The seven transmembrane helices of serpentine receptors comprise a conserved switch that relays signals from extracellular stimuli to heterotrimeric G proteins on the cytoplasmic face of the membrane. By substituting histidines for residues at the cytoplasmic ends of helices III and VI in retinal rhodopsin, we engineered a metal-binding site whose occupancy by Zn(II) blocked activation of the receptor from activating a retinal G protein, Gt (Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) Nature 383, 347–350). Now we report engineering of metal-binding sites bridging the cytoplasmic ends of these two helices in two other serpentine receptors, the β2-adrenoreceptor and the parathyroid hormone receptor; occupancy of the metal-binding site by Zn(II) markedly impairs the ability of each receptor to mediate ligand-dependent activation of Gα, the stimulatory regulator of adenylyl cyclase. We infer that these two receptors share with rhodopsin a common three-dimensional architecture and an activation switch that requires movement, relative to one another, of helices III and VI; these inferences are surprising in the case of the parathyroid hormone receptor, a receptor that contains seven stretches of hydrophobic sequence but whose amino acid sequence otherwise shows no apparent similarity to those of receptors in the rhodopsin family. These findings highlight the evolutionary conservation of the switch mechanism of serpentine receptors and help to constrain models of how the switch works.

Serpentine receptors are key signaling molecules that relay extracellular signals from hormones and sensory stimuli to heterotrimeric G proteins located on the cytoplasmic face of the plasma membrane. Ligand-activated receptors activate G proteins by promoting exchange of GTP for GDP bound to the α subunit (Gα) of the heterotrimer, causing liberation of both α-GTP and free βγ complexes, which in turn activate effector enzymes and ion channels (1, 2). Patterns of conserved amino acid sequence distinguish three separate families of serpentine receptors in mammals; these include the rhodopsin-like receptors, with more than 1000 members, and two smaller families, related to the secretin receptor or to metabotropic glutamate receptors, respectively (3–6). Although the three families share no similarities of primary structure, all members of each family activate heterotrimeric G proteins, and all contain seven stretches of hydrophobic amino acids, which are thought to be folded into a bundle of transmembrane α-helices.

Baldwin et al. (7) have proposed a three-dimensional model of the transmembrane helices of receptors in the rhodopsin family. Based on analysis of the amino acid sequences of ~500 rhodopsin-like receptors and guided by a projection density map of frog rhodopsin (8), the model places each individual helix in the density map and specifies its position relative to the lipid bilayer, tilt in the plane of the membrane, and position and orientation relative to other helices. Thus experiments that define distance constraints between specific positions in individual helices can test the model and may enhance its precision. In addition, the model provides a starting point for designing experiments to determine the molecular mechanism by which the helix bundle transmits signals across the membrane from ligand to G protein. In such a mechanism, it seems likely that occupancy of the ligand-binding pocket induces a switch-like movement in the relative positions of two or more helices.

At present we have only a fragmentary notion of how one part of such a receptor switch may work. Site-directed spin labeling experiments with retinal rhodopsin suggest that activation causes the cytoplasmic end of helix VI to move, as a rigid body, away from helix III (9). In accord with this idea, activation of rhodopsin is blocked by either of two kinds of biochemical constraints that prevent movement of helices III and VI relative to one another. These constraints include disulfide bonds (9) or a metal ion bridge (10), engineered by substituting cysteines or histidines, respectively, at appropriate positions in the two helices.

Does ligand-induced separation of helices III and VI play a key role in activation of other serpentine receptors? To answer this question, and to test the generality of the Baldwin-Scherler model, we have constructed Zn(II) bridges connecting the cognate helices of two additional serpentine receptors, the β2 adrenoreceptor (β2AR) and the parathyroid hormone recep-

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1 The abbreviations used are: β2AR, β2-adrenoreceptor; PTH, parathyroid hormone; PTHR, parathyroid hormone receptor; Gs, trimeric G protein that stimulates adenylyl cyclase; Gt, trimeric G protein that mediates vision in rod cells (also called transducin); CYP, cytochrome P450; GTPγS, guanosine 5’-3-O-(thio)triphosphate; TM, transmembrane; CHO, Chinese hamster ovary.

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tor (PTHR). The βAR, one of the best studied serpentine receptors, belongs to the rhodopsin family but is stimulated by different ligands (norepinephrine or epinephrine, rather than light-activated retinal) and activates a different G protein (G$_i$ rather than G$_s$). A member of the secretin-like receptor family, the PTHR regulates calcium homeostasis, is stimulated by a polypeptide hormone, and activates both G$_i$ and G$_o$; its primary structure shows no resemblance to that of either rhodopsin or the βAR (11), whereas amino acid sequences of the latter two receptors are 16% identical. Thus the PTHR furnishes an opportunity to probe a distinct family of serpentine receptors, whose evolutionary relation to the rhodopsin family is unknown.

The results of our experiments indicate that both the βAR and the PTHR share with rhodopsin a conserved structure and activation mechanism. Thus it is likely that the secretin-like and rhodopsin families evolved from a common serpentine precursor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium H21, minimal essential medium, fetal bovine serum, and E.coli strain DH5α were obtained from Gibco-BRL; HyClone. Earle’s balanced salt solution (EBSS), penicillin, streptomycin and fetal calf serum were obtained from Flow Laboratories. COS-7 and CHO cells were maintained in Dulbecco’s modified Eagle’s medium H21 or modified Eagle’s (minimal essential medium) α medium, containing 10% fetal bovine serum, Fungizone, and 10 μg/ml gentamycin. Transient transfections with wild type and mutant receptors were performed using a DEAE-dextran/adenovirus method as described (12).

