The function of structural domains of the β-adrenergic receptor were probed by studying the ability of tryptic fragments of the receptor to catalyze the binding of guanosine-5′-O-(3-thiotriphosphate) (GTPγS) to the GTP-binding regulatory protein, Gs. β-Adrenergic receptor purified from turkey erythrocites was treated with trypsin under non-denaturing conditions. Such treatment decreased β-adrenergic ligand binding activity by only 15–25%. Active components of the limit digest were repurified by affinity chromatography on alprenolol-agarose and then reconstituted with purified Gs, into unilamellar phospholipid vesicles. After reconstitution, the proteolyzed receptor was able to catalyze agonist-stimulated binding of GTPγS to Gs, at a rate and extent equivalent to that of the nonproteolyzed receptor. The proteolyzed receptor was also partially activated upon reduction by dithiothreitol, as previously reported for the intact receptor (Pedersen, S. E., and Ross, E. M. (1985) J. Biol. Chem. 260, 14150–14157). The repurified, active tryptic digest contained two detectable peptides. One, of approximately 2 × 10^4 Da, contained either four or five of the amino-terminal membrane-spanning domains plus the intervening hydrophilic loops but not the amino-terminal extracellular, glycosylated peptide. The second, of 9,000–10,000 Da, was composed essentially of the two carboxyl-terminal membrane-spanning domains and the intervening extracellular, hydrophilic loop. These data indicate that most of the large intracellular hydrophilic loop and the hydrophilic, carboxyl-terminal region of the receptor are not necessary for the agonist-stimulated regulation of Gs.

The β-adrenergic receptor is one member of a large class of cell surface receptors that control the activity of GTP-binding regulatory proteins (Gs) involved in transmembrane signaling. The β-adrenergic receptor regulates Gs, which mediates the activity of adenylate cyclase (1). Other receptors that promote the accumulation of cyclic AMP also act upon Gs, and receptors that mediate production of other intracellular signals are appropriately selective for different G proteins. Thus, rhodopsin interacts with transducin (Gs) to cause activation of the retinal cyclic GMP phosphodiesterase (2). The muscarinic cholinergic receptor can activate both Gs and Gs, (3, 4), leading to phospholipid hydrolysis, inhibition of adenylate cyclase, and the activation of K+ channels (5).

The recent sequencing of cDNAs for two β-adrenergic receptors (6, 7) and two muscarinic cholinergic receptors (8, 9) has also indicated that these receptors are homologous both to each other and to rhodopsin. The sequence homology among the G protein-coupled receptors is not uniform but rather is concentrated in seven strongly hydrophobic regions. In the case of rhodopsin, these hydrophobic regions each span the membrane bilayer, such that the glycosylated amino terminus is on the luminal face of the disc membrane and the carboxyl terminus is on the cytoplasmic face (10; see also Fig. 1). Sequence homology and the presence of N-linked glycosylation sites near the amino termini suggest that the β-adrenergic and muscarinic cholinergic receptors are similarly folded. The conservation of sequence in the membrane-spanning regions argues for their functional importance, but details of their function are unknown.

The functions of the hydrophilic regions of the receptors have been only partially defined. The binding of G proteins presumably occurs on the hydrophilic, cytoplasmic face; the presence of certain homologous sequences in the first and second hydrophilic loops of the receptors make them likely sites for this interaction. The third intracellular, hydrophilic loop in these receptors displays relatively little conservation of sequence and its function is not well understood. Kühn and Hargrave (11) and Litman et al. (12) found that a single tryptic cleavage in this loop of rhodopsin did not adversely affect light-stimulated control of transducin but that more extensive proteolysis here inhibited regulatory activity of the bleached rhodopsin molecule. In a conceptually similar study using site-directed mutagenesis, Dixon et al. (13) showed that deletion of a serine residue at a potential cyclic AMP-dependent protein kinase substrate site did not decrease β-adrenergic regulation of adenylate cyclase. However, deletion of most of the large intracellular loop inhibited the receptor’s ability to activate Gs. Because the mutant receptor retained β-adrenergic ligand binding activity, these authors speculated that this loop may be involved in binding to Gs. Removal of all or most of the intracellular carboxyl terminal region of either rhodopsin or of the β-adrenergic receptor seems to have little effect on the regulatory function of these proteins (11–13).

