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# Computational Mapping of the Conformational Transitions in Agonist Selective Pathways of a G-Protein Coupled Receptor

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***Abstract:*** The active state conformation of a G-protein coupled receptor (GPCR) is influenced by the chemical structure and the efficacy of the bound ligand. Insight into the active state conformation as well as the activation pathway for ligands with different efficacies is critical in designing functionally specific drugs for GPCRs. Starting from the crystal structure of the $\beta$2-adrenergic receptor, we have used coarse grain computational methods to understand the modulation of the potential energy landscape of the receptor by two full agonists, two partial agonists, and an inverse agonist. Our coarse grain method involves a systematic conformational spanning of the receptor transmembrane helices followed by an energy minimization and ligand redocking in each sampled conformation. We have derived the activation pathways for several agonists and partial agonists, using a Monte Carlo algorithm, and these are in agreement with fluorescence spectroscopy measurements. The calculated pathways for the full agonists start with an energy downhill step leading to a stable intermediate followed by a barrier crossing leading to the active state. We find that the barrier crossing involves breaking of an interhelical hydrogen bond between helix5 and helix6, and polarization of the binding site residues by water facilