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Evolutionary Trace and Molecular Dynamics Simulation of the Beta2 Adrenergic Receptor Mutation-induced Activation*

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Abstract Beta2 adrenergic receptor (β_2 AR) is one member of G protein coupled receptors (GPCRs), which is a key pharmaceutical target in the treatment of asthma. Evolutionary trace (ET) method was employed to analyze AR sequences and 44 conserved residues were identified. Then molecular dynamics (MD) simulation of the β_2 wild-type receptor, D130N active mutant and D79N inactive mutant were carried out and tried to explore the structural/dynamic features characterizing functionally different states of the receptor, by means of investigating ET identified conserved basic residues in the wild-type receptor and its two mutants. Particularly, it was found that the departing of D130 from R131 of DRY motif and approaching to K149 are highly correlated with the receptor activation, and the movement of helix 2, 4 and 6 upon receptor activation is inferred from the observation that R151 and K270 interact with other residues in the receptor active state on the basis of little change of the side chain orientations. The results might provide further insights into the activating mechanism of β_2 AR mutants, as well as the molecular bases of the diseases induced by the mutations of the receptor.

Key words adrenergic receptor, constitutive activity, evolutionary trace, G protein coupled receptors(GPCR), homology modeling

The adrenergic receptors (ARs) belong to the superfamily of G-protein-coupled receptors (GPCRs), which share a common topology, a seven-transmembrane helix bundle [1]. There are basically three subclasses of ARs, α_1, α_2, and β ARs. This paper focus on β_2-adrenergic receptor (β_2 AR), which is a key pharmaceutical target in the treatment of asthma. So far, most GPCR 3D structures are still unknown except for the bovine photoreceptor rhodopsin [2]. Fortunately, the ARs have been thoroughly studied in the past years, as a result, sufficient experimental data has been accumulated for us to theoretically investigate β_2 AR[3,4].

The β_2 AR is a challenging system because naturally occurring activating or inactivating mutations of this receptor may result in a wide variety of human diseases. Therefore, molecular models are employed to generate hypotheses regarding the functionally different mutants of the receptor, on a basis of the theory that the specific sequence of the receptor determines local structure and interactions between proximal residues. Scheer, *et al.*[5] investigated the single residue mutation of α_{1b} AR and found that mutation of Arg of the DRY motif lying in the intracellular extension of transmembrane 3 (TM3) to several other amino acids caused inhibition of stimulated signaling, while the basal signaling activity increased with the residue mutated to Lys. Furthermore, for β_2 AR, an analog of α_{1b} AR in the ARs family, R131 of the DRY motif is observed to be of great importance for the receptor activation[4]. Here, we pick two of β_2 AR mutants, D130N active mutant carrying a substitution of asparagine for the highly conserved aspartic acid of the DRY motif[4] and D79N inactive mutant which is a highly conserved residue of

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helix 2 in AR family[6], to analyze the influence of R131 on the receptor activation and the mechanism of the receptor constitutive activity (CA).

Based on the fact that rhodopsin is the only GPCR member whose 3D structure has been published, we take a combined strategy for modeling β_2 AR and its mutants, homology modeling for the transmembrane domains and ab initio modeling for the loops. Transmembrane regions are modeled using rhodopsin as template (PDB entry 1F88)[2]. Both rhodopsin and β_2 AR are members of rhodopsin-like Class A GPCRs. Biogenic amine ligands of β_2 AR are analogs of the retinal structure, with cationic ammonium groups at one end and a ring-like structure at the other. Moreover, there is ~23% sequence identity between transmembrane domains of the two receptors, and it is observed that proteins descending from the same ancestor are quite similar in structure despite the low sequence identity[7]. Additionally, we exploit structural information derived from a wide analysis of the GPCRs[8] and available data of β_2 AR[9], to challenge the model. As for the extracellular and intracellular loops, we use de novo modeling approach, based on the evidence that the loops are quite flexible in structure and there is little sequence similarity between rhodopsin and β_2 AR in loops.

