High Affinity Agonistic Metal Ion Binding Sites within the Melanocortin 4 Receptor Illustrate Conformational Change of Transmembrane Region 3*

Malin C. Lagerström†, Janis Klovins‡, Robert Fredriksson‡, Davids Fridmanis§, Tatjana Haitina‡, Maria K. Ling‡, Magnus M. Berglund‡, and Helgi B. Schioth‡

From the †Department of Neuroscience, Uppsala University, BMC, Box 593, 751 24, Uppsala, Sweden and §Biomedical Research and Study Centre, University of Latvia, Ratsupites 1, LV1067 Riga, Latvia

We created a molecular model of the human melanocortin 4 receptor (MC4R) and introduced a series of His residues into the receptor protein to form metal ion binding sites. We were able to insert micromolar affinity binding sites for zinc between transmembrane region (TM) 2 and TM3 where the metal ion alone was able to activate this peptide binding G-protein-coupled receptor. The exact conformation of the metal ion interactions allowed us to predict the orientation of the helices, and remodeling of the receptor protein indicated that Glu100 and Ile104 in TM2 and Asp122 and Ile125 in TM3 are directed toward a putative area of activation of the receptor. The molecular model suggests that a rotation of TM3 may be important for activation of the MC4R. Previous models of G-protein-coupled receptors have suggested that unlocking of a stabilizing interaction between the DRY motif, in the cytosolic part of TM3, and TM6 is important for the activation process. We suggest that this unlocking process may be facilitated through creation of a new interaction between TM3 and TM2 in the MC4R.

The G-protein-coupled receptors (GPCRs) require a membrane to maintain their functionality and structural integrity. This makes crystallization of these receptors difficult, hampering structural determination. Crystallization of the first mammalian GPCR, the bovine rhodopsin, was an important breakthrough (1). Earlier three-dimensional models of GPCRs were mainly based on cryoelectron microscopy data generated from bacteriorhodopsin (2), assuming a common fold in the transmembrane (TM) regions. Such assumptions had clear limitations as bacteriorhodopsin is not a GPCR and has no sequence homology to human GPCRs. Even though the bovine rhodopsin model is detailed, it is not clear how applicable it is for different GPCRs, considering the large variety of these receptors in the human genome. Today it is still an unrealistic task to crystallize the hundreds of GPCRs found in the human genome mainly due to the difficulties in obtaining material suitable for solubilization and crystallization studies. Site-directed mutagenesis has played an important role in determining the putative interaction of a ligand to a single amino acid within a GPCR. Such interactions, however, may not always be informative about the orientation of the helix bundle, which is crucial for building structural models. Moreover, the results of alanine replacement studies in most cases cannot discriminate between specific ligand-receptor interactions and changes that cause specific conformational alterations that perturb the binding. This is particularly evident when the ligand is a flexible molecule like a peptide or a protein. One alternative approach for studying the three-dimensional structure of GPCRs is construction of a high affinity zinc-binding site between the helices, using two His residues facing each other (3). Such artificial intrahelical and interhelical binding sites have been used effectively to determine the orientation and exact distances between the α-helices of the tachykinin, opioid, and the β-adrenergic receptor families (4–6). Moreover, in two previous studies (6, 7), an interhelical binding site has been created that allowed the metal ion to act as an agonist and activate a GPCR. The coordination of the metal ion binding sites is well characterized in numerous x-ray structures of soluble proteins, and the distances between the chelating atoms and the metal ion are known, providing excellent specific information regarding the orientation of the relative helices (3, 8).

The MC4R is a Gαi-coupled receptor belonging to the group of rhodopsin-like class α receptors (9). The MCRs seem to be remarkably well conserved through evolution (10). The MC4R is exclusively expressed in brain, particularly in hypothalamus, and has an important role for central regulation of food intake and energy balance. Agonistic stimulation of the receptor reduces food intake, whereas antagonists are among the most potent orexigenic agents available (11). This receptor, and the closely related MC3R, also has an important role in regulating the metabolic rate and general body weight homeostasis. The MC4R is thus one of the most pursued targets for the development of drugs to treat obesity and anorexia (12).

