Critical Role of Electrostatic Interactions of Amino Acids at the Cytoplasmic Region of Helices 3 and 6 in Rhodopsin Conformational Properties and Activation*

Eva Ramon1, Arnau Cordomí2, Laia Bosch1, Eugeni Yu. Zernii†, Ivan I. Senin*, Joan Manyosa†, Pavel P. Philippov†, Juan J. Pérez‡, and Pere Garriga‡

From the 1Centre de Biotecnologia Molecular, Departament d’Enginyeria Química, Universitat Politècnica de Catalunya, 08222 Terrassa, Catalonia, Spain, 2Centre de Biotecnologia Molecular, Departament d’Enginyeria Química, Universitat Politècnica de Catalunya, 08028 Barcelona, Catalonia, Spain, 3Department of Cell Signalling, A. N. Belozersky Institute of Physico-Chemical Biology, M.V. Lomonosov Moscow State University, 119992 Moscow, Russia, and 4Unitat de Biofísica, Departament de Bioquímica i de Biologia Molecular and Centre d’Estudis en Biofísica, Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain

The cytoplasmic sides of transmembrane helices 3 and 6 of G-protein-coupled receptors are connected by a network of ionic interactions that play an important role in maintaining its inactive conformation. To investigate the role of such a network in rhodopsin structure and function, we have constructed single mutants at position 134 in helix 3 and at positions 247 and 251 in helix 6, as well as combinations of these to obtain double mutants involving the two helices. These mutants have been expressed in COS-1 cells, immunopurified using the rho-D4 antibody, and studied by UV-visible spectrophotometry. Most of the single mutations did not affect chromophore formation, but double mutants, especially those involving the T251K mutant, resulted in low yield of protein and impaired 11-cis-retinal binding. Single mutants E134Q, E247A, and E247A showed the ability to activate transducin in the dark, and E134Q and E247A enhanced activation upon illumination, with regard to wild-type rhodopsin. Mutations E247A and T251A (in E134Q/E247A and E134Q/T251A double mutants) resulted in enhanced activation compared with the single E134Q mutant in the dark. A role for Thr251 in this network is proposed for the first time in rhodopsin. As a result of these mutations, alterations in the hydrogen bond interactions between the amino acid side chains at the cytoplasmic region of transmembrane helices 3 and 6 have been observed using molecular dynamics simulations. Our combined experimental and modeling results provide new insights into the details of the structural determinants of the conformational change ensuing photoactivation of rhodopsin.

Rhodopsin is the leading model for the G-protein-coupled receptor (GPCR) superfamily, which includes over 1000 membrane proteins (1). The three-dimensional structure of rhodopsin, characterized by seven transmembrane helices and a conserved disulfide bridge at the extracellular region, has been determined by x-ray crystallography (2–4). Upon rhodopsin activation by light absorption, the native chromophore 11-cis-retinal, bound through a protonated Schiff base linkage to Lys296, is isomerized to its all-trans configuration. This photoreaction causes a conformational change in the protein that leads to the formation, through a series of short lived photointermediates, of the active state metarhodopsin II. Structures of some of these intermediates have been recently determined (5–8). Most of these changes are concentrated on the transmembrane domain of the protein involving amino acids in the binding pocket of the retinal chromophore, which are efficiently transmitted to the cytoplasmic domain. The structural connection among several regions of rhodopsin (in the transmembrane and cytoplasmic domains) allows the interaction to its G-protein, transducin (Gt), which is activated, and initiates the visual transduction cascade (9, 10). The structural details underlying the signal transmission process, going from retinal isomerization to G-protein activation, have not been determined and still remain for the most part a main scientific question to be answered.

Based on the proposed existence of a common activation mechanism, which has been correlated with the presence of a set of conserved residues among the GPCR superfamily, nomenclature has been established for an easy comparison among their amino acid sequences (11). The highly conserved triplet Glu134–Arg135–Tyr136 (corresponding to 3.49–3.50–3.51 according to this general numbering code), in the C-terminal part of helix 3, plays a critical role in the rhodopsin photoactivation process (12, 13). In particular, the Glu134–Arg135 ionic couple has been shown to be very important for the activation of Gt (14), and any changes in this interaction can alter

*This work was supported in part by Spanish Ministry of Education Grants SAF2005-08148-C04-01 (to J. J. P.) and SAF2005-08148-C04-02 (to P. G.) and in part by NATO Scientific Programme, INTAS Grant 03-51-4548 (to I. I. S., J. M., and P. G.), FEBS fellowship program (to E. Yu. Z.), and Russian Foundation for Basic Research Grants 06-04-48018, 04-04-04001 (to P. P. P.), and 06-04-48761 (to I. I. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Present address: Center for Membrane Biology, Dept. of Biochemistry and Molecular Biology, University of Texas Health Science Center, Houston, TX 77030.
2 Recipient of doctoral fellowship from the Universitat Politècnica de Catalunya.
3 To whom correspondence should be addressed. Tel.: 34-937398998; Fax: 34-937398225; E-mail: pere.garriga@upc.edu.

4 The abbreviations used are: GPCR, G-protein-coupled receptors; Gt, transducin; UV-Vis, UV-visible; DM, dodecyl maltoside; MD, molecular dynamics; 9-mer, peptide corresponding to the last nine amino acids of the C-terminal region of rhodopsin; WT, wild type; DPPC, dipalmitoylphosphatidylcholine; GTPγS, guanosine 5′-O-(3-thiotriphosphate).
Examples of these receptors are rhodopsin (20–22), GPCR have been described upon mutation of these residues. Formational changes causing constitutive activity of some positions 3.49 and 3.50 has been reported previously, and conformational and functional properties of the receptor.

In this work, the study of this electrostatic network, involving positions Glu\textsubscript{134}, Arg\textsubscript{135}, Glu\textsubscript{247}, and Thr\textsubscript{251}, has been undertaken. Specifically, single and double mutants involving Glu\textsubscript{134} in helix 3 and Glu\textsubscript{247} and Thr\textsubscript{251} in helix 6 have been constructed, purified, and characterized spectroscopically, functionally, and through molecular modeling. The results presented here provide experimental evidence for an important role of these amino acids in the functional status of the visual photoreceptor rhodopsin. Some of the double mutants show clear activity in dark conditions, particularly E134Q/T251A, and point to a key role of Thr\textsubscript{251} in the electrostatic network. The results provide experimental evidence, for the first time, for the involvement of Thr\textsubscript{251} in the conformational changes accompanying rhodopsin activation and highlight the importance of this residue in the structure and function of rhodopsin. The results are discussed in terms of the dynamic behavior of the different models of a selected subset of mutants as compared with the behavior of wild-type (WT) rhodopsin through molecular dynamics (MD) simulations.

