# Fat Cell $\beta_1$-Adrenergic Receptor: Structural Evidence for Existence of Disulfide Bridges Essential for Ligand Binding†

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ABSTRACT: Agents that react chemically with sulfhydryl groups of proteins modify the response of adenylate cyclase to stimulation by β-adrenergic agonists. *N*-Ethylmaleimide, an agent that alkylates sulfhydryl groups, inactivates both the catalytic moiety of adenylate cyclase and the stimulatory, regulatory guanine nucleotide binding protein $N_s$ of rat fat cells but fails to affect binding of antagonists to the β-adrenergic receptor [Malbon, C. C., Graziano, M. P., & Johnson, G. L. (1984) *J. Biol. Chem. 259*, 3254-3260]. Treating membranes of rat fat cells with dithiothreitol or β-mercaptoethanol, agents that reduce disulfide bridges of proteins, results in a loss of binding of β-adrenergic radioligands to the receptor. The specific binding of radioligands to β-adrenergic receptors that are solubilized in digitonin is affected similarly by treatment with disulfide bridge reducing agents. β-Adrenergic receptor purified from rat fat cells and treated with β-mercaptoethanol (10%) and then subjected to gel electrophoresis in the presence of sodium dodecyl sulfate migrates as a $M_r$ 67 000 peptide [Cubero, A., & Malbon, C. C. (1984) *J. Biol. Chem. 259*, 1344-1350]. In the absence of disulfide bridge reducing agents, however, the purified receptor exhibits greater electrophoretic mobility, migrating as a peptide with $M_r$ 54 000. Treating the native form of the purified receptor with β-mercaptoethanol (0.1-10%) or dithiothreitol (0.1-10 mM) decreases the ability of the receptor to bind β-adrenergic ligands, decreases the electrophoretic mobility of the receptor, and results in receptor peptides migrating with molecular weight ranging from 54 000 to 67 000 when subjected to gel electrophoresis in the presence of sodium dodecyl sulfate. Treating purified receptor with *N*-ethylmaleimide (10 mM) does not alter the electrophoretic mobility of the receptor ($M_r$ 54 000) on polyacrylamide gels under nonreducing conditions. These data demonstrate for the first time the existence of intramolecular disulfide bridges in β-adrenergic receptors and suggest that the integrity of these disulfide bridges is essential for the binding of ligands by the receptor.

The β-adrenergic catecholamines regulate a broad spectrum of physiological processes (Stiles et al., 1984), including lipolysis (Fain, 1973). Many of the effects of β-adrenergic hormones are believed to be mediated via the activation of adenylate cyclase and generation of intracellular cyclic AMP (Sutherland & Rall, 1960). The β-adrenergic receptor (R), the catalytic moiety of adenylate cyclase (C), and a stimulatory, regulatory guanine nucleotide binding component ($N_s$)[1] that transduces agonist binding to R into activation of C constitute the three major components of the adenylate cyclase system through which stimulatory hormones act [for review, see Ross & Gilman (1980)]. The mechanism by which agonist binding activates the β-adrenergic receptor remains obscure.

One approach to the study of receptor activation has been to investigate the effects of agents that react chemically with sulfhydryl groups and disulfide bridges of proteins. This approach albeit indirect has provided several important insights. Both C and $N_s$ have been shown to be inactivated by treatment with *N*-ethylmaleimide, an agent that alkylates sulfhydryl groups of proteins (Howlett et al., 1978; Orly & Schramm, 1978; Ross et al., 1978; Stadel & Lefkowitz, 1979; Korner et al., 1982; Northup et al., 1983). C displays greater sensitivity than $N_s$ to inactivation by treatment with *N*-ethylmaleimide in several systems including rat fat cells (Malbon et al., 1984). In contrast to C and $N_s$, the β-adrenergic receptor of rat fat cells displays little sensitivity to *N*-ethylmaleimide. Treating fat cell membranes with 3 mM *N*-ethylmaleimide abolishes the activity of C, reduces the activity of $N_s$ by 90%, but does not affect the ability of R to bind β-adrenergic antagonists (Malbon et al., 1984).

β-Adrenergic receptors do display sensitivity to *N*-ethylmaleimide when membranes are exposed to *N*-ethylmaleimide and a β-adrenergic agonist simultaneously. A marked decrease in specific binding of β-adrenergic radioligands is observed in membranes treated simultaneously with *N*-ethylmaleimide and isoproterenol (Bottari et al., 1979; Vauquelin et al., 1980; Vauquelin & Maguire, 1980; Heidenreich et al., 1982). Treating membranes with either β-adrenergic agonists or *N*-ethylmaleimide alone or with *N*-ethylmaleimide in combination with a β-adrenergic antagonist fails to affect the ability of the receptor to bind radioligands (Bottari et al. 1979; Vauquelin et al., 1979; Vauquelin & Maguire, 1980; Heidenreich et al., 1982). These data suggest the possibility that the binding of β-adrenergic agonists results in the exposure of or generation of a sulfhydryl group in the receptor, in $N_s$, or perhaps in both that is essential for receptor binding of β-adrenergic ligands and is sensitive to reaction with *N*-ethylmaleimide

β-Adrenergic receptors appear to be sensitive to disulfide bridge reducing agents such as dithiothreitol. Both the $K_{act}$

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[1] Abbreviations: $N_s$, guanine nucleotide binding stimulatory regulatory component of adenylate cyclase; DHA, dihydroalprenolol; SDS, sodium dodecyl sulfate; IABP, [(iodoazido)benzyl]pindolol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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of isoproterenol for the stimulation of adenylate cyclase and the ability of β-adrenergic receptors to bind radioligands have been shown to be decreased in membranes exposed to dithiothreitol (Lucas et al., 1978; Vauquelin et al., 1979). However, it is not clear from these data whether or not the effects of disulfide bridge reducing agents on both of these parameters are a reflection of the action of the reducing agent upon R, $N_s$, or both R and $N_s$. In the present study, we investigate the existence of disulfide bridges and sulfhydryl groups in one component of the system, the β-adrenergic receptor, using pure $\beta_1$-adrenergic receptor isolated from rat fat cells. The data presented demonstrate directly the existence of intramolecular disulfide bridges in the β-adrenergic receptor.

## Experimental Procedures

*Receptor Preparations.* Isolation of rat fat cells, preparation of highly purified membranes from these cells, and solubilization of membranes with digitonin were performed as described earlier (Culbero & Malbon, 1984).

*Radioligand Binding Assay.* Measurements of β-adrenergic receptors in fat cell membranes, digitonin extracts prepared from these membranes, and purified preparations of receptor were performed with the high-affinity, β-adrenergic antagonist (−)-[$^3$H]dihydroalprenolol as described previously (Malbon et al., 1978; Cubero & Malbon, 1984). β-Mercaptoethanol, dithiothreitol, and *N*-ethylmaleimide when employed were added directly to the incubation mixture containing radioligand and receptor preparations.

*Purification of β-Adrenergic Receptor.* β-Adrenergic receptors were purified from rat fat cell membranes by affinity chromatography and high-pressure size-exclusion liquid chromatography as described earlier (Cubero & Malbon, 1984).

*Radioiodination of Purified Receptor.* Purified β-adrenergic receptors were radiolabeled with Na$^{125}$I and chloramine T (Greenwood et al., 1969) and then collected by precipitation with an ice-cold solution of 10% trichloroacetic acid. Alternatively, radioiodination was catalyzed by lactoperoxidase (Marchalonis, 1969). Following radiolabeling catalyzed by lactoperoxidase, the receptor was applied to a Sephadex G-50 column (0.5 × 20 cm, Bio-Rad Laboratories) that was preequilibrated and run in a buffer composed of 100 mM $KH_2PO_4/K_2HPO_4$ (pH 7.0), 200 mM NaCl, and 0.05% digitonin. The chromatography was performed at 22 °C. Fractions (0.1 mL) were collected, and the peak of radiolabeled receptor was identified by liquid scintillation spectrometry.

*Polyacrylamide Gel Electrophoresis.* Samples were subjected to electrophoresis on homogeneous, 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate (Laemmli, 1970), under reducing and nonreducing conditions.

*Autoradiography.* Radioiodinated β-adrenergic receptors were incubated in 0.125 M Tris-HCl, pH 6.8, 4% SDS, and 20% glycerol in the absence or presence of the indicated concentrations of dithiothreitol, β-mercaptoethanol, or *N*-ethylmaleimide for 30 min at 37 °C and then subjected to gel electrophoresis. Following electrophoresis the gels were fixed, stained, destained, and dried as described (Malbon, 1982). Proteins were visualized by autoradiography of the dried gels on Kodak XAR-5 film.

*Silver Staining.* Purified, unlabeled β-adrenergic receptors were incubated in 0.125 M Tris-HCl, pH 6.8, 4% SDS, and 20% glycerol in the absence or presence of 10% β-mercaptoethanol for 30 min at 37 °C and then subjected to gel electrophoresis. Silver staining of the fixed gel was performed with the Bio-Rad silver stain kit according to the method of Merril et al. (1981).