In this study we constructed a molecular model of the human MC4R based on the crystal structure of rhodopsin (1). We also inserted His residues into several positions in the receptor in order to generate metal ion binding sites. We were able to create artificial high affinity zinc sites where the zinc ion was able to concentration dependently increase cAMP levels. The results were used to construct a refined model for the MC4R.
protein that suggests that interaction between TM2 and TM3 is important for the activation process of this receptor.

EXPERIMENTAL PROCEDURES

Molecular Modeling—The model was based on the coordinates from the high-resolution structure of bovine rhodopsin (1). The TM regions were reconstructed to avoid problems with structural features derived from specific amino acid sequences found in the rhodopsin receptor but not in the MC4R or vice versa. The resulting TM (1–7) boundaries were between positions Gln110 and Ile298, Phe222 and Ile304, Ile233 and Ile306, Val257 and Tyr311, Ser269 and Met322, Asn345 and Leu366, and Ser382 and Tyr393. These TM regions were postulated to be helical, respectively. The positions of the three first amino acids in the cytosolic part of the helices in the rhodopsin model were used to orientate the helical segments in relation to each other. Segments of the extra- and intracellular parts of the receptor were excluded from the model due to length differences between the rhodopsin and the MC4R and due to inaccuracy in computer predictions of random coil structures. Modeling was performed with Sybyl 6.4 software (Tripos, Germany) running on work station O2 (Silicon Graphics, Mountain View, CA). Helices were given ideal a-helical conformation, and charges were assigned and helical structure refined using energy minimization in the subroutine Powell. Minimizations were carried out until convergence at 0.05 kcal/mol A energy gradient. After two cycles of successive minimizations steps were reached (maximum iteration 1000, non-bonded cut-off = 8 Å, dielectric constant = 1). Initial optimization was carried out using simplex to decrease the number of iterations required for convergence. The whole model was then minimized giving a receptor model with the energy of 288.3 kcal/mol. Refinements of the model were made after mutational data and then re-minimized giving a final energy of ~294.6 kcal/mol. Distance measurements were made between the a-carbons of the pin-pointed residues. Construction of Receptor Mutants—Point mutations were introduced in the human MC4R by PCR in two steps using Pfu polymerase (Invitrogen). Specific end primers containing HindIII (Amersham Biosciences, Little Chalfont, UK) sites were used together with mutated internal primers in two steps using the following conditions: 30 s at 95 °C for one cycle, then 30 s at 95 °C, 40 s at 50 °C and 1 min at 72 °C for 40 cycles followed by 5 min at 72 °C. In the first step, overlapping fragments containing the desired mutation were generated. Fragments were purified using Qiagen Gel Extraction MiniElute kit (Qiagen, Stockholm, Sweden) and subsequently used as templates for a second PCR amplification to obtain the full-length mutated receptor. These fragments were digested with HindIII and XhoI, purified with QiAquick PCR purification kit (Qiagen), and ligated into the modified pCEP4 turbo expression vector. The integrity of each construct was controlled with restriction analysis and DNA sequencing using ABI PRISM Dye Terminator cycle sequencing kit version 2.0 (Applied Biosystems) according to the manufacturer’s recommendations. The extension products were analyzed on an ABI PRISM-310 Automated Sequencer (Applied Biosystems). Sequences were analyzed using Sequencer 3.0 (Gene Codes) and found to be identical to the sequence of the desired MC4R mutant.

