presence

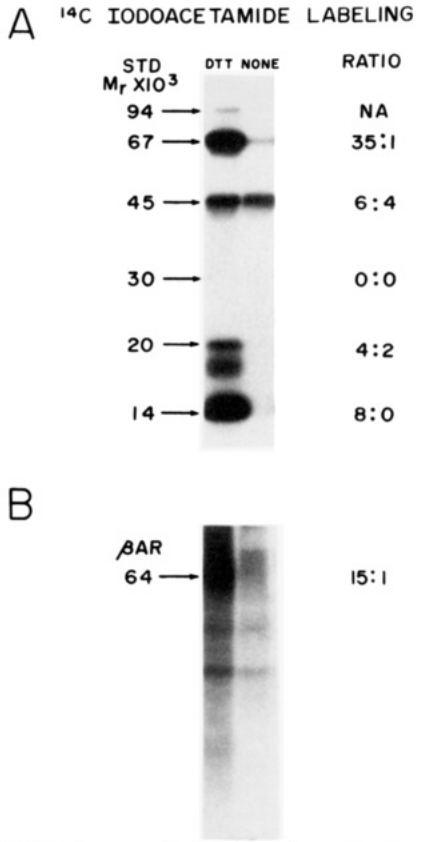

FIGURE 1: [^{14}C]Iodoacetamide labeling of cysteines in reduced and nonreduced βAR. (A) To test the validity of this method for estimating the number of disulfide bonds in the βAR, radioalkylation was first carried out using reduced (DTT) and nonreduced (NONE) proteins for which the extent of disulfide bonding has been established previously. These proteins are the same as those used as molecular weight markers (STD). Samples were denatured with 6 M guanidine hydrochloride, reduced (where indicated, DTT), labeled with [^{14}C]-iodoacetamide, and subjected to SDS–PAGE and autoradiography (1-week exposure) as detailed under Experimental Procedures. The predicted ratio of labeling for these proteins (Brown et al., 1966, 1974; Thompson & Fisher, 1978; Sciaky et al., 1976; Vanaman et al., 1970) is given (RATIO) and represents the alkylation of cysteines in the reduced vs nonreduced (disulfide-bonded) state. The experimentally derived ratio values are from scanning densitometry of the autoradiograph. The expected ratio was obtained for all standard proteins except soybean trypsin inhibitor (M_r ~20 000), possibly due to incomplete recovery of smaller polypeptides after molecular sieve chromatography (Sephadex G-25). NA, ratio not available. (B) Radioalkylation was carried out as in panel A, but using pure βAR as a substrate (1-week exposure). βAR was denatured in the presence (DTT) or absence of reducing agent. The M_r ~64 000 receptor (βAR, 64) is indicated. The M_r ~40 000 band is the result of degradation during purification and storage of the receptor. In multiple experiments, labeling occurs with a ratio of 15:1 or greater in the reduced vs nonreduced sample. The ratio values were obtained by scanning densitometry of the autoradiographs, as well as by visual comparison with lanes in which $^1/_{15}$th or $^3/_{15}$th of the fully reduced sample was loaded (data not shown). Variable labeling in the absence of DTT may reflect palmitoylation of Cys^{341} in a portion of our purified βAR preparations (O'Dowd et al., 1989). The expected ratios of labeling for each lane are given, assuming 1 free cysteine in the free sulfhydryl state and 14 cysteines in the disulfide-bonded state of the βAR.

but not in the absence of a bound ligand, it is assumed that a group in the binding site has been protected. Indeed, protection is observed to follow a pharmacological order of potency and stereospecificity identical with that typically observed for competition of β-adrenergic radioligand binding: (−)-alprenolol > (−)-isoproterenol > (+)-isoproterenol > (−)-norepinephrine. Moreover, protection by a biologically inactive antagonist such as alprenolol suggests that simple occupancy, rather than an agonist-dependent conformational change, is responsible for

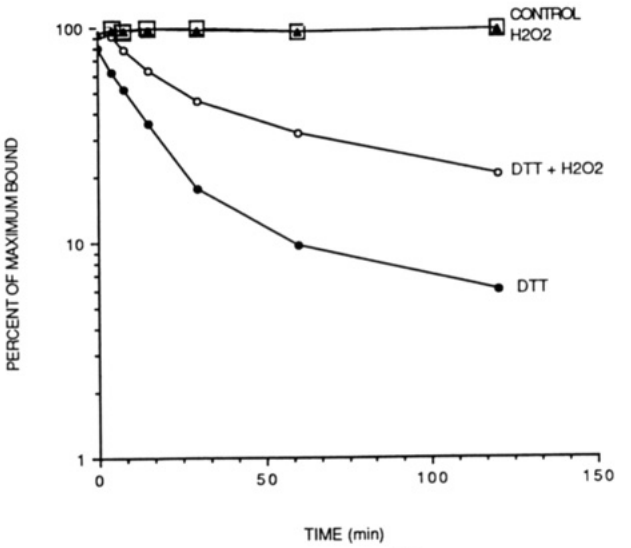

FIGURE 2: Mechanism of DTT inhibition of ^{125}I-CYP binding to pure βAR: time course and reversibility of DTT inhibition of binding. As detailed under Experimental Procedures, 10 pM pure βAR was incubated with (○, ●) or without (□, ▲) 10 mM DTT for the time indicated and then diluted 10-fold with a buffer containing 600 pM ^{125}I-CYP in the presence (○, □) or absence (●, ▲) of 20 mM hydrogen peroxide (H_2O_2). The samples were allowed to equilibrate for 3 h at 23 °C and then chromatographed on Sephadex G-50 to separate bound from free ligand. Receptor binding is expressed as a percent of the maximum bound (~5 fmol/0.5 mL).

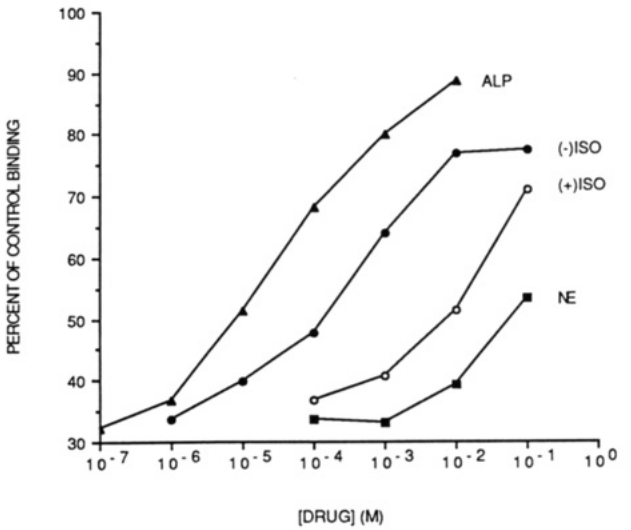

FIGURE 3: Mechanism of DTT inhibition of ^{125}I-CYP binding to pure βAR: prevention of the DTT-dependent loss of βAR ligand binding by β-adrenergic ligands. As detailed under Experimental Procedures, 10 pM pure βAR was equilibrated for 1 h at 23 °C with various concentrations of four different ligands (DRUG) and then treated with 10 mM DTT for an additional 30 min. The ligands used are (−)-isoproterenol [(●) (−)ISO], (+)-isoproterenol [(○) (+)ISO], (−)-norepinephrine [(■) NE], and (−)-alprenolol [(▲) ALP]. The incubation was stopped by dilution and chromatography on Sephadex G-50 to remove ligand and DTT, and the number of binding sites remaining for an aliquot of the sample was assessed with ^{125}I-CYP as before. Receptor binding is expressed as a percent of the maximum bound (~2.5 fmol/0.5 mL). The base line (30% of control binding) represents binding after DTT treatment in the absence of ligand. While higher than "physiological" concentrations of these drugs are required to preserve binding in the presence of DTT, this phenomenon is typically observed for competitive binding of two agents to a single binding site (in this case, drug and DTT).

the protection observed. These data suggest a sensitivity to DTT of at least one critical disulfide bond within the ligand binding site of the receptor.

Surprisingly, the results presented in Figure 2 indicate that

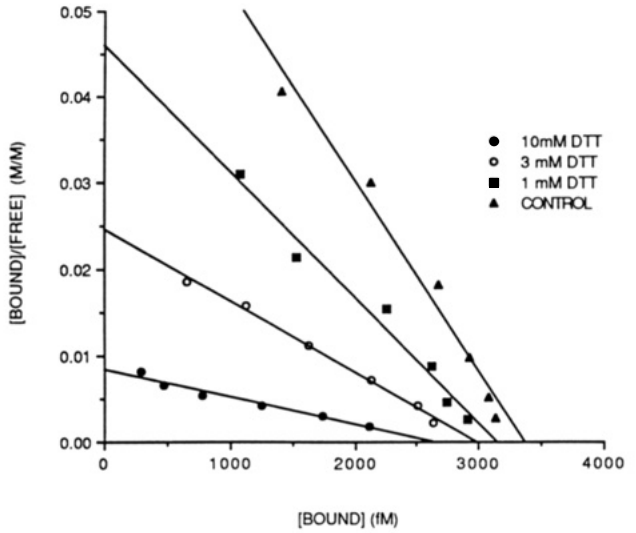

FIGURE 4: Mechanism of DTT inhibition of ^{125}I-CYP binding to pure βAR: competitive inhibition of ligand binding by DTT. As detailed under Experimental Procedures, pure βAR ($\sim$1.7 fmol/0.5 mL) was equilibrated with varying concentrations of ^{125}I-CYP in the absence (▲) or presence of DTT, at final concentrations of 10 mM (●), 3 mM (○), or 1 mM (□). Scatchard analysis (Scatchard, 1949) was performed; estimated K_d values for ^{125}I-CYP binding calculated for each condition are as follows: 325 pM (10 mM DTT), 120 pM (3 mM), 70 pM (1 mM), and 40 pM (none).

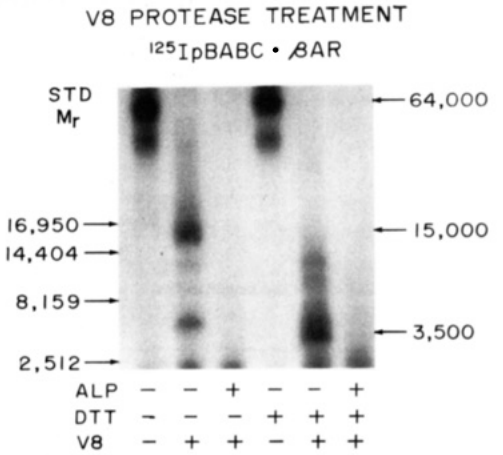

FIGURE 5: Effect of DTT on the pattern of limit V8 protease cleavage of ^{125}I-pBABC–βAR. βAR was labeled with ^{125}I-pBABC in the presence (+) or absence (−) of alprenolol (ALP) to define specificity. Samples were then incubated with (+) or without (−) V8 protease (V8) in the presence (+) or absence (−) of 25 mM DTT and then subjected to SDS–urea–PAGE and autoradiography as detailed under Experimental Procedures. The mobilities of the specifically labeled native βAR (64 000), partially digested βAR (15 000), and the limit digestion product (3500) are indicated. The faint M_r $\sim$40 000 band observed in the absence of V8 protease is the result of degradation during purification and storage of the βAR. Also given are the mobilities of the molecular weight standards (STD). These are horse heart myoglobin fragments of 16 950 (residues 1–153), 14 404 (1–131), 8159 (56–131), and 2512 (132–153).

the DTT-dependent loss of βAR binding is nonlinear when plotted as a function of log activity remaining vs time. When there is one essential disulfide bond being reduced, the log of the rate of inactivation is expected to be linear (Means & Feeney, 1971). However, differences in the reactivity of multiple disulfide bridges would be reflected in just such a nonlinear log rate of inactivation. These findings suggest that there may be two or more critical disulfide bonds being reduced in the absence of bound ligand.

