Cooperative Conformational Changes in a G-protein-coupled Receptor Dimer, the Leukotriene B$_4$ Receptor BLT1*

Received for publication, May 4, 2004, and in revised form, September 7, 2004
Published, JBC Papers in Press, September 9, 2004, DOI 10.1074/jbc.M404941200

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We have used an isolated receptor, the leukotriene B$_4$ receptor BLT1, to analyze the mechanism of receptor activation in a G-protein-coupled receptor dimer. The isolated receptor is essentially a dimer whether the agonist is present or not, provided the detergent used stabilizes the inactive dimeric assembly. We have produced a receptor mutant where Cys$^{97}$ in the third transmembrane domain has been replaced by a serine. This mutation leads to an $\sim$100-fold decrease in the affinity for the agonist. 5-Hydroxytryptophan has then been introduced at position 234 in the C97A mutant sixth transmembrane domain. Agonist binding to the labeled receptor is associated with variations in the fluorescence properties of 5-hydroxytryptophan due to specific agonist-induced conformational changes. The C97A mutant labeled with 5-hydroxytryptophan has then been associated with a wild-type receptor in a dimeric complex that has been subsequently purified. The purified complex activates its G-protein partner in a similar manner as the wild-type homodimer. Due to the difference in the affinity for the agonist between the wild-type and mutant protomers in this dimer, we have been able to reach a state where one of the protomers, the mutant, is in its unliganded state, whereas the other, the wild type, is loaded with the agonist. We show that agonist binding to the wild-type receptor induces specific changes in the conformation of the unliganded protomer, as evidenced by the variations in the emission of the 5-hydroxytryptophan residue in the mutant receptor. These data provide a direct demonstration for agonist-induced cooperative conformational changes in a GPCR dimer.

G-protein-coupled receptors (GPCRs)$^\dagger$ are versatile biological sensors that are responsible for the majority of cellular responses to hormones and neurotransmitters as well as for the senses of sight, smell, and taste (1, 2). Signal transduction is associated with a set of changes in the tertiary structure of the receptor that are recognized by the associated G-protein (3). A growing body of evidence point to the fact that GPCRs exist as homo- or heterodimers (4–6). However, the role of dimerization in receptor function and the molecular mechanisms associated with receptor activation in the dimeric assembly are less well understood. Receptor dimerization is, in some cases, required for a correct addressing of the receptor to the membrane. This is clearly demonstrated for class C GPCRs such as the GABA$_B$ receptor (7–10) and for some class A receptors (11–13). There is also evidence that receptor dimerization and activation are intrinsically associated. In this context, an interesting mechanism to consider is trans-activation. In this mechanism, ligand binding to one of the monomers in the dimeric assembly results in activation of the other receptor within the dimer. Trans-activation is well documented for class C GPCRs, in particular for the GABA$_B$ receptor. In this case, it is clearly demonstrated that one subunit, GB1, binds the agonist, whereas the other, GB2, activates the G-protein (14–16). The situation is not that clear for the other classes of receptors. In the case of the LH/CG receptor, co-expression of a mutant receptor defective in hormone binding and another mutant defective in signal generation rescued hormone-activated cAMP production (17, 18). Similarly, Carillo et al. (19) have shown, using a series of Go fusion proteins and specific mutant of the histamine H1 and $\beta_{1}$-adrenergic receptors, that co-expression of two nonfunctional but complementary fusion constructs reconstituted agonist-mediated signaling. All of these results point to the fact that dimers of these receptors could function via trans-activation. Finally, dimerization is also likely to be required for an efficient interaction with intracellular partners such as the G-protein (20) and certainly plays a role in receptor internalization (21, 22).

We have produced the leukotriene B$_4$ receptor BLT1 as a functional protein isolated in a detergent medium (23). Binding of the agonist to the isolated receptor is associated with a series of specific conformational changes leading to the active state of the receptor (23). Isolated BLT1 therefore appears as a particularly convenient in vitro model system to analyze the relationship between dimerization and activation. By using a receptor dimer where one of the protomers displays a lower affinity for the agonist, we establish here that agonist-induced activation of one of the monomers in the dimeric assembly affects the conformation of the unliganded monomer. This clearly indicates that cooperative conformational changes occur in this GPCR dimer.