In this study, we have used proteolysis of the β-adrenergic receptor to test the importance of three readily cleaved domains, the extracellular, amino-terminal region that contains bound carbohydrate, the large intracellular loop, and the carboxyl-terminal intracellular region. We find that removal

(Received for publication, April 27, 1987)
of these three domains by proteolysis followed by affinity repurification yields two noncovalently bound fragments that can both bind to \( \alpha \)-adrenergic ligands and stimulate guanine nucleotide binding to G, in response to \( \alpha \)-adrenergic agonists or to reduction by thiols.

**EXPERIMENTAL PROCEDURES**

**Materials**—(-)-Propranolol, alprenolol, (z)-cyanopindolol, and Lubrol 12 A9 were gifts from Ayerst Laboratories, Hassele Pharmaceuticals, Dr. G. Engel of Sandoz Pharmaceuticals, and ICI, Ltd. TPCK-treated trypsin was purchased from Sigma or Millipore. Soy trypsin inhibitor, tosyl-lysyl-chloromethyl ketone, and disopropylfluorophosphate were from Sigma. GTPyS from Boehringer Mannheim was a gift from P. Cure of England Nuclear. \([\text{25}^1]\)ICYP was prepared according to Engel et al. (15). \([\gamma^32P]\)ATP, synthesized according to Johnson and Walschel (16), was a gift from J. Gregory and Dr. M. H. Cobb of this department. Phosphatidylethanolamine and phosphatidylserine were purchased from Avanti Polar Lipids. Molecular weight standards were purchased from Pharmacia (high molecular weight) or Bethesda Research Laboratories (low molecular weight). Protein kinase C (Ca\(^{2+}\)/phospholipid-dependent enzyme), partially purified from rat brain according to Cobb (9), was phosphorylated using protein kinase C. Phosphorylation was carried out for 30 min at 30°C in medium containing 20 mM Hepes (pH 8.0), 5 mM MgCl\(_2\), 0.5 mM EDTA, 1 mM CaCl\(_2\), and 1–30 \( \mu \)M [\(\gamma^32P]\)ATP (100–1500 cpm/fmol). Phosphorylation was monitored by separating the labeled receptor on SDS-polyacrylamide electrophoresis gels and scintillation counting of the labeled band after its localization by autoradiography.

**Preparation and Assay of \( \beta \)-Adrenergic Receptor—**\( \beta \)-Adrenergic receptor was purified from turkey erythrocytes as described previously (19). Soluble receptor was assayed in digitonin solution according to the binding of the antagonist \([\text{25}^1]\)IICYP using centrifugal gel filtration to separate free and bound ligand (20). Reconstituted receptor was assayed using affinity filtration on glass fiber filters to remove unbound ligand (18, 20). Proteolytic fragments of the receptor were purified by HPLC on a 0.45 x 150 mm Synchron C\(_4\) column eluted with a gradient of 0.1% trifluoroacetic acid to 50% 1-propanol in 0.06% trifluoroacetic acid (6).

**Preparation and Assay of G, G.-**Purified \( \alpha \)-adrenergic receptor, G, was reconstituted into unilamellar phospholipid vesicles composed of phosphatidylethanolamine and phosphatidylserine on a \( \alpha \)-adrenergic agonist (6, 7), rhodopsin (10), and the muscarinic cholinergic receptor (8, 9) suggests that the largest amount of sequence homology, both identity and conservation, occurs within the proposed membrane-spanning regions. We reasoned that these regions are likely to be important for the integrity of the structure and/or function of the receptor. It also seemed likely that their hydrophobic nature might limit their accessibility to proteases. Fig. 2 shows the proteolysis products that were obtained when purified \( \beta \)-adrenergic receptor from turkey erythrocytes was treated at room temperature with trypsin in digitonin solution. The untreated receptor displays two bands, a minor species at \( M, \sim 52,000 \) and a major species at \( M, \sim 42,000 \). The \( 42,000 \)-Da species is at least partially deglycosylated, probably by proteolysis of 15–20 amino acids near the amino terminus (27, 28). The peptide that usually behaves as an \( M, \sim 42,000 \) silver-stained band concomitant with the appearance of smaller species. An intermediate of \( M, \sim 25,000 \) preceded the appearance of an \( M, \sim 19,000 \) product. An \( M, \sim 36,000 \) precursor of the \( M, \sim 25,000 \) peptide is barely visible in the figure, as is a poorly stained \( M, \sim 11,000 \) fragment. Treatment with high concentrations of trypsin did not result in complete proteolysis of the receptor; a detectable digest resulted even at 7.5 mg/ml of trypsin (not shown). The limit digest contained a major silver-stained band concomitant with the appearance of smaller species. An intermediate of \( M, \sim 25,000 \) preceded the appearance of an \( M, \sim 19,000 \) product. An \( M, \sim 36,000 \) precursor of the \( M, \sim 25,000 \) peptide is barely visible in the figure, as is a poorly stained \( M, \sim 11,000 \) fragment. Treatment with high concentrations of trypsin did not result in complete proteolysis of the receptor; a limit tryptic digest resulted even at 7.5 mg/ml of trypsin (not shown).