Expression of Mutant Receptor in HEK 293-EBNA—Stable-cell line expressing the different constructs were made from HEK 293-EBNA cells through transfection with 15 μg of plasmid-DNA using FuGENE™ Transfection reagent (Roche Applied Science) diluted in Opti-MEM medium (Invitrogen) according to the manufacturer’s recommendations. After transfection, cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with 2% serum protein-free (BioWhittaker) supplied with 10% fetal calf serum (Invitrogen), 2.4 μM t-glutamic acid (Invitrogen) and 250 μg/ml gentamicin (Invitrogen), penicillin/ streptomycin (100 units of penicillin, 100 μg of streptomycin/ml) (Invitrogen) for 48 h before addition of 200 μg/ml hygromycin B (Invitrogen). The cells were harvested in 1 x PBS after 3 weeks of selection. Membrane Preparation—After the harvest, the cells were homogenized using an UltraTurrax. The cell suspension was centrifuged for 3 min at 1300 rpm, and the supernatant was recentrifuged for 15 min at 15,000 rpm. The cell pellet was resuspended in binding buffer containing 50 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, 1 mM CaCl2, and 150 mM NaCl.

Receptor Binding Assays—Binding studies were performed using a buffer containing 50 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, 1 mM CaCl2, 150 mM NaCl (for optimization of buffer system see Supplemental Material, 1A–C), in a final volume of 100 μl for 3 h at room temperature using 10–20 μg of membrane preparation per well, aiming at 60–70% specific binding. Competition studies were performed with various concentrations of ZnCl2 (Sigma) or non-labeled NDP-MSH peptide (Neosystem, France) included in the incubation mixture along with 0.6 nM 125I-labeled NDP-MSH. Nonspecific binding was defined as the amount of radioactivity remaining bound to the cell homogenate after incubation in the presence of 1000 nM unlabeled NDP-MSH. Incubation was terminated by filtration through GF/C filters, Filtermat A (Wallac Oy, Turku, Finland), which had been pre-soaked in 0.3% polyethyleneimine (Sigma), using a TOMTEC Mach III cell harvester (Orange, CT). The filters were washed with 5.0 ml of 50 mM Tris-HCl, pH 7.4, per well at 4 °C and dried at 60 °C. The dried filters were then treated with Melt-iLex A (PerkinElmer Life Sciences) melt-on scintillator sheets and counted with Wallac 1450 (Wizard automatic Microbeta counter). The results were analyzed with Prism 3.0 GraphPad (San Diego, CA). All competition assays were performed in duplicate and repeated at least three times. Non-transfected HEK 293-EBNA cells did not show any specific binding for 125I-labeled NDP-MSH. The radioligand NDP-MSH was labeled by the chloramine-T method and purified by high performance liquid chromatography. Protein concentrations were measured using a Bio-Rad Protein Assay (Bio-Rad).

cAMP Assay—cAMP assay was assayed on semi-stable HEK 293-EBNA cells pre-treated for 15 min at 37 °C with 250 μM isobutylmethylxanthine (Sigma). For antagonist studies, 10–20 μg of cells were incubated in a 300-μl reaction volume with ZnCl2 at three different concentrations together with various concentrations (serial dilutions) of NDP-MSH for 1 h at 37 °C. All points were done in duplicate, and the measurements for each concentration were repeated at least three times. Agonistic studies were performed with various concentrations (serial dilutions) of NDP-MSH or ZnCl2. Non-transfected HEK 293-EBNA showed no cAMP response to NDP-MSH or ZnCl2. The incubations were terminated by addition of 25 μl of 4.4 M perchloric acid and neutralized by addition of 45 μl of 5 M KOH. Levels of cAMP were then measured using radioassay (Amershams Biosciences).