EXPERIMENTAL PROCEDURES

Materials—The antagonist molecule 5bs, (1S,3S*)-1-hydroxy-3-((3S,R,R*)-3-hydroxy-7-phenylhept-1-en-1-yl)cylohexane-1-N,N-dimethylacetamide, was synthesized as described by Poudreil et al. (24). LTB$_4$ was purchased from BIOMOL Laboratories; 5HW and hexadecyl-$\beta$-p-maltoside were from Sigma. Alexa Fluor 350 carboxylic acid succinimidyld ester and Alexa Fluor 488 carboxylic acid succinimidyld ester were from Molecular Probes, Inc. (Eugene, OR). Buffer A contained 12.5 mM sodium borate, 50 mM KCl, 2 mM LDAO, pH 7.8. Buffer B contained...
Site-directed Mutagenesis—The C97A mutation was introduced in both the wild-type receptor and the W41L, W83L, W142L, W161L mutant by PCR-mediated mutagenesis using the QuikChange multisitedirected mutagenesis kit (Stratagene) and, as a template, either the wild-type BLT1 or the BLT1W41L, W83L, W142L, W161L constructs described by Banerès et al. (23). Mutations were confirmed by nucleotide sequencing.

**R-R Purification**—We used here the wild-type BLT1 and BLT1W41L,W83L,C97A,W142L,W161L construct described previously (23). In both cases, a stop codon (TGA) was inserted by site-directed mutagenesis immediately after the sequence encoding the receptor to prevent expression of the His tag sequence at the receptor C terminus. We inserted an S tag encoding sequence (Novagen) at the 5′-end of the sequence encoding BLT1 and a hexahistidine encoding sequence at the 5′-end of the sequence encoding BLT1W41L,W83L,C97A,W142L,W161L. These two tags were inserted between the NdeI and BamHI sites in the pET21b-BLT1 vector (23). The BLT1 receptor was expressed and purified, under denaturing conditions, as previously described (23), except that 1.25% (w/v) sarcosyl was added to the denaturing buffer to increase the solubilization ratios. 5HW was incorporated in the liganded receptor better than detergents with short chain (23). The 5HW-labeled protein was purified under denaturing conditions under the same conditions as the wild-type receptor (23). The wild-type (R) and mutant (R_S) receptors were mixed in equimolecular amounts before refolding, and the protein was refolded as described in Banerès et al. (23). Unfolded proteins were discarded as described in Banerès et al. (23), and the functional receptor dimers were further purified on a 5–20% isokinetic sucrose gradient. The conformational features of the recovered protein were systematically checked by CD and shown to be similar to those previously reported (23). For R-R purification, the refolded proteins were first immobilized on an S-protein-agarose column (Novagen) previously equilibrated in buffer B. A first protein fraction (F1; see Fig. 4) eluted during washing with buffer B. The retained proteins were then eluted with buffer B containing 1 M MgCl₂. The protein fraction recovered under these conditions was used to assess the purity of the recovered BLT1 construct (21). A first protein fraction (F2; see Fig. 4) was eluted during the washing step with buffer B. Elution was then carried out with buffer B containing 100 mM imidazole to recover the last protein fraction (F3; see Fig. 4). F1, F2, and F3 were extensively dialyzed in buffer B.

**Ligand Binding Assays—**LTB₄ binding assay was performed as previously described (20). The titration data were analyzed using the PRISM software (GraphPad Inc.) by considering a set of usual models for describing the ligand-receptor interactions.

**GTP-γS Binding Assays—**[³²P]GTP-γS binding assays were performed as previously described (20). Data are presented as percentage of bound GTP-γS normalized to the binding obtained with the LTB₄-saturated R-R complex (20).

**Affinity Fluorescence Labeling—**The receptor purified in its unfolded state was extensively dialyzed in 12.5 mM sodium borate, pH 7.8, 1.25% (w/v) sarcosyl to remove urea while maintaining the receptor soluble. This pH value was determined from a series of labeling reactions carried out at different pH (ranging from 7 to 9) to define the optimal value for labeling only the protein N terminus and not the lysyl residues. The fluorescent probes were added to the protein solution (reactive reagent/protein molar ratio 10:1), and the reaction was carried out at room temperature for 5 h under constant stirring. The conjugate was then purified by gel filtration (Sephadex G-50 column (10 × 300 mm) equilibrated in 12.5 mM sodium borate, pH 7.8, 1.25% (w/v) sarcosyl). The relative efficiency of the labeling reaction was determined by measuring the absorbance of the protein at 276 nm (23) and that of the dye at its absorbance maximum (346 and 495 nm for Alexa Fluor 550 and 488, respectively). A ratio of 0.7–0.8 of fluorescent probe per receptor molecule was routinely found. The effectiveness of the N-terminal labeling was verified by proteolytic digestion of the receptor and N-terminal sequencing.