**Membrane Preparation**—Membranes from COS-7 or CHO cells, transfected with cDNAs encoding PTHRs or βARs, respectively, were prepared by a modification of a previously described method (12). Briefly, cells were harvested, lysed in a buffer containing 20 mM NaHepes, pH 7.4, with protease inhibitors (phenylmethylsulfonyl fluoride, bacitracin, pepstatin, and leupeptin), and homogenized by passing 12 times through a 27-gauge needle. Membrane fractions were obtained by centrifugation at 4 °C, first at 900 × g for 10 min and then at 100,000 × g for 30 min. The membranes were stripped of GTP-binding proteins essentially as described (13), by incubation in 6 M urea buffered by 25 mM NaHepes, pH 7.4, for 30 min on ice, followed by sedimentation at 100,000 × g for 30 min at 4 °C. A second urea wash and centrifugation, the membranes were reconstituted in 250 mM sucrose, 5 mM Tris/HCl, pH 7.4, frozen in liquid nitrogen, and stored at −70 °C.

**G Protein Purification**—α$_i$, was purified from cytosol of SF9 cells infected with baculovirus encoding the wild type protein, exactly as described (14). In some experiments we used His$_i$-tagged α$_i$, purified without detergents in two steps at 4 °C. SF9 cell cytosol, prepared by nitrogen cavitation, was passed over a nickel-charged HitrapChelate column (5 ml bed volume), and α$_i$ was eluted with 10 mM imidazole, followed by chromatography on a HitrapQ column (5 ml bed volume) with a NaCl gradient. Gβγ was purified from SF9 cells using His$_i$-tagged α$_i$, as described (15).

**G Activation**—Exchange of GTP for GDP bound to G$_s$ was measured by a modification of a previously described procedure (14, 16). Briefly, membranes containing receptors (~5 nm) were preincubated with purified α$_i$ (50 nm) and βγ (100 nm) for 15 min on ice in a buffer containing 20 mM NaHepes, pH 7.6, 1 mM Tris/HCl, pH 7.6, 100 mM NaCl, 0.1 mM ascorbic acid, 2 mM MgCl$_2$, 1 μM GDP, and 1 mM β-mercaptoethanol. Assays were initiated by addition of agonist and 1 μM GTP-$\gamma$-S (10$^5$ cpm per tube), in a total volume of 20 μl. After incubation for the indicated times at 30 °C, reactions were terminated by adding 400 μl of ice-cold stop solution containing 20 mM Tris/HCl, pH 8, 100 mM NaCl, and 10 mM MgCl$_2$, and filtered over nitrocellulose membranes on a vacuum manifold; filters were then washed 5 times with 250 μl of stop solution. Radioactivity was quantitated by liquid scintillation in a β-counter. Nonspecific binding (binding to the filter in the presence of 10 μM isoproterenol) was less than 10% of total binding. Specific binding was defined as the difference between total binding and nonspecific binding.

**Ligand Binding**—Binding of 125I-cyanopindolol (CYP) was determined as described (17). Binding was initiated by suspending membranes (5 μg of protein in a final volume of 500 μl) in a mixture of 10 mM-CYP (5 μl), increasing concentrations of isoproterenol, a buffer consisting of 25 mM NaHepes, pH 7.6, 0.05% (w/v) bovine serum albumin, and 0.1 mM ascorbic acid. Zn(II) (10 μM) and GTP-$\gamma$-S (30 μM) were present or absent, as indicated. Nonspecific binding was assessed in the presence of 10 μM isoproterenol. Reactions were conducted for 45 min at 30 °C, stopped by adding 2 ml of ice-cold binding buffer, and filtered over Whatman GF/C filters. Membranes used for binding assays were not exposed to serum. Results were analyzed by non-linear regression.

**Alignments Using the Evolutionary Trace Method**—In the absence of recognizable sequence identity, the evolutionary trace method can be used to align positions so as to match their functional importance during evolution. Here, 58 animal visual opsins, 56 adrenergic receptors, and 33 members of the secretin-like family were gathered from Swiss-Prot version 34.0. These sequences are shown in Table I. The seven transmembrane regions, recognizable by their hydrophobicity, were excised from the rhodopsin and adrenergic receptors. PILEUP (from the GCG8.0 Wisconsin Sequence Analysis Package) then produced an alignment and a sequence identity dendrogram of each helix. The evolutionary trace computed for each position of each helix its evolutionary trace method can be used to align positions so as to match their functional importance during evolution. Here, 58 animal visual opsins, 56 adrenergic receptors, and 33 members of the secretin-like family were gathered from Swiss-Prot version 34.0. These sequences are shown in Table I. The seven transmembrane regions, recognizable by their hydrophobicity, were excised from the rhodopsin and adrenergic receptors. PILEUP (from the GCG8.0 Wisconsin Sequence Analysis Package) then produced an alignment and a sequence identity dendrogram of each helix. The evolutionary trace computed for each position of each helix its evolutionary trace method can be used to align positions so as to match their functional importance during evolution. Here, 58 animal visual opsins, 56 adrenergic receptors, and 33 members of the secretin-like family were gathered from Swiss-Prot version 34.0. These sequences are shown in Table I. The seven transmembrane regions, recognizable by their hydrophobicity, were excised from the rhodopsin and adrenergic receptors. PILEUP (from the GCG8.0 Wisconsin Sequence Analysis Package) then produced an alignment and a sequence identity dendrogram of each helix. The evolutionary trace computed for each position of each helix its evolutionary trace method can be used to align positions so as to match their functional importance during evolution.

**RESULTS**

**Receptor Activation Assay**—To assess activation of G$_i$ by wild type and mutant receptors, we measured ligand-dependent binding of radioactive GTP-$\gamma$-S in a mixture containing pure α$_i$, pure βγ, and urea-washed membranes from cells expressing the recombinant receptor. Washing the membranes with urea, for the time periods used (30°C), reactions were terminated by addition of GTP-$\gamma$-S to the membranes without inactivating the recombinant serpentine receptors. We expressed wild type and mutant versions of the PTHR and the βAR in COS-7 cells and CHO K1 cells (which lack the endogenous β-adrenoreceptors present in COS-7 membranes), respectively.

In the βAR assay, GTP-$\gamma$-S binding required the presence of receptor, α$_i$, βγ, and the agonist, isoproterenol (Fig. 1A). The effect of isoproterenol was rapid (complete within 3 min; Fig. 1B), saturable by increasing concentrations of α$_i$ (Fig. 1C) or βγ (not shown), and dependent on concentration (Fig. 1D); the EC$_{50}$ for isoproterenol, 68 nM, is comparable to values reported previously (e.g. Ref. 17). Isoproterenol increased GTP-$\gamma$-S binding 5–20-fold in different experiments; at maximal stimulation, 2

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radioactive GTPγS bound to 10–30% of the total αs present in the assay. Concentrations of αs and βγ required in the PTHR assay (not shown) were similar to those in the β2AR assay, but a longer time (10 min) was required for maximal PTH-dependent binding of GTPγS.

Choosing Sites for Histidine Substitutions—In the β2AR and PTHR we sought to reproduce metal-binding sites cognate to the site we had constructed in rhodopsin (10). To do so, we used the alignments of helix III and VI amino acid sequences shown in Table II. Similar primary structures made it straightforward to align these segments of the β2AR with those of rhodopsin. In TM III of the β2AR we substituted a histidine for Ala-134, which is cognate in sequence to Val-138 of rhodopsin, a position that participated in the helix III–helix VI metal ion-binding site we created in that receptor (10). In TM VI, we substituted histidine, in separate mutant receptors, for each of six consecutive amino acids. These six residues (Table II) cover more than a full turn of the putative α helix; they include Leu-272, which is cognate to the position in helix VI of rhodopsin (residue 251) at which a substituted histidine participated in the engineered metal ion-binding site (10).

In the absence of obvious similarities of amino acid sequence, aligning the PTHR and rhodopsin sequences (Table II) was more difficult. We based this alignment on an evolutionary trace analysis (18, 21), as described under “Experimental Procedures.” This method has already successfully identified Gα surfaces that interact with βγ and with serpentine receptors (21, 22). Briefly, the analysis assumes that shared structures

| Opsins | Adrenergic | Secretin-like | No. |
|--------|------------|---------------|-----|
| OPS1_CALVI | A1AA HUMAN | CALR HUMAN | 1 |
| OPS1_DROME | A1AA RAT | CALR FIG | |
| OPS1 DROP | A1AB HUMAN | CALR RAT | |
| OPS1_LIMPO | A1AB MESAU | CRF2 RAT | |
| OPS2_DROME | A1AB RAT | CRFR HUMAN | |
| OPS2 DROP | A1AC BOVIN | CRFR MOUSE | |
| OPS2_LIMPO | A1AC HUMAN | CRFR HUMAN | |
| OPSB_ANOCA | A1AC RAT | GIPR HUMAN | |
| OPSB_ASTFA | A2AA HUMAN | GIPR MESAU | |
| OPSB_BOVIN | A2AA MOUSE | GIPR RAT | 10 |
| OPSB_CARAU | A2AA FIG | GLFR HUMAN | |
| OPSB_CHICK | A2AA RAT | GLFR RAT | |
| OPSB_GECGE | A2AB HUMAN | GLR HUMAN | |
| OPSB_HUMAN | A2AB MOUSE | GLR RAT | |
| OPSB_MOUSE | A2AB RAT | GRFR HUMAN | |
| OPD_ALLMI | A2AC DIDA | GRFR MOUSE | |
| OPD_ANOCA | A2AC HUMAN | GRFR HUMAN | |
| OPD_ASTFA | A2AC FIG | GFR R RAT | |
| OPD_BOVIN | A2AC RAT | PACR HUMAN | |
| OPD_CANFA | A2AD HUMAN | PACR RAT | 20 |
| OPD_CARAU | A2AR CARAU | PTTR HUMAN | |
| OPD_CHICK | A2AR CANFA | PTTR DIDMA | |
| OPD_KRIG | B1AR HUMAN | PTTR HUMAN | |
| OPD_CYPAC | B1AR MACMU | PTTR MOUSE | |
| OPD_HUMAN | B1AR MELGA | PTTR RAT | |
| OPD_LAMIA | B1AR MOUSE | PTTR FIG | |
| OPD_LOLFO | B1AR RAT | SCRC HUMAN | |
| OPD_MOUSE | B2AR CANFA | SCRC R RAT | |
| OPD_OCTDO | B2AR HUMAN | VIPR HUMAN | |
| OPD_POMMI | B2AR MESAU | VIPR RAT | 30 |
| OPD_PROCL | B2AR MOUSE | VIPS HUMAN | |
| OPD_RABT | B2AR RAT | VIPS MOUSE | |
| OPD_RANCA | B3AR BOVIN | VIPS R RAT | |
| OPD_RANPI | B3AR HUMAN | | |
| OPD_RAT | B3AR MOUSE | | |
| OPD_SHEEP | B3AR RAT | | |
| OPD_SPESP | B4AR MELGA | | |
| OPD_TODPA | D1DR CARAU | | |
| OPD_XENLA | D3DR CEREA | | |
| OPG_ASTFA | D3DR HUMAN | | 40 |
| OPG_CARAU | D3DR MOUSE | | |
| OPG_CHICK | D3DR RAT | | |
| OPG_GECGE | D4DR RAT | | |
| OPG_HUMAN | DADR DIDMA | | |
| OPH_ASTFA | DADR HUMAN | | |
| OPH_CARAU | DADR FIG | | |
| OPS_ASTFA | DADR RAT | | |
| OPS_CALJA | DADR XENLA | | |
| OPS_CHICK | DBDR RAT | | |
| OPS_COLL | DBDR XENLA | | 50 |
| OPS_ANOCA | DOPR DROME | | |
| OPS_ASTFA | HH1R CAYPO | | |
| OPS_CARAU | HH1R RAT | | |
| OPS_CHICK | HH2R CANFA | | |
| OPS_HUMAN | HH2R CAYPO | | |
| OPSU_BRAKE | HH2R RAT | | |
| OPSV_CHICK | | | |
| OPSV_XENLA | | | 58 |
and molecular mechanisms dictate similarly located interfaces between helices and therefore similar patterns of functionally important residues in each helix; this should be true even if the sequences themselves show no identical amino acids. The analysis (to be described in detail elsewhere) identified apparently functionally important positions (as indicated by patterns of sequence conservation) in serpentine receptors related to rhodopsin and compared distributions of these positions to those of similarly important positions in receptors related to the PTHR. The evolutionary trace approach revealed putative structural and functional similarities between the rhodopsin and secretin-like receptor families. Based on the analysis, we substituted histidines for Leu-303 in helix III and at each of six consecutive positions in helix VI of the PTHR (Table II). In addition, we tested the Zn(II) sensitivity of mutants containing the histidine at position 301 in helix III of the PTHR (Table II).