RESULTS

Molecular Modeling and Placement of Initial Mutations—A preliminary molecular model of the MC4R was generated using the coordinates from the high-resolution structure of bovine rhodopsin (1). We deliberately did not take into account any constraint from previous mutagenesis studies on the MCRs (13–15). Based on our preliminary model, His residues were inserted into several positions close to the extracellular parts of the receptor. These positions were predicted to be facing the binding pocket, thus enabling the formation of a zinc-binding site possible to induce activation of the receptor or to inhibit ligand binding thereon. In this way we created the following single mutants (among a number of others): MC4R-I103H and MC4R-I104H at the top of TM2, MC4R-I125H at the top of TM3, and MC4R-F284H and MC4R-N285H at the top of TM7. In the preliminary model Ile104 was oriented toward TM3, 11 Å away from the TM3 backbone. Position Ile103 was in close proximity to Ile104 but turned toward the lipid bilayer. Ile125 was facing TM2, 14 Å from the a-carbon of Ile104 but one helical turn up. Position Phc284 was placed in TM7, facing TM3 at a distance of 17 Å from the a-carbon of Ile125. Asn285 was positioned in TM7, 18 Å away from the backbone of Ile104 in TM2. Naturally occurring zinc-binding residues, His and Asp, in these areas of the TM regions were also investigated by creating Ala-substituted mutants: MC4R-D122A (TM3), MC4R-D126A (TM3), MC4R-H283A (TM7), and MC4R-D289A (TM7).

Receptor Binding Analysis of Initial Mutants—At first we optimized the ligand binding conditions (see Supplemental Material, 1C). The binding results show that the wild-type MC4R bound zinc with about 20 μM affinity (Table 1). This natural binding site for zinc in the human MC4R was reported during our studies by another group (16). These results are in agreement with ours. The introduction of the His residues listed above did not affect the binding of NDP-MSH significantly. However, MC4R-I1104H (TM2) and MC4R-I125F (TM3) both gained 26-fold in affinity for zinc compared with the wild-type receptor (Table 1). Moreover, the MC4R-I1103H mutant in TM2
showed a 49-fold increase in zinc affinity. We also found an increase in affinity to zinc for two mutants in TM7, MC4R-F284H and MC4R-N285H, 8- and 19-fold, respectively. As mentioned above some alanine mutations were made on residues that may have the capability to create a high affinity binding site for zinc. Several of these mutants showed reduced zinc binding affinity without greatly influencing the NDP-MSH binding. The MC4R-D122A (TM3) lost all detectable affinity to zinc but maintained its affinity to NDP-MSH. The MC4R-I104H/D122A showed a 49-fold increase in zinc affinity. We also found an increase in zinc affinity to zinc at 0.7 M (Table II). We subsequently introduced whether zinc was forming an interhelical or intra-helical agonistic binding site between the His in position 104 and a naturally occurring amino acid residue in TM3 or TM2. It is of interest, zinc induced cAMP production with a potency of 5.9 μM upon exposure to zinc in our assay. Our antagonistic studies unveiled an inhibitory effect of zinc at 32.9 μM (see Table II and Fig. 1) in accordance with the binding studies (Table I). In agreement with several previous studies, NDP-MSH stimulation showed an agonistic response of 8.9 nM for the wild-type construct. The single mutants mentioned above responded agonistically to NDP-MSH displaying decreased EC50 values in comparison with the wild-type receptor (Table III). Cells expressing these constructs showed no increase in basal cAMP production. Interestingly, zinc induced cAMP production with a potency of 5.9 μM (Table III) in cells expressing the MC4R-I104H mutant, whereas cells transfected with MC4R-I103H showed no agonistic response. Moreover, zinc was able to inhibit the agonistic response of NDP-MSH, in cells expressing the MC4R-I103H mutant, with a Ki value of 42.1 μM (Table II). We subsequently investigated whether zinc was forming an interhelical or intra-helical agonistic binding site between the His in position 104 and a naturally occurring amino acid residue in TM3 or TM2. The most likely residues according to the original model were Asp122 in TM3 and Glu100 in TM2. There was also a possibility for an intrahelical interaction between I125H and Asp122. However, we were able to show that the His-substituted MC4R-I125H construct showed an agonistic response of 3.9 μM upon exposure to zinc. According to the original model, one highly plausible residue for interhelical interaction with the His in position 125 was Glu100 in TM2. There was also a possibility for an intrahelical interaction between I125H and Asp122. However, we were able to show that the His-substituted MC4R-I125H construct showed an agonistic response of 3.9 μM upon exposure to zinc. According to the original model, one highly plausible residue for interhelical interaction with the His in position 125 was Glu100 in TM2. There was also a possibility for an intrahelical interaction between I125H and Asp122. However, we were able to show that the His-substituted MC4R-I125H construct showed an agonistic response of 3.9 μM upon exposure to zinc. According to the original model, one highly plausible residue for interhelical interaction with the His in position 125 was Glu100 in TM2. There was also a possibility for an intrahelical interaction between I125H and Asp122. However, we were able to show that the His-substituted MC4R-I125H construct showed an agonistic response of 3.9 μM upon exposure to zinc. According to the original model, one highly plausible residue for interhelical interaction with the His in position 125 was Glu100 in TM2. There was also a possibility for an intrahelical interaction between I125H and Asp122. However, we were able to show that the His-substituted MC4R-I125H construct showed an agonistic response of 3.9 μM upon exposure to zinc.
its own longitudinal axis ~76° counterclockwise, making the interacting residues in TM3 facing the corresponding residues in TM2 (Fig. 3, A and B). The distance between the α-carbon of Asp122 in TM3 and I104H in TM2 was calculated to 10 Å. The corresponding distance between the α-carbon of Gru100 in TM2 and I125H in TM3 was estimated to be 8 Å, both interactions clearly within the maximum chelating distance of 12.3–14.8 Å. The distances in the perimeter of the helical bundle were measured as described in Fig. 3D. Comparison between orientations in the crystallized bovine rhodospin receptor and the remodelled human MC4R shows a clear spatial agreement (1) (Fig. 3C). TM1 and TM2 show agreement regarding the orientations of the highly conserved residues Asn62 (TM1) and Asp90 (TM2). The rhodospin model and our new model also share the same spatial arrangement for Asp298 and Tyr302 in the well conserved (D/N)PXXY motif in the bottom of TM7. The highly conserved Asp in TM2, which corresponds to Asp122 in bovine rhodospin, is pointing toward the cavity between TM7, TM1, TM2, and TM3 as in the rhodospin model, where it is participating in an interaction with Asp298 in TM7 (1), and Trp258 in the well conserved CWXP motif in TM6 is directed toward the binding cavity. This amino acid is believed to interact with the C13-methyl group of retinal in the bovine rhodospin model (1).

**DISCUSSION**

Initially, we created a preliminary model of the MC4R based on the crystal structure of the bovine rhodospin receptor. Subsequently, His residues were inserted into several positions in order to create artificial metal ion binding sites. The most interesting mutagenesis results were obtained from those at the extracellular parts of TM2 and TM3. Three of our mutants, MC4R-I103H (TM2), MC4R-I104H (TM2), and MC4R-I125H (TM3), remarkably increased the zinc affinity of the receptor by 25–50-fold. Moreover, zinc was able to induce cAMP production with a potency of 5.9 μM in cells expressing the MC4R-I104H mutant, whereas cells transfected with MC4R-I103H showed no agonistic response. Furthermore, zinc was able to inhibit the agonistic response of NDP-MSH in cells expressing the MC4R-I103H mutant, with a Ki value of 42.1 μM (Fig. 1). Interestingly, the single His-substituted MC4R-I125H construct also showed an agonistic response of 3.9 μM upon exposure to zinc. The residues highly plausible to interact with the His-substituted positions were subsequently replaced by Ala which is unable to bind zinc. Strikingly, the double mutant MC4R-I104H/D122A was unable to respond to zinc, whereas the double mutant MC4R-I104H/E100A showed an agonistic response to zinc at

**FIG. 2.** A, generation of cAMP in response to Zn2+ for receptor mutant MC4R-I104H (■), MC4R-I104H/E100A (○), and MC4R-I104H/D122A (▲). Each point represents the average ± S.E. of values in duplicate. B, generation of cAMP in response to Zn2+ for receptor mutant MC4R-I125H (■), MC4R-I125H/E100A (▲), and MC4R-I125H/D122A (○). Each point represents the average ± S.E. of values in duplicate. Non-transfected HEK293-EBNA cells showed no cAMP response to Zn2+ (data not shown).