**Fluorescence Measurements—**Fluorescence emission spectra were recorded at 20 °C on an Eclipse spectrophotometer (Cary) with an excitation bandwidth of 18 nm (bandpass 2 nm) for the 5HW fluorescence measurements and 350 nm (bandpass 2 nm) for the fluorescence resonance energy transfer (FRET) experiments. For the excitation spectra, the emission was collected at 340 nm. For the emission spectra of the agonist-loaded receptor, emission was recorded 30 min after adding the ligand. Protein concentrations in the 10⁻⁸ to 10⁻³ M range were used. Buffer B was used in all measurements. Buffer contributions were subtracted under the same experimental conditions.

**RESULTS**

**BLT1 Dimerization—**We previously showed that agonist-loaded BLT1 was essentially a dimer, whereas the free receptor corresponded to a mixture of monomer and dimer (20). As we previously discussed, this could be due to agonist-induced dimerization or could translate the fact that the unliganded dimer is less stable than the agonist-loaded one so that it partly dissociates in a detergent medium. To address these different possibilities on an experimental basis, we explored the stability of the ligand-free BLT1 dimer in the presence of different detergents. The monomer/dimer ratio was defined by chemical cross-linking, as previously described (20). The results obtained for some of these detergents are given in Fig. 1. The amount of receptor dimer in the absence of agonist is clearly dependent on the detergent used. Whereas, as previously reported (20), the agonist-free receptor corresponds to a mixture of monomer and dimer in the presence of LDAO or β-d-dodecyl maltoside, the unliganded receptor is essentially dimeric when reconstituted with detergents such as hexadecyl-β-D-maltoside (Fig. 1). BLT1 was also found to be dimeric when reconstituted in proteoliposomes (not shown). This indicates that BLT1 is likely to be essentially dimeric but that the unliganded dimer is less stable than the agonist-loaded one so that it dissociates depending on the detergent used (see “Discussion”). Hexadecyl-β-D-maltoside has the same head group as the widely used β-d-dodecyl maltoside detergent but displays a longer alkyl chain (16 and 12 atoms of carbon, respectively), and this may have an effect on dimer stability. Indeed, we previously showed that detergents with long hydrophobic chains stabilized the purified receptor better than detergents with short chain (23). We used in the following work hexadecyl-β-D-maltoside that allows a maximal stabilization of the receptor dimer, even in the absence of agonist.

**BLT1 C97A Mutant—**We produced a receptor mutant where Cys⁹⁷ in the third TM domain has been substituted by an alanine. Cys⁹⁷ was identified on the basis of photoaffinity labeling experiments as being part of the receptor ligand-binding pocket and directly responsible for specific BLT1-LTB₄ interactions. Substituting Cys⁹⁷ for an alanine does not affect the structural features of the complex, as inferred from circular dichroism measurements (data not shown). However, as expected on the
basis of the photolabeling data, the C97A mutant is characterized by an ~100-fold decrease in the affinity for LTB₄ (Kₐ = 2.4 x 10⁻⁷ and 1.8 x 10⁻⁹ µM for the mutant and wild-type receptors, respectively) (Fig. 2).

We then analyzed the ability of the C97A mutant of BLT1 to be specifically activated by the agonist. We previously established that agonist binding to a receptor mutant where all of the tryptophan residues, besides Trp²³⁴ in TM6, had been replaced by leucines (W41L,W83L,W142L,W161L mutant) is associated with specific changes in the fluorescence emission spectrum of the receptor (23). These changes are probably due to a difference in the immediate environment of Trp²³⁴ between the inactive and active states of the receptor. We introduced here the C97A mutation in the W41L,W83L,W142L,W161L mutant of BLT1. We previously showed that all the Trp → Leu mutations, with the exception of W234L, resulted in no significant alteration of the Kₐ value for the agonist binding to its WT agonist (23). The W41L,W83L,C97A,W142L,W161L mutant therefore displays the same affinity for LTB₄ as the wild-type C97A mutant. We then produced this mutant with 5HW instead of tryptophan at position 234 (see above), one should expect, after refolding, a mixture of both Rₐ and Rₕ receptor conformations induced by agonist binding therefore provide a convenient method for monitoring changes in Rₐ conformation.