The complementary study of the experimental and modeling features of the mutants provides new insights into the nature of the electrostatic network that keeps rhodopsin in its inactive conformation, allowing the dissection of the individual role of Glu\textsubscript{134}, Glu\textsubscript{247}, and Thr\textsubscript{251} in the cytoplasmic domain of rhodopsin.

**EXPERIMENTAL PROCEDURES**

**Materials**—All buffers and chemicals were purchased from Panreac and Sigma. Oligonucleotides were obtained from Operon (Qiagen). Enzymes for site-directed mutagenesis by PCR were purchased from Stratagene and rho-1D4 antibody from the National Cell Culture Facilities. Cyanogen bromide-4B-Sepharose and phenylmethylsulfonyl fluoride were obtained from Amersham Biosciences and New England Biolabs. Dodecyl maltoside (DM) was purchased from Anatrace. 11-cis-Retinal was synthesized at Moscow State University. The nonapeptide corresponding to the last nine residues of the C-terminal region of rhodopsin (9-mer) was synthesized in the Laboratori de Síntesi de Peptíds (Universitat de Barcelona).

Buffers are defined as buffer A (1.8 mM KH\textsubscript{2}PO\textsubscript{4}, 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 137 mM NaCl, 2.7 mM KCl, pH 7.2), buffer B (buffer A + 1%DM + 100 mM phenylmethylsulfonyl fluoride), buffer C (buffer A + 0.05%DM), buffer D (2 mM NaH\textsubscript{2}PO\textsubscript{4} + 0.05%DM, 0.05%DM, 1%DM, 10%DM).
Cytoplasmic Regions of Helices 3 and 6 of Rhodopsin

pH 6.0), and buffer D + the nonapeptide (buffer D + 100 μM 9-mer) for protein elution from the chromatographic column.

Construction, Expression, and Purification of Rhodopsin Mutants—For single mutations, site-directed mutagenesis was performed using cassette-based strategy replacing the PstI/MluI fragment of pMT4 (36) for an oligonucleotide containing the single mutation E134Q, E247A, and E247Q. PCR was used for obtaining single mutants at position Thr251 and double mutants. Plasmid DNA was analyzed by restriction analysis (using EcoRI and MluI) and followed by DNA sequencing using the dye-deoxy chain-terminated method (constructed rhodopsin mutants are shown in Table 1). WT and mutant opsin genes were expressed in transiently transfected COS-1 cells as described (37). Transfected cells were harvested between 60 and 72 h after the addition of DNA and washed twice with buffer A. The cells were then incubated with 20 μM 11-cis-retinal for 5 h for reconstitution, solubilized in buffer B for 1 h, and finally centrifuged at 35,000 rpm for 35 min. All these procedures were carried out at 4 °C. Rhodopsin was purified from the supernatant using rho-1D4-Sepharose and incubated overnight at 4 °C. Sepharose was subsequently washed twice with buffer C and twice with buffer D and finally was incubated with buffer D + the nonapeptide for 30 min at 4 °C for rhodopsin elution. Rhodopsin concentration was determined by using a molar extinction coefficient value ε560 of 40,600 M⁻¹ cm⁻¹.

UV-visible (UV-Vis) Absorption Spectroscopic Assays—All measurements were made on a Cary 1E spectrophotometer (Varian, Australia), equipped with a water-jacketed cuvette holder connected to a circulating water bath. Temperature was controlled by a Peltier accessory connected to the spectrophotometer. All spectra were recorded, in the 250–650 nm range, with a bandwidth of 2 mm, a response time of 0.5 s, and a scan speed of 180 nm/min. For the photobleaching and acidification of rhodopsin, samples were bleached by using a 150-watt power source with a 495 nm cutoff filter. Dark-adapted rhodopsin samples were illuminated for 20 s to ensure complete photobleaching, and the absorption spectrum was recorded 2 min later.

Rhodopsin thermal stability in the dark was followed by monitoring the loss of Aₘₐₓ in the visible region as a function of time, at constant temperature (45 °C). Complete spectra were recorded every 5 min. Spectra were normalized and fitted to single exponential functions using SigmaPlot version 8.02 to derive the τ₁/₂ values. Hydroxylamine treatment was performed by adding hydroxylamine (pH 7.0) to the samples at a final concentration of 30 mM at 20 °C.

Gt Activation Assay—Gt activation levels were determined by incorporation of radioactive GTPγ[35]S in Gt molecules induced by WT rhodopsin or its mutants essentially as described previously (38). Briefly, the reaction mixture, containing 1 μM Gt, 20 nm rhodopsin, 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM MgCl₂, 2 mM dithiothreitol, 0.012% DM, and 3 μM GTPγ[35]S, was bleached for 30 s by using a 150-watt power source with a 495 nm cutoff filter. Samples were incubated at room temperature for 1 h, and the reactions were stopped by addition of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 10 mM EDTA. The dark activity of the proteins was determined in exactly the same way but without illumination. The unbound GTPγ[35]S was removed by microfiltration. The amount of GTPγ[35]S bound to Gt was determined by Cerenkov counting using a TRI-CARB 2100TR scintillation counter from Packard Instrument Co.

MD Simulations—Atomic coordinates of rhodopsin were retrieved from the Protein Data Bank (entry 1GZM) (18). In contrast to other previously published crystal structures (2–4), this structure contains the coordinates of all the amino acids of the intradiscal and cytosolic loops of rhodopsin. The N-terminal acetyl group and the two palmitoyl chains at the cytoplasmic end of the receptor that are covalently linked to two consecutive cysteine residues were also included in the present model. In contrast, the C-terminal region after the palmitoylated cysteines, at the end of the short helix 8, was not included. Side chains of all the amino acids were considered in the protonation state they exhibit as free amino acids in water at pH 7, with the exception of Asp[83] and Glu[122] that were treated as protonated and neutral, respectively, according to experimental evidence (39).

The process of protein embedding was performed as reported elsewhere (40). Specifically, the protein was placed in a box containing a mixture of DPPC lipids and water molecules generated and equilibrated according to the procedure described previously (41). The simulation box had an initial size of 10.3 × 8.0 × 10.2 nm³ (xyz), and organized in such a way that the first two dimensions corresponded to the bilayer plane. The area per lipid was 0.64 nm². Before protein insertion, the box contained 256 lipids and ~17,000 water molecules. After the protein was inserted in the center of the box, all water molecules with oxygen atoms closer to 0.40 nm from a non-hydrogen atom of the protein and all lipid molecules with at least one atom closer to 0.25 nm from a non-hydrogen atom of the protein were removed, resulting in a final system that contained 198 lipids and ~16,000 water molecules.