**RESULTS**

Comparison of the primary amino acid sequences of the \( \beta \)-adrenergic receptor (6, 7), rhodopsin (10), and the muscarinic cholinergic receptor (8, 9) suggests that the largest amount of sequence homology, both identity and conservation, occurs within the proposed membrane-spanning regions. We reasoned that these regions are likely to be important for the integrity of the structure and/or function of the receptor. It also seemed likely that their hydrophobic nature might limit their accessibility to proteases. Fig. 2 shows the proteolysis products that were obtained when purified \( \beta \)-adrenergic receptor from turkey erythrocytes was treated at room temperature with trypsin in digitonin solution. The untreated receptor displays two bands, a minor species at \( M, \sim 52,000 \) and a major species at \( M, \sim 42,000 \). The \( 42,000 \)-Da species is at least partially deglycosylated, probably by proteolysis of 15–20 amino acids near the amino terminus (27, 28), and may also be proteolysed at the carboxyl terminus (6). At increasing concentrations of trypsin, there was a gradual loss of the predominant \( M, \sim 42,000 \) silver-stained band concomitant with the appearance of smaller species. An intermediate of \( M, \sim 25,000 \) preceded the appearance of an \( M, \sim 19,000 \) product. An \( M, \sim 36,000 \) precursor of the \( M, \sim 25,000 \) peptide is barely visible in the figure, as is a poorly stained \( M, \sim 11,000 \) fragment. Treatment with high concentrations of trypsin did not result in complete proteolysis of the receptor; a detectable digest resulted even at 7.5 mg/ml of trypsin (not shown). The limit digest contained a major silver-stained band with a mobility similar to that of trypsin inhibitor. This fragment, which usually behaves as an \( M, \sim 19,000 \) peptide, will be referred to below as fragment A. The second component is a poorly stained \( M, \sim 11,000 \) peptide, referred to as fragment B. The band at \( M, \sim 27,000 \) in the right-most lanes is trypsin. This pattern of limit proteolysis resembles...
buffer containing 3% 2-mercaptoethanol was added, and the samples that displayed by rhodopsin (11, 12).

Part of each sample was diluted 600-fold with the same buffer containing 0.1 mg/ml soy trypsin inhibitor and assayed for [125I]ICYP-binding activity as described under "Experimental Procedures." Concentrated diisopropylfluorophosphate (0.13 mg/ml final concentration) was added to the remainder. After 10 min at 0 °C, one-half volume of 3-fold concentrated SDS dissociation buffer containing 3% 2-mercaptoethanol was added, and the samples were incubated at 37 °C for 30 min. Samples were analyzed on 12% polyacrylamide gels and silver stained as described. The band that appears at ~27,000 Da is trypsin, and the narrow band at Mr ~37,000 is a trypsin-resistant contaminant.

that displayed by rhodopsin (11, 12).

Surprisingly, receptors treated as described above retained their ability to bind β-adrenergic ligands. In the limit digest, the capacity to bind the β-adrenergic antagonist [125I]ICYP was decreased by only 23% (Fig. 2). In other experiments, in which no fragments of Mr >19,000 were found, the loss of binding capacity has been as little as 15% (not shown). The affinities for [125I]ICYP and for competing β-adrenergic ligands were also not substantially altered in the limit tryptic digest (not shown).

Similar patterns of proteolysis were observed when the receptor was treated with chymotrypsin, thermolysin, or subtilisin under nondenaturing conditions (not shown). Each protease produced limit digests that contained a prominent silver-stained band of Mr ~19,000~24,000 on SDS-polyacrylamide gels. These proteolyzed receptors also retained the ability to bind β-adrenergic ligands.

