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# Molecular Characterization of a Purified 5-HT<sub>4</sub> Receptor

A STRUCTURAL BASIS FOR DRUG EFFICACY\*

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**Serotonin 5-HT<sub>4(a)</sub> receptor, a G-protein-coupled receptor (GPCR), was produced as a functional isolated protein using *Escherichia coli* as an expression system. The isolated receptor was characterized at the molecular level by circular dichroism (CD) and steady-state fluorescence. A specific change in the near-UV CD band associated with the GPCR disulfide bond connecting the third transmembrane domain to the second extracellular loop (e2) was observed upon agonist binding to the purified receptor. This is a direct experimental evidence for a change in the conformation of the e2 loop upon receptor activation. Different variations were obtained depending whether the ligand was an agonist (partial or full) or an inverse agonist. In contrast, antagonist binding did not induce any variation. These observations provide a first direct evidence for the fact that free (or antagonist-occupied), active (partial- or full agonist-occupied) and silent (inverse agonist-occupied) states of the receptor involve different arrangements of the e2 loop. Finally, ligand-induced changes in the fluorescence emission profile of the purified receptor confirmed that the partial agonist stabilized a single, well-defined, conformational state and not a mixture of different states. This result is of particular interest in a pharmacological perspective since it directly demonstrates that the efficacy of a drug is likely due to the stabilization of a ligand-specific state rather than selection of a mixture of different conformational states of the receptor.**

G-protein-coupled receptors are versatile biological sensors. They are responsible for the majority of cellular responses to hormones and neurotransmitters, as well as sight, smell, and taste senses (1, 2). Signal transduction is specifically associated with GPCR<sup>1</sup> activation. Although significant progress has been made within the last few years in dissecting GPCR-mediated

signal transduction pathways, understanding of the mechanisms underlying receptor activation is still hampered by the lack of information at the molecular level (3, 4). This is largely due to the fact that very few expression systems have proven satisfactory for producing these receptors in a functional state and with sufficient yields for biophysical studies to be carried out (5–7). Most of the systems that have been developed to elucidate the mechanism of GPCR activation therefore essentially rely on the use of purified rhodopsin and  $\beta_2$ -adrenergic receptor (3, 4, 8). Interestingly, most of the results obtained so far report on the conformational events occurring at the level of the cytoplasmic side of the receptors. In contrast, few reports give indications on the possible conformational rearrangements certainly occurring in the extracellular part of the receptor, in particular in the extracellular loops.

Several models have been developed to conceptualize the mechanisms of activation (9, 10). The two-state model and the extended ternary model assumes that the receptor exists in an equilibrium between a resting state (R) and an active (R\*) state that stimulates the G protein. Agonists preferentially enrich the R\* state, whereas inverse agonists select for the R state. In this simple model, the efficacy of a drug can be explained by its relative affinity for the two states. Recent structural and spectroscopy findings revealed that GPCRs (rhodopsin, 5-HT<sub>4</sub>, and opiate receptors) probably exist in at least three states: an R<sub>g</sub> (ground) state that corresponds to the totally inactive “silent” state, an R state (without ligand) that is able to activate to some extent G-proteins, although with a low efficacy, and an R\* state that corresponds to the active state stabilized by full agonists (8, 11–13). It is also possible that antagonists stabilize a specific state different from R (13, 14).

We have recently described a model system composed of the leukotriene B<sub>4</sub> receptor BLT1 isolated in a detergent medium that allowed us to show that formation of the leukotriene B<sub>4</sub>-BLT1 complex is associated with a specific conformational adaptations of the receptor (15). Here we report on the molecular characterization of the mouse 5-HT<sub>4(a)</sub> receptor produced in *Escherichia coli*. Using an expression strategy similar to that described in the case of BLT1, we produced 5-HT<sub>4(a)</sub> as a functional protein isolated in a detergent-medium. The isolated receptor constituted a useful model system to investigate the molecular mechanisms underlying receptor activation using biophysical methods. We used CD and steady-state fluorescence to analyze the changes induced by different ligands on the receptor

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<sup>1</sup> The abbreviations used are: GPCR, G-protein coupled receptor; 5-HT, 5-hydroxytryptamine; e2, second extracellular loop; DMPC, dimyristoyl-phosphatidylcholine; RASSL, receptor activated solely by

synthetic ligands; TM, transmembrane; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid; NTA, nitrilotriacetic acid.

conformation. We report evidence that the conformational changes involved in both receptor activation and inactivation affect the geometrical features of its second extracellular loop. Furthermore, we also provide direct evidence for the occurrence of multiple and well defined conformational states of the 5-HT<sub>4(a)</sub> receptor depending on whether the ligand is a partial, full, or inverse agonist.

#### EXPERIMENTAL PROCEDURES

**Materials and Buffers**—RO116-1148, RS 100235, and RS 67333 were generously donated by Roche Applied Science. BIMU-8 was kindly provided by Roche Applied Science. [<sup>3</sup>H]GR 113808 was from Amersham Biosciences. GR 113808 was from GlaxoSmithKline. All detergents were from Calbiochem. DMPC and cholesteryl hemisuccinate were from Avanti Lipids. All other reagents were purchased from Sigma. The oligonucleotides were synthesized by Eurogentec. Buffer A contained 25 mM Tris-HCl, pH 7.8, containing 500 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride. Buffer B contained 25 mM Tris-HCl, pH 7.8, 6 M urea, 0.2% (w/w) SDS, 0.2 mM 2-mercaptoethanol. Buffer C contained 25 mM Tris-HCl, pH 7.8, 6 M urea. Buffer D contained 12.5 mM sodium borate, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 6 M urea, 1.25% (w/v) sarcosyl, pH 8.0. Buffer E contained 12.5 mM sodium borate, 25 mM NaCl, 2 mM MgCl<sub>2</sub>, pH 9.2, containing 1% DMPC, 1% CHAPS, and 0.02% cholesteryl hemisuccinate. Buffer F contained 12.5 mM sodium borate, 25 mM KCl, pH 7.8, containing 1% DMPC, 1% CHAPS, and 0.02% cholesteryl hemisuccinate. Buffer G contained 12.5 mM sodium borate, 25 mM KCl, pH 7.8, containing 1 mM *n*-dodecyl-β-D-maltoside and 0.5 mM lauryldimethylamino oxide.