In the context, we perform evolutionary trace (ET) analysis on the seven helices regions of the AR sequences, to identify the conserved amino acids, which may be potentially associated with receptor activation[10]. Then, we carry out molecular dynamics (MD) simulated mutagenesis to investigate the pattern of microscopic configurations in different functional states of β_2 AR and the concerted structural change when the receptor switches from inactive state to active state. Particularly, the roles of R131 and the other ET identified conserved basic residues are investigated, in β_2 AR wild-type, D130N active mutant and D79N inactive mutant, to provide further insights into the activating mechanism of β_2 AR mutants as well as to infer the molecular bases of diseases induced by mutations of β_2 AR.

1 Materials and methods

1.1 Evolutionary trace (ET)

The evolutionary trace (ET) method[10] discovers evolutionarily conserved residues in structurally similar but functionally divergent protein families. It is mainly based on two observations: First, proteins descending from the same ancestor are remarkably similar in structure, with backbone deviations within 2 Å even when sequence identity falls to 25%[7]. Second, the key residues under evolutionary pressure tend to maintain their functional integrity and undergo fewer mutations than functionally less important amino acids[11]. Here, we exploit ET to analyze ARs sequences. 105 adrenergic receptor sequences of α_1, α_2, β_1, β_2, β_3 AR subgroups are obtained from GPCRDB[6]. Each subgroup is aligned with CLUSTAL W[12] separately, and their helices segments are excised and then concatenated, to produce a gapless alignment of 105 sequences containing seven transmembrane helices. These residues identified by ET are very likely to be functionally important in helix rearrangement during the receptor activation[13].

The residues completely invariant in the whole ARs family are termed conserved, and β_2 AR specific residues refer to those varying only between subgroups. Afterwards, the basic residues are identified, to explore the roles of the residues in receptor mutation-induced activation.

1.2 Model building

The transmembrane (t) domains as well as the extracellular (e) and intracellular (i) loops included in the input receptor model, are reported in Figure 1. The length of the seven-helices is determined on the basis of multiple sequence alignment and available experiment data.

The β_2 AR structure model is obtained by means of the program Modeller 7v7[14], consisting of the procedure started with homology modeling on the transmembrane domains of seven helices using rhodopsin crystal structure (PDB code 1F88)[2] as template, followed by ab initio modeling on the intracellular and extracellular loops, and subsequently the whole structure refined. A disulfide bridge is allowed to be formed between C106 and C184, according to previous works. Currently, we omit modeling the amino and carboxyl terminal regions. The input structures of the β_2 AR wild-type, the D130N active mutant and the D79N inactive mutant for energy minimization are almost the same, except for the mutant positions.

The minimized structures of the wild-type β_2 AR and two mutants are inserted into a lipid bilayer constituted by 128 molecules of dipalmitoylphosphatidylcholine (DPPC), which are fully equilibrated. The overlapping lipid molecules are

deleted, leaving a gap between the receptor and lipid molecules. To preserve the electric neutrality of the system, balancing Cl^- ions are added to the bulk solvent on either side of the bilayer. These ions replaced water molecules at the most favorable electrostatic potential positions. The charges of the ionized groups are assigned assuming standard amino acid protonation state at pH 7. Histidines are considered in the neutral form. The total number of atoms in the system is ～16000.

Helix 1
v G M G I V M S L I V L A I V F G N V L V I T a i 58
i1
A K F E R L Q T V T N Y 70
Helix 2
f i T S L A C A D L V M G L A V V P F G A A H I L 95
e1
M K M W T F G N F W C 106
Helix 3
E F W T S I D V L C V T A S I E T L C V I A V D R Y V 133
i2
A I T S P F K Y Q S L L T K N K A 150
Helix 4
R V I I L M V W I V S G L T S F L P I Q M H W Y 174
e2
R A T H Q E A I N C Y A N E T C C D F F T N Q 197
Helix 5
A Y A I A S S I V S F Y V P L V I M V F V Y S R V F q e a k r q l q k 232
i3
I D K S E G R F H V Q N L S Q V E Q D G R T G H G L R R S S 262
Helix 6
k f c l k e h k a l k t L G I I M G T F T L C W L P F F I V N I V H V I 298
e3
Q D N L I R K 305
Helix 7
E V Y I L L N W I G Y V N S G F N P L I Y C r s p 330

Fig. 1 The topology of β_2 AR is reported above

The bilayer/solvent boundary is marked by the switch from upper case to lower case.

1.3 Molecular dynamics simulation

Minimizations of the system are carried out by using 20 000 steps of steepest descent, followed by conjugate gradient minimization, successive 20 ps MD runs with decreasing positional restraints force constants on the solutes (K_{posres} = 1 000, 100, 50 and 10 kJ·mol^{-1}·nm^{-2}) prior to the 10 ns production runs. Once the system is minimized, 3 ns is required to reach equilibrium owing to the large system size. Then, the last 5 ns of the whole equilibrated trajectory (3～10 ns) is extracted for analysis. The SPC water model is used. Periodic boundary conditions are applied. We use particle mesh Ewald (PME) [15] to treat the electrostatic interactions, and the cutoff distance for van der Waals and electrostatic interactions is 0.9 nm. The time step is 2 fs with the LINCS algorithm[16] to constrain bond lengths. A constant pressure of 100 kPa is used with a coupling constant of τ_p = 1.0 ps. Water, protein, lipid and Cl^- ions are coupled separately to a template bath at 300 K using a coupling constant τ_T = 0.1 ps. Coordinate sets are saved every 1.0 ps for analysis.

We choose using Gromacs forcefield for modeling. Lipid parameters are from Berger *et al* [17]. The lipid-protein interaction exploits GROMOS parameters. All simulations are performed with GROMACS v3, on a 64 CPU[18]. Analysis is performed with the programs in the GROMACS suite. Structural diagrams are prepared using VMD[19].

The internal consistency of the β_2 AR models is checked by means of PROCHECK and WHAT IF programs, as well as available experimental data.

2 Result and discussion

2.1 Evolutionary trace analysis

The seven helices regions of 105 adrenergic receptor sequences are aligned. Totally 44 invariant residues are identified in the transmembrane domain of the AR family (Table 1), most of which are lying in the cytosolic halves of the domain [13]. The conserved residues include critical GPCR motifs and residues, such as DRY in helix 3, NPXXY motif in helix 7, and D79 in helix 2. Four of the total five prolines in the seven helices regions of β_2 AR are highly conserved in the AR family except for the proline in helix 2, indicating that the proline kink motif is well maintained in AR family. The importance of the helix 3, 5, 6 and 7 is inferred from the finding that there are more conserved residues in helix 3, 5, 6 and 7 than the other helices, which is consistent with the observation that the ligand binding residues such as D113, S204, S207, F289 and N293 are lying in these four helices[20] and that these helices undergo rigid-body motions upon receptor activation[4].

Table 1 ET identified residues in the seven helices regions of the receptor

Location	Conserved residues in the AR family[1)]	β_2 AR class specific residues[2)]
H1	G50 N51 V54	S41 A57 I 58
H2	L75 A76 A78 D79	S74 L80 L84 V86
H3	D113 V114 L115 T118 S120 I121 L124 C125 D130 R131 Y132	W109 T110 S111 C116 V117 A119 A128
H4	W158 S165 P168	K149 R151 S161 T164 I169 Y174
H5	Y199 S204 S207 F208 P211 Y219	N196 Y209 M215 V219 S220 R221 V222 K227
H6	L275 G280 F282 C285 W286 P288 F289 F290	K267 K270 A271 L272 T274 I278 N293
H7	W313 G315 Y316 N318 S319 N322 P323 I325 Y326	N312 C327 S329 P330 D331 R333

1) The residues are completely invariant in the whole AR family, and those underlined residues are highly conserved in the whole rhodopsin-like Class A GPCRs. 2) The residues are invariant in β_2 AR and vary only between subgroups.