The area per lipid was 0.64 nm². Before protein insertion, the box contained 256 lipids and ~17,000 water molecules. After the protein was inserted in the center of the box, all water molecules with oxygen atoms closer to 0.40 nm from a non-hydrogen atom of the protein and all lipid molecules with at least one atom closer to 0.25 nm from a non-hydrogen atom of the protein were removed, resulting in a final system that contained 198 lipids and ~16,000 water molecules. Removal of these atoms introduces small voids between the protein and water or lipid molecules that are easily removed during the first step of the equilibration process (see below), in which a progressive adjustment of the lipid bilayer and water molecules to the protein takes place. Next, in the system with WT rhodopsin, 113 water molecules were selected randomly and replaced by 57 sodium and 56 chloride ions, providing a neutral system with a concentration of about 0.2 M of both sodium and chloride ions (fairly similar to that found in biological organisms). For the simulations of mutants, single water molecules were converted to ions when necessary, to keep the electroneutrality of the system.

All the computer simulations reported in this study were performed using a parallel version of the GROMACS 3.2 package (42, 43). The system was subjected to periodic boundary conditions in the three coordinate directions. Temperature was kept constant at 323 K (well below the gel/liquid crystalline phase transition temperature of 314 K) using separate thermostats for the protein, water, ions, and lipid molecules (44). The time constant for the thermostats was set to 0.1 ps except for water, for which a smaller value of 0.01 ps was used. The pressure in the three coordinate directions was kept at 0.1 MPa.
TABLE 1
Mutant rhodopsins constructed to study the importance of Glu<sup>134</sup>, Glu<sup>247</sup>, and Thr<sup>251</sup> on the stability and activity of rhodopsin

Single mutants were constructed using cassette mutagenesis, and double mutants were obtained by PCR.

| Single mutants | Double mutants |
|----------------|----------------|
| E134Q          | E134Q/E247A    |
| E247A          | E247A/E247Q    |
| E247Q          | E247Q/T251A    |
| T251A          | E247A/T251A    |
| T251K          | E247Q/T251K    |

results
Expression, Purification, and Spectral Characterization of Rhodopsin Mutants—The rhodopsin mutants obtained at the cytoplasmic boundaries of helices 3 and 6 (Table 1) were expressed in COS-1 cells, immunopurified with the monoclonal rho-1D4 antibody, and spectrophotometrically characterized by means of UV-Vis spectroscopy (Fig. 2). The A<sub>280</sub>/A<sub>500</sub> spectral ratios, which reflect chromophore regeneration and protein yield after purification, are shown in Table 2. All mutants studied showed no hydroxylamine sensitivity in the dark, and their photobleaching and acidification properties were very similar to those of WT rhodopsin (data not shown).

Mutations E134Q, E247A, E247Q, E134Q/E247A, and E134Q/E247Q—These mutants were expressed to an extent similar to that of WT rhodopsin according to their absorbance spectra (Fig. 2, A and C). All mutants were regenerated with 11-cis-retinal to form a chromophore with spectral features similar to those of WT (λ<sub>max</sub> at 500 nm). E247A and E247Q showed a chromophore formation similar to WT rhodopsin, whereas E134Q and E134Q/E247A showed some differences judged from the A<sub>280</sub>/A<sub>500</sub> ratios (Table 2). On the other hand, the double mutant E134Q/E247Q displayed impaired 11-cis-retinal binding (Table 2). This reduction may be interpreted as lack of accessibility of the retinal to the binding pocket as a result of the mutations, to some slight degree of structural misfolding affecting the proper conformation of the ligand-accepting receptor or to a lower stability of the mutant in detergent...
**Cytoplasmic Regions of Helices 3 and 6 of Rhodopsin**

**TABLE 3**

| Rhodopsin | $t_{1/2}$ (min) |
|-----------|----------------|
| WT        | 65             |
| E134Q     | 22             |
| E247A     | 29             |
| E247Q     | 13             |
| E134Q/E247A | 13         |
| E134Q/E247Q | 14         |

Thermal stability of WT and mutant rhodopsins

Spectra of rhodopsin (either WT or mutants) in Buffer C + 0.05% DM were recorded every 5 min at 45 °C. $A_{280 \text{ nm}}$ at different times were fitted to single exponential functions using SigmaPlot version 8.02, and the $t_{1/2}$ values were determined.

---

solution. In any case, this reduction does not reflect gross conformational perturbations and it may be assumed that the overall conformation of the mutants is similar to WT rhodopsin, as observed for the same maximum absorbance at $\lambda = 500$ nm (Fig. 2).

The positions where the mutations have been introduced (3.49 and 6.30 according to a generic numbering system for GPCR (11)) were also studied in the $\beta_2$-adrenergic receptor (30) and proposed to play a critical role in the conformational rearrangement involving helix 3 and helix 6 that is required for rhodopsin activation (12). The results obtained with the $\beta_2$-adrenergic receptor suggested that ionic interactions between 3.49, 3.50, and 6.30 may constitute a common switch governing activation of rhodopsin-like receptors. Furthermore, the features of the mutants, with regard to charge neutralization and changes in electrostatic interactions, showed a good correlation with the extent of constitutive activity observed for the mutants (30). In this study, some degree of dark activity was detected for some of the mutants studied (see "Gt Activation of Rhodopsin Mutants").

The thermal stability of these mutants, in the dark, was measured at 45 °C (Table 3). The general observed trend is that the mutants are less stable than WT. Lack of hydroxylamine reactivity indicated that the mutants present a compact structure around the Schiff base linkage, as would be expected because of the far away location of the mutations referred to the Schiff base environment (data not shown).

**Mutations T251A, T251K, E134Q/T251A, and E134Q/T251K**—We explored for the first time the functional role of the polar amino acid Thr$^{251}$ (in helix 6) in the electrostatic interaction with Glu$^{134}$ and Glu$^{247}$, in native rhodopsin structure and function, by introducing mutations at this site. The single mutants T251A and T251K were analyzed to determine the involvement of Thr$^{251}$ (6.34) in the rhodopsin activation mechanism. Protein yield after purification from COS-1 was about half that of the WT under the same conditions (Fig. 2B).

This suggests that the mutant proteins are expressed in the COS-1 cell line to a lower extent than the WT. The mutant rhodopsins show spectroscopic features of the WT concerning the $\lambda_{\text{max}}$ which is located at 500 nm, but their $A_{280}/A_{500}$ ratios are slightly higher than those of the WT (Table 2).