**5HT<sub>4(a)</sub> Receptor Cloning**—The synthetic cDNA was produced as described for BLT1 (15). The oligonucleotides used in the last PCR step introduced an *Ava*I restriction site followed by a factor Xa cleavage site and an acid cleavage site (16) at the 5'-end of the receptor encoding sequence and *Xho*I restriction site at its 3'-end. After *Ava*I and *Xho*I digestion, the cDNA fragment was ligated into the similarly digested pET31b vector (Novagen). The expression vector obtained, named here pET31b-5HT<sub>4(a)</sub>, was verified on the basis of restriction digestion mapping and nucleotide sequencing. The construct encodes a fusion protein composed of ketosteroid isomerase followed by m5-HT<sub>4(a)</sub> receptor and then a His tag, with a factor Xa and an acid cleavage site between ketosteroid isomerase and 5-HT<sub>4(a)</sub>.

**Site-directed Mutagenesis**—The C93S,C184S, and C93S,C184S mutants were obtained by PCR-mediated mutagenesis using the QuikChange™ multisite-directed mutagenesis kit (Stratagene) and the pET31b-5HT<sub>4(a)</sub> vector as a template. Mutations were confirmed by nucleotide sequencing. The cysteine mutant proteins were produced as described below for the wild-type receptor. No significant differences in the refolding ratios were obtained for these mutants, consistent with the fact that the cysteine mutations in the e2 loop do not affect the secondary structure of the receptor.

**5-HT<sub>4(a)</sub> Expression and Purification**—The pET31b-5HT<sub>4(a)</sub> vector described above was introduced in the *E. coli* host strain Rosetta(DE3)pLysS (Novagen). The transformed bacteria were grown at 37 °C to an absorbance of 1 at 600 nm in 2YT medium containing 100 mg/liter ampicillin. Expression was induced by adding isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.8 mM. 0.2% glucose was also added at this step. Induction was allowed to proceed for 4 h. The cells were harvested by centrifugation (4,000 × *g* for 30 min), resuspended in buffer A and lysed by sonication. Centrifugation (27,000 × *g* for 30 min) afforded an insoluble protein fraction that was washed twice in buffer A containing 1 M urea and then solubilized in buffer B. The solubilized proteins were loaded onto a Ni<sup>2+</sup>-NTA-agarose column. After washing with buffer B, a linear imidazole gradient (0–200 mM) in the same buffer was applied to elute the polyhistidine-tagged recombinant protein. The eluted fractions containing the fusion protein, as assessed by SDS-PAGE analysis, were pooled and extensively dialyzed against buffer C. The N-terminal ketosteroid isomerase fusion partner was removed by incubation of the isolated unfolded fusion protein in acidic conditions, as described by Ryan *et al.* (15). Alternatively, the fusion protein was cleaved with factor Xa. For this, the ketosteroid isomerase-5HT<sub>4(a)</sub> fusion protein was extensively dialyzed in a 50 mM Tris-Cl, 50 mM NaCl, 1 mM CaCl<sub>2</sub>, 5 mM lauryldimethylamino oxide, pH 6.5, and digested (enzyme/substrate ratio, 0.5 units of enzyme/mg of protein) for 3 h at 20 °C. The reaction was then stopped by adding phenylmethylsulfonyl fluoride at a final concentration of 1 mM, and the reaction mixture was extensively dialyzed in buffer B. In either case (acid or factor Xa), up to 60–70% of the fusion

protein was cleaved. The cleavage mixture was then loaded on a Superdex 200 column (10 × 300 mm; Amersham Biosciences) equilibrated in the same buffer. The protein content in each fraction eluted from the column was determined by SDS-PAGE on a 15% polyacrylamide gel (16). The fractions containing cleaved 5-HT<sub>4(a)</sub> receptor were pooled.

**Refolding Assays**—The systematic search for refolding conditions was carried out as described by Banères *et al.* (15). The urea-denatured His-tagged receptor was loaded on a Ni<sup>2+</sup>-NTA matrix at different protein concentrations, and then refolding was carried out by applying a linear gradient to remove urea under a variety of buffer conditions (see "Results"). Dissociation of the receptor from the matrix was then achieved by adding 100 mM imidazole to the refolding buffer. After extensive dialysis in the same buffer, the protein solution was clarified by centrifugation (15,000 × *g* for 1 h at 20 °C), and the protein amount in the resulting supernatant was estimated by UV absorption spectrometry (see below). The refolding ratio was defined as the ratio of the amount of soluble protein after centrifugation to that initially loaded on the column.

**5-HT<sub>4(a)</sub> Receptor Refolding**—The urea-denatured His-tagged 5-HT<sub>4(a)</sub> receptor was loaded on a Ni<sup>2+</sup>-NTA matrix and immobilized at a protein/resin ratio of ~0.3 mg of protein/ml of hydrated Ni<sup>2+</sup>-NTA-agarose. The resin was previously equilibrated in buffer D. After extensive washing with buffer D, refolding was achieved by applying a linear gradient from buffer D urea to buffer E. Dissociation of the receptor from the matrix was then achieved with buffer E containing 100 mM imidazole. After extensive dialysis in buffer F, the eluted proteins were loaded on a Superdex 200 column (10 × 300 mm) previously equilibrated in buffer F. Elution was carried out with the same buffer at a 1 ml/min flow rate. The nonaggregated material (chemical cross-linking evidence) eluted from the Sephacryl column was then pooled and loaded a 10–30% isokinetic concave exponential sucrose gradient in buffer F. Centrifugation carried out for 7 h at 200,000 × *g* (Beckman SW41 rotor). The sedimentation pattern was monitored with a turbulence-free flow cell from Molecular Instruments. The nonaggregated fractions recovered from the sucrose gradient were pooled and dialyzed against buffer F. The active receptor was then purified on a GR 113808-affinity column. The ligand was immobilized on an Affi-Gel 15 (Bio-Rad) matrix as described for GR 119566X (17). The receptor was recirculated (flow rate 0.5 ml/min) on the matrix for at least 12 h, and then washing was carried out with buffer F. The receptor was then eluted with buffer F containing 1 mM 5-HT. 5-HT was removed from the eluate by extensive dialysis against buffer F. In the case of the cysteine mutants, the decrease in the affinity for GR 113808 (0.5 and 50 nM for the wild-type and double mutant, respectively) due to the replacement of the Cys residues in the e2 loop by serines ensured that the last purification step only led to an enrichment in active receptor instead of a full purification. The contaminant inactive receptor fraction corresponded to 10–15% of the total protein, as inferred from GR 113808 binding measurements (not shown).