*Protein Determination.* Protein content of particulate membranes and extracts was determined by the method of Lowry et al. (1951).

*Molecular Weight Determinations.* The relative molecular weights ($M_r$) of proteins separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate were established with either the use of [$^{14}$C]methylated molecular weight standards [myosin (heavy-chain) (200 000), phosphorylase *b* (92 500), bovine serum albumin (68 000), ovalbumin (43 000), α-chymotrypsinogen (25 700), β-lactoglobulin (18 400), and cytochrome *c* (12 300)] or the use of unlabeled molecular weight standards [myosin (heavy-chain) (200 000), β-galactosidase (116 250), phosphorylase *b* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400)].

*Materials.* Rats were purchased from Taconic Farms (Germantown, NY). Materials used were purchased from the following sources: crude bacterial collagenase (*Clostridium hystolyticum*, type I, lot 43H044) from Worthington; Na$^{125}$I (sp act. 2000 Ci/mmol) and (−)-[$^3$H]DHA (sp act. 104 Ci/mmol) from New England Nuclear; digitonin, (±)-propranolol, and *N*-ethylmaleimide from Sigma Chemical Co.; dithiothreitol and β-mercaptoethanol from Aldrich Chemical Co.; [$^{14}$C]methylated molecular weight standards from Bethesda Research Laboratories; unlabeled molecular weight standards and silver stain kit from Bio-Rad Laboratories.

## Results

The effects of agents that react chemically with sulfhydryl groups or disulfide bridges of β-adrenergic receptors in fat cell membranes and digitonin extracts prepared from these membranes were examined by the use of equilibrium binding techniques and the high-affinity, β-adrenergic antagonist radioligand (−)-[$^3$H]dihydroalprenolol (DHA). Specific binding of (−)-[$^3$H]DHA to fat cell membranes and to soluble extracts of these membranes displayed pharmacological properties consistent with those expected of $\beta_1$-adrenergic receptors (Williams et al., 1976; Malbon et al., 1978; Cubero & Malbon, 1984). Specific binding of (−)-[$^3$H]DHA to fat cell membranes was decreased by the inclusion of disulfide bridge reducing agents such as dithiothreitol or β-mercaptoethanol in the incubation mixture (Figure 1, panels A and B). Treatment with 10 mM dithiothreitol decreased specific binding of (−)-[$^3$H]DHA to membranes by 50%, while treatment with 10% β-mercaptoethanol abolished receptor binding altogether. The specific binding of (−)-[$^3$H]DHA to soluble extracts was equally sensitive to these effects of disulfide bridge reducing agents (Figure 1, panels A and B). Treating particulate membranes or soluble extracts with 0.1 or 1 mM *N*-ethylmaleimide, an agent that alkylates "free" sulfhydryl groups (Knauf & Rothstein, 1971), did not affect the ability of either preparation to bind specifically the (−)-[$^3$H]DHA (Figure 1, panel C). A slight reduction in ligand binding activity was routinely observed when 10 mM *N*-ethylmaleimide was included in the incubation mixture. The basis for this loss of ligand binding resulting from treatment with 10 mM *N*-ethylmaleimide remains obscure.

Scatchard plots of specific binding of (−)-[$^3$H]DHA to fat cell membranes are curvilinear with upward concavity (Figure 2) (Malbon et al., 1978; Cabelli & Malbon, 1979; Guidicelli et al., 1979). Scatchard plots of ligand binding to membranes exposed to 1 mM dithiothreitol or 1% β-mercaptoethanol were also curvilinear with upward concavity but were displaced toward the origin with respect to the control curve. These data suggest that the reduction of one or more disulfide bridges of the β-adrenergic receptor by either 1 mM dithiothreitol or 1%