Double mutants E134Q/T251A, and E134Q/T251K were constructed and spectroscopically analyzed. These mutants were purified in lower amounts than WT (Fig. 2C). The $A_{280}/A_{500}$ ratios are different for both mutants as follows: in the case of E134Q/T251A, the ratio is similar to that of WT, whereas E134Q/T251K has a slightly higher ratio when compared with WT rhodopsin (Table 2).

**Mutations E247A/T251A, E247A/T251K, E247Q/T251A, and E247Q/T251K**—Double mutants replacing amino acids in the same helix 6 (at 247 and 251 positions, which are about one helical turn apart in this helix), E247A/T251A and E247A/T251K, were obtained and spectrally characterized (Fig. 2D). Both mutants were obtained at lower yields and showed impaired 11-cis-retinal binding with regard to WT rhodopsin. Similar features were also observed for mutants E247Q/T251A and E247Q/T251K (Fig. 2D). Specifically, the mutation E247Q/T251K produces the most destabilizing effect in the protein resulting in a higher $A_{280}/A_{500}$ ratio, which is larger than that of mutant E247A/T251K (Table 2). This suggests that there is steric hindrance imposed by the bulkier Gln side chain at position 247, in contrast to the case of Ala substitution in the E247A/T251K, which appears to be better tolerated.

**Gt Activation of Rhodopsin Mutants**—The spectral characterization shows that most of the mutants studied can be expressed, purified, and regenerated with 11-cis-retinal to an extent not very different from WT rhodopsin. This suggests that the overall conformation of these mutants in the dark state is not dramatically altered. Therefore, it was of great interest to analyze the functional characteristics of these mutants in terms of their ability to activate Gt.

The Gt activation experiments were carried out with detergent-solubilized and purified WT and mutant rhodopsins by means of a classical radioactive assay. Samples were analyzed both under dark and light conditions in all cases, and the data reported correspond to the final activation (after 1 h) detected in the two conditions. The Gt activation data for all the mutants studied are summarized in Table 4.

**TABLE 4**

| Rhodopsin | Gt activation (mol GTP$\gamma$S/mol Rho) |
|-----------|----------------------------------------|
|           | Dark                                  | Light-activated |
| Wild type | 0.99 ± 0.09                            | 10.37          |
| E134Q     | 1.32 ± 0.12                            | 13.17 ± 1.08   |
| E247A     | 2.02 ± 0.05                            | 13.12 ± 1.34   |
| E247Q     | 1.50 ± 0.34                            | 9.95 ± 0.96    |
| T251A     | 1.21 ± 0.25                            | 7.72 ± 0.35    |
| T251K     | 1.09 ± 0.36                            | 8.09 ± 0.36    |
| E134Q/E247Q | 0.52 ± 0.22                        | 10.37 ± 1.34   |
| E134Q/E247A | 2.04 ± 0.85                      | 13.32 ± 2.95   |
| E134Q/T251A | 5.81 ± 1.45                  | 14.03 ± 0.26   |
| E134Q/T251K | 0.91 ± 0.49                  | 23.69 ± 0.98   |
| E247A/T251A | 1.38 ± 0.33                  | 5.60 ± 0.47    |
| E247Q/T251A | 0.31 ± 0.10                  | 5.98 ± 0.15    |
| E247A/T251K | 0.62 ± 0.10                  | 2.07 ± 0.10    |
in dark conditions (53). On the other hand, the activity of the mutants in the content of COS-1 cell membranes was found to be lower than when they are purified in detergent solution (54). This may be due to an increased conformational flexibility of the mutants in the detergent-solubilized state that facilitates physical interaction with Gt in the recognition event that takes places in the activation mechanism.

In the case of the E134Q mutant, we found a small increase in dark activity as well as increased activity in light conditions (of ~30%) which was similar to that previously reported (54, 55). For the Glu247 mutations in helix 6, we found a different behavior for the E247A and E247Q. In the latter case, the light activation is not increased with regard to WT rhodopsin and in the dark appears to be only slightly above the E134Q level. It appears that mutations at Ala result in higher levels of dark activity, as will be discussed below. Mutations at Thr251 result in a somehow lower activity with regard to WT under light conditions.

**Double Mutants Involving Amino Acids of Helices 3 and 6 (Interhelical)** —In the case of the double mutants (Fig. 3B and Table 4), a combined effect can also be observed. Addition of E134Q mutation to any of the single mutants in helix 6 (in the E134Q/E247A, E134Q/E247Q, E134Q/T251A, and E134Q/T251K mutants) resulted in an increase of Gt activation in light conditions, except T251K which showed a clear decrease to about 40% of the WT activity. The E134Q/T251A double mutant shows a clear and definite synergistic effect when compared with the corresponding single mutants both in the dark (50%) and in the light (230%). This is the first time that experimental evidence is provided for the decisive involvement of Thr251 in rhodopsin helix 6 in the electrostatic network at the cytoplasmic ends of helices 3 and 6.

A synergistic effect was observed previously for mutations in the transmembrane domain like G90D/M257Y (56) or E113Q/M257Y (57). In the case of the double mutant E134Q/M257A, this was proposed to have a 50% activity in the dark. We observe a similar effect for the E134Q/T251A double mutant. However, in our case this is the first time where mutations at the cytoplasmic boundaries show such dark activity. Mutations at the homologous position of Thr251 in other GPCR were shown to be constitutively active (31, 58 – 60).

**Double Mutants Involving Only Amino Acids of Helix 6 (Intrahelical)** —We studied double mutants E247A/T251A, E247Q/T251A, and E247Q/T251K. All of these mutants showed reduced Gt activation under light conditions, particularly E247A/T251K which showed only 20% activity (Table 4).

In the case of the samples under light conditions, the possibility that different metarhodopsin II decay rates are in part responsible for the observed differences in transducin activation by the different mutants cannot be excluded.

**Molecular Modeling Studies** —The analysis of the crystal structure of rhodopsin (2–4, 17, 18) reveals that residues Glu134, Glu247, and Thr251 are part of a network of hydrogen bonds located at the cytoplasmic sides of helices 3 and 6 as shown in Fig. 1. The use of MD simulations allows a better characterization of these interactions, providing insight into the structural effects caused by the different mutations on WT rhodopsin and their effect on the activation process of the receptor.

The amino acid sequence corresponding to the cytoplasmic side of helices 3 and 6 is particularly abundant in charged residues (Table 5). Thus, it is expected that the structure is sensitive to alterations on the charge of the side chains (Table 6). Hydrogen bond interactions involving the residues on the cytoplasmic sides of helices 3 and 6, as well as on the second and third cytoplasmic loops, are listed in Table 7. A more detailed description of the molecular computations on the WT and mutants follows.