**Protein Concentration Measurements**—All protein concentrations were determined by UV spectrometry using extinction coefficients determined by the method of Gill and von Hippel (19).

**Circular Dichroism Measurements**—The CD spectra are the average of five scans using a bandwidth of 2 nm, a step width of 0.2 nm and a 0.5-s averaging time per point. Protein solutions at concentrations in the 10<sup>-6</sup> to 10<sup>-5</sup> M range were used. Cell path lengths were 1.00 ± 0.01 and 2 ± 0.01 mm (far- and near-UV measurements, respectively). [θ] are mean residue (*M<sub>r</sub>* = 115) molar ellipticities. Deconvolution was carried out using the Convex Constraint Analysis procedure adapted to membrane proteins (20, 21). In the case of the cysteine-to-serine mutant, the near-UV CD measurements were carried out at higher protein concentrations (in the 150 μM range) to compensate the decreased affinity of the mutant for the BIMU-8 agonist. In this case, 1.00-mm path lengths giving rise to absorbance values in the 1 range were used to avoid any saturation of the photomultiplier. Three independent measurements with different protein preparations were systematically carried out. The values given in Figs. 4B and 6B are to the mean value calculated from these three independent experiments. The error bar corresponds to the S.D. from the mean value.

**Fluorescence Measurements**—Fluorescence emission spectra were recorded at 20 °C on an Eclipse spectrofluorimeter (Cary) with an excitation wavelength of 298 nm (bandwidth 2 nm). Protein concentrations in the 10<sup>-6</sup> to 10<sup>-5</sup> M range were used. For emission spectra of the ligand-loaded receptor, emission was recorded 30 min after adding the ligand at a 1:1 molar ratio. Three independent measurements with different protein preparations were systematically carried out. The values given in Fig. 7B are to the mean value calculated

from these three independent experiments. The *error bar* corresponds to the S.D. value. Buffer contributions were subtracted under the same experimental conditions. We systematically checked that the ligand used neither significantly contributed to the emission spectra upon excitation at 290 nm nor displayed a significant absorbance in the 300–360-nm range.

**Ligand Binding Assays**—The ligand binding quantitative parameters were determined by equilibrium dialysis. Dialysis cells from Dianorm were used with two 800- $\mu$ l cavities separated by 12–14-kDa molecular mass cut-off dialysis membrane (Spectra/Por). Experiments were performed at 20 °C and incubated for 24 h, sufficient time to ensure equilibrium as determined by following the time course for approach to equilibrium in control experiments. Buffer F was used in all dialysis experiments with protein concentration in the  $10^{-10}$  M range. A series of experiments in the same conditions but in the absence of protein were carried out in parallel to measure nonspecific binding. [ $^3$ H]GR 113808 concentrations after equilibrium dialysis were determined by scintillation counting. Competition experiments were carried out in the same conditions, in the presence of 0.6 nM [ $^3$ H]GR 113808 and increasing concentrations of competitor compound. The titration data were analyzed using the PRISM software (Graphpad Inc.) by considering a set of usual models for describing the ligand-receptor interactions.

## RESULTS

**Production of the Recombinant 5-HT<sub>4(a)</sub> Receptor**—Mouse 5-HT<sub>4(a)</sub> receptor was produced as a recombinant protein in *E. coli*. The approach used was similar to that previously developed for BLT1 (15). It consisted in (i) obtaining high expression yields through the use of a synthetic cDNA optimized for bacterial expression, (ii) accumulating the protein in the insoluble inclusion bodies to avoid toxicity effects associated with membrane addressing, and (iii) searching optimal refolding conditions of the receptor purified from the inclusion bodies.

We tested here the ability of different protein partners to optimize the addressing of the receptor to the inclusion bodies. These fusion partners were the T7 tag sequence we used for BLT1 (15), glutathione *S*-transferase that had been used for producing different GPCRs (22), and ketosteroid isomerase, a highly hydrophobic protein produced in large amounts as insoluble aggregates in the *E. coli* cytoplasm (23). It is to be noted that no periplasmic protein such as maltose-binding protein were tested, since we intended to specifically accumulate the receptor in the inclusion bodies. Although some expression was observed with the T7 tag sequence or glutathione *S*-transferase, the highest expression levels were systematically achieved when ketosteroid isomerase was fused to the N terminus of the receptor. As expected, the ketosteroid isomerase-5-HT<sub>4(a)</sub> fusion protein was exclusively accumulated in the inclusion bodies with no indication of membrane addressing of the receptor. Lack of membrane addressing limited the toxicity of the expressed protein thus allowing high fermentation rates to be achieved. The fusion protein was recovered from the inclusion bodies under denaturing conditions (*i.e.* in the presence of both urea and detergents) (see “Experimental Procedures”) and purified by immobilized metal affinity chromatography on a Ni<sup>2+</sup>-NTA column. Typically, 5–8 mg of purified protein were recovered from one liter of bacterial culture. The next step consisted in removing the ketosteroid isomerase fusion partner. After cleavage of the link between the fusion protein and the receptor (see “Experimental Procedures”), the cleaved ketosteroid isomerase and 5-HT<sub>4(a)</sub> as well as the remaining uncleaved fusion protein were separated through a size exclusion chromatographic step under denaturing conditions.