Finally, we analyzed the dimerization properties of Rₐ by chemical cross-linking, as described above for the wild-type receptor. No difference was observed between the mutant and wild-type receptor at the dimerization level: Rₐ, when reconstituted in hexadecyl-β-n-maltoside, is essentially in a dimeric state in the absence or presence of its agonist (not shown). Therefore, represents a receptor with the same properties than the wild-type receptor in terms of activation and dimerization but with a decreased affinity for its agonist.

**Rₐ-Rₕ Dimer—** In order to produce a receptor dimer containing a single Rₐ molecule, we selectively labeled R and Rₐ with two different purification tags, namely an S tag and a His tag. Briefly stated (see "Experimental Procedures" for details), both receptors were mixtures refolded in equimolecular amounts and then refolded. Since Rₐ displays the same structural features and dimerization properties as the wild-type receptor (see above), one should expect, after refolding, a mixture of both Rₐ-Rₐ and Rₐ-Rₕ homodimers as well as the Rₐ-Rₕ "heterodimer." The latter was purified through two successive steps involving the two different affinity tags on R and Rₐ (Fig. 4). The protein complex obtained after the second purification step on the nickel column (F3; see Fig. 4 for the definition of the fractions) displays two classes of agonist binding sites (Fig. 5). One is of high affinity, identical to that of the wild-type receptor, and the other displays an affinity close to, although slightly higher than (see "Discussion"), that of the C97A mutant. A series of cross-linking experiments were also carried out to assess the stoichiometric features of the protein complex in the F3 fraction. As expected, a single species was observed after chemical cross-linking with an electrophoretic mobility compatible with that of a receptor dimer (see inset in Fig. 5A), indicating that the major species obtained under such conditions is the dimeric assembly. All of these data indicate that the protein complex purified under such conditions is the Rₐ-Rₕ "heterodimer." The interesting feature with the Rₐ-Rₕ complex is that the difference in the affinity for LTB₄ between R and Rₐ makes it possible to reach a state where only one of the proteomes, R, is loaded with the agonist (see titration plot in Fig. 5).

![Fig. 2](image_url) **Fig. 2.** Ligand-binding and activation properties of the wild-type and mutant BLT1 receptor. A, direct binding of LTB₄ to isolated BLT1 (open circles) and BLT1W41L,W83L,C97A,W142L,W161L (closed circles). The binding ratio corresponds to the ratio of bound LTB₄ per receptor dimer. Ligand binding measurements were carried out as described under "Experimental Procedures."

![Fig. 3](image_url) **Fig. 3.** Rₐ agonist-induced activation. A, excitation spectra of the W41L,W83L,C97A,W142L,W161L mutant in the absence (profile 1) and presence (profile 2) of 5HW labeling. 5HW labeling is clearly visible by the shoulder in the excitation spectrum that makes possible a specific excitation at 315 nm. B, fluorescence emission spectra of Rₐ in the absence (profile 1) and presence (profile 2) of LTB₄, obtained by specifically exciting 5HW at 315 nm. Fluorescence measurements were carried out as described under "Experimental Procedures."
We confirmed R-R0 dimerization using FRET. For these measurements, R0 and R were labeled at their N terminus with a fluorescence donor (Alexa Fluor 350) and acceptor (Alexa Fluor 488) molecule, respectively. To label only the N terminus of the proteins, the labeling reaction was carried out in vitro at a pH value where only the N-terminal amine and not the lysyl ε-amino group is protonated and therefore able to react with the probe (see “Experimental Procedures”). We then analyzed the fluorescence transfer properties of each of the protein complexes obtained from the two successive purification steps on the S-protein-agarose and Ni²⁺-nitrilotriacetic acid columns (see Fig. 4). As shown in Fig. 6, a significant energy transfer signal was observed only for the protein complex in the second peak eluted from the Ni²⁺-nitrilotriacetic acid column (F3). This indicates that both the wild-type and the mutant receptor are associated in the dimeric complex, confirming the conclusion inferred from the ligand-binding data.