Zn(II) Sensitivity of β2-AR Receptors—To our surprise, a relatively low concentration of Zn(II) (IC \(_{50} \sim 10 \mu M\)) inhibited the ability of the wild type β2-AR to activate G\(_s\) (Fig. 2). This suggested that the wild type receptor contains a cryptic endogenous site where Zn(II) can bind and block activation. One partner in such a site may be a naturally occurring histidine (His-269) in helix VI (see Table II). Several observations suggest that this residue participates in a metal-ion-binding site. Replacement of His-269 by an alanine reduced the sensitivity of the receptor to inhibition by Zn(II). Moreover, substitution of a histidine for Ala-134 in helix III increased the Zn(II) sensitivity of the receptor containing His-269 ∼10-fold (Fig. 2 and Table III). A 30-fold higher Zn(II) concentration was required for half-maximal inhibition of the control receptor, containing the histidine substituted in helix III but lacking histidine at position 269 in helix VI (Table III). Taken together, these observations indicate that histidines in helices III and VI can form a Zn(II) bridge that inhibits activation by the β2-AR and that the metal-binding site is in fact tridentate, involving an unidentified third amino acid (see below) somewhere nearby.

We next tested the abilities of histidines placed at successive positions around helix VI to cooperate with the histidine substituted at position 134 in helix III (Fig. 2 and Table III). Zn(II) blocked receptor activation (IC \(_{50} \sim 1 \mu M\)) when the histidine in helix III (red in Fig. 3A) was paired with a histidine at position 268, 269, or 272 (yellow in the same figure). Relative to a receptor lacking His-269 and the substituted histidine in helix III, a histidine at position 268 or 272 in helix VI produced a receptor with intermediate sensitivity to inhibition by Zn(II) (IC \(_{50} \sim 10 \mu M\); Table III); this suggests that residues at all three positions (268, 269, and 272) may cooperate with the same cryptic residue to produce a bidentate metal-binding site with intermediate Zn(II) binding affinity. In contrast, a histidine at each of the other positions (270, 271, or 273; green in Fig. 3A) produced a receptor that was quite insensitive to Zn(II) inhibition (IC \(_{50} \sim 30 \mu M\); Table III), suggesting that these positions cannot cooperate either with the cryptic residue or with the histidine at position 134 of helix III to form a metal-binding site. According to the Baldwin-Schertler model, positions 268, 269, and 272 are clustered on one side of helix VI (Fig. 3A). Moreover, histidines at positions 268 and 272 would occupy locations one turn apart in an α-helix, in keeping with evidence (23) that the cytoplasmic end of helix VI in rhodopsin projects as an α-helix beyond the sequence that is buried in the lipid core of the membrane.

We have not identified the cryptic third member of this putative tridentate binding site. A likely possibility is Asp-130, which forms part of a highly conserved DRY/ERY motif in helix III; Asp-130 is one turn (4 residues) away from the site of our histidine substitution (position 134).

Zn(II) Sensitivity of PTH Receptors—We first probed the PTHR with histidines substituted at positions that correspond (according to the evolutionary trace analysis) to those we mutated in the β2-AR, that is Leu-303 in helix III and six positions in helix VI. Each of the helix III–helix VI double histidine mutants activated G\(_s\) poorly, however, even in the absence of Zn(II) (results not shown). To our surprise, each of the helix VI substitutions by itself (in the absence of a substitution at position 303 in helix III) produced a functioning receptor that was inhibited by Zn(II). The susceptibility to Zn(II) turned out to depend on a histidine residue naturally present in helix III of the PTHR, at position 301. Replacement of the histidine at position 301 by alanine produced receptors whose sensitivity to Zn(II) was unaffected by histidines substituted at any of the six positions in helix VI (Table III). The Zn(II) sensitivity of this H301A mutant (lacking histidines substituted into helix VI) was identical to that of the wild type PTHR, that is, to that of a receptor with a histidine at position 301 but no histidine at any of the six positions in helix VI (Table III).

From these results we infer that a histidine at position 301 in helix III can partner with histidines at six different positions in helix VI to form metal-binding sites that inhibit the ability of the receptor to activate G\(_s\). Table III shows that the Zn(II) sensitivity of two of these putative metal-binding sites is much greater than those of others: combinations of His-301 in helix III with histidines at positions 401 or 402 in helix VI created receptors that were 20- or 80-fold more sensitive to Zn(II) than the wild type receptor (Table III and Fig. 2).

Fig. 3B depicts the Baldwin-Schertler receptor model, highlighting the predicted positions of histidines that create metal-binding sites in the PTHR. In the model the key histidine (red) in helix III of the PTHR, His-301, is located on the opposite face

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**Fig. 1.** β2-AR-dependent activation of G\(_s\) in a reconstituted system. Urea-washed membranes from CHO cells expressing the recombinant β2-AR were treated with or without isoproterenol in the presence of G\(_s\) subunits, and binding of [\(^{35}\)S]GTP\(_S\) to α\(_s\), was measured, as described under “Experimental Procedures.” A, binding of [\(^{35}\)S]GTP\(_S\) after incubating membranes for 4 min at 30 °C in the presence or absence (as indicated) of isoproterenol (1 μM), α\(_S\) (50 nM), and β\(_g\) (100 nM); the concentration of β\(_2\)-AR (assessed by binding radioactive antagonist) was 5 nM, except for the two columns on the right, which represent incubations of membranes from cells not expressing the β\(_2\)-AR. The total volume of each incubation was 20 μL. The bars represent means of duplicate determinations, shown in circles. This experiment is representative of four separate experiments using two different membrane preparations. B–D, binding of [\(^{35}\)S]GTP\(_S\) under conditions identical to those described for A, except that incubations were conducted for different times (B), at different concentrations of α\(_s\), (C), or at different concentrations of isoproterenol (D). Values in B represent determinations in the presence or absence of 1 μM isoproterenol (filled and open symbols, respectively). C, the concentrations of isoproterenol (1 μM), β\(_2\) adrenoreceptors (5 nM), and G\(_s\)β\(_y\) (250 nM) were fixed.
The evolutionary trace method (18) was used to align the sequences of amino acid residues in helices III and VI, as described under “Experimental Procedures.” The concentrations of both isoproterenol and PTH were 1 μM. Circles and bars represent means ± S.E. of 3–5 separate experiments.

Fig. 2. Effect of different concentrations of Zn(II) on Gα activation (assessed by binding of [35S]GTPγS) stimulated by wild type and mutant β2AR (top) and PTHR (bottom). Concentration of [35S]GTPγS, receptors, α5 and β2 are described under “Experimental Procedures.” The concentrations of both isotropenol and PTH were 1 μM. Circles and bars represent means ± S.E. of 3–5 separate experiments.

of this helix from the position that creates a Zn(II)-binding pocket in the β2AR (position 134 in Fig. 3A); as noted above, substitution at a different site was necessary because histidine at position 303 inactivated the PTHR. Nonetheless, the ability of His-301 to form Zn(II)-binding sites in cooperation with histidines in helix VI agrees both with the Baldwin-Schertler model and with the alignment of PTHR sequence with those of receptors in the rhodopsin family. This is because the α-carbon of each histidine substituted in helix VI would be almost equidistant in the model from α-carbons at either position 301 or 303 of helix III.

Fig. 3 highlights an apparent difference between the β2AR and the PTHR. All six histidines in helix VI of the PTHR enhanced its sensitivity to inhibition by Zn(II) (Fig. 3B), whereas in the β2AR only three of the six histidine residues tested did so (Fig. 3A). This may indicate that the cytoplasmic end of helix VI in the PTHR is more mobile than its counterpart in the β2AR. It should be pointed out, however, that substitutions at two of the helix VI sites in the PTHR (indicated by yellow balls in Fig. 3B) produce receptors that are much more sensitive to inhibition by Zn(II). One of these, at position 401, is precisely cognate to the position in the β2AR where histidine substitution (L272H) induced the greatest sensitivity to Zn(II); in rhodopsin a histidine substituted (T251H) at the corresponding position also produced a Zn(II)-sensitive receptor (10).

**Agonist Binding Affinity of Wild Type and Mutant β2ARs—** Interaction of Gα with the β2AR is known to enhance the receptor’s affinity for agonists (for review, see Ref. 24), presumably via a conformational change transmitted to the ligand-binding pocket from the G protein-binding cytoplasmic surface of the receptor. The effect of Gα on agonist binding affinity can be reversed by adding a GTP analog, such as GTPγS, as shown for the β2AR(H269A) mutant in Fig. 4; this reversal is thought to reflect GTP-induced dissociation of α5 from β2 and of both α5-GTP and βγ from the receptor. If occupancy of the Zn(II) metal-binding site in appropriate β2ARs prevents them from interacting with Gα, Zn(II) should partially or completely mimic the effect of GTPγS. This prediction was not fulfilled (Fig. 4). In the absence of GTPγS, addition of Zn(II) (10 μM) caused small increases (2-3-fold) in the apparent agonist binding affinities of both the β2AR(H269A) mutant and the β2AR(H134/H269) double mutant; addition of GTPγS caused equivalent decreases in agonist binding affinities of both mutant receptors, measured either in the absence or presence of 10 μM Zn(II), despite the ~30-fold difference in sensitivity of the same receptors to inhibition by Zn(II) (Table III). From these results we infer that occupancy of Zn(II) of the metal-binding site does not prevent the receptor from associating with Gα, although it does block agonist-dependent activation of Gα by the receptor.

It is worth noting that failure of Zn(II) to affect agonist binding affinity of the β2AR(H134/H269) double mutant rules out the possibility that the cation inhibits stimulation of Gα by denaturing the receptor.

**DISCUSSION**

In these experiments we engineered potential metal-binding sites into two serpentine receptors as probes for elucidating the structure and molecular mechanism of the receptor switch. This approach, which has been applied to serpentine receptors (25–27) and many other proteins (28), depends upon the ability of Zn(II) (or certain other metals) to be chelated by side chains of two or more amino acids in a protein. Because the imidazole group of histidine chelates metals rather well, potential Zn(II) bridges are often constructed by substituting histidines at appropriate positions in a mutant protein. A Zn(II) bridge between appropriately oriented histidines in separate structural elements of the protein will link the two elements together. If Zn(II) inhibits a function of such a mutant protein, we infer that normal function requires movement of one or both of the two structural elements, relative to the other. For this inference to be valid, Zn(II) must inhibit function of the mutant protein at a considerably lower concentration than that required to inhibit function of the wild type protein; moreover, neither histidine substitution should mediate the Zn(II) effect on its own, and the histidine substitutions should not alter function of the mutant protein in the absence of Zn(II). Our results meet these criteria.

From the effects of Zn(II) bridges in the β2AR and the PTHR, we infer that helices III and VI of each receptor lie close to one another and also that the two helices must move, relative to one another, for the receptor to activate the G protein. These inferences extend our understanding of the serpentine receptor switch in four ways. First, experiments with the β2AR confirm
Ligand-stimulated activation of Gs was assessed by measuring the specific binding of [35S]GTPγS to recombinant αs, as described under "Experimental Procedures." Results indicate the maximal ligand-induced binding of [35S]GTPγS (fmol per tube) and the Zn(II) concentration that inhibited activation by 50% (IC50, μM). Data for each receptor construct represent mean values from 3 to 12 separate experiments, conducted on 2-6 different membrane preparations. Distances between the α-carbons of mutated residues are those specified in the Baldwin-Schertler model (7).

![Table III](image)

| TM-III | TM-VI |
|--------|-------|
| **β2-Adrenoceptor** | **PTH receptor** |
| Maximum | IC50 | Mutation | α-Carbon distance | Maximum | IC50 | Mutation | α-Carbon distance |
| fmol | μM | A | fmol | μM | fmol | μM | fmol | μM |
| H369A | 169 | 32 | 163 | 29 | 1.1 | Y397H | 10.7 | 84 | 39 | 101 | 11 | 3.5 |
| E688H | 126 | 9.1 | 178 | 0.9 | 10 | R398H | 12.7 | 33 | 94 | 8.8 | 3.8 |
| H269 | 212 | 7.5 | 144 | 0.7 | 11 | L272H | 154 | 8.2 | 101 | 0.7 | 12 |
| K270H | 81 | 27 | 157 | 25 | 1.1 | K273H | 12.4 | 30 | 149 | 32 | 0.9 |
| A271H | 93 | 31 | 143 | 28 | 1.1 | |
| L272H | 8.76 | 23.1 | |
| K273H | 12.48 | 23.1 | |

that the Baldwin-Schertler structural model, based on density maps made from rhodopsin, applies to other receptors in the rhodopsin family of serpentine receptors. Second, experiments with the PTHR show that a member of the secretin-like family of serpentine receptors shares a common three-dimensional structure and activation mechanism with receptors in the rhodopsin family. Third, our results confirm the notion (10, 29) that ligand-induced movement of helix VI relative to helix III is necessary for the receptor to catalyze efficient replacement by GTP of GDP bound to the G protein. Finally, however, the surprising failure of Zn(II) bridges to alter the agonist binding affinity of β2AR mutants (Fig. 4) indicates that relative movement of helix VI versus helix III is not required for the G protein to associate with the receptor and to regulate the affinity of its ligand-binding site.

A Test of the Baldwin-Schertler Model—In known three-dimensional structures, α-carbons of histidines that form Zn(II) bridges are found no more than 13 Å apart (30, 31). The Baldwin-Schertler model (7) predicts that the α-carbon of the amino acid (Ala-134) replaced by histidine in helix III of the β2AR (Fig. 3) lies within 8-10.3 Å of the α-carbons of helix VI positions at which a second histidine substitution makes β2AR/(A134H) sensitive to inhibition by Zn(II). Although details of the coordination of the metal ion remain to be elucidated, these results confirm a key prediction of the model, that the cytoplasmic ends of helices III and VI are located relatively close to one another. Abundant evidence has already indicated proximity of the cytoplasmic ends of helices III and VI in retinal rhodopsin; this evidence includes the Zn(II) bridge we engineered into rhodopsin (10), distances between the helices as assessed with site-directed spin labels (9), and formation of disulfide bonds between cysteines substituted into the same regions of these helices (9).

These geometrical constraints constitute useful tests of the Baldwin-Schertler model. This is because projection density maps obtained from electron cryomicroscopic studies of rhodopsin (8) indicate probable locations and tilts of α-helices, but the low resolution of these maps does not make it possible to identify a specific density with a specific helix or to know whether the density map is being viewed from the cytoplasmic or the extracellular side. The Baldwin-Schertler model, however, does identify the helices and predicts that they are arranged in a clockwise fashion, as viewed from the cytoplasm (7, 32). Effects of Zn(II) sites on the β2AR mutants, along with earlier biochemical studies of rhodopsin (9, 10), support the predicted proximity of helices III and VI as well as the predicted clockwise arrangement. Baldwin identified helix III with the centrally located density of the rhodopsin map (32), based on its amino acid sequence; among all the helices, the hydrophilic and conserved residues of helix III show the least tendency to be distributed on one face; this is the pattern expected of a helix in the center of the helix bundle, where it must interact with other helices on all sides. If the central density corresponds to helix III, the Zn(II) bridges indicate a clockwise arrangement of the helices; this is because in the opposite (counterclockwise) arrangement, helix VI would be too far (25 Å) from helix III for formation of a Zn(II) bridge (not shown).

Other data can similarly be explained only if the helices are ordered in a clockwise fashion. In the tachykinin NK-1 receptor, agonist binding was blocked by either of two bidentate Zn(II) sites on the extracellular side of the helix bundle (between helix III and helix II or helix V) (26); formation of these Zn(II)-binding sites would have been much less likely in a counterclockwise helix bundle. In addition, functional folding of chimeric muscarinic receptors required alteration of threonine residues in helices I and VII, suggesting that these residues meet at an interface between these two helices (33). The Zn(II) bridges we engineered into the β2AR involve positions in helices III and VI cognate to positions that participated in function-inhibiting Zn(II) bridges (10) engineered into retinal rhodopsin. Thus it is likely that these two receptors share both a similar three-dimensional architecture and a highly conserved activation switch, even though they stimulate different G proteins, Gs and Gt, respectively. By extension, this architecture and switch mechanism are probably common to all receptors in the rhodopsin family. The same overall inference
FIG. 3. Mutated positions and Zn(II) bridges. Ribbons represent the seven helices of serpentine receptors in the rhodopsin family, according to the Baldwin-Schertler model (7). Balls represent β-carbons of the positions where histidine substitutions were tested for their ability to participate in Zn(II) bridges. A, β2AR, in which a histidine at position 134 (red) in helix III formed a Zn(II) bridge with histidines at each of three positions (yellow) in helix VI, but did not form bridges with three other substituted histidines (green) in helix VI. B, PTHR, in which a histidine at position 301 (red) in helix III formed a Zn(II) bridge with histidines at each of six positions in helix VI; two helix VI positions (yellow) formed bridges at especially low Zn(II) concentrations, whereas four (green) formed bridges at intermediate Zn(II) concentrations.

can be drawn from the effects of engineered metal-binding sites on the extracellular sides of two other receptors in this family (25–27); the engineered metal ion-binding site between helices V and VI on the extracellular side of the tachykinin NK-1 receptor (25, 26) was duplicated by mutations at cognate positions in the same helices of the α-opioid receptor (27).