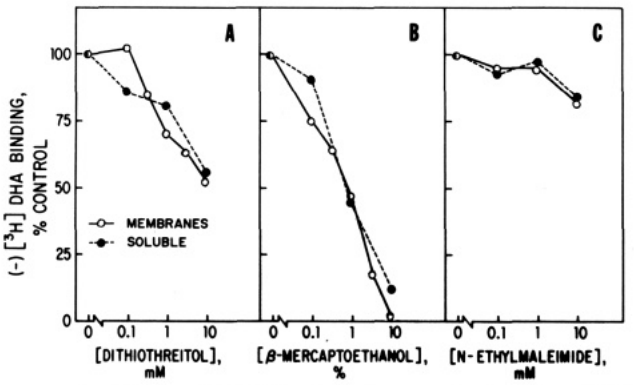


FIGURE 1: Effect of dithiothreitol, β-mercaptoethanol, and *N*-ethylmaleimide upon (−)-[$^3$H]dihydroalprenolol binding to fat cell membranes and to β-adrenergic receptors solubilized in digitonin. Binding of (−)-[$^3$H]DHA to fat cell membranes and to digitonin-solubilized extracts was assayed at 10 and 100 nM radioligand, respectively, as described under Experimental Procedures. Dithiothreitol, β-mercaptoethanol, and *N*-ethylmaleimide were included in the incubation mixtures where indicated. The specific binding of (−)-[$^3$H]DHA to membranes and to soluble preparations was 0.2 and 0.5 pmol/mg of protein, respectively, as determined by competition studies using 10 μM (±)-propranolol. Data are expressed as the mean values from two separate experiments each assayed in triplicate. Variance among the triplicates was routinely less than 5%.

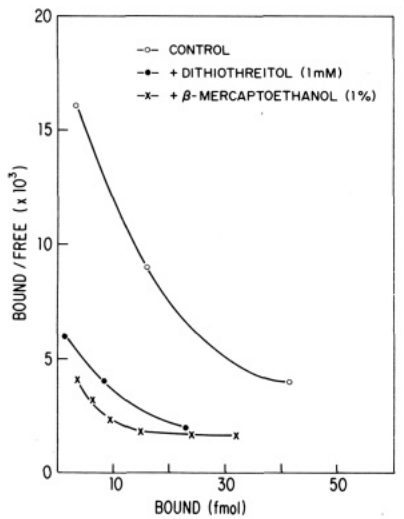


FIGURE 2: Scatchard analysis of the steady-state binding of (−)-[$^3$H]dihydroalprenolol to membranes of rat fat cells: effects of dithiothreitol and β-mercaptoethanol on binding. Binding of (−)-[$^3$H]DHA to fat cell membranes at increasing concentrations of radioligand (2–100 nM) was performed as described under Experimental Procedures. The data were analyzed by the method of Scatchard (1949) and are expressed as the mean values of triplicate determinations from a single experiment performed twice with essentially identical results.

β-mercaptoethanol results in an apparent loss of the capacity of the receptor to bind ligand.

These radioligand binding studies provide indirect evidence that the integrity of one or more disulfide bridge(s) in the receptor is essential for the receptor to display specific binding of a β-adrenergic antagonist. Our ability to purify fat cell $\beta_1$-adrenergic receptors to apparent homogeneity permitted a more direct investigation of the effects of disulfide bridge reducing agents on β-adrenergic receptor structure. Purified, radioiodinated receptor that was treated with β-mercaptoethanol (10%) migrated as a $M_r$ 67 000 peptide during gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 3A, lane 1). Interestingly, in the absence of treatment with a disulfide bridge reducing agent, the purified receptor migrated with greater electrophoretic mobility as a peptide