Scatchard analysis (Scatchard, 1949) of ^{125}I-CYP binding in the presence of various concentrations of DTT gives straight lines with nonequal slopes and similar intercepts at the abscissa (Figure 4). Because DTT does not form a stable complex with target cysteine groups, and reduced disulfide bonds are readily re-formed under oxidizing conditions, DTT falls into the category of reversible protein-modifying reagents. Thus, insofar as the DTT-dependent reduction of disulfide bonds is presumed to lead to a reversible inhibition of ligand binding, Michaelis–Menten kinetics can be applied and these data interpreted to indicate a competitive mode of inhibition of ^{125}I-CYP binding. However, while competitive inhibition of ligand binding represents strong evidence for a binding site directed effect of DTT treatment, we have not found conditions which result in complete recovery of ligand binding following exposure to DTT. This may be a consequence of the irreversible loss of some ligand binding sites due to denaturation, and may explain the nonequal intercepts at the abscissa, indicating a small contribution of noncompetitive inhibition as well.

In order to provide additional physical evidence for a disulfide bond near the ligand binding site, we conducted proteolytic mapping experiments on reduced or nonreduced affinity-labeled βAR. This approach is similar to that used in our previous assignment of the site of incorporation of the β-adrenergic-specific antagonist ^{125}I-pBABC to the second transmembrane segment of the βAR (Dohlman et al., 1988). Since the binding site disulfide bonds and the site of incorporation of ^{125}I-pBABC are presumed to be close to one another, reduction of disulfide bonds might be required to achieve limit proteolysis of the affinity-labeled βAR. Indeed, as shown in Figure 5, while the reduced form of the affinity-labeled receptor is completely degraded to a fragment of M_r $\sim$3500 (Figure 5, lane 5), proteolysis of the nonreduced form of the receptor results in only partial degradation, predominantly to a M_r $\sim$15 000 species (Figure 5, lane 2). It thus appears that some of the V8 protease sensitive sites closest to the binding site of ^{125}I-pBABC are conformationally hidden in the nonreduced state, presumably due to steric hindrance by disulfide bonding. These results, together with data presented in Figures 2–4 showing competitive inhibition by DTT of ligand binding and protection from inhibition by β-adrenergic-specific ligands, provide strong evidence for disulfide bonding at or near the ligand binding site, the integrity of which is essential for normal binding of ^{125}I-CYP.

Evidence for a Role of Extracellular Cysteines in the Ligand Binding Function. In an attempt to localize the disulfide-bonded cysteines determined to be critical for ligand binding, we turned to the technique of site-directed mutagenesis and expression of the βAR gene. Our goal was to identify substitutions of individual cysteines which would most closely mimic the effects on ^{125}I-CYP binding observed after DTT treatment of pure βAR. Specifically, such mutations might exhibit a decreased binding affinity for ^{125}I-CYP, and possibly a reduced number of total binding sites. On the basis of our topographical model of the βAR discussed earlier, it was predicted that the DTT-sensitive cysteines at the binding site would be located in the hydrophobic transmembrane regions. Thus, a series of mutants were prepared in which the codons for potential binding site cysteines were replaced with that for valine. Valine was chosen because it is a hydrophobic amino acid, unlikely to disrupt folding of the hydrophobic transmembrane domains. The cysteines substituted include the four postulated to lie within the hydrophobic transmembrane segments ($Cys^{77,116,125,285}$), as well as the four in the hydrophilic loops connecting transmembrane segments II and III (Cys^{106}) and IV and V ($Cys^{184,190,191}$). The role of cytoplasmic cysteines was not examined here, since they are un-