**WT and E134Q, E247A, E247Q, and T251K Single Mutants** —In native rhodopsin, the positively charged Arg135 establishes strong interactions with residues Glu134, Glu247, and Thr251 along the entire simulation either with one of the negatively charged residues of helix 3 (Glu236) or with one negatively charged and one polar residue of helix 6 (Glu247 and Thr251). The involvement of Thr251 is supported by the increased activity exhibited by the Thr251 single mutant reported in this study and the effect of an analogous residue in the β2-adrenergic receptor (30). A set of hydrogen bonds among residues at the boundary between helix 6 and the third cytoplasmic loop (involving Glu236, Ser240, Thr242, Thr243, Gln244, and Lys248) was observed. The removal of the negative charge at position 134 in mutant
TABLE 5
Sequence of bovine rhodopsin at the region of the cytoplasmic sides of helices 3 and 6
These parts of the sequence contain a large number of charged residues. The signs (*) and (·) indicate the charged and the polar residues, respectively. The lengths of the secondary motifs have been taken from Ref. 18. H3 and H6 account for helices 3 and 6, respectively; C2 and C3 for the second and the third cytoplasmic loops; Cyt and Int indicate whether the part of the sequence is located on the cytoplasmic or the intradiscal side, respectively.

| Helix | Cyt | Int |
|-------|-----|-----|
| H3    | 106 |     |
| C2    | 141 |     |
| C3    | 234 |     |
| H6    | 241 |     |

TABLE 6
The net charge at helices 3 and 6 and their average relative distances in the studied mutants compared to WT
The separations are given in nm and related to the value observed in WT.

|        | E134Q | E247Q | T251K | E134Q/E247A | E134Q/T251A | E134Q/T251K | E247Q/T251K |
|--------|-------|-------|-------|-------------|-------------|-------------|-------------|
| Net total charge |       |       |       |             |             |             |             |
| H3      | 0     | +1    | 0     | +1          | +1          | +1          | 0           |
| H6      | 0     | 0     | +1    | +1          | 0           | +1          | +2          |
| H3-H6 separation | -0.8  | -0.9  | 2.4   | -0.3        | -0.6        | 0.0         | -0.9        |

E134Q has effects on the competition to form hydrogen-bonding interactions between helices 3 and 6. Specifically, the E134Q mutation induces a remarkable preference of Arg135 for residues located in helix 6. Thus, after a short simulation time, the interaction between residues Gln134 and Arg135 is lost, whereas Arg135–Glu247 and Arg135–Thr251 interactions remain stable. Therefore, the interaction between the cytoplasmic side of helices 3 and 6 is stronger than in WT rhodopsin. Glu134 forms a hydrogen bond with His152 on helix 4, with the drop of Arg135 side chain toward helix 3 moving away from helix 3. There are also two interactions as follows: Glu239–Ser240 and Ser240–Thr242 at the boundary between the third cytoplasmic loop and helix 6, which can be observed during most part of the trajectory. These interactions are already present in the WT, although to a lower extent.

The T251K mutation provides an additional positive charge at the end of helix 6, a region with a large number of charged residues as follows: three positively charged residues (Lys245, Lys248, and Arg252) and two negatively charged ones (Glu247 and Glu249), as shown in Table 5. The presence of Lys251 forces a rearrangement of the neighboring positively charged residues, driven by electrostatic repulsion. Consequently, the Arg135–Glu247 interaction vanishes after 2.5 ns, with Glu134–Arg135 the only remaining hydrogen bond interaction involving residue Arg135. Therefore, the effect of this mutation is similar to that of E247Q, favoring the Arg135–Glu134 interaction. Lys251 interacts mainly with Glu239 and Gln137 on the third cytoplasmic loop, but also with Thr229 in helix 5. Moreover, Glu247 exhibits a hydrogen bond interaction with Gln312 at the short cytoplasmic helix 8 during a long time of the trajectory, not observed in any other simulation. As a consequence of these rearrangements induced by the new positively charged chain at position 251, a set of hydrogen bonds appears to be favored involving residues located either on helix 6 or on the third cytoplasmic loop (Glu232, Gln236, Gln237, Glu239, Ser240, and Lys248).

Nevertheless, the only hydrogen bond detected in WT rhodopsin is Glu239–Ser240, although for shorter times, as well as in the remainder of the mutants. These new interactions are a consequence of the positive charge density at the end of helix 6, suggesting that T251K can significantly alter the conformation of the cytoplasmic ends of helices 3 and 6 and the third cytoplasmic loop by modifying the hydrogen bond network between them. Because none of the mutants involving this single mutation (including the double ones) exhibited increased activation, neither in the dark nor in the light conditions, it may be regarded as a deleterious one in terms of protein function, probably by constraining the conformation of the receptor in a functionally defective state.

Double Mutants E134Q/E247A, E134Q/T251A, E134Q/T251K, and E247Q/T251K—The E134Q/E247A double mutation adds two net positive charges with regard to the WT and, more important, removes the two counter-charges of Arg135 (Glu134 and Glu247). As shown above, the hydrogen bond interactions with Arg135 are kept in E247Q and lost in the E134Q single mutant, respectively. Similarly, in the double mutant the interaction Arg135–Gln134 is too weak to be kept without the
Hydrogen bonds at the cytoplasmic sides of helices 3 and 6
Gray scale indicates percentage of trajectory present: 0–20% (white), 20–40% (light gray), 40–60% (medium gray), 60–80% (dark gray), and 80–100% (black). The limits of helices and loops are taken from Ref. 18. The analysis has been performed on the last 2 ns of each trajectory using a cutoff of 0.45 nm.