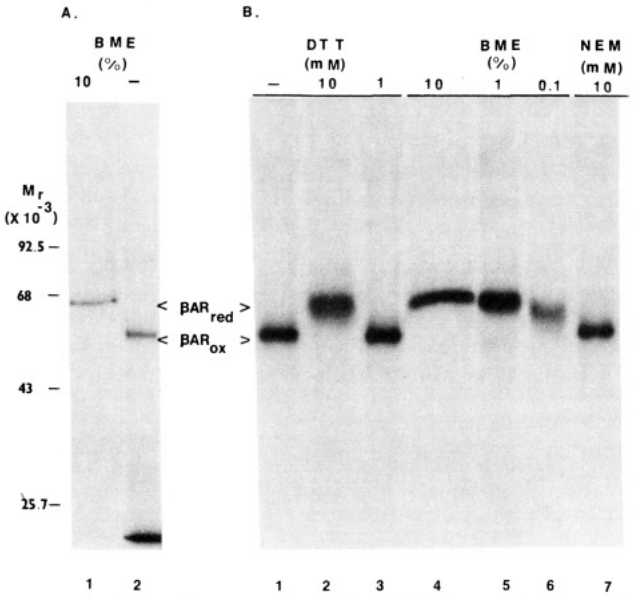


FIGURE 3: Autoradiogram of purified, radioiodinated β-adrenergic receptor that was treated with disulfide bridge reducing agents or *N*-ethylmaleimide and then subjected to gel electrophoresis in the presence of sodium dodecyl sulfate. (Panel A) A 0.3-mL aliquot (3.2 ng of protein) of purified $\beta_1$-adrenergic receptor was lyophilized to 0.1 mL in a Savant Speed Vac and then radioiodinated with lactoperoxidase. The radioiodinated receptor was treated with (lane 1) or without (lane 2) 10% β-mercaptoethanol and subjected to gel electrophoresis as described under Experimental Procedures. The autoradiogram is a 3-day exposure. (Panel B) A 0.3-mL aliquot (15 ng of protein) of purified $\beta_1$-adrenergic receptor was lyophilized to 0.1 mL and then radioiodinated with chloramine T. The radioiodinated receptor was treated with dithiothreitol, β-mercaptoethanol, or *N*-ethylmaleimide as indicated and then subjected to gel electrophoresis as described under Experimental Procedures. The autoradiogram is a 2-day exposure. $\beta AR_{red}$ denotes purified β-adrenergic receptor in the reduced state; $\beta AR_{ox}$ denotes purified β-adrenergic receptor in the oxidized state.

with $M_r$ 54 000 (Figure 3A, lane 2). Native, purified receptor subjected to gel electrophoresis in sodium dodecyl sulfate and stained with the silver reagents also was found to migrate as a peptide of $M_r$ 54 000 in the absence of treatment with disulfide bridge reducing agents, while observed to migrate as a peptide of $M_r$ 67 000 in the presence of 10% β-mercaptoethanol (Figure 4, lanes 1 and 3). These data demonstrate that the increased migration of the radioiodinated receptor as a $M_r$ 54 000 peptide is due to preexisting intramolecular disulfide bridges and is not the result of oxidizing conditions used for iodination. Treating the purified receptor with dithiothreitol (10 mM) or β-mercaptoethanol (0.1–10%) decreased the electrophoretic mobility of the peptide, whereas exposing the receptor to 10 mM *N*-ethylmaleimide was without effect on its electrophoretic mobility (Figure 3B, lanes 2–7). The purified, radioiodinated receptor treated with increasing concentrations of β-mercaptoethanol and subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate migrated as a broadened band with diminished electrophoretic mobility.

The effects of chemical reduction of disulfide bridges on both radioligand binding to and electrophoretic mobility of the receptor were examined with β-adrenergic receptor purified from rat fat cells. Specific binding of (−)-[$^3$H]DHA to β-adrenergic receptors was decreased by the inclusion of β-mercaptoethanol in the incubation mixture (Figure 5, panel B). Treating the receptor with increasing concentrations of β-mercaptoethanol also decreased the mobility of the receptor upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 5, panel A). These observations

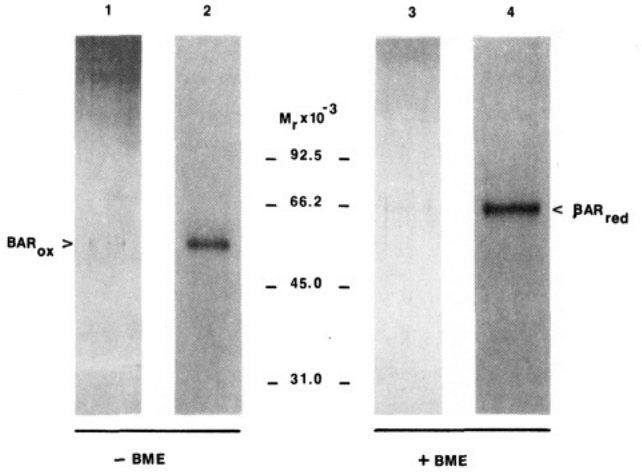


FIGURE 4: Silver stain and autoradiogram of purified β-adrenergic receptor treated with disulfide bridge reducing agents and then subjected to gel electrophoresis in the presence of sodium dodecyl sulfate. (Lanes 1 and 3) A 1.0-mL aliquot (200 ng of protein) of purified $\beta_1$-adrenergic receptor was concentrated to 0.1 mL in an Amicon Centricon-30. The receptor was then divided into two 0.05-mL aliquots and treated without (lane 1) or with (lane 3) 10% β-mercaptoethanol, subjected to gel electrophoresis, and stained with silver as described under Experimental Procedures. (Lanes 2 and 4) A 0.3-mL aliquot of purified receptor was lyophilized to 0.1 mL and radioiodinated with lactoperoxidase. The radioiodinated receptor was treated without (lane 2) or with (lane 4) 10% β-mercaptoethanol and subjected to gel electrophoresis as described under Experimental Procedures. The autoradiogram is a 3-day exposure. $\beta AR_{red}$ denotes purified β-adrenergic receptor in the reduced state; $\beta AR_{ox}$ denotes purified β-adrenergic receptor in the oxidized state.