**Reconstitution of the Recombinant 5-HT<sub>4(a)</sub> Receptor**—The refolding scheme of the unfolded receptor obtained as described above was similar to the one that we described in the case of BLT1 (15). We used a matrix-assisted refolding procedure in which the refolding was carried out with the protein immobilized on a Ni<sup>2+</sup>-NTA matrix (16). In our hands, this method has

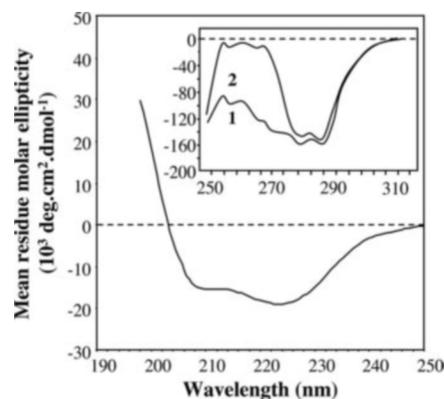


FIG. 1. **Structural characterization of the purified 5-HT<sub>4(a)</sub> receptor.** Shown is the far-UV spectrum of free 5-HT<sub>4(a)</sub>. *Inset*, near-UV spectrum of the wild-type receptor (*profile 1*) and of the double mutant C93S,C184S (*profile 2*).

systematically allowed better refolding yields than dialysis probably because of the physical separation of the refolding polypeptides on the column that limits nonspecific protein-protein interactions and thus aggregation (24). We investigated, in a systematic step-by-step manner, the biochemical parameters that could minimize aggregation. The factors tested were the detergent nature and concentration, temperature, ionic strength, presence of ionic or nonionic additives, presence of reducing/oxidizing reagents, and protein concentration, as previously described (15). A refolding yield was calculated for each of the conditions tested. This yield corresponded to the ratio of the amount of soluble protein after and before refolding (see “Experimental Procedures”). Receptor refolding was also assessed at the qualitative level by analyzing both the conformational features of the refolded receptor (far-UV CD evidence) and its ligand-binding properties (equilibrium dialysis; see below). On the basis of these assays, we defined a refolding condition combining a series of biochemical parameters in an optimal manner (see buffer E under “Experimental Procedures”) that allowed a recovery yield of about 10–15% to be achieved. It is to be noted that one of the most crucial parameters to ensure receptor refolding was, as expected for a membrane protein, the chemical nature of the detergent. The best results were obtained when the protein was reconstituted in mixed DMPC/CHAPS micelles in the presence of cholesterol. The active receptor was further purified by ultracentrifugation and ligand-immobilized affinity chromatography (see “Experimental Procedures”). Under such conditions, 0.3–0.5 mg of protein were obtained per liter of culture.

**Secondary and Tertiary Fold of Recombinant 5-HT<sub>4(a)</sub> Receptor**—We carried out a first characterization of the refolded 5-HT<sub>4(a)</sub> receptor by circular dichroism. As shown in Fig. 1, the far-UV spectrum in the 200–250-nm range was characteristic of a folded protein with a high content of secondary structure (~50% of  $\alpha$ -helix; see deconvolution description under “Experimental Procedures”). The CD spectrum in the near-UV region (250–310 nm) included a negative broad band centered at 265 nm (Fig. 1, *profile 1*). The position of this band as well as the absence of any fine structure suggested the contribution of a disulfide chromophore (25). To unambiguously assess the origin of this CD band, we replaced the cysteines that were supposed to be connected by a disulfide bridge in the 5-HT<sub>4(a)</sub> receptor (*i.e.* Cys<sup>93</sup> and Cys<sup>184</sup>) (26, 27) by a serine. Upon mutating either of these cysteines (not shown) or both residues (Fig. 1, *profile 2*), the intensity of the CD band at ~265 nm dramatically decreased. No other major alterations in the CD spectrum of the receptor were observed, indicating that these

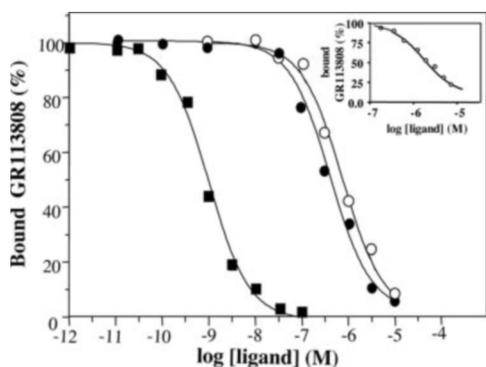


FIG. 2. **Ligand-binding properties of isolated m5-HT<sub>4(a)</sub> receptor.** Competition binding in presence of 0.6 nM of [<sup>3</sup>H]GR 113808. The assays were carried out using equilibrium dialysis as described under “Experimental Procedures.” Results are expressed as a percentage of the specific binding in the absence of a competing ligand. *Closed squares*, R01148; *closed circles*, 5-HT<sub>4(a)</sub>; *open circles*, BIMU-8; *inset*, competition binding of BIMU-8 to the C93S,C184S mutant in presence of 100 nM [<sup>3</sup>H]GR 113808.

mutations did not drastically affect the overall structural arrangement of the receptor. No additional modification of the receptor near-UV CD spectrum was observed upon treating the C93S,C184S mutant with the nonchiral reducing agent 2-mercaptoethanol. This indicated that the disulfide-associated CD band essentially resulted from the contribution of the Cys<sup>93</sup>–Cys<sup>184</sup> bridge.