**FIG. 3.** The orientation of TM1–TM7 of human MC4R in our re-defined computer model, originally based on the inactive crystallized bovine rhodospin receptor (1). Positions involved in zinc binding in TM2 and TM3 and seen from the extracellular side of the membrane before (A) and after the remodeling (B). TM3 had to be turned 76° counterclockwise in order to demonstrate the interaction with TM2. The figure shows that the spatial orientations of well conserved residues are preserved between the new MC4R model and the bovine rhodospin model (C). D, the distances between TM regions and between zinc-interacting residues in MC4R. Color denotation for the helices is as follows: TM1, yellow; TM2, orange; TM3, red; TM4, purple; TM5, dark blue; TM6, light blue; and TM7, green. The molecular model of the re-modeled human MC4R is shown. The amino acid residues on the border of the helices and the extracellular loops are marked with arrows and positions. Sites subjected to histidine replacements appear as yellow on green and naturally occurring residues involved in activation of the receptor as blue on purple (E).
0.7 μm (Fig. 2A). This indicates that I104H is forming an agonistic interhelical zinc site with Asp$^{122}$, connecting TM2 and TM3 horizontally. Since MC4R-I104H/E100A could respond to zinc, the possibility of a solitary intrahelical site was unlikely. It was evident that the agonistic response derived by the single mutant MC4R-I125H was due to Glu$^{100}$ (TM2) since the double mutant MC4R-E100A/I125H was unable to respond to zinc. There was also a possibility for an intrahelical interaction between I125H (TM3) and Asp$^{122}$ (TM3). However, this did not appear to be the case since the double mutant MC4R-I125H/D122A responded to zinc with a potency of 2.2 μm (Fig. 2B). Taken together this shows that we have created two separate interhelical agonistic binding sites for zinc between TM2 and TM3 in the human MC4R. This clearly shows that this area of the receptor is crucial for activation, and the data prompted us to take a closer look at this area in the model. The two agonistic interhelical zinc interactions provide coordinates that define the spatial orientation both horizontally and vertically. Therefore, the active conformation of TM3 is ~76° counterclockwise as compared with the initial conformation. Since the initial MC4R model (Fig. 3A) was based on the inactive conformation of bovine rhodopsin, the results from the activation studies (Fig. 3B) could be superimposed on this model, thereby suggesting how the transition from the inactive to the active state could affect this part of the MC4R.

The agonistic zinc-binding sites between I104H (TM2) and Asp$^{122}$ (TM3) and between Glu$^{100}$ (TM2) and I125H (TM3) show how these amino acids are facing each other, creating an important part of the binding pocket for the MSH ligand, which is illustrated in Fig. 3E. The exact positioning of the zinc-binding sites together with the computer-generated receptor model, the proposed rigidity of the α-helix, and the calculated distance for a zinc-binding site allowed us to predict the orientation of the remaining residues in the two helices. Thus, Asp$^{126}$, Ile$^{129}$, Ser$^{132}$, and Leu$^{133}$ in TM3 may likely face the internal space. These residues in the upper half of this TM region could thus act as interaction points for the ligand. It is interesting that during our work, Yang et al. (14) showed that mutation L133M transformed a classical MC4R-selective antagonist, SHU9119, to an agonist, without affecting the binding of NDP-MSH. Furthermore, position Asp$^{126}$ has in other studies, besides ours, been identified as a critical amino acid for MSH-ligands (14, 16, 18). Recently, MC4R-I125K was shown to be present as a missense mutation in obese patients where the mutant receptor had totally lost its ability to bind NDP-MSH and/or stimulate cAMP production (18), further strengthening the importance of this area for activation. Our computer model and intracellular results display how Glu$^{100}$ and Ile$^{104}$ in TM2 are pointing toward TM3, whereas Ile$^{103}$ is turned toward the lipid membrane. An Ala replacement of Glu$^{100}$ in TM2 reduces the affinity for NDP-MSH remarkably, indicating the relevance for this position in ligand-binding. In several naturally occurring phenotypes, this amino acid is found to be mutated to Lys, inducing constitutive activation of the melanocortin 1 receptor (MC1R). This unique Glu/Lys mutation has been linked to hair and fur colors in mice (19) and more recently to feather color in chickens (20). Furthermore, MC4R-N97D, found as a homoyzgote missense mutation in obese patients, has been shown to be unable to bind NDP-MSH (18). Taken together, these findings further support our suggestion about the orientation of the helices and strengthen our hypothesis regarding the importance of TM2 and TM3 in receptor activation.