G-protein Activation by the R-R0 Dimer—We then checked whether the mutation in the R-R0 complex affected the coupling to the G-protein. For this, we analyzed the ability of the heterodimer to catalyze GDP/GTP exchange at the level of Goαs as previously reported for the R-R homodimer (20). As shown in Fig. 7, the nonhydrolyzable GTPγS analog does not significantly bind to Goαβγ when the R-R heterodimer is devoid of its agonist. In contrast, saturating the ligand-binding sites of R-R with LTβ leads to the binding of GTPγS to an amount comparable with that obtained with the wild-type homodimer (Fig. 7). As expected, no GTPγS binding occurred in the presence of the 5βa antagonist. Moreover, as previously reported in the case of the R-R homodimer, this is a specific effect, since nearly no G-protein activation was observed when Goαs was used instead of Goα (not shown). As stated above, the GTPγS binding profiles are similar for the R-R and R-R0 complexes, indicating that the R-R0 heterodimer is able to activate the G-protein in the same way as the R-R homodimer does.

Activation of R0 in the R-R0 Dimer—We then analyzed the activation of R0 in the R-R0 dimer at different LTβ concentrations. As described above, in this “heterodimer,” only the R0 protomer includes a 5HW residue. Under such conditions, it is possible to selectively follow the conformational changes of R0 in the R-R0 complex by measuring the fluorescence variations upon specifically exciting the 5HW residue at 315 nm, with no contribution of the tryptophan residues in R. We therefore carried out a parallel titration experiment by measuring both LTβ binding to R-R0 and the changes in the emission properties of 5HW in R0. As clearly shown in Fig. 8, filling the high affinity binding sites in R with LTβ is associated with a significant increase in the emission of 5HW in R0. Fitting this fluorescence variation to a single-site hyperbolic function showed EC₅₀ closely related to the affinity value determined for the high affinity sites in R from direct LTβ binding measurement. This strongly indicates that the effects observed on the emission properties of 5HW in R0 are the direct consequence of the binding of LTβ to R. It is to be noted that in this state, the R protomer is, as expected, in the active conformation (intrinsic fluorescence data with an R-R0 complex where only R contains 5HW; not shown). A subsequent change in the emission properties of 5HW, of lesser amplitude, was observed after loading the low affinity sites in R0 with LTβ, indicating that a subsequent conformational adaptation occurs after binding of the agonist to R0 in the R-R0 dimer. In contrast to what is observed...
above were specific to the agonist. All of these observations

Stimulation of GTP

Data are presented as the percentage of bound GTP

receptor activation (20, 23), it appears as a convenient

occurs in a lipid bilayer with regard to ligand binding and

receptor BLT1, to analyze the activation mechanism in a GPCR

protomer in the R

with LTB4, no change in the emission properties of 5HW was

observed upon binding of an antagonist molecule (5ba) to the

R-R0 dimer (not shown), indicating that the effects observed

above were specific to the agonist. All of these observations

clearly show that specific changes in the conformation of the R0

protomer in the R-R0 complex occur upon activation of R.

DISCUSSION

We have used here a purified receptor, the leukotriene B4

receptor BLT1, to analyze the activation mechanism in a GPCR
dimer. Since the isolated receptor is likely to recapitulate what

occurs in a lipid bilayer with regard to ligand binding and

receptor activation (20, 23), it appears as a convenient in vitro

model system to analyze the relationship between dimerization

and activation. By using a receptor dimer where one of the

protomers displays a lower affinity for the agonist, we show

agonist-induced activation of one of the monomers in the
dimeric assembly induces specific changes in the conformation

of the unliganded monomer.

The data presented in this work indicate that detergent-
solubilized BLT1 is essentially a dimer independently of the

presence of the ligand, provided we use a detergent that fully

stabilizes the dimeric assembly. In agreement with this conclu-
sion, the detergent-isolated receptor reconstituted in a lipid

environment is dimeric whatever the conditions are (not shown).
This is consistent with the view that, at least for class A GPCRs with small ligands that bind within the seven trans-
membrane helix bundle, ligand binding does not significantly

alter dimerization (25–27). Our data suggest that the dimer

composed of the receptor in its inactive state is less stable than

that in the active state, since it dissociates in vitro depending on

the detergent used. This could indicate that the active and

inactive dimers involve different protein/protein contacts, prob-
able as a consequence of the conformational changes associated

with receptor activation. However, in the absence of accurate

structural data on the BLT1-BLT1 assembly, it is difficult to

further discuss our observations on a firm molecular basis.