Table I: Binding Parameters for Cysteine Mutants[a]

construct	domain	B_{max} (pmol/mg)	K_d (pM) ^{125}I-CYP	K_i (nM) (−)ISO	(+)ISO	NE	EPI
WT		24	11	0.130	4.0	5.8	0.360
CysΔVal^{77}	II	29	13	0.290	6.9	23	1.1
CysΔVal^{106}	II–III	0.45	173	3.5	210	110	471
CysΔVal^{116}	III	9.8	25	0.090	2.4	4.2	0.30
CysΔVal^{125}	III	18	11	0.460	16	27	4.1
CysΔVal^{184}	IV–V	0.71	47	1.8	55	320	16
CysΔVal^{190}	IV–V	1.2	124	3.4	153	130	55
CysΔVal^{191}	IV–V	0.68	167	2.9	672	120	57
CysΔVal^{285}	VI	1.7	19	0.040	2.1	2.4	0.095
NT		0.08					

[a] To identify the DTT-sensitive cysteines which may be located at the ligand binding site, eight different mutants were constructed in which a Val for Cys substitution was made, as indicated and as detailed under Experimental Procedures. ^{125}I-CYP binding for the expressed receptor was evaluated in COS-7 membranes (0.06–4.8 μg of protein/condition, ~2 pM βAR). B_{max} and K_d values were determined by varying ^{125}I-CYP concentrations and competing with alprenolol (10 μM). Low-affinity (non-G-protein-coupled) K_i values were determined in the presence of 100 μM GTP by displacing 20 or 130 pM ^{125}I-CYP with various concentrations of agonists: (−)ISO, (−)-isoproterenol; (+)ISO, (+)-isoproterenol; NE, (−)-norepinephrine; EPI, (−)-epinephrine (Cheng & Prushoff, 1973). All of the binding data (saturation analysis with ^{125}I-CYP and agonist competition curves) were analyzed by nonlinear least-squares regression, as previously described (DeLean et al., 1982). Underlined are B_{max} values <5% of wild type, as well as K_d and K_i values >10-fold that of wild-type-transfected receptor. The presumed location of each residue is given as a transmembrane segment number, or as a loop connecting two transmembrane segments. Values given are representative of two to three experiments with similar results. NT, nontransfected. WT, wild-type control transfected receptor.

likely to be at the ligand binding site, and were demonstrated previously not to be required for normal ligand binding (Kobilka et al., 1987b; Dixon et al., 1988; O'Dowd et al., 1988, 1989).

As shown in Table I, we have performed detailed ligand binding studies and analyzed these by computer modeling techniques. Of the eight Cys for Val substitution mutants analyzed, four display either normal (CysΔVal^{77,125}) or only partially diminished (CysΔVal^{116,285}) binding levels (B_{max}). These are localized to the hydrophobic transmembrane segments of the protein. In contrast, mutations of each of the four cysteine residues in the extracellular domains of the βAR result in markedly diminished levels of ^{125}I-CYP binding (i.e. <5% of wild type; CysΔVal^{106,184,190,191}). We interpret these data to reflect decreased expression of these mutant receptors at the cell surface. More significant is the observation that receptors with mutations of the transmembrane cysteines bind the β-adrenergic antagonist and agonists with normal affinities (Table I), while replacement of cysteines within the extracellular domains results in a markedly decreased affinity for the ligands tested. Following substitution of extracellular cysteines, binding affinities for the radiolabeled antagonist ^{125}I-CYP are reduced 4–16-fold. Affinities for the agonists (−)-norepinephrine, (−)-epinephrine, and the (−) and (+) stereoisomers of isoproterenol are reduced 14–1400-fold. Since substitution of cysteines in transmembrane domains resulted in binding affinities nearly comparable to the wild-type βAR, these data indicate that the four (or more) DTT-sensitive cysteines critical for the binding function of the receptor cannot be those in the transmembrane segments, as had been predicted. Furthermore, our findings suggest that alteration in the processing or membrane insertion of these receptors, as well as profound changes in the determinants for normal ligand binding affinities, occur in receptors in which extracellular cysteines have been mutated. It would thus appear that extracellular disulfide-bonded cysteines are critically important for forming or stabilizing the ligand binding site, and are most likely to be the same as those sensitive to DTT treatment.