To assess the functional importance of the 44 ET identified residues, we reviewed the mutations of residues in adrenergic receptors obtained from t-GRAP mutant database[6] and found that mutations of most of the residues had functional effects, i.e., affecting ligand binding, causing constitutive activity or folding defects, affecting G protein coupling or signaling. Besides, 41 β_2 AR specific residues are identified (Table 1), which seem not so important as conserved residues by site mutagenesis data.

While the segment of β_2 AR 266 LKEHKAL 272 region lying in the extension of helix 6 is replaced with the homologous region of α_{1b} AR, LFKFSREKKAA, the chimeric receptor acquires constitutive activity (CA)[21], which may be partly attributed to the increase of basic residue percentage in the segment, from 28.6% (2/7) in β_2 AR to 36.4% (4/11) in α_{1b} AR. Therefore, to assess the importance of basic residues, totally eight basic residues, R131, K149, R151, R221, K227, K267, K270 and R333 are picked out in ET identified residues, which are highly conserved in the whole AR family or conservatively substituted between R and K based on the conservation analysis of the basic residues. The result is displayed in Figure 2.

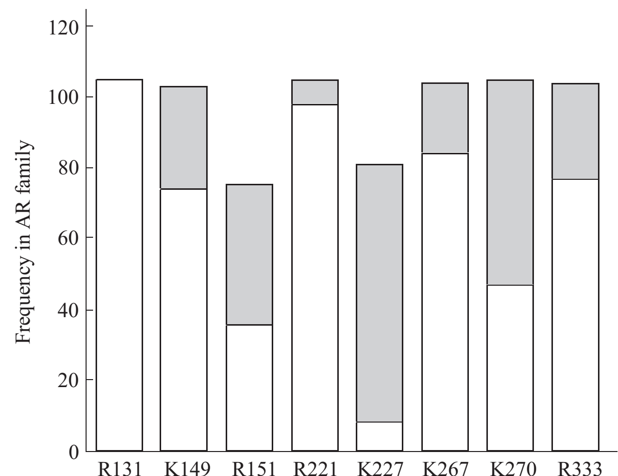

Fig. 2 The conservation of basic residues at sites analogous to β_2 AR′s R131, K149, R151, R221, K227, K267, K270 and R333 in the AR family

□: R; ■: K.

The mutation of these conserved residues may influence the structure of the receptor and make the receptor gain CA or maintained in the inactive state[6].

Consequently, we investigate the eight basic residues carefully in the study, to explore the role of R131 and the other seven conserved residues in receptor mutation-induced activation.

2.2 Structural/dynamic features of the wild-type β_2 AR and its mutants

β_2 AR mediates a wide variety of physiological process, including regulation of lipolysis, and thermogenesis by catecholamines. It is evident that a limited number of single amino acid mutations of the receptor may cause the receptor to acquire CA or lose function. So far, a lot of work has been done on different mutants of β_2 AR, which provides information for exploring the receptor structural change between different functional states[6].

To infer the structural/dynamic features which may characterize functionally different receptor states, the MD trajectories of the wild-type β_2 AR have been compared with those of the D130N active mutant and the D79N inactive mutant. In the context, we mainly focus on the conserved basic residues lying in the cytosolic extensions of helices identified by ET, R131, K149, R151, R221, K227, K267 K270 and R333, to compare the MD trajectories of the wild-type β_2 AR and the two mutants. Table 2 displays the interactions between these basic residues and some polar amino acids in the wild-type receptor, the D130N active mutant and the D79N inactive mutant. It is known that there are basically four kinds of interactions maintaining the protein structure, salt bridge, hydrogen bonding, van der Waals and hydrophobic interactions. In particular, salt bridge and hydrogen bonding interactions are more powerful. So here we mainly compute the interactions involving some of the polar amino acids in the environment of these conserved basic residues.