**Ligand Binding Properties of the Recombinant Receptor**—The structural homogeneity of the purified receptor was assessed with specific ligands. The recombinant detergent-solubilized receptor displayed a single class of binding sites for the radiolabeled antagonist [<sup>3</sup>H]GR 113808 with a  $K_d$  value of ~0.5 nM and a stoichiometric ratio of 0.9–1 ligand molecule/receptor. Unlabeled 5-HT ligands inhibited specific [<sup>3</sup>H]GR 113808 binding to the detergent-solubilized receptor (Fig. 2). The rank order of potency obtained for the purified receptor (*i.e.* R0116-1148 > 5-HT > BIMU-8) (see Fig. 2), was similar to that reported for the same receptor expressed in cell membranes (26). The  $K_d$  values for R0116-1148, 5-HT, and BIMU-8, inferred from the competition profiles in Fig. 2, were 0.2, 80, and 90 nM, respectively.

**Ligand-induced Changes in the Receptor Conformation**—Using CD, we subsequently investigated whether the isolated 5-HT<sub>4(a)</sub> receptor underwent a conformational adaptation upon interaction with different ligands. The changes induced by the ligand on receptor conformation were visualized by calculating the difference between the spectrum of the ligand-loaded receptor and the sum of the spectra of the free ligand and receptor recorded at the same concentrations (see schematic representation in Fig. 3). The ligand-loaded state of the receptor was obtained by adding the ligand to the purified receptor at a 1:1 molar ratio. The protein concentrations used in the CD experiments (in the 10<sup>-6</sup> to 10<sup>-5</sup> M range) should result in a full loading of the ligand-binding pocket of the receptor if one takes into account the  $K_d$  and stoichiometry values inferred from the ligand-binding assays (see above).

We first analyzed the effects of the binding of a full agonist (BIMU-8) on the conformation of 5-HT<sub>4(a)</sub>. A clear difference spectrum was observed in the near-UV region (250–300 nm; Fig. 4A). The difference spectrum essentially included a broad band centered at 265 nm. As shown in Fig. 4B, the S.D. calculated from three independent experiments was much lower than the amplitude of the signal in the difference spectrum, indicating that these difference spectra are spectroscopically significant. The position of the 265-nm band as well as the absence of any vibronic fine structure indicated that it could

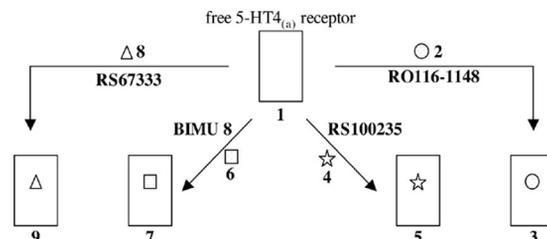


FIG. 3. **5-HT<sub>4(a)</sub> receptor conformational adaptation upon ligand binding.** Schematic representation of different species considered for the CD measurements in Fig. 4. BIMU-8 is the agonist, RO116-1148 the inverse agonist, RS 100235 the neutral antagonist and RS 67333 the partial agonist.

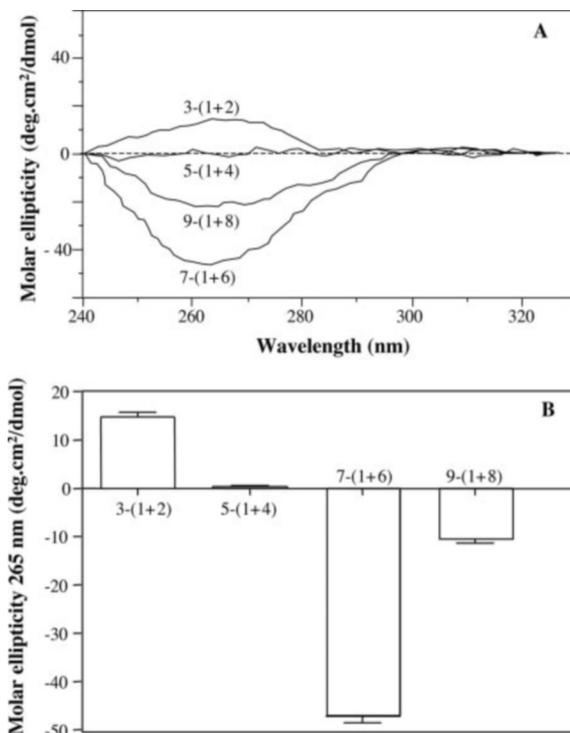


FIG. 4. **5-HT<sub>4(a)</sub> receptor conformational adaptation upon ligand binding as assessed by CD difference spectroscopy.** A, difference spectrum in the 240–320-nm range obtained after subtracting the sum of the individual CD spectra of free receptor and ligand from the spectrum of the receptor-ligand complex. The different molecular species used for these calculations are indicated under each profile (see Fig. 3 for a definition of the species). B, molar ellipticities at 265 nm inferred from the difference spectra in A. The error bar corresponds to the S.D. from the mean value calculated from three independent experiments.

originate from the contribution of the Cys<sup>90</sup>–Cys<sup>184</sup> disulfide bridge. To test this hypothesis, we compared the near-UV spectra of the agonist-free and agonist-loaded C93S,C184S mutant. This mutant still bound its ligands, although with a lower affinity (its  $K_d$  for BIMU-8 was 8.5 μM, as compared with 0.09 μM for the wild-type receptor). As expected, the difference CD spectrum obtained with this mutant was a null spectrum in both the far- and near-UV regions (not shown), indicating that the band observed in the difference spectrum with the wild-type receptor was exclusively due to the contribution of the Cys<sup>93</sup>–Cys<sup>184</sup> bridge. It is to be noted that in the case of the double mutant receptor, the protein solutions still contained ~10–15% of inactive receptor (see “Experimental Procedures”). However, the presence of ~85–90% of active protein would have been sufficient for an effect to be observed on the CD spectra were these mutants to give a signal variation upon agonist binding (compare the amplitude of the signal

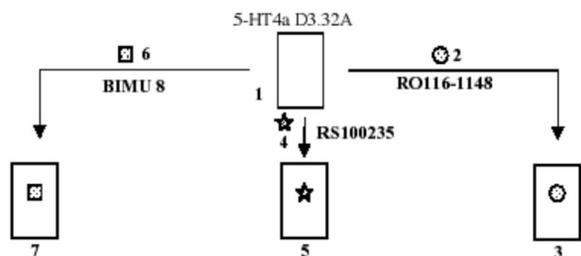


FIG. 5. Ligand-induced conformational changes of the  $D^{3.32A}$  5-HT $_{4(a)}$  mutant. Shown is a schematic representation of different species considered for the CD measurements in Fig. 6. BIMU-8 is the agonist, RO116-1148 is the inverse agonist, and RS 100235 is the neutral antagonist.

and that of the experimental error in Fig. 4B).