Two main three-dimensional models of the closely related MC1R have been reported (21, 22), both based on bacteriorhodopsin and the low resolution structure of bovine rhodopsin (2). These two models show dissimilar positioning of the ligand binding pocket. Prusis et al. (21) highlights the importance of TM1, TM2, and TM7 in ligand binding, whereas Haskell-Luevano et al. (22) shows a more general binding pocket involving all TM regions but with emphasis on TM4 and TM5. None of these models has been re-evaluated after the presentation of the high resolution structure of bovine rhodopsin (1). Both MC1R models suggest involvement of Glu$^{100}$ (corresponds to Glu$^{114}$ in MC1R) in ligand binding. Prusis et al. (21) directs Glu$^{100}$ against TM1, whereas the Haskell-Luevano model shows Glu$^{100}$ directed in space between TM2 and TM7 in a binding pocket that spans to TM4 and TM5. The directions of the well conserved (D/N)P$^{XX}$ motif in TM7 and CWXP motif in TM6 are consistent between our model and the Prusis model, whereas the CWXP motif in TM6 in the Haskell-Luevano model seems to be shifted about 180° counterclockwise. It is likely that these MC1R models can be improved by taking advantage of our model of MC4R that takes advantage of the defined orientation of the TM2 and TM3.

The involvement of TM3 in the activation process, for GPCRs in general, has been suggested by several previous publications where the attention has been drawn to the well conserved DRY motif close to the cytosolic end (23–25). Previous studies (26, 27) have indicated a stabilization of the inactive conformation of GPCRs by an ionic interaction between the Arg, within the motif in TM3, and a residue close to the cytosolic end of TM6. We suggest that this central interaction between TM3 and TM6 is disrupted by the 76° counterclockwise turn of TM3, initiated by an interaction between TM2 and TM3 in the MC4R (Fig. 3). The initiating process for the adrenergic β2-receptor (ADRB2), on the other hand, has been suggested to involve ligand binding to a cluster of highly conserved aromatic residues close to the presumed Pro-kink, which may trigger the receptor activation through the “toggle switch” (28). Evidence has also been shown for a clockwise rotation and/or tilt of TM6, away from TM3, toward TM5 for ADRB2 (29, 30). It is probable that TM3 serves as a common unit within the activation machinery. A slight counterclockwise turn of TM3 during the activation process can also be interpreted from older data for the ADR1B receptor (25). Subsequently, the turn of TM3 might lead to the previously suggested (31, 32) outward movements primarily of TM6, but also TM2 and TM7, which changes the environment in the cytosolic part of the receptor and thereby subsequently creates and enlarges a G-protein binding cavity on the inside of the receptor.

In summary, we have created agonistic sites between TM2 and TM3 within a GPCR that is one of the most pursued targets of drug development. The double nature of the sites allowed us in a unique fashion to predict the three-dimensional structure and activation mechanisms of the receptor. This analysis has enabled us to predict potential areas of activation within the MC4R. It is likely that this template may serve as a guide for further mutagenesis and three-dimensional structure analyses, and also be important for the development of agonistic ligands for the MC4R.