In Table 2, we can find that some of the structural features of the wild-type β_2 AR are also shared by the D79N inactive mutant. In this mutant, similar to the wild-type receptor, R131 of the conserved DRY motif interacts with D130 and E268, which is consistent with the results of site mutation analysis on β_2 AR[3]. The distance between the γ carbon atom of D130 and the ζ carbon atom of R131 fluctuates around an average of 7.0 Å during the last 5 ns of MD simulation, comparable to the mean value of 6.0 Å in the wild-type receptor (Figure 3, 4). In the D130N active mutant, the mutation of D130 to an asparagine residue induces local structural change in the environment of R131. The N130-R131 distance mainly oscillates around a value of 10.5 Å (Figure 3, 4). This perturbation releases the salt bridge interaction found in the wild-type and the D79N inactive mutant between D130 and R131[4]. The data suggests that stabilizing conformational constraints have been disrupted in the D130N mutant, providing indirect structural evidence for the concept that β_2 AR is maintained preferentially in an inactive configuration by a network of stabilizing, intramolecular interactions.

Table 2 Interactions of some polar amino acids with the conserved basic residues in helix cytosolic extensions in the minimized structures of β_2 AR wild-type, the D130N active mutant and the D79N inactive mutant

Residue1)	Location	Wild-type2)	D130N2)	D79N2)
R131	H3	R131- D130 R131- E268	R131- E268	R131- D130 R131- E268
K149	H4		K149- N130	
R151	H4	R151- Y70	R151- T73 R151- S74	R151- Y70
R221	H5	R221- Y132	R221- Y132	R221- Y132
K227	H5	K227- Q224	K227- Q224	K227- Q224
K267	H6	K267- E268	K267- E268	K267- E268
K270	H6	K270- E225 K270- Q229	K270- Y219	K270- E225 K270- Q229
R333	H7	R333- Q337	R333- Q337	R333- Q337

1) All the residues listed above are lying in the cytosolic extensions of the helices, R131 is highly conserved in the ARs family, and the others are conservatively substituted between R and K. 2) Only the salt bridge interactions and H-bonding interactions between the conserved basic residues and some polar amino acids are computed for the wild-type and two mutants.

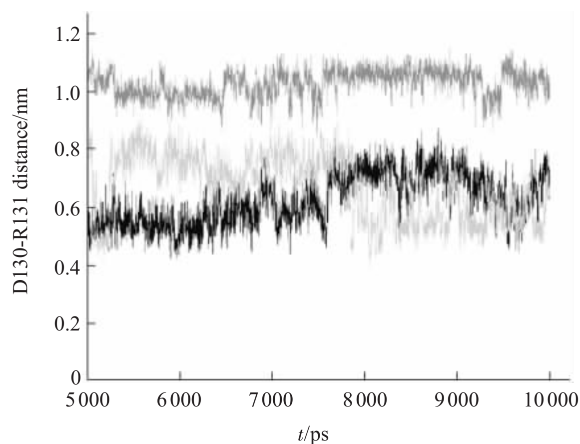

Fig. 3 Traces of the distances between the γ carbon atom of D/N130 and the ζ carbon atom of R131 measured over the 5000 structures collected during the last 5 ns of the 10 ns MD trajectories of the wild type $β_2$ AR, the active mutant D130N and the inactive mutant D79N

—: Wild-type receptor; —: D130N active mutant; —: D79N inactive mutant.