Although the geometric constraints imposed by histidine-histidine Zn(II) bridges (30, 31) confirm the low resolution model (7) proposed by Baldwin and colleagues, they do not enhance its precision. The helix VI substitutions that did enhance sensitivity appear to be situated on one face of the helix (Fig. 3), as the model would predict. The low “resolution” of these experiments, however, is evident from comparing the effects of Zn(II) on mutant β2ARs versus the predicted α-carbon distances between the positions substituted in helix III and helix VI. The model shows short α-carbon distances (~8.7 Å) for two of the mutants that were strongly inhibited by Zn(II) and considerably longer distances (~12.5 Å) for two of the mutants without enhanced sensitivity to inhibition by Zn(II); the model predicted an intermediate distance (10.3 Å) for the other two mutants, only one of which showed enhanced sensitivity to Zn(II).

The PTHR Versus Rhodopsin and the β2AR—In mammals, the receptors coupled to G proteins can be grouped into three families, which resemble, respectively, rhodopsin, the secretin receptor, and “metabotropic” receptors for glutamate (4–6, 32). Within each of these families, deduced amino acid sequences of individual receptors show clear-cut patterns of conserved and identical amino acids. No such sequence conservation or identity has been found in comparisons of receptors in any one of the three families versus receptors in another. Receptors in all three families do, however, exhibit seven stretches of hydrophobic amino acids, each of which is thought to constitute an α-helix that crosses the plasma membrane (4–6, 32).

The shared seven-helix topology suggests the hypothesis that all three receptor families evolved from a common precursor and share a common three-dimensional architecture and mechanism for transducing signals from the agonist-binding site to the G protein. Based on this hypothesis we used the evolutionary trace approach (18) to align sequences in the secretin-like receptor family with apparently cognate positions in helices III and VI of the rhodopsin family (for details, see “Experimental Procedures” and “Results”). As an initial test of the hypothesis, we compared effects of metal-binding sites engineered into a member of each family, the PTHR and the β2AR, respectively. The results suggest that these two receptors and, by extension, the two receptor families do share a common origin in evolution, as well as similar three-dimensional architectures and switching mechanisms.

The effects of Zn(II) on the PTHR mutants indicate that the cytoplasmic ends of helices III and VI are close to one another in this receptor, much like the corresponding helices of the β2AR and other receptors in the rhodopsin family. Indeed, the “strongest” Zn(II) bridges (those at which the lowest Zn(II) concentrations inhibit G protein activation) link positions that would be quite close to one another in a PTHR that conforms to the Baldwin-Schertler model and the alignment based on the evolutionary trace approach, that is, α-carbon distances between position 301 in helix III and positions 401 or 402 in helix VI are 7.3 or 10.3 Å, respectively (Table III); these are within the range of distances (30, 31) that allow histidine residues to...
form metal-binding sites. In comparison to the wild type PTHR, histidine substitutions at these two positions in helix VI markedly enhanced Zn(II) sensitivity (20- and 83-fold, respectively). Substitutions at the other four positions tested in helix VI also increased Zn(II) sensitivity, although to a lower degree (3.5–5.3-fold; Table III). We do not know how histidines at six positions around the entire circumference of helix VI can form Zn(II) bridges with a single histidine in helix III. Indeed, residues 397–402 in the PTHR may not belong to an α-helix at all; alternatively, the putative helix may be unusually flexible. The latter possibility is consistent with a rhodopsin experiment (9), in which a cysteine at each of five positions in helix VI could form a disulfide bond with a cysteine in helix III.

Overall, Zn(II) sensitivities of the PTHR mutants generally agree with the Baldwin-Schertler model, which would predict distances between the appropriate positions ranging from 7.3 to 12.5 Å (see Table III). Accordingly, we propose that receptors in the secretin receptor-like family share the overall three-dimensional architecture of receptors in the rhodopsin family. Extension of the model to this second family of receptors was not anticipated (7) but is in keeping with the basic topology shared by the two families and with their apparently similar signaling functions.

Donnelly (4) has proposed a molecular model for the transmembrane helices of another member of the secretin-like receptor family, the glucagon-like peptide 1 receptor. In this model, as compared with the Baldwin-Schertler model, helix III is less buried within the helix bundle; helix VII, lying closer to the receptor core, is located between helices III and VI. Donnelly’s tentative model, which includes loops and tilts of helices, predicts much longer α-carbon distances between positions 301 (helix III) and positions 401 or 402 (helix VI) of the PTHR, 20.73 and 20.71 Å, respectively. These distances substantially exceed those that allow histidines to form effective metal ion-binding sites (28). Consequently, our results are much more congruent with the Baldwin-Schertler model than with that of Donnelly.