Discussion

This report is one of a series of biochemical studies aimed at characterization of the structural and functional relationships of the βAR. From our initial elucidation of the gene structure and the encoded amino acid sequence of the hamster βAR (Dixon et al., 1986a), a number of testable hypotheses have emerged regarding the molecular basis for hormone binding specificity, regulation by specific protein kinases (leading to phosphorylation and desensitization), and effector coupling. A topography of seven membrane-spanning domains was proposed based on the deduced amino acid sequence and analogy with the homologous protein rhodopsin (Dixon et al., 1986a), and was subsequently tested through limited proteolysis experiments (Dohlman et al., 1987a). On the basis of identification of the sites of incorporation of β-adrenergic affinity ligands (Dohlman et al., 1988; Wong et al., 1988), as well as site-directed mutagenesis studies (Dixon et al., 1986b), the hormone binding site is believed to include the "hydrophobic pocket" formed by the bundle of membrane-spanning segments, analogous to the site of covalent binding of retinal to opsin (Ovchinnikov, 1982). This may be the functional basis for the stretches of remarkably homologous sequence found to be concentrated within the hydrophobic domains of the various members of the G-protein-coupled receptor family (Dohlman et al., 1987b). Another structural feature shared among the G-protein-coupled receptors is the presence of a number of highly conserved cysteine residues (Dohlman et al., 1987b). Accordingly, biochemical experiments were designed to examine the role of disulfides in maintaining receptor structure and binding function. Through in vitro mutagenesis, we have examined the molecular basis for the results of these experiments. Our findings have interesting functional implications given the known primary structure and the presumed topology of the βAR.

The data in Figure 1 showing differential labeling with [^{14}C]iodoacetamide in the presence or absence of DTT suggest extensive disulfide bridging in the βAR. The competitive and reversible nature of DTT inhibition of ligand binding to the βAR (Figures 2 and 4), the specific protection by β-adrenergic ligands of these effects (Figure 3), and also the requirement of DTT to achieve limit proteolysis of affinity-liganded receptor (Figure 5) all point to the involvement of one or more critical disulfide bridges for the ligand binding function of βAR. Indeed, the kinetics of inactivation (Figure 2) suggests at least two pairs of disulfide-bonded cysteines essential for normal binding. The systematic analysis by site-directed mutagenesis of all potential cysteines critical for the ligand binding function has identified the four extracellular cysteines to be the most likely sites of action of DTT. These conclusions are partially

corroborated by earlier data from site-directed mutagenesis (Dixon et al., 1988), in which two mutations of cysteine residues ($Cys^{106,184}$) were reported to affect binding of (−)-isoproterenol.

As presented in Table I, binding parameters for eight mutations of potential binding site cysteines were evaluated. It is clear from these data that four of the eight mutants display markedly lower binding affinities for ^{125}I-CYP and for a number of agonists, as well as significantly reduced levels of expression. Despite the decreased B_{max} for four of these mutants, ligand binding in the plasma membrane is sufficient (450–1200 fmol/mg of protein) to measure agonist affinities by competition with ^{125}I-CYP. Thus, while there is considerable biochemical and genetic evidence that seven transmembrane domains assemble to form the ligand binding pocket, the markedly altered ligand binding affinities for the mutant receptors expressed suggest that extracellular disulfide-bonded cysteines are important for the functional integrity of the ligand binding site. These disulfides may contribute directly to ligand binding specificity, or they might play a structural role in maintaining the correct topology for ligand binding.

These findings also serve to highlight the complexity of interpreting the changes in ligand binding properties which are often observed for site-directed mutants. One commonly heard criticism of such studies is that changes which affect a particular function may simply reflect some nonspecific alteration in receptor conformation, stability, or processing, and thus such studies can best provide information about which residues are *not* important for that particular function. Indeed, at least one posttranslational modification event, disulfide bond formation, is prevented by substitution of cysteine residues. As reflected in lower B_{max} values for some of these mutants, this appears to result in a lower number of functional receptors reaching the plasma membrane. In light of such caveats, perhaps more meaningful (and unexpected) is the observation that the cysteine residues that fall within the hydrophobic transmembrane domains appear *not* to be required for normal expression and ligand binding properties. Indeed, our radioligand binding experiments indicate no significant change in the B_{max}, affinity, specificity, or stereoselectivity exhibited by mutations at these sites.