The trypsin-treated receptor also retained its ability to regulate Gs. In preliminary experiments, a limit digest of the sort shown in Fig. 2 was reconstituted into phospholipid vesicles with purified Gs, using established techniques (19). In these vesicles, the addition of the β-adrenergic agonist isoproterenol stimulated the rate of GDP-GTP binding to Gs to the same extent as observed for vesicles that contained untreated receptor (data not shown). This unexpected result prompted more complete characterization of the identities and functions of the proteolytic peptides that were derived from the receptor.

**Composition of the Limit Tryptic Digest of the β-Adrenergic Receptor**—The limit tryptic digest of the receptor contained two peptide components with Mr >6000 that could be identified by silver staining (Fig. 2). The Mr ~19,000 fragment A was isolated from the total digest by reverse-phase HPLC as described (6), identified by its mobility on SDS-polyacrylamide gels, and subjected to automated sequence analysis. The amino-terminal sequence of fragment A, which could be interpreted for 15–20 residues, begins at Arg309, indicating that tryptic cleavage occurred after Arg309 (6, confirmed in this study).

Because of uncertainty in assigning an exact molecular size to fragment A based only on its electrophoretic mobility on SDS gels, the location of its carboxyl terminus was approximated according to its differential retention or loss of defined antigenic determinants. Antisera were raised against the synthetic peptides listed in the legend to Fig. 1 and used to probe Western blots that contained the untreated receptor, fragment A, and intermediate proteolytic fragments (Fig. 3). These immunoblots showed that both the untreated receptor (lanes 1, 4, and 6) and a Mr ~25,000 proteolytic intermediate (lanes 3, 5, and 7) contained peptides I, II, and V. Fragment A, shown in lane 2 and as a slightly larger precursor in lane 3, contained the sequence only of peptide I, on the second extracellular loop. Antisera against peptides II or V, on the major intracellular loop, did not cross-react with fragment A in the samples shown in lanes 5 and 7, although fragment A was clearly apparent in these samples by silver staining (not shown). It is likely that the loss of immunoreactivity against peptide II and peptide V in fragment A reflects their proteolysis rather than an alteration of their tertiary structure. Both the proteolyzed receptor and its fragments were denatured before and during electrophoresis and the antisera were initially raised against short peptides.

These data indicate that fragment A includes at least the four amino-terminal membrane spanning domains and the intervening loops. It may also contain the fifth membrane-spanning region but does not extend significantly beyond Cys244. This maximum length would predict a true molecular
weight of ~24,000 for fragment A. Termination in the second extracellular loop (ArgZos) would predict a molecular weight of 19,500. Neither is inconsistent with the electrophoretic weight of 24,000 for fragment A. Termination in the second by reverse-phase HPLC and subjected to sequence analysis, the amino terminus was found to be ValZm within the major intracellular loop. Sequence data were interpretable for over 25-amino acid residues in three experiments. The apparent molecular size of fragment B suggested that it includes the two carboxyl-terminal membrane-spanning sequences and the intervening extracellular loop but a minimal amount of the intracellular carboxyl-terminal region. This assignment is also supported by the differential retention and loss of antigenic determinants. Fragment B prepared from radiolabeled receptor was specifically immunoprecipitated by antipeptide VII serum, indicating that it contains most of the seventh membrane-spanning region. This minimum length was confirmed by automated Edman sequencing of the major CNBr peptide of fragment B. Readable sequence was obtained through AspZos, just beyond the carboxyl terminus of peptide VII. However, antisera against peptide VIII that cross-reacted with the intact receptor did not react with fragment B. Therefore, peptide B terminates just to the carboxyl-terminal side of the seventh membrane span and before ArgZos. These data are consistent with tryptic cleavage between ArgZos-ArgSos, an area that is rich in basic residues.

To provide a more convenient monitor of the proteolysis of the receptor and to facilitate the detection of specific sites on tryptic fragments, the receptor was labeled with 32P using protein kinase C and [γ-32P]ATP. The time course of tryptic proteolysis of the [32P]phosphorylated receptor is shown in Fig. 4, using an acrylamide gradient gel to separate the small peptides. Initially there was a loss of the 32P-labeled receptor bands at M, ~52,000 and 42,000. This loss paralleled the formation of the two 32P-labeled proteolytic intermediates that electrophorese at apparent M, ~36,000 and 25,000. At later times, the conversion of these intermediates to fragment A coincided with the loss of all peptide-bound 32P from the gel, indicating that neither fragments A nor B was phosphorylated. These data suggest that at least one site of protein kinase C-catalyzed phosphorylation of the β-adrenergic receptor resides on the large intracellular loop, which is removed in the proteolytic cleavage of the apparent M, ~25,000 intermediate to fragment A (Figs. 3 and 4).