We then compared the difference CD spectra obtained when the ligand was a full agonist (BIMU-8), an inverse agonist (RO116-1148), a partial agonist (RS 67333), and a neutral antagonist (RS 100235). As shown in Fig. 4, the difference spectrum in the near-UV region observed with the inverse agonist again displayed a broad band corresponding to the contribution of the disulfide chromophore. However, both the sign and intensity of the difference spectrum obtained with either the agonist or the inverse agonist were totally different (Fig. 4), indicating that the handedness of the disulfide chromophore in the full agonist-loaded (hereafter called R<sup>f</sup> state) and in the inverse agonist-loaded (hereafter called R<sub>g</sub> state) states were different (see “Discussion”). In contrast, no significant signal in the difference spectrum was observed with an antagonist molecule (RS 100235) (Fig. 4), indicating that the conformation of the receptor was similar either free or bound to the antagonist (hereafter called the R state), at least as far as the disulfide bond geometry is considered.

We previously described a 5-HT $_{4(a)}$  mutant ( $D^{3.32A}$ ) in TM 3 that has two interesting properties (12, 28). The first one is that it is a RASSL, which is activated by compounds like BIMU-8 but not by 5-HT. The second one is that inverse agonists such as RO116-1148 behave on this mutant as neutral antagonists. The  $D^{3.32A}$  mutant was produced as an isolated protein. No significant difference in the receptor production was observed, compared with what was obtained for the wild-type receptor. The purified  $D^{3.32A}$  mutant still bound both BIMU-8 and RO116-1148 ( $K_d = 87.2$  and  $12.1$  nM, respectively). We compared here, again using CD, the effects induced by the binding of the full agonist BIMU-8, the inverse agonist RO116-1148, and the neutral antagonist RS 100235 (Fig. 5) on the conformation of purified ( $D^{3.32A}$ ) 5-HT $_{4(a)}$ . The difference CD spectra obtained in the case of the binding of BIMU-8 to the  $D^{3.32A}$  and wild-type receptors were similar (Fig. 6), indicating that this agonist induced the same conformational changes on both receptors. However, in contrast to what was observed with the wild-type receptor, no difference was observed between the CD spectra of the free and inverse agonist-loaded states of the  $D^{3.32A}$  mutant (Fig. 6). Besides providing a molecular basis to the *in vivo* data on the absence of effect of RO116-1148 on the  $D^{3.32A}$  mutant, this observation confirms that the difference spectra obtained with RO116-1148 on wild-type receptor was effectively due to a specific change of the disulfide bridge conformation rather than to the binding of the compound.

Finally, we determined the difference spectrum of the receptor fully loaded with a partial agonist (RS 67333) and that of the free receptor. Again, a difference was observed in the intensity of the disulfide chromophore (Fig. 4). This change had the same pattern as the one observed with the full agonist but

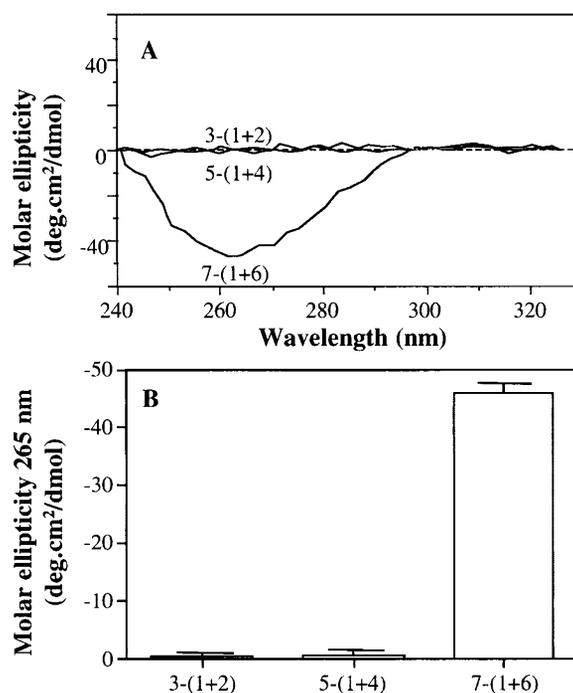


FIG. 6. Conformational adaptation of the  $D^{3.32A}$  mutant upon ligand binding as assessed by CD difference spectroscopy. A, difference CD spectrum in the 240–320-nm range obtained after subtracting the sum of the individual CD spectra of free receptor and ligand from the spectrum of the receptor-ligand complex. The different molecular species used for these calculations are indicated under each profile (see Fig. 5 for a definition of the species). B, molar ellipticities at 265 nm inferred from the difference spectra in A. The error bar corresponds to the S.D. from the mean value calculated from three independent experiments.

was not as intense. This could be due either to a difference in the conformational features induced by full and partial agonist or to the presence of a mixture of inactive and activate states of the receptor (see below).