To monitor BLT1 activation, we have used the variations in

the emission properties of Trp234 in TM6. These changes are

specifically associated with the agonist-induced changes in re-

ceptor conformation (23). Agonist binding to the purified recep-
tor is accompanied by a significant increase in the fluorescence

emission intensity of Trp234. As previously discussed (23), this

increase could be due to a decreased polarity of the immediate

surrounding of Trp234 in the active conformation of the recep-
tor. Another explanation would be that LTB4 binding increases

the population of Trp234 conformational substates with higher

lifetimes, and this could also account for the increase in fluo-

rescence intensity (28). Whatever the exact explanation is, the

agonist-induced change in the fluorescence properties of Trp234

provides us with a probe to specifically follow the changes in

the conformation of the BLT1 receptor. To specifically follow
the activation of R0 with no contribution of the Trp residues in

R, we selectively labeled R0 with 5HW at position 234. As

stated under “Results,” the advantage of this strategy is that

5HW can be selectively excited between 310 and 320 nm in the

presence of several Trp residues. The fluorescence of the single

5HW in R0 can therefore be selectively monitored in the pres-

ence of the five Trp residues of R. Under these conditions, 5HW

appears as a site-specific probe for conformational modifica-

tions in R0.

We then produced a “heterodimer” containing a wild-type

protomer (R) and a protomer (R0) whose affinity for LTB4 is
decreased as the result of the mutation of Cys97. This residue

has been identified on the basis of photolabeling experiments

as being part of the receptor ligand-binding pocket and directly

responsible for specific BLT1-LTB4 interactions.2 This cer-
tainly explains the decrease in the affinity of the receptor for its

agonist observed for the C97A mutant. The existence of the

R-R0 complex was confirmed by FRET. Indeed, a significant

fluorescence transfer was observed when R0 and R were labeled

at their N terminus with a fluorescence donor and acceptor,

respectively. It is to be noted that a difference in FRET effi-
ciency was observed between the unliganded and agonist-

loaded complexes (not shown), although no change was ob-
served in the dimer amount, as assessed by chemical cross-

linking. This indicates that, as previously discussed (4), the

changes in the fluorescence transfer properties observed upon

agonist binding are likely to be associated with a different

relative arrangement of the protomers in the inactive and

active complexes. Such differences in the topological features

of the dimer could be related to the difference in the stability

between the unliganded and the agonist-loaded BLT1 dimer.

We also analyzed whether the R-R0 heterodimer was able to

activate the G-protein in the same way as the RR dimer. We

previously showed that, upon saturation of the BLT1 ho-
momandimer ligand-binding sites with LTB4, GDP-GTP exchange

occurs at the level of GoGDP, as assessed by GTPγS binding (20).
No difference was observed in this work between the R-R and

R-R0 dimers in the ability to activate the G-protein, indicating

that the dimeric complex with one mutated subunit at the level

of the ligand-binding site is still fully able to interact, in a

specific manner, with its G-protein partner. These data clearly

indicate that the heterodimeric species produced here displays

the same characteristics as the wild-type homodimer in terms of

coupling to the G-protein partner. It is to be noted that this

observation opens the way to the analysis of the relationship

between the different steps in the conformational adaptation of

the receptor, as described here, and the interaction with the

G-protein (work in progress).

The RR heterodimer obtained under such conditions dis-

plays two classes of agonist binding sites corresponding to the

ligand-binding sites in R (high affinity site) and R0 (low affinity

site). The difference in the relative affinities for the agonist of

the two protomers within the same dimeric complex allowed a

sequential filling of the agonist-binding sites of R-R0. It is to be

noted that a 2–3-fold increase in the affinity of R0 for LTB4 in

the R-R0 dimer was observed in comparison with what is meas-

ured for the R-R0 heterodimer. This could be due to the changes

in R0 conformation upon filling the ligand-binding sites in R

(see below). Indeed, a careful analysis of the LTB4 binding

profiles in Fig. 2 clearly indicates that some positive cooperat-
activity occurs in the binding of LTB₄ to the stabilized receptor dimer. These cooperative effects, as well as their molecular significance, were not further investigated in this work and will not be discussed here. This will require a more detailed ligand binding analysis with the isolated dimeric receptor in the absence and presence of G-proteins (work in progress).