| residue | number | motif | residue | number | motif | WT | E134Q | E247Q | T251K | E134Q/E247A | E134Q/T251A | E134Q/T251K | E247Q/T251K |
|---------|--------|-------|---------|--------|-------|-----|-------|-------|-------|-------------|-------------|-------------|-------------|
| E/Q     | 134    | H3    | R       | 135    | H3    |     |       |       |       |             |             |             |             |
| E/Q     | 134    | H3    | HIS     | 152    | H4    |     |       |       |       |             |             |             |             |
| E/Q     | 134    | H3    | E/Q     | 247    | H6    |     |       |       |       |             |             |             |             |
| E/Q     | 134    | H3    | T/K/A   | 251    | H6    |     |       |       |       |             |             |             |             |
| R       | 135    | H3    | E       | 150    | H4    |     |       |       |       |             |             |             |             |
| R       | 135    | H3    | E/Q     | 247    | H6    |     |       |       |       |             |             |             |             |
| R       | 135    | H3    | T/A     | 251    | H6    |     |       |       |       |             |             |             |             |
| Y       | 136    | H3    | Q       | 225    | H5    |     |       |       |       |             |             |             |             |
| R       | 147    | C2    | E       | 239    | C3    |     |       |       |       |             |             |             |             |
| Y       | 223    | H5    | K       | 248    | H6    |     |       |       |       |             |             |             |             |
| Y       | 223    | H5    | R       | 252    | H6    |     |       |       |       |             |             |             |             |
| T       | 229    | H5    | T/K/A   | 251    | H6    |     |       |       |       |             |             |             |             |
| T       | 229    | H5    | T/K/A   | 251    | H6    |     |       |       |       |             |             |             |             |
| E       | 232    | C3    | Q       | 236    | C3    |     |       |       |       |             |             |             |             |
| Q       | 237    | C3    | K       | 248    | H6    |     |       |       |       |             |             |             |             |
| Q       | 237    | C3    | T/K/A   | 251    | H6    |     |       |       |       |             |             |             |             |
| E       | 239    | C3    | S       | 240    | C3    |     |       |       |       |             |             |             |             |
| E       | 239    | C3    | T       | 243    | H6    |     |       |       |       |             |             |             |             |
| E       | 239    | C3    | K       | 245    | H6    |     |       |       |       |             |             |             |             |
| E       | 239    | C3    | K       | 248    | H6    |     |       |       |       |             |             |             |             |
| E       | 239    | C3    | T/K/A   | 251    | H6    |     |       |       |       |             |             |             |             |
| S       | 240    | C3    | T       | 242    | H6    |     |       |       |       |             |             |             |             |
| S       | 240    | C3    | T       | 243    | H6    |     |       |       |       |             |             |             |             |
| S       | 240    | C3    | K       | 248    | H6    |     |       |       |       |             |             |             |             |
| Q       | 244    | H6    | K       | 248    | H6    |     |       |       |       |             |             |             |             |
| Q       | 244    | H6    | T/K/A   | 251    | H6    |     |       |       |       |             |             |             |             |
| E/Q     | 247    | H6    | E       | 239    | C3    |     |       |       |       |             |             |             |             |
| E/Q     | 247    | H6    | T/K/A   | 251    | H6    |     |       |       |       |             |             |             |             |
| E/Q     | 247    | H6    | Q       | 312    | H8    |     |       |       |       |             |             |             |             |
| K       | 248    | H6    | E       | 249    | H6    |     |       |       |       |             |             |             |             |
| E       | 249    | H6    | R       | 252    | H6    |     |       |       |       |             |             |             |             |
negative charge. The E247A mutation only allows the residue to interact with Arg135 through its backbone carbonyl. As a consequence of this, both interactions vanish, destroying the ionic lock associated with the D(E)RY motif and keeping only the interaction Arg135–Thr251 when compared with WT, although exhibiting shorter residence times. The removal of two negative charges forces hydrogen bond donors on the third loop to be closer to the cytoplasmic end of helix 6 to compensate for the lack of the negative charge, similarly to the effect observed when a positive charge is added in the case of T251K. Therefore, a set of interactions involving residues Glu239, Ser240, and Thr243 already observed in the WT are reinforced, specifically those involving Glu239 (Glu239–Thr243 and Glu239–Ser240), at the boundary between the third cytoplasmic loop and helix 6.

In the case of the E134Q/T251A double mutant, there are two simultaneous effects. On the one hand, as observed for the E134Q single mutant, removal of the negative charge at position 134 favors the Arg135–Glu247 interaction, and on the other hand the T251A mutation removes the hydrogen bond between Arg135 and residue 251. The present analysis shows a decrease of the fraction of trajectory where Arg135 and Glu247 are interacting (about 25%). As a result of all these rearrangements, new hydrogen bonds between Lys248 on helix 6 and both Thr229 on helix 5 and Gln247 on the third cytoplasmic loop are formed and kept during most of the trajectory analyzed. Additionally, interactions involving Ser240 already present in WT are reinforced. The existence of these new hydrogen bonds and the reinforcement of others already existing in the WT may explain the high level of dark activity observed for this mutant (see Fig. 3 and Table 4).

The E134Q/T251K double mutant implies removal of the negative charge at position 134. This causes impaired interaction with Arg135, although this interaction is still observed in about half of the snapshots analyzed. This situation is different from the other mutants containing Gln134 studied (E134Q and E134Q/T251A), where the interaction is completely lost. The reason for this specific behavior can be associated with the net +2 charge of the mutant, which requires a large number of residues with negative charge density to be closer. In some snapshots, where Gln134 does not interact with Arg135, it is involved in a hydrogen bond with His152. Obviously, the absence of Thr251 results in the loss of the interaction with Arg135. Additionally, the presence of Lys251 weakens the Glu247–Arg135 interaction, because it competes with Arg135 for Glu247, as in the other mutants containing T251K. Here, Lys251 also interacts with Glu244. Although the interactions involving Arg135 are significantly weakened or lost, when compared with the WT, a network of hydrogen bonds between residues at helix 6 and the third cytoplasmic loop, already present in the WT, is sensibly enhanced as follows: Glu239–Ser240, Glu239–Thr243, Ser240–Thr242, and Ser240–Thr243.

Finally, the E247Q/T251K double mutant involves mutations of two amino acids in the same helix. In this case, the side-chain replacements introduce a net charge of +2 at helix 6, resulting in five positively charged residues within two helix turns with only a single negatively charged side chain (see Table 5). The suppression of the negative charge at position 247 and the introduction of an additional positively charged residue at position 251 breaks the Arg135–Gln247 interaction, leaving Lys251–Gln247 as the predominant interaction. Additionally, Gln247 interacts with Glu249 on the third cytoplasmic loop. The results are somehow similar to those described for the single mutant E247Q, but the changes in the interaction patterns are larger because of the existence of an additional positive charge in the region. Arg135 and Lys251 share the same counter-ion (Glu134) with the Arg135–Glu134 interaction being pre-dominant during the trajectory analyzed. The interactions involving Glu239, Ser240, Thr242, and Thr243 at the boundary between helix 3 and the third loop become favored.

**Distance between Helices 3 and 6**—Table 6 shows the average relative distances between the cytoplasmic ends of helices 3 and 6 obtained from the simulations. Interestingly, the alterations on the hydrogen bond patterns described in the preceding section do not necessarily imply changes in the relative distances between helices. Specifically, the differences are smaller than 0.1 nm in all cases except for the T251K mutant. The results show that single mutants E134Q, E247Q, and T251K exhibit similar, shorter, or larger distances, respectively, compared with WT. Specifically, because residue 134 in helix 3 does not interact with helix 6, the removal of the negative charge of the E134Q mutation gives the smaller change. The results for E247Q clearly show that the loss of the electrostatic “lock” does not lead to a larger separation of the helices. On the contrary, here they even become slightly closer. T251K exhibits the largest separation between helices 3 and 6 because of the additional volume and/or charge at this position after the mutation. The different results observed for the double mutants, and specifically for those containing T251K, suggest that rhodopsin has mechanisms to rearrange the relative orientations of the helices. This can be done by rigid body motions of helices or helical fragments and lead to long range effects. Overall, the analysis performed here suggests that despite being important for maintaining the receptor in its inactive state, other residues, in addition to Glu134, Glu247, and Thr251, are involved in maintaining the cytoplasmic sides of helices 3 and 6 together. This is in agreement with the observation that Arg135 is not critical for receptor function, but it is important for stabilizing receptors in the inactive conformation (61).