**Fluorescence-monitored Conformational Changes**—Since the data reported above indicated different conformations of the receptor depending on the ligand considered, we subsequently studied ligand-induced conformational changes using intrinsic fluorescence. The recombinant 5-HT $_{4(a)}$  includes 7 tryptophan residues that were used to monitor ligand-induced receptor conformational changes. These measurements were carried out with ligands that do not interfere with the receptor fluorescence in the spectral region selected (see “Experimental Procedures”). As shown in Fig. 7, the recombinant receptor in its free state displays a broad fluorescence spectrum that certainly translates the contribution of the different Trp residues. Upon adding the BIMU-8 agonist, a significant change was observed in the fluorescence spectrum of the receptor associated with an overall increase in the fluorescence intensity, with no significant change in the emission maxima. In contrast, no significant change in the fluorescence spectrum of the receptor was observed upon adding the inverse agonist RO116-1148, indicating that the conformational changes observed by CD are not associated with a significant change in the surrounding of any of the receptor Trp residues. We finally recorded the fluorescence emission spectrum of the receptor loaded with the partial agonist RS 67333. As is the case with the full agonist, the binding of the partial agonist to the recombinant receptor is associated with a change in the emission properties of 5-HT $_{4(a)}$ . However, these changes were totally different from those observed with the full agonist, since, in this case, these changes were associated with a slight decrease in the emission intensity and a slight change in the emission maxima. As noted for the CD

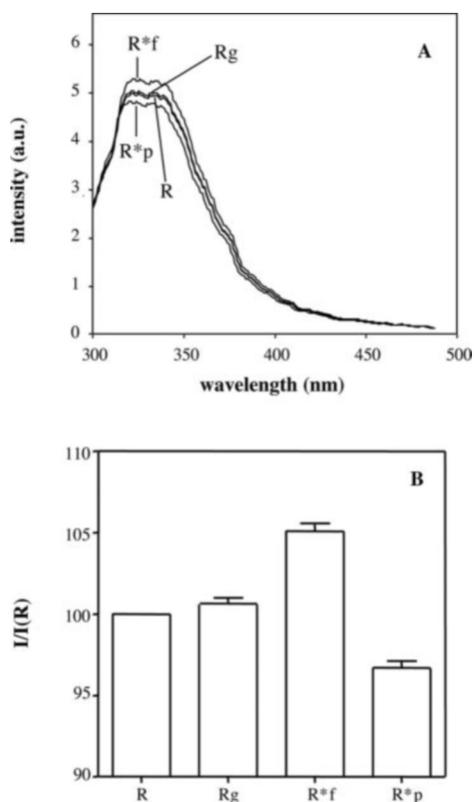


FIG. 7. **Ligand-induced changes in 5-HT<sub>4(a)</sub> conformation monitored by fluorescence.** A, Trp emission spectra of m5-HT<sub>4(a)</sub> in the absence of ligand (R) or in the presence of the full agonist BIMU-8 (R\*f), of the inverse agonist RO116-1148 (Rg), or in the presence of the partial agonist RS 67333 (R\*p). B, normalized variations in the emission intensity of the isolated receptor in the presence of different ligands inferred from the emission spectra in A. The emission intensity of R, R\*f, Rg, or R\*p (see above for a definition of the different states) at 329 nm was normalized to the emission intensity measured for R at the same wavelength. The error bar corresponds to the S.D. from the mean value calculated from three independent experiments.

measurements, the S.D. calculated from three independent experiments was much lower than the differences in the emission spectra observed for the different conformational states, indicating that these differences are spectroscopically significant. The fact that a decrease in the emission intensity was observed in the latter case compared with the Rg and R states whereas the fully active state displayed a higher emission intensity ruled out the fact that the conformational state induced by the binding of the RS 67333 partial agonist corresponds to a mixture of R\*f and Rg (or R). Indeed, in the latter case, one should have observed a fluorescence emission spectrum with an intermediate intensity between those of the R\*f and Rg (or R) spectra.

#### DISCUSSION

The 5-HT<sub>4(a)</sub> receptor was produced in *E. coli* in sufficient quantities to provide the first structural characterization in solution. The use of an adequate fusion partner appeared to be a critical step to reach an efficient addressing of the receptor to the inclusion bodies. As detailed under "Results," the best results were obtained with ketosteroid isomerase. The systematic search for convenient refolding conditions with the Ni<sup>2+</sup>-NTA-immobilized receptor also appeared to be a crucial step to obtain the protein in a folded, functional state. It is to be noted that a significant refolding was obtained only through the use of mixed lipid/detergent micelles, in contrast to what had been described with the BLT1 receptor that can be refolded in the presence of the sole lauryldi-

methylamino oxide detergent (15). However, in the latter case, the used of mixed micelles also significantly improved the refolding ratios.<sup>2</sup>

The purified 5-HT<sub>4(a)</sub> receptor was characterized by a well defined structural organization, as deduced from the far-UV and near-UV regions of its CD spectrum. It also bound the [<sup>3</sup>H]GR 113808 antagonist in a specific and saturable manner. A stoichiometric ratio of ~1 ligand molecule/purified receptor was inferred from the binding data. This indicates that the receptor population after refolding and purification was functional. The *K<sub>d</sub>* values obtained for the binding of agonists to the isolated recombinant receptor were systematically lower than those measured for the receptor in a cellular environment as opposed to the observation made with the antagonist. This was probably due to the uncoupling from the G-proteins, as previously reported for isolated BLT1 (29). In the case of BLT1, high affinity agonist binding was recovered by reconstituting the purified receptor with G-proteins (29). Thus, the affinity of the recombinant 5-HT<sub>4(a)</sub> for the agonist may probably be higher upon adding G-proteins (work in progress with recombinant G $\alpha_s$ ).