Phosphorylation in this region is probably on Ser79, Thr277, or Ser31 according to the specificity of protein kinase C (29–31). The fragments containing the 32P label must have been cleaved to M, <25,000, or they would have been detected in these or other experiments.

Preliminary experiments suggest that the receptor is also phosphorylated by protein kinase C between ArgZos and the carboxyl terminus, most likely between Ser415 and Ser421. However, the extent of phosphorylation remains uncertain and the site or sites have not been independently determined.

Autophosphorylated protein kinase C or 32P-labeled contaminants do not give rise to labeled peptides that interfere with this analysis. When purified autophosphorylated protein kinase C was treated with trypsin, its proteolysis was rapid, and there was no overlap of either silver-stained or autoradiographic bands with those of the receptor (not shown).

Affinity Chromatographic Repurification of the Trypsin-treated Receptor—For further studies of adrenergic ligand binding and the regulation of G, by the proteolyzed receptor, the active components of the limit tryptic digest were repurified by β-adrenergic affinity chromatography on alprenolol-agarose (18). This process should retain only those peptides that are directly involved in ligand binding or those that remain noncovalently associated with ligand-binding peptides. As a marker for the removal of the major cytoplasmic domains, receptors were labeled with 32P prior to digestion with trypsin. As shown in Fig. 5, A and B, the β-adrenergic ligand-binding activity of trypsin-treated or untreated receptors were similarly adsorbed and eluted from alprenolol-agarose. However, there was a 60% loss of 32P radioactivity in the peak fractions from the limit tryptic digest relative to the same fractions of the untreated receptor. Much of the remaining 40% was evidently eluted nonspecifically when the temperature of the column was raised (Fig. 5 legend). Similar results were obtained in three experiments.

When identical volumes of these peak fractions from alprenolol-agarose chromatography were analyzed by SDS-pol-
yacrylamide gel electrophoresis, the silver-stained bands of the native β-adrenergic receptor (Fig. 5C) and of fragment A (Fig. 5D) were clearly visible. Fragment B was also visible on the original silver-stained gel. However, only the M r, ~42,000 band in the sample of untreated receptor retained detectable 32P label (Fig. 5C). No 32P was associated with either fragment A or B, even though the autoradiograph of the trypsin-treated preparation (Fig. 5D) was exposed more than twice as long as was that of the intact receptor. Although pale, diffuse bands could be observed at this exposure, they did not correspond to the silver-stained bands or to previously identified tryptic peptides of the receptor. From these data, it appears that the major cytoplasmic loop (loop 5-6) of the proteolyzed receptor did not coelute with the ligand binding activity and fragments A and B.

Regulatory Activity of Trypsin-treated β-Adrenergic Receptor—The ability of trypsin-treated receptor to regulate the binding of guanine nucleotides to G, was assayed after the coreconstitution of both proteins into phospholipid vesicles. Aliquots of the limit tryptic digest of the receptor and of untreated receptor were 32P-labeled, affinity purified as described above, and reconstituted with purified G,. Both batches of vesicles contained similar amounts of both G, and of β-adrenergic ligand-binding activity. As shown in Fig. 6, the β-adrenergic agonist isoproterenol stimulated the binding of GTPγS to a similar extent and with a similar rate in the vesicles that contained the proteolyzed and repurified receptor as in the vesicles that contained untreated but repurified receptor. The rates of agonist-stimulated binding (inserts) were comparable to those reported previously (19) and multiple molecules of G, were stimulated by a single receptor in several experiments. This complete experiment has been repeated twice with and twice without prior phosphorylation of the receptor with the same results (data not shown).
The β-adrenergic receptor is stimulated in its regulatory activity by reduction of one or more intramolecular disulfide bonds. The thiol-reduced receptor promotes the binding of GTPγS to Gβ, in the absence of agonist; the reduced and agonist-ligated receptor is more effective than the agonist-ligated receptor alone (32). The trypsin-proteolized and affinity-repurified receptor retained its positive response to reduction, as shown in Fig. 7. Reduction by dithiothreitol potentiated the activation of the receptor by the agonist isopropylbenzylxanthine to approximately the same relative extent as was observed with the untreated receptor. These data imply that the tryptic core of the receptor retains the disulfide bond that mediates the receptor’s regulatory activity. Tryptic fragment A contains at least one disulfide that may serve this function. However, the proteolyzed receptor was essentially unresponsive to treatment with thiol in the absence of agonist (Fig. 7). Little stimulation of GTPγS binding by the reduced, antagonist-ligated receptor was observed in this or other experiments. Although reduction of the receptor does decrease it stability to thermal denaturation, the partial loss of responsiveness to thiols in the proteolyzed receptor does not appear to be a result of denaturation. In separate experiments (not shown), the stability at 30°C of the ICYP-binding capacity of the dithiothreitol-reduced receptor before and after tryptic cleavage was unchanged.