Acknowledgments—We thank Jan Andersson and Anna Otto, Uppsala University, for assistance with molecular modeling and distance calculations.

REFERENCES
1. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Steinkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
2. Schertler, G. F., Villa, C., and Henderson, R. (1993) Nature 362, 770–772
3. Elling, C. E., Thirstrup, K., Nielsen, S. M., Hjorth, S. A., and Schwartz, T. W. (1997) Annu. N. Y. Acad. Sci. 814, 142–151
4. Thirstrup, K., Elling, C. E., Hjorth, S. A., and Schwartz, T. W. (1996) J. Biol. Chem. 271, 7675–7678
5. Elling, C. E., and Schwartz, T. W. (1996) EMBO J. 1, 6213–6219
6. Elling, C. E., Thirstrup, K., Holst, B., and Schwartz, T. W. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 12322–12327
Agonistic Metal Ion Binding Sites in MC4R

7. Holst, B., Elling, C. E., and Schwartz, T. W. (2000) Mol. Pharmacol. 58, 263–270
8. Christianson, D. W. (1991) Adv. Protein Chem. 42, 281–355
9. Fredriksson, R., Lagerstrom, M. C., Lundin, L. G., and Schioth, H. B. (2003) Mol. Pharmacol. 63, 1296–1273
10. Ringholm, A., Fredriksson, R., Poliakova, N., Yan, Y.-L., Postlethwait, J. H., Larhammar, D., and Schioth, H. B. (2002) J. Neurochem. 82, 6–18
11. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
12. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
13. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
14. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
15. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
16. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
17. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
18. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
19. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
20. Kask, A., Mutulis, F., Muceniece, R., Pahkla, R., Mutulis, I., Wikberg, J. E., Rago, L., and Schioth, H. B. (2002) J. Neurochem. 82, 661–68
21. Prusis, P., Schioth, H. B., Muceniece, R., Herryk, P., Afshar, M., Hubbard, R. E., and Wikberg, J. E. (1997) J. Mol. Graph Model. 15, 307–317
22. Haskell-Luevano, C., Sawyer, T. K., Trumpf-Kallmeyer, S., Bikker, J. A., Humblet, C., Gantz, I., and Hubry, V. J. (1996) Drug Des. Discov. 14, 197–211
23. Gabork, Z., Jagadeesh, G., Zhang, M., Spat, A., Catt, K. J., and Hunyady, L. (2003) Endocrinology 144, 2220–2228
24. Alewijnse, A. E., Timmerman, H., Jacobs, E. H., Smit, M. J., Roovers, E., Coteceha, S., and Leurs, R. (2000) Mol. Pharmacol. 57, 890–898
25. Scheer, A., Costa, T., Fanelli, P., De Benedetti, P. G., Mhaeauty-Kodja, S., Aduin, L., Nenniger-Tosato, M., and Coteceha, S. (2000) Mol. Pharmacol. 57, 219–231
26. Gresley, P. J., Fanelli, F., Rosier, O., Aduin, L., and Coteceha, S. (2002) Mol. Pharmacol. 61, 1025–1032
27. Franke, R. R., Konig, B., Sakmar, T. P., Khorana, H. G., and Hofmann, K. P. (1990) Science 250, 123–125
28. Shi, L., Liapakis, G., Xu, R., Guarnieri, F., Ballesteros, J. A., and Javitch, J. A. (2002) J. Biol. Chem. 277, 40989–40996
29. Ghanouni, P., Steenhuis, J. J., Farrens, D. L., and Kobulka, B. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5997–6002
30. Ballesteros, J. A., Jensen, A. D., Liapakis, G., Rasmussen, S. G., Shi, L., Gether, U., and Javitch, J. A. (2001) J. Biol. Chem. 276, 29171–29177
31. Hubbell, W. L., Cafiso, D. S., and Altenbach, C. (2000) Nat. Struct. Biol. 9, 735–739
32. Khorana, H. G. (2000) Proc. Conversat. Biomol. Stereodyn. 11, 1–16