Functional Role of Helices III and VI in Activating the G Protein—How does Zn(II) prevent receptors with the appropriate histidine substitutions from activating the G protein? A straightforward interpretation, previously applied to the inhibitory effect of a cognate Zn(II) bridge in rhodopsin (10), is that the Zn(II) ion prevents movement of helix VI relative to helix III; the relative motion of the two helices results from activation of the serpentine receptor switch and is necessary for effective catalysis of GDP-GTP exchange on the G protein. This notion is strongly supported by results of site-directed spin labeling experiments (9), which were interpreted as showing that photo-oxidation of rhodopsin causes the cytoplasmic end of helix VI to move 10–15 Å away from helix III and to rotate on its own axis, in a clockwise direction.4

Our speculative extension of this scenario (29) includes two additional inferences: the stimulus-induced separation of helix VI from helix III opens a cleft or pocket in the cytoplasmic surface of the receptor, and occupancy of the cleft by the C-terminal tail of the G protein’s α subunit is required for the receptor to catalyze GDP-GTP exchange. The first of these additional inferences implies that the Baldwin-Schertler model represents the inactive conformation of serpentine receptors, and is in keeping with both the spin labeling results and our experiments with metal-binding sites. The second inference, that helix VI interacts specifically with the C terminus of Gα, was suggested by results of an experiment (34) that tested the ability of chimeric muscarinic receptors to interact with chimeric Gα subunits; a 4-residue epitope in helix VI functionally complemented a similarly short sequence in the Gα C terminus. One of the complementing receptor residues in that study (34) is cognate to residue 272 of the β2AR, a position at which substitution of a second histidine made the β2AR(A134H) mutant susceptible to inhibition by Zn(II) (Table III); this raises the possibility that a Zn(II) bridge may inhibit G protein activation not only by immobilizing helix VI relative to helix III but also by steric hindrance.

Agonist Binding Affinities of Mutant β2ARs—Surprisingly, Zn(II) failed to reduce the agonist binding affinity of the histidine-substituted β2AR mutants whose ability to activate Gs was sensitive to inhibition by Zn(II) (Fig. 4). We had expected the contrary result, based on the scenario for G protein activation described above, in combination with a number of observations in many laboratories. Thus in the absence of added guanine nucleotide the β2AR (like many other receptors) exhibits an enhanced affinity for binding agonists (reviewed in Ref. 24). This high affinity is thought to result from association of the receptor with the appropriate G protein, because addition of GTP analogs reduces agonist binding affinity and at the same time causes G protein α and βγ subunits to dissociate from each other and from the receptor. The affinity of the β2AR for agonists is similarly low in cells genetically lacking the α subunit of Gα (35, 36). In contrast, neither GTP analogs nor genetic absence of the G protein affect the receptor’s affinities for binding pharmacological antagonists. The ability of the G protein to enhance binding of agonists, but not antagonists, suggests a reciprocal interaction between the receptor-G protein interface and the agonist-binding pocket. A similar reciprocity is thought to account for the ability of retinal transducin (Gt) to stabilize a spectral form of photorhodopsin called metarhodopsin II (see references in Ref. 10); that is, hormone and light induce a conformational change that enhances affinity of their respective receptors for binding G proteins, and G proteins reciprocate by enhancing stability of this agonist-bound “activated” conformation of the receptors. For this reason, we had expected Zn(II) bridges to prevent Gs from increasing the affinity of the β2AR for binding agonist, just as the same bridges inhibited agonist-induced activation of Gs.

Instead, Zn(II) had little or no effect on agonist binding affinity (Fig. 4); wild type and mutant receptors showed nearly identical affinities for binding isoproterenol, whether or not Zn(II) was present. Moreover, a GTP analog reduced isoproterenol binding affinity to the same degree in both wild type and mutant receptors, again in a fashion that was unaffected by Zn(II). Thus transmission of conformational change from the G protein to the ligand-binding site is unaffected by the same Zn(II) bridges that inhibit transmission of conformational change in the other direction, from the ligand-binding site to the G protein. Moreover, the Zn(II) bridges prevent the G protein from activating the receptor but not from interacting with the receptor.

Can these asymmetric effects of Zn(II) tell us something useful about how the receptor switch works? If Zn(II) bridges prevent movement of helix VI relative to helix III, as described above, then this movement does not mediate the effect of G protein on agonist binding affinity. For example, Zn(II)-induced immobilization of helices III and VI may prevent the receptor from promoting release of GDP from the G protein trimer, whereas the GDP-bound form of the trimer interacts with a separate site on the cytoplasmic face of the receptor to initiate the conformational change that enhances agonist binding affinity. In this regard, several rhodopsin mutants furnish an instructive precedent (37, 38); these mutations prevent the

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4 The spin labels were attached to a cysteine substituted at position 139 in helix III and to individual cysteines engineered into positions cognate to those where we substituted histidines in helix VI of the β2AR and the PTHR.
receptor from promoting GDP release from the trimer (38), but their association with the trimer nonetheless stabilizes the metarhodopsin II spectral form of rhodopsin. The locations of these mutations suggest that, like the engineered Zn(II) bridges, they affect a function mediated by the cytoplasmic ends of helices III and VI. One of the mutations substituted a different sequence (which included the cytoplasmic end of helix III) for a part of the second intracellular loop of rhodopsin; the other deleted most of the third intracellular loop, including the cytoplasmic end of helix VI.

Peptides representing the 11 C-terminal amino acids of two different Gα subunits are reported (39, 40) to reproduce the effects of G proteins on ligand-binding sites of the corresponding receptors. Such a peptide from αi stabilized the metarhodopsin II state of rhodopsin (39), and the cognate peptide from αs enhanced affinity of the β2AR for binding isoproteol (40). These results conflict with the proposal (29), described above, that the C termini of Gα subunits associate with a cleft between helices III and VI on the cytoplasmic face of the receptor: if Zn(II) bridges prevent formation of such a cleft, how can a G protein use the C terminus of Gα to regulate the ligand-binding site?

One possibility is that our interpretation (29) of the reduction of the binding and activation assays be performed under similar conditions. This has proved difficult because (a) the high background of guanine nucleotide-binding proteins in intact membranes can obscure receptor-catalyzed binding of GTPγS, and (b) we have found it extremely difficult to reconstitute high affinity receptor binding by adding pure G protein subunits to receptors in vitro.

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