**DISCUSSION**

This study represents an initial attempt to map the functional domains of the β-adrenergic receptor in terms of its primary structure. In general such an undertaking would require either direct spectrophotometric or crystallographic data on the tertiary structure of the protein. In this case, however, assignment is facilitated because the receptor is structurally homologous and functionally similar to rhodopsin, which has been studied in great detail (reviewed in Ref. 10). The homology among mammalian and avian β-adrenergic receptors, rhodopsins from several sources, and two forms of the muscarinic cholinergic receptor is concentrated primarily in seven hydrophobic regions (see 6, 8). In the case of rhodopsin, these sequences are known to span the membrane bilayer and are arranged approximately normal to the membrane in a quasi-globular structure (see 10). Specific amino acid residues can therefore be inferred to lie on the cytoplasmic or extracellular face of the receptor or within the hydrophobic core region. The sensitivity of the β-adrenergic receptor to proteolysis in regions that are predicted to extend into the aqueous medium is consistent with this assignment of tertiary structure.

Within this hypothetical tertiary structure, it is of interest to determine the location of the regulatory binding domains on the receptor’s surface, for agonist, G protein, kinases, oxidases, or phosphatases, oxidizing or reducing agents, and cytoskeletal components. The Gα-Gβ interface presumably lies on the cytoplasmic side of the plasma membrane. If the receptor binds directly to the α subunit of Gα, which is relatively hydrophilic (33), the Gβ-Gγ regulating domain will presumably be composed of one or more of the hydrophobic cytoplasmic loops or the carboxyl-terminal region. Such an interaction is consistent with a receptor’s ability to discriminate among G proteins, which differ primarily in their α subunits (5). If, however, a receptor binds to the βγ subunits, which bind directly to phospholipid bilayers and are not water soluble (33), the the Gα-Gβ subunit domain may include more hydrophobic parts of the intramembranous core of the protein. Interaction of receptor and βγ is suggested, but not proven, by data showing that the βγ subunits of transducin are required for the binding of transducin to bleached rhodopsin (54, see also 2).

The data presented here essentially rule out the hydrophilic carboxyl-terminal domain and most of the largest intracellular hydrophilic loop as being directly involved with the regulation of Gβ. That the carboxyl-terminal region is not required for the regulation of Gβ is consistent with previous studies showing that proteolytic removal of the small carboxyl-terminal domain of rhodopsin is not required for the light-dependent regulation of transducin (11, 12). It also agrees with a recent study showing that deletion of the cDNA that encodes this region of the β-adrenergic receptor does not compromise the ability of the truncated receptor to regulate Gβ (13). The finding that the proteolytic removal of approximately 5000 Da of the major intracellular loop also does not diminish the regulatory activity of the receptor was somewhat more surprising. Dixon et al. (13) recently reported that mutational deletion of 33 amino acids from this loop yielded a β-adrenergic receptor that retained ligand binding activity but had lost the ability to activate adenylate cyclase. Kühn and Hangrave (11) showed that proteolytic cleavage of this loop in rhodopsin blocked the light-dependent binding of transducin to disc membranes and Litman et al. (12) showed that similar cleavage inhibited the activation of the retinal phosphodiesterase. A single proteolytic cleavage in this loop was not inhibitory. The disparity between our data and the data on rhodopsin may perhaps be explained by the fact that this loop in rhodopsin is significantly shorter than the homologous region of the β-adrenergic receptor, and constraints on its structure may therefore be more rigorous. Proteolysis of rhodopsin may also have been more extensive. Because the proteolytic cleavage of this loop in the present study is roughly equivalent to the deletion produced by Dixon and co-workers (13), it is more surprising that the proteolyzed receptor retained activity while the mutant receptor did not. The simplest interpretation of this discrepancy is that the genetic deletion imposes alterations in the tertiary structure of the mutant receptor, i.e. the loss of activity in the mutant reflects
a disseminated conformational change. Proteolysis of the native, wild-type receptor might leave a correctly folded conformation otherwise unaltered. This interpretation agrees with more recent data (35) showing that individual mutational deletion of three smaller segments of the loop does not inactivate the receptor as long as the total deletion is kept small.