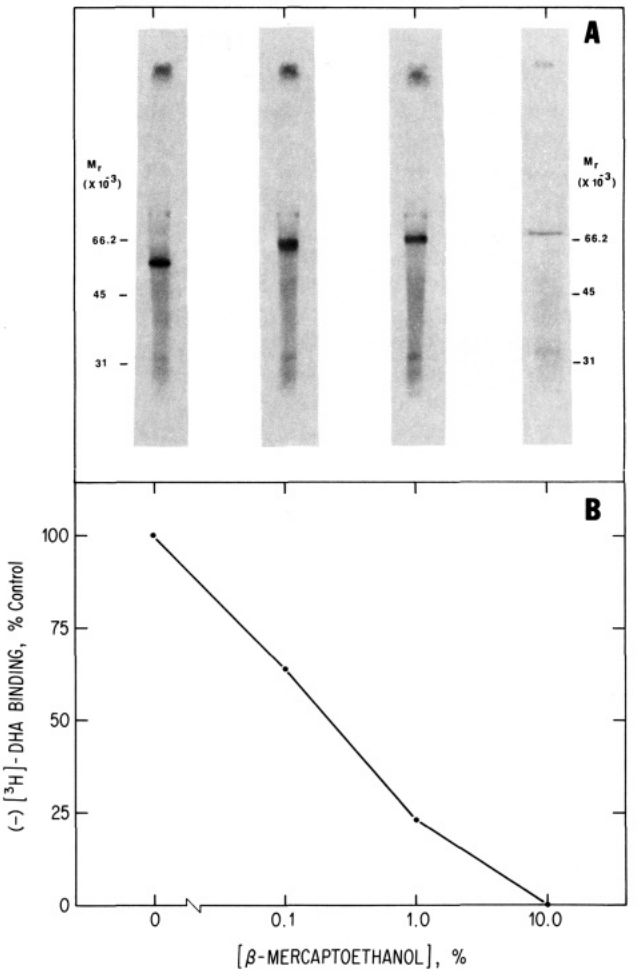


FIGURE 5: Effect of β-mercaptoethanol on the mobility of purified, radioiodinated β-adrenergic receptor subjected to gel electrophoresis in the presence of sodium dodecyl sulfate (panel A) and on the specific binding of (–)-[$^3$H]DHA to purified β-adrenergic receptors (panel B). (Panel A) A 0.1-mL aliquot of purified receptor was radioiodinated with chloramine T. The radioiodinated receptor was treated with the indicated concentrations of β-mercaptoethanol and subjected to gel electrophoresis in the presence of sodium dodecyl sulfate as described under Experimental Procedures. The autoradiogram is a 1-day exposure. (Panel B) Binding of (–)-[$^3$H]DHA to purified β-adrenergic receptors was assayed at 100 nM radioligand as described under Experimental Procedures. β-Mercaptoethanol was included in the incubation mixture at the indicated concentrations. Data are expressed as the mean values of triplicate determinations from a single experiment performed twice with essentially identical results.

support the tenet that the integrity of intramolecular disulfide bridges is essential for the expression of ligand binding by the β-adrenergic receptor.

It was of interest to ascertain if the change in electrophoretic mobility that results from exposure of the receptor to a disulfide bridge reducing agent was a general phenomenon that is characteristic of other membrane proteins or a phenomenon unique to the β-adrenergic receptor. A sample of β-adrenergic receptor that was purified up to the second pass of high-pressure size-exclusion liquid chromatography and contained other membrane proteins was radioiodinated, treated either with or without dithiothreitol or β-mercaptoethanol, and then subjected to gel electrophoresis. The autoradiogram of the gel revealed that the β-adrenergic receptor was the only major peptide in this preparation that displayed decreased mobility upon gel electrophoresis following treatment with a disulfide bridge reducing agent (Figure 6).