The data presented here show that agonist-induced activation of one of the protomers in the receptor dimer induces specific changes in the conformation of the unliganded protomer, as assessed by the changes in the fluorescence properties of 5HW in the TM6 segment of the receptor. An alternative explanation would be to consider a domain-swapped model for the BLT1 dimer. In this model, the TM3 domain with the C97A mutation and the TM6-labeled domain would be located in two different protomers as a result of domain swapping. However, we have built a series of “heterodimeric” species with rescuing mutations within TM3 and TM6. No evidence compatible with a swapped geometry of the BLT1 dimer has been obtained with these species. Moreover, the same results were obtained when both the affinity-decreasing mutation and the 5HW residue were introduced in the same TM domain (not shown). All of these data indicate that the effects observed here certainly are not the consequence of domain swapping.

The question that arises is whether the conformation of Rₐ after filling the ligand binding sites in R with LTB₄ is that of the ligand-loaded active state of the receptor. The changes in the fluorescence properties of 5HW observed upon binding of the agonist to R in the R-Rₐ dimer are of the same nature as those observed upon direct binding of the agonist to the Rₐ protomer, indicating that the conformation of Rₐ in the half-loaded dimer could be close to the active one. However, it must be emphasized that the fluorescence changes are associated with very local changes in the environment of the Trp²⁹⁴ residue, so that different conformations could lead to closely related fluorescence spectra. Moreover, a subsequent change, although of minor amplitude, was observed in the 5HW fluorescence features after the filling of the Rₐ ligand-binding sites in R-Rₐ. This clearly indicates that a subsequent conformational adaptation step, associated with agonist binding to the receptor, is required to reach the fully active conformation. The fact that a given protomer does not reach its fully activated state upon binding of the agonist to the other protomer in the dimeric assembly could explain recent observations with the vasopressin receptors where the identity of the activated protomer within the heterodimer determines the fate of the internalized receptors (29).

Another aspect that has also to be considered is the kinetic one. It is important to emphasize that the experiments reported here have all been carried out under thermodynamic equilibrium conditions. Therefore, we cannot ascertain if the kinetic features of receptor activation through the binding of a single ligand to the dimer are similar to those resulting from the filling of both agonist-binding sites. This aspect of receptor activation will require a time-resolved analysis of receptor activation in the dimer (work in progress).

On these bases, the agonist half-loaded dimer could represent an intermediate conformational state between the inactive and fully activated dimeric assembly, with one of the protomers fully activated and the other one in a conformation close to the active one. If this is the case, such a cooperative conformational adaptation in the receptor dimer could facilitate the formation of a fully active GPCR dimer and/or lead to a higher diversity in the coupling to different intracellular partners that could be dependent on different conformational states of the receptor dimeric assembly.


3 J.-L. Banères, unpublished data.
situation is less clear in the case of class A receptors where the ligand binds directly to the transmembrane helix bundle. As stated above, all of the data presented here are consistent with the idea that in such a receptor, BLT1, activation of one of the protomers induces some changes in the conformation of the other one in the dimer. A possibility would be that the changes in the orientation of the TM domains induced in one protomer by the binding of the agonist lead to changes in the protein/protein interface in the dimer that would induce a change in the orientation of the TM6 domain of the other protomer. However, the exact molecular explanation for such a mechanism will require more structural information on the topological features of the BLT1-BLT1 dimer.

Finally, whether both protomers need to be in the active state to bind their ligand to activate the G-protein remains an open question. A receptor dimer with a single functional sub-unit can lead to in vivo signaling (19), indicating that activation of the receptor dimer could occur even when a single protomer is in the agonist-loaded state. As shown in this work, the conformational features of the unliganded protomer in the half-loaded receptor do not appear to be strictly identical to those in the fully active state. However, if one considers a complex composed of a single G-protein per receptor dimer (20), it could be possible that a single activated receptor selectively interacts with its Gα-protein partner, whereas the other protomer would only ensure the stability of the complex, by interacting with either a different region of Gα or with the βγ complex, as recently proposed (34, 35). If this is the case, activation of the Gα-interacting receptor protomer could be sufficient for triggering a specific ligand-induced response. However, all of these models will require more molecular data on the receptor-G-protein complex to be obtained (work in progress with the isolated receptor and purified G-proteins).

Acknowledgments—We thank J. Parello for constant constructive discussions and J.-P. Pin for a critical reading of the manuscript and highly beneficial suggestions. We thank J.-P. Girard for the gift of the antagonist 5bα.

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