The most difficult aspect of critically analyzing the present data is determining which peptide fragments of the receptor may remain associated in the active, repurified limit tryptic digest. The amino termini of fragments A and B have been determined directly and their carboxyl termini have been reasonably well defined based on molecular size, sequence of internal CNBr peptides, and retention or loss of specific immunogenic determinants. They are not covalently bound to each other because they can be separated on SDS-polyacrylamide gels or by reverse phase HPLC without reduction of disulfides. The proteolytic digestion or the chromatographic removal of the bulk of the intracellular loop is strongly suggested by the loss of specific immunological determinants (Fig. 2, for example) and the loss of radioactivity after labeling with 32P (Figs 4 and 5). The carboxyl terminal domain was also cleaved at least twice. Conversion of the 52,000-Da receptor to the 42,000-Da species removes at least the two most carboxyl-terminal CNBr fragments, indicating cleavage at or before Lys926C (6). Further cleavage with trypsin either destroys peptide VIII or cleaves the remaining carboxyl-terminal peptide, which contains peptides VIII, to small enough fragments that they are undetectable on polyacrylamide gels. Based on experience with peptides produced by treatment with pepsin in dilute formic acid, peptides smaller than the glucagon or insulin standards can be resolved, fixed, and stained by the methods used here, suggesting that other products of trypptic cleavage are either very small (<20 amino acids) or not detectable by silver staining.

We have no data that argue whether the glycosylated amino-terminal peptide, which is cleaved from the receptor by trypsin after Arg929, remains covalently associated with fragments A and B after affinity chromatography and reconstitution. The only available antigenic determinant in this region (peptide III) is itself cleaved by trypsin rendering it undetectable. Relatively convincing data argue that amino-terminal glycopeptide, at least through Asn4, is removed when the M, ~52,000 receptor is converted to the M, ~42,000 form (27, 28, 36). Whether the amino-terminal region Ser4-Arg929 is involved in ligand binding remains uncertain.

These data, taken together, suggest that the seven membrane-spanning regions and the shorter connecting loops form a compact core structure that is active, quite stable, and resistant to proteases. If the large intracellular loop and the carboxyl-terminal region of G protein-linked receptors are not involved in G-protein binding, one wonders what their function may be. This question is intensified by the observations that there is minimal homology among receptors in these two regions (6–9) and that the sequences in these regions are unusual. The carboxyl-terminal domain of rhodopsin is the site of phosphorylation by rhodopsin kinase, which leads indirectly to inactivation of rhodopsin (37–38, and references therein). A similar role has been proposed for this region of the β-adrenergic receptor and for potential sites of phosphorylation by cyclic AMP-dependent protein kinase that are found in the large intracellular loop and carboxyl-terminal region (7, 39). Whether phosphorylation and desensitization are the principal functions of these regions and whether phosphorylation of these sites can be assigned to specific kinases remains to be seen.

Because of their asymmetric orientation in the bilayer, the
definition of specific structural domains, and the presence of several interaction sites, the G protein-coupled receptors are attractive models for correlating specific structures with specific protein-protein interactions involved with transmembrane signaling. The success of positive results obtained here and in several of the mutants described by Dixon and co-workers (13) allows strong inferences about what structures are not required for specific functions. Inferences about the role of a specific domain based on the loss of a function is more difficult to substantiate. Because these receptors are quite specific in recognizing ligands and G proteins, the chemical and mutational alteration of their selectivity for ligands may yield a clearer mapping of their structure-function relationships.

Acknowledgments—We are grateful to Dr. John Burnier, of Genentech, Inc., for preparing antigenic peptides and to Dr. Clive Slaughter, of the Department of Biochemistry, University of Texas Health Science Center, for performing the peptide sequencing. We thank S. Rozmiarek for excellent technical assistance, B. Strifer for purification of β-adrenergic receptor, K. C. McFarland for raising some of the antisera, and J. Cordero for preparation of the manuscript.

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Regulatory Domains of the β-Adrenergic Receptor