**DISCUSSION**

Previous studies have suggested that the cytoplasmic regions of transmembrane helices 3 and 6 of the GPCR are engaged in a network of electrostatic interactions that stabilizes their inactive states. In rhodopsin this would involve Arg135 in helix 3. Thus, mutation at Arg135, as in the R135Q mutant, could affect these interactions leading to a conformation that would have some features of the activated receptor. However, mutations at this site did not result in dark activity of the mutated rhodopsins (21, 54, 62). This suggested that the network of interactions was more complex than expected and stimulated our interest for other residues, such as Thr251 in helix 6. Therefore, we have constructed and analyzed rhodopsins with mutations at other residues involved in this network (namely Glu134, Ser240, and Thr243).

---

5 A. Cordomi, E. Ramon, P. Garriga, and J. J. Pérez, unpublished data.
Glu$^{247}$, and Thr$^{251}$), but our discussion of the results takes into account the proposed role for Arg$^{135}$ in this specific set of interactions (although no mutant at this position was used in this study).

The detailed analysis of the structural and functional properties of mutants at the Glu$^{247}$ and Thr$^{251}$ positions in helix 6, and combining these with the previously studied E134Q mutant in helix 3, provides experimental evidence for the importance of the electrostatic network around the cytoplasmic boundaries of helices 3 and 6 of rhodopsin in the conformational and functional properties of the receptor. Our results support the existence of such a network and indicate, for the first time, that Thr$^{251}$ plays a key role in it. A clear synergistic effect is found for the E134Q/T251A double mutant that shows a significant level of dark activity (as well as a large increase of activity in light condition) in comparison with the corresponding individual single mutants. Previous studies have also shown synergistic effects on Gt activation in rhodopsin, but in that case the amino acids were more deeply buried into the transmembrane domain. This is also the first time where such an effect is reported for amino acids clearly at the cytoplasmic domain in rhodopsin. The dark activity of the E134Q/T251A double mutant suggests that the combination of a neutral nonpolar side chain at position 251 (like in T251A) and a charge at position 247 leads to a rhodopsin conformation that permits activation of Gt in dark conditions and stresses the critical role of amino acid interactions in this region (possibly including residues in the cytoplasmic loop as detected in the MD simulations).

The analysis of the MD simulations performed in this study provides insight into the dynamic nature of the residue interactions within the protein structure, providing a basis for the interpretation of the experimental results of the mutants presented. The analysis outlines important differences, as a result of the side chain replacements, in terms of hydrogen bond patterns between residues in the region around the cytoplasmic sides of helices 3 and 6 and in the third cytoplasmic loop. Specifically, every mutant studied exhibits a different hydrogen bond profile and distinct to that found in WT. Moreover, the profile exhibited by double mutants cannot be predicted from the effects observed for the corresponding single mutants, suggesting that the protein is extremely sensitive to subtle conformational rearrangements. The network of interactions found in WT rhodopsin around the ERY motif becomes weakened in all the mutants studied, and this seems to be partly compensated by the additional hydrogen bond involving helix 6 and the third loop.

In summary, the results presented in this study provide experimental evidence that the interaction among Glu$^{134}$, Arg$^{135}$, Glu$^{247}$, and Thr$^{251}$, between helices 3 and 6, respectively, is crucial in keeping rhodopsin in the inactive state. Arg$^{135}$-Glu$^{134}$ interaction is important so that Arg$^{135}$ adopts the necessary conformation that favors the previous interaction. A clear new finding is that the additional hydrogen bond between helices 3 and 6 with Arg$^{135}$-Thr$^{251}$ contributes to the stabilization of the inactive state. Thus, removal of this interaction in the E134Q/T251A double mutant results in high levels of activity in dark and light conditions. Spectral and functional characterization and MD simulations taken together highlight the importance of the so-called “ionic lock” at the cytoplasmic ends of helices 3 and 6 in rhodopsin, and allow the proposal of a key role for Thr$^{251}$ in this electrostatic network for the first time. Our results suggest that the mutants can affect the rhodopsin activation process as follows: (i) by alleviating the network of interactions in the region around the ERY motif, favoring rhodopsin activation, and/or (ii) by altering the conformation of the third loop, disrupting the interaction with the G-protein.

Acknowledgments—We thank Kevin Ridge for critical reading of the manuscript. We are grateful to Barcelona Supercomputer Center for the generous allocation of computer time granted to the project “Molecular Dynamics Simulations of G-protein-coupled Receptors.”