## Discussion

The components of the adenylate cyclase system that are involved in transducing β-adrenergic catecholamine binding into activation of the catalytic moiety display differing sensitivities to agents that react chemically with sulfhydryl groups or disulfide bridges. The catalytic moiety is sensitive to *N*-ethylmaleimide at concentrations that do not affect β-adrenergic binding activity (Stadel & Lefkowitz, 1979; Malbon et al., 1984). *N*-Ethylmaleimide has also been shown to have effects on the activity of $N_s$ (Korner et al., 1982) and more directly on the β subunit of this component (Northup et al., 1983). Exposing membranes to *N*-ethylmaleimide in concert with β-adrenergic agonists, but not antagonists, results in a decrease in radioligand binding to β-adrenergic receptors (Bottari et al., 1979; Vauquelin et al., 1980; Vauquelin & Maguire, 1980; Heidenreich et al., 1982). These results suggest the possibility that agonist binding to β-adrenergic receptors results in the exposure or generation of a sulfhydryl group in $N_s$, in the β-adrenergic receptor, or in both that is sensitive to *N*-ethylmaleimide. In the present study fat cell membranes treated with either dithiothreitol or β-mercaptoethanol displayed a decrease in specific radioligand binding. Digitonin-solubilized receptors of fat cell membranes also displayed a loss of binding activity upon exposure to either dithiothreitol or β-mercaptoethanol. Earlier, Vauquelin et al. (1979) reported that β-adrenergic receptors solubilized from turkey erythrocyte membranes with digitonin also displayed a loss of receptor binding activity once exposed to dithiothreitol. Thus, the effects of agents that react chemically with either sulfhydryl groups or disulfide bridges on the stimulation of the hormone-sensitive adenylate cyclase system may reflect their actions upon either $N_s$, the β-adrenergic receptor, or both.

The availability of pure $\beta_1$-adrenergic receptor from rat fat cells has permitted us to investigate directly the role of disulfide bridges in at least this component of the system. Purified, β-adrenergic receptor subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate under

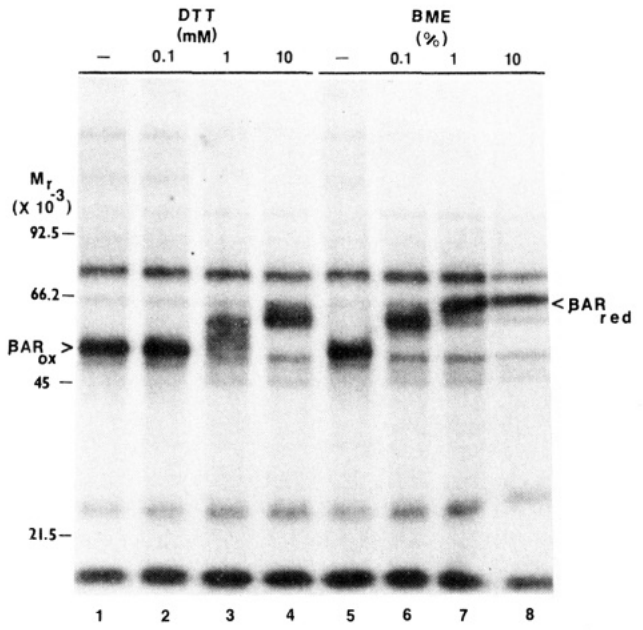


FIGURE 6: Autoradiogram of a radioiodinated, partially purified preparation of β-adrenergic receptor treated with disulfide bridge reducing agents and then subjected to gel electrophoresis in the presence of sodium dodecyl sulfate. A 0.4-mL aliquot (50 ng of protein) of a partially purified preparation of fat cell β-adrenergic receptor was lyophilized to 0.1 mL and radioiodinated with lactoperoxidase. The radioiodinated samples were treated with or without dithiothreitol or β-mercaptoethanol and then subjected to gel electrophoresis as described under Experimental Procedures. The autoradiogram is a 3-day exposure. $\beta AR_{red}$ denotes β-adrenergic receptor in the reduced state; $\beta AR_{ox}$ denotes purified β-adrenergic receptor in the oxidized state.

reducing conditions migrated as a single peptide $M_r$ 67 000. However, when subjected to gel electrophoresis under nonreducing conditions, this receptor migrated with increased mobility as a peptide with $M_r$ 54000. Purified receptor treated with *N*-ethylmaleimide, in contrast, migrated as a $M_r$ 54 000 peptide when subjected to gel electrophoresis in the presence of sodium dodecyl sulfate under nonreducing conditions. These observations suggest that it is the modification of disulfide bridges rather than of "free" sulfhydryl groups that is essential to the change in electrophoretic mobility of the receptor.

Treating purified receptor with β-mercaptoethanol decreased specific binding of radioligand to the receptor in a dose-dependent fashion. This effect of β-mercaptoethanol on radioligand binding to the purified receptor parallels the effect of this reducing agent on the apparent mobility of the receptor upon gel electrophoresis in the presence of sodium dodecyl sulfate. Increasing concentrations of this disulfide bridge reducing agent produced decreases in both the binding activity of the receptor and the mobility of the receptor on polyacrylamide gels in the presence of sodium dodecyl sulfate. These data demonstrate that the chemical reduction of disulfide bridges within the receptor results in an attenuation and eventual abolition of the ability of the receptor to bind β-adrenergic antagonists.

Recently, we have investigated the effects of incubating purified β-adrenergic receptor with isoproterenol and *N*-ethylmaleimide simultaneously on the mobility of the receptor subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate under nonreducing conditions. An agonist-induced change in the electrophoretic mobility of the purified β-adrenergic receptor has not been observed.[2] Previously, Vauquelin & Maguire (1980) demonstrated that functional $N_s$ is required to observe a decrease in ligand binding in membranes incubated simultaneously with *N*-ethylmaleimide and a β-adrenergic agonist. An agonist-induced alteration of the electrophoretic mobility of the purified receptor under nonreducing conditions may well require the presence of functional $N_s$.

Rashidbaigi et al. (1983) reported that two peptides ($M_r$ 55 000 and 65 000) of membranes of S49 wild-type mouse lymphoma cells were specifically labeled by the β-adrenergic photoaffinity label [([$^{125}$I]iodoazido)benzyl]pindolol (IABP). Additionally, it was observed in their study that exposure of cells to epinephrine resulted in the preferential loss of labeling of the $M_r$ 55 000 peptide. Recently, these investigators reported that photoaffinity labeling of the $M_r$ 55 000 peptide also was lost preferentially upon treatment of the S49 wild-type mouse membranes with 1 mM dithiothreitol (Clark et al., 1983). Specific photoaffinity labeling of both peptides by IABP was abolished by treatment of the membranes with 10 mM dithiothreitol (Clark et al., 1983). The nature of the $M_r$ 55 000 species of receptor identified with IABP has not been elucidated. Strulovici & Lefkowitz (1984) speculated that the $M_r$ 55 000 form represents a degradation product of the $M_r$ 65 000 receptor peptide and that this $M_r$ 55 000 species is more efficiently cleared from the plasma membrane upon agonist-induced desensitization. Alternatively, we propose that the $M_r$ 55 000 peptide identified upon photolysis with IABP represents receptor that was not completely reduced prior to electrophoresis. The conditions employed by Rashidbaigi et al. (1983) to reduce the solubilized membranes may not have been optimal and may have resulted in an incomplete reduction of the β-adrenergic receptor. The results reported in the present study suggest that complete reduction of the receptor requires 4–5-fold higher concentrations of β-mercaptoethanol than those employed in the studies of Rashidbaigi et al. (1983). Cross-linking of peptide bonds within the receptor by photolysis also may prevent complete unfolding of the receptor even upon complete chemical reduction. In addition, our observations call into question prior speculations by ourselves (Cubero & Malbon, 1984) and others (Stiles et al., 1982) that a minor $M_r$ 55 000 peptide observed in purified and photolabeled preparations of β-adrenergic receptor, respectively, represents a proteolytic fragment of the $M_r$ 67 000 form of the receptor. An equally plausible explanation for the appearance of the $M_r$ 55 000 form is that of incomplete chemical reduction of the receptor prior to electrophoresis on polyacrylamide gels.

Recently, we have purified the $\beta_2$-adrenergic receptors from S49 mouse lymphoma cells (George & Malbon, 1985) and rat liver (Graziano et al., 1985). Both of these $\beta_2$-adrenergic receptors migrate as peptides with $M_r$ 65 000–67 000 when subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate under reducing conditions. Under nonreducing conditions, these receptors migrate as peptides with $M_r$ 55 000.[3] These data demonstrate the existence of intramolecular disulfide bridges in both $\beta_1$- and $\beta_2$-adrenergic receptors. The observation by Rashidbaigi et al. (1983) that exposure of cells to epinephrine resulted in the preferential loss of the photolabeling of the $M_r$ 55 000 peptide by IABP taken in context with the present data suggests the interesting possibility that agonist binding may be promoting the exposure or generation of free sulfhydryls and a net reduction of the native receptor. We speculate that cleavage

[2] C. P. Moxham and C. C. Malbon, unpublished observations.

[3] S. T. George, M. P. Graziano, and C. C. Malbon, unpublished observations.

of intramolecular disulfide bridges and/or exposure of free sulfhydryl groups may be involved in the activation of the β-adrenergic receptor by agonists.

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