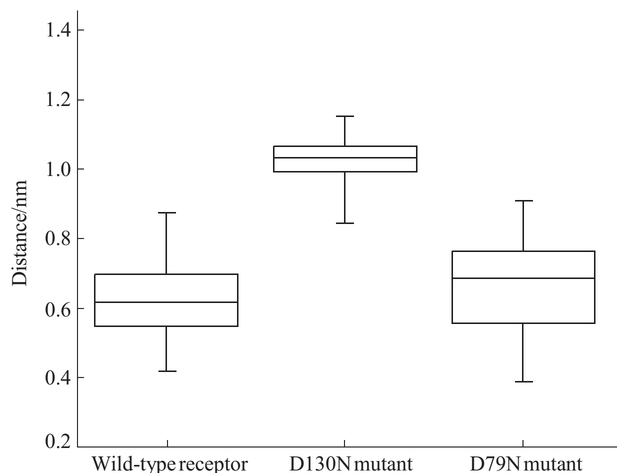

Fig. 4 Summary of simulations of the wild-type $β_2$ AR, and the two mutants

For each receptor, the maximum, upper quatile, median, lower quatile and minimum distances of the γ carbon atom of D/N130 and the ζ carbon atom of R131 are plotted.

Moreover, the side chain orientation of D130 and R131 in the receptor different functional forms is displayed in Figure 5. In D130N active mutant, N130 projects inwards and R131 points outwards to the solvent, opposite to the situations in the wild-type receptor and the D79N inactive mutant, where D130 points outwards and R131 is kept buried with respect to the cytosol. This is in agreement with the results of site mutation analysis on $α_{1b}$ AR, which describe that the shift out of Arg of DRY motif to the cytosol is characteristic of the receptor activation induced by activating mutations[5].

K149 is in the cytosolic extension of helix 4. The hydrogen-bonding interaction between K149 and N130 is formed in the D130N active mutant. The detachment of N130 from R131 and approaching to K149 may suggest the key structural change of the receptor switching from inactive state to active state. In Figure 5, K149 reorient from exposure to burial with respect to the cytosol in the D130N active mutant, as compared with the wild-type and the D79N inactive mutant. Therefore, when the receptor is inactive, K149 is exposed to the solvent, and there is a salt bridge between R131 and D130, which constrains R131 buried in the seven-helix bundle. While the receptor is activated by single amino acid mutation of D130 to D130N, K149 becomes buried to interact with N130, and the interaction of R131 and N130 is released, which release the structural constraints of R131 at the same time, and drive R131 to move outwards.

R151 is close to K149. In the D130N active mutant, the detachment of R151 from Y71 and the approaching to T73 and S74 (Table 2) may be due, at least in part, to the relative movement between helix 2 and helix 4. Furthermore, there is no obvious change

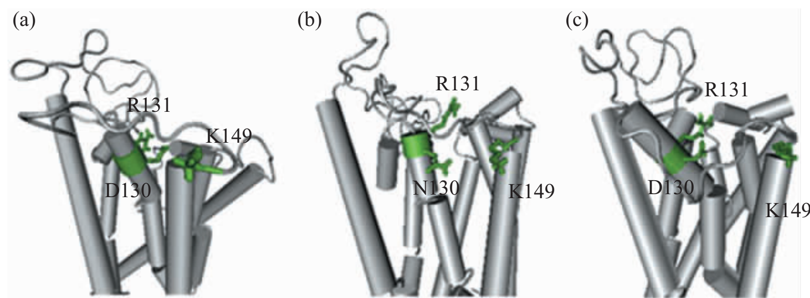

Fig. 5 Side view of the minimized structure of wild-type $β_2$ AR and its two mutants

Wild-type $β_2$ AR (a), its active mutant D130N (b) and inactive mutant D79N (c), are displayed in a direction parallel to the membrane surface, showing the side chain orientation of the residues D/N130, R131 of the DRY motif and K149. Only the cytosolic domains and the cytosolic halves of the seven helices are represented.

of the side chain orientation of R151, which strengthens the above hypothesis, that is, the gain of the partnership between R151 and T73, S74 may mostly result from the relative motion between helix 2 and helix 4 rather than the side chain reorientation of R151. The interaction of K149 and N130 in the D130N active mutant may also correlate with the helix movement, which drives K149 into close proximity to N130.