Most GPCRs display a conserved disulfide bridge that connects the extracellular tip of TM 3 and the extracellular loop e2. The topology of this disulfide bridge can be deduced from the crystal structure of rhodopsin (30). The CD spectrum of the purified 5-HT<sub>4(a)</sub> receptor displayed a broad *negative* band centered at ~265 nm corresponding to the contribution of the unique Cys<sup>93</sup>-Cys<sup>184</sup> disulfide bridge in this receptor. The fact that this band was totally absent in the CD spectrum of the double mutant C93S,C184S unambiguously demonstrated its origin. The sign of the CD bands associated with a disulfide chromophore, in the near-UV region, is directly related to the handedness of the CSSC motif (25). BLT1, another recombinant GPCR expressed in *E. coli*, was also characterized by a broad *negative* band at ~265 nm (15). This suggests that the handedness of the conserved disulfide bridge might be a topological invariant as a consequence of the specific clustering of the TM domain. A more detailed analysis of the CD properties associated with the disulfide bridges in isolated GPCRs is in progress.<sup>3</sup>

Since the sign of the CD bands associated with the disulfide chromophore, in the near-UV region, is directly related to the handedness of the CSSC motif, any change in the geometrical parameters of the disulfide bridge should be accompanied by a change in its CD properties (25). Such a change was observed here upon ligand binding to the detergent-solubilized 5-HT<sub>4(a)</sub> receptor. At this point, the fact that the differences observed here could be due to an induced optical activity of the ligand should be excluded since the same kind of difference spectra (large band with no fine vibronic structure) were observed with ligand molecules of different chemical nature. As stated above, the handedness of the disulfide bridge is likely to be associated to the specific clustering of the TM domain. Any change in the relative orientation of the TM helices is therefore likely to lead to a change in the geometrical features of the e2 loop and therefore to a change in the handedness of the bridge. Interestingly, in the three-dimensional model recently proposed for the active structure of rhodopsin (31), a change in the orientation of the e2 loop is observed compared with the crystal structure of rhodopsin complexed to 11-*cis*-retinal. This is associated with a significant change in the dihedral angle of the conserved disulfide bridge. Our results therefore provide direct experimental data on such a rearrangement of the e2 loop upon receptor activation.

<sup>2</sup> J.-L. Banères, unpublished results.

<sup>3</sup> J.-L. Banères and J. Parello, manuscript in preparation.

Another explanation could be that the e2 loop participates in ligand binding in a different manner depending on the nature of the ligand. This would lead to different orientations of the loop and therefore to different arrangements of the disulfide bridge. A counterion switch from Glu-113 to Glu-181 has been recently shown to be associated with the transition from in the dark to Meta I states of rhodopsin (32). This results in a reorganization of the e2 loop that is coupled to movements of helix III through the conserved disulfide bond. The e2 loop has also been shown to possibly contribute to the binding site in the dopamine D<sub>2</sub> receptor (33). Whatever the exact molecular explanation is, our data clearly demonstrate that a conformational rearrangement of the e2 loop occurs upon shifting from the resting to either the active or the silent state of the receptor.

Our data show that the handedness of the disulfide bridge, and therefore the conformational features of the e2 loop, is similar in the free receptor or the receptor occupied by an antagonist (R state). In contrast, the handedness of the disulfide bridge is totally distinct for the receptor liganded with a full agonist or an inverse agonist, clearly demonstrating the occurrence of two different receptor conformations corresponding to the R\*f and Rg states, respectively. The specificity of the difference spectrum observed with the inverse agonist and the existence of the Rg state was confirmed by their disappearance when the experiment were done with the RASSL (D<sup>3-32</sup>A) known to be not sensitive to inverse agonists (12, 28). The proposal of a specific Rg state is in accordance with previous data on others receptors such as rhodopsin (8), 5-HT<sub>4(a)</sub> (12), and opiate receptors (13). Of special interest is the molecular demonstration that the free state of the receptor (R), at least when isolated in a detergent medium in the absence of any G-protein, corresponded to a well defined conformation (R) and not to a mixture of active (R\*f) and silent (Rg) states.

Our fluorescence emission data also report ligand-induced conformational changes of the isolated receptor. It must be emphasized here that the S.D. calculated from independent experiments was much lower than the differences observed for the different conformational states, indicating that the differences observed are spectroscopically significant. The fully activated state was associated to an increase in the emission intensity. This increase could be due to a decreased polarity of the immediate surrounding of one (or several) of the receptor Trp residues in the active conformation of the receptor (34). Another explanation would be that agonist binding increases the population of Trp conformational substates with higher lifetimes, and this could also account for the increase in fluorescence intensity (35). No significant change was observed between the emission spectra of R and Rg. This indicates that the conformational changes induced by the binding of the inverse agonist, as evidenced by the CD measurements, do not affect the immediate surrounding of the receptor Trp residues. In contrast, the R\*p state displayed an emission spectrum with a decreased intensity compared with that of R (and Rg). This spectrum could not therefore correspond to a mixed contribution of R\*f and R (or Rg). Indeed, in this case, a fluorescence spectrum with an emission intensity higher than that of R should have been obtained. Our fluorescence emission data results thus clearly indicate that the conformational state of the partial agonist-liganded receptor (R\*p) was homogenous and did not correspond, in the case of the isolated receptor, to a mixture of active (R\*f) and inactive (R and/or Rg) states. This suggests that the efficacy of a partial agonist is probably not due to the existence of a mixture between R\*f and R plus Rg states (due to a differential affinity of the full and partial agonist for the different states

as predicted by the two-state model) but rather to the existence of a given receptor conformation. In recent studies on the conformation of the  $\beta_2$ -adrenergic receptor, a similar conclusion was reached (14, 36). Finally, plasmon waveguide resonance measurements with the opioid receptor also provided evidence for distinct receptor conformations induced by full and partial agonists (13), suggesting that this could be a common feature of the GPCR family. Interestingly, the AMPA (L- $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionate) glutamate receptor also adopts specific and well defined conformational states upon partial and full agonist binding (37).

Finally, the question of whether the activation (or inactivation) of the receptor occurs through a sequence of conformational changes, as proposed for the  $\beta_2$ -adrenergic receptor (14, 38), is difficult to assess at this point of our study. Indeed, both the CD and the fluorescence measurements were carried out at the thermodynamic equilibrium. It is also to be noted here that CD is a method that provides global conformational information. The results presented here cannot, therefore, discriminate between a single conformation for each state and a population of closely related states.

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