Similarly, Khorana and colleagues have elucidated the role of cysteines in the functionality of bovine rhodopsin (Karnik et al., 1988). A series of mutants was prepared in which Cys residues were replaced by Ser, and then evaluated for their levels of expression, pattern of glycosylation, retinal binding, and coupling to transducin. Only substitutions of Cys^{110} and Cys^{187}, localized to the intradiscal (topologically extracellular) domains of rhodopsin, were characterized by abnormally lower levels of protein expression, as well as incomplete glycan processing and an inability to bind 11-*cis*-retinal. Their conclusions, consistent with our findings, are that a disulfide bond in the intradiscal domain may be critical in directing correct helix–helix interactions that form the 11-*cis*-retinal binding pocket. Whereas the βAR has four Cys residues in this domain ($Cys^{106,184,190,191}$), rhodopsin has only three ($Cys^{110,185,187}$).

Finally, Fraser (1989) has quite recently reported on mutagenesis of several of the same cysteines ($Cys^{77,190,191,285}$) examined here. Similar agonist and antagonist binding affinities were observed for each of the mutants examined by both groups, though relative B_{max} values differed in some cases. While our experiments were performed with a transient transfection system, Fraser's use of stable transfected cells may have resulted in the arbitrary selection of some clones bearing a high copy number of the βAR gene, and which as a consequence displayed relatively higher B_{max} values.

Through biochemical techniques and the method of site-directed mutagenesis, a number of disulfide-bonded cysteines near the ligand binding site have been identified. The location of these residues indicates a role for extracellular domains, in addition to the membrane-spanning domains, in forming the ligand binding pocket of the βAR. These results suggest a complex interaction of multiple transmembrane domains assembling to form the functionally active βAR, and that this assembly is critically dependent on the formation of disulfide bonds in the extracellular regions which connect these domains.

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Satellite DNAs Contain Sequences That Induce Curvature[†,‡]

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ABSTRACT: The repeating units of mouse, rat, and α-monkey satellites have been cloned. All three show properties that are characteristic of curved DNA: (i) their migration in polyacrylamide gels is slower than predicted from their sequences, and (ii) they appear as curved molecules when visualized by electron microscopy. All three satellite repeats contain runs of $d(A\cdot T)_{n\geq 3}$ residues that are likely to be responsible for their curvature. From analysis of 20 different satellite DNA sequences, we conclude that, in satellite DNA, adenine residues show a high tendency to cluster in groups of three or more.

A significant proportion of eukaryotic genomic DNA is formed by tandemly repeated satellite DNA sequences (Brutlag, 1980). Their monomeric units are variable in length and quite complex in sequence. Several functions have been proposed for satellite DNA (Singer, 1982). Satellite DNAs are universally, although not exclusively, associated with regions of constitutive heterochromatin (Brutlag, 1980; John & Miklos, 1979). In particular, a centromeric location has been demonstrated for many satellites (Peacock et al., 1978), and a role in centromeric chromatin condensation during metaphase has been proposed for mouse satellite DNA (Lica et al., 1986). It has also been suggested that satellite DNAs might be involved in some gene amplification events (Bostock & Clark, 1980) as well as in spindle attachment during metaphase (Avila et al., 1983).

The molecular basis of the peculiar properties shown by satellite chromatin, such as transcriptional inactivation and high degree of condensation, is still unknown. Nuclear proteins that appear to interact preferentially with satellite DNA sequences have been detected in several eukaryotic cells (Hsieh & Brutlag, 1978; Levinger & Varshavsky, 1982; Garreau & Williams, 1983; Strauss & Varshavsky, 1984). In particular, a non-histone nuclear protein that recognizes runs of $d(A\cdot T)_{n\geq 5}$

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