REFERENCES
1. Palczewski, K. (2006) Annu. Rev. Biochem. 75, 743–767
2. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
3. Teller, D. C., Okada, T., Behnke, C. A., and Palczewski, K. (2001) Biochemistry 40, 7761–7772
4. Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M., and Shichida, Y. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5982–5987
5. Salom, D., Lodowski, D. T., Stenkamp, R. E., Le Trong, I., Golczak, M., Jastrzebska, B., Harris, T., Ballesteros, J. A., and Palczewski, K. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 16123–16128
6. Salgado, G. F., Struts, A. V., Tanaka, K., Krane, S., Nakanishi, K., and Brown, M. F. (2006) J. Am. Chem. Soc. 128, 11067–11071
7. Ruprecht, J. J., Mielke, T., Vogel, R., Villa, C., and Scherler, G. F. (2004) EMBO J. 23, 3609–3620
8. Nakamichi, H., and Okada, T. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 12729–12734
9. Shichida, Y., and Morizumi, T. (March 1, 2006) Photochem Photobiol. 10.1562/2006-3-22-IR-854
10. Oldham, W. M., and Hamm, H. E. (2006) Q. Rev. Biophys. 39, 117–166
11. Ballesteros, J. A., and Weinstein, H. (1995) Methods Neurosci. 25, 366–428
12. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W., and Khorana, H. G. (1996) Science 276, 768–770
13. Patel, A. B., Crocker, E., Eilers, M., Hirshfeld, A., Sheves, M., and Smith, S. O. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10048–10053
14. Franke, R. R., König, B., Sakmar, T. P., Khorana, H. G., and Hofmann, K. P. (1990) Science 250, 123–125
15. Arnis, S., Fahmy, K., Hofmann, K. P., and Sakmar, T. P. (1994) J. Biol. Chem. 269, 23879–23881
16. Jansen, J. I., Mulder, W. R., De Caluwe, G. L., Vlak, J. M., and DeGrip, W. J. (1991) Biochim. Biophys. Acta 1089, 68–76
17. Kaushal, S., Ridge, K. D., and Khorana, H. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4024–4028
18. Li, J., Edwards, P. C., Burghammer, M., Villa, C., and Schertler, G. F. X. (2004) J. Mol. Biol. 343, 1409–1438
19. Okada, T., Sugihara, M., Bondar, A. N., Elstner, M., Entel, P., and Buss, V. (2004) J. Mol. Biol. 342, 571–583
20. Fahmy, K., Sakmar, T. P., and Siebert, F. (2000) Biochemistry 39, 10607–10612
21. Cohen, G. B., Yang, T., Robinson, P. R., and Oprian, D. D. (1993) Biochemistry 32, 6111–6115
22. Kim, J. M., Altenbach, C., Thurmond, R. L., Khorana, H. G., and Hubbell, W. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14273–14278
23. Scheer, A., Fanelli, F., Costa, T., De Benedetti, P. G., and Coteccia, S. (1996) EMBO J. 15, 3566–3578
24. Alewijsne, A. E., Timmerman, H., Jacobs, E. H., Smit, M. J., Roovers, E., Coteccia, S., and Leurs, R. (2000) Mol. Pharmacol. 57, 890–898
Cytoplasmic Regions of Helices 3 and 6 of Rhodopsin

25. Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 4625–4635
26. Ringkananont, U., Van Durme, J., Montanelli, L., Ugrashul, F., Yu, Y. M., Weiss, R. E., Refetoff, S., and Grasberger, H. (2006) Mol. Endocrinol. 20, 893–903
27. Arora, K. K., Cheng, Z., and Catt, K. J. (1997) Mol. Endocrinol. 11, 203–212
28. Li, J., Huang, P., Chen, C., de Riel, J. K., Weinstein, H., and Liu-Chen, L. Y. (2001) Biochemistry 40, 12039–12050
29. Rasmussen, S. G., Jensen, A. D., Liapakis, G., Ghanouni, P., Javitch, J. A., and Gether, U. (1999) Mol. Pharmacol. 56, 175–184
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. Parma, J., Duprez, L., Van Sande, J., Cochaux, P., Gervy, C., Mockel, J., Dumont, J., and Vassart, G. (1993) Nature 365, 649–651
32. Greasley, P. J., Fanelli, F., Rossier, O., Abuin, L., and Cotecchia, S. (2002) Mol. Pharmacol. 61, 1025–1032
33. Kosugi, S., Mori, T., and Shenker, A. (1998) Mol. Pharmacol. 53, 894–901
34. Huang, P., Li, J., Chen, C., Visiers, I., Weinstein, H., and Liu-Chen, L. Y. (2001) Biochemistry 40, 12039–12050
35. Shapiro, D. A., Kristiansen, K., Weiner, D. M., Kroeze, W. K., and Roth, B. L. (2002) J. Biol. Chem. 277, 11441–11449
36. Franke, R. R., Sakmar, T. P., Oprian, D. D., and Khorana, H. G. (1988) J. Biol. Chem. 263, 2119–2122
37. Oprian, D. D., Molday, R. S., Kaufman, R. J., and Khorana, H. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8874–8878
38. Senin, I. I., Bosch, L., Ramon, E., Zernii, E. Y., Manyosya, J., Philippov, P. P., and Garriga, P. (2006) Biochem. Biophys. Res. Commun. 349, 345–352
39. Fahmy, K., Jager, F., Beck, M., Zvyaga, T. A., Sakmar, T. P., and Siebert, F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10206–10210
40. Cordomi, A., Edholm, O., and Perez, J. J. (2007) J. Comp. Chem. 28, 1017–1030
41. Lindahl, E., and Edholm, O. (2000) Biophys. J. 79, 426–433
42. Berendsen, H. J. C., van der Spoel, D., and Vandenren, R. (1995) Comput. Phys. Commun. 91, 43–56
43. Lindahl, E., Hess, B., and van der Spoel, D. (2001) J. Mol. Model. 7, 306–317
44. Berendsen, H. J. C., Postma, J. P. M., DiNola, A., and Haak, J. R. (1984) J. Chem. Phys. 81, 3684–3690
45. Miyamoto, S., and Kollman, P. A. (1992) J. Comput. Chem. 13, 952–962
46. Jorgensen, W. L., Maxwell, D. S., and Tirado-Rives, J. (1996) J. Am. Chem. Soc. 118, 11225–11236
47. Berger, O., Edholm, O., and Jahnig, F. (1997) Biophys. J. 72, 2002–2013
48. Hofsass, C., Lindahl, E., and Edholm, O. (2003) Biophys. J. 84, 2192–2206
49. Wohletz, J., and Edholm, O. (2004) Biophys. J. 87, 2433–2445
50. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983) J. Chem. Phys. 79, 926–935
51. Hayashi, S., Tajkhorshid, E., and Schulten, K. (2002) Biophys. J. 83, 1281–1297
52. Saam, J., Tajkhorshid, E., Hayashi, S., and Schulten, K. (2002) Biophys. J. 83, 3097–3112
53. Han, M., Lou, J., Nakanishi, K., Sakmar, T. P., and Smith, S. O. (1997) J. Biol. Chem. 272, 23081–23085
54. Acharya, S., and Karuk, S. S. (1996) J. Biol. Chem. 271, 25406–25411
55. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8309–8313
56. Kim, J. M., Altenbach, C., Kono, M., Oprian, D. D., Hubbell, W. L., and Khorana, H. G. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12508–12513
57. Han, M., Smith, S. O., and Sakmar, T. P. (1998) Biochemistry 37, 8253–8261
58. Van Sande, J., Parma, J., Tonacchera, M., Swillens, S., Dumont, J., and Vassart, G. (1995) J. Clin. Endocrinol. Metab. 80, 2577–2585
59. Ren, Q., Kurose, H., Lefkowitz, R. J., and Cotecchia, S. (1993) J. Biol. Chem. 268, 16483–16487
60. Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 1430–1433
61. Flanagan, C. A. (2005) Mol. Pharmacol. 68, 1–3
62. Shi, W., Sports, C. D., Raman, D., Shirakawa, S., Osawa, S., and Weiss, E. R. (1998) Biochemistry 37, 4869–4874