Similar to the wild-type and the D79N inactive mutant, R221, K227, K267 and R333 interact with the same polar residue in the D130N active mutant, and there is subtle change of the side chain orientation of these residues. It is difficult to define the roles of these basic residues here. Therefore, further studies need to be done to explore the roles of these residues in the receptor mutation-induced activation.

There are interactions between K270 and E225, Q229 in the wild-type and the D79N inactive mutant, while in the D130N active mutant, the interaction of K270 with Y219 is highly favored with little orientation change of the side chain of K270. It is implied that the approaching of K270 to Y219 may partly result from the vertical displacement of helix 6 upwards toward the membrane bilayer, which is believed to be one of the structural/dynamic features characterizing the active forms of the receptor[22].

Therefore, the above analysis suggests the structural/dynamic features which characterize functionally different receptors. When the receptor is inactive, D130 interacts with R131 and constrains R131 in a buried condition. While the receptor is activated by single amino acid mutation of D130 to D130N, N130 departs from R131 and approaches to K149. Moreover, R151 and K270 trace the helix movement upon the receptor activation, providing further sight into the activating mechanism of β_2 AR. Additionally, there is no much difference of the residues R221, K227, K267 and R333 in the receptor active state and the inactive state, and their roles correlated with the receptor activation need to be further explored in the following studies.

3 Conclusion

The results of this study provide us with the molecular mechanism underlying the activation of β_2 AR induced by single residue mutation from perspective of sequence and structure.

ET analysis identified 44 conserved residues in AR family, most of which are key for the receptor to function based on the evidence of site mutagenesis data from t-GRAP mutant database. The comparative analysis of R131, K149, R151, R221, K227, K267 K270 and R333 lying in the cytosolic extensions of helices allows us to explore the structural/dynamic features of wild-type receptor and two functionally different mutants (D130N active and D79N inactive). When the receptor is inactive, there is a salt bridge between D130 and R131 of the conserved DRY motif, constraining R131 in the buried condition. When the receptor is activated by single amino acid mutation of D130 to asparagine, N130 departs from R131 and approaches to K149. In consequence, R131 becomes exposed to the solvent.

The structural change is mainly characterized by the movement of helix 2, 4 and 6, inferred from the observation that R151 and K270 interact with some other residues in the receptor active state. More importantly, there is little change of the side chain orientation. However, the roles of R221, K227, K267 and R333 remain elusive, and need to be more carefully investigated later.

Despite the fact that the model presented here is reliant on many simplifications and is limited, due, at least in part, to the ab initio modeling of structures of extracellular and long intracellular loops, it provides useful suggestions for new experiments aiming at investigating the mutation-induced activation mechanism of β_2 AR, as well as of the other members of the rhodopsin family of GPCRs.

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应用进化踪迹及分子动力学模拟研究 β2 肾上腺素受体突变活性*

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摘要 β_2 肾上腺素受体 (β_2 adrenergic receptor，β_2 AR) 是 G 蛋白耦联受体 (G protein coupled receptors，GPCRs) 超家族中的一员，也是研究治疗哮喘的关键药物受体靶标. 采用进化踪迹 (evolutionary trace，ET) 方法分析肾上腺素受体家族跨膜区片段序列，识别出了 44 个保守的残基，然后将 β_2 肾上腺素受体以及受体 D130N 活性突变体、D79N 失活突变体进行分子动力学模拟，试图找出与受体不同功能状态相关的结构动力学特征. 发现受体 DRY motif 中的 D130 远离 R131 而转向 K149 残基这一结构特征与受体活性高度关联，此外，从残基相互作用的变化推断出了受体 helix 2，4 and 6 伴随着受体活化而发生的运动. 这些研究结果对进一步探索 β_2 肾上腺素受体突变体的激活机制以及所诱发疾病的分子机理提供了依据.

关键词 肾上腺素受体，组成性活性，进化踪迹方法，G 蛋白耦联受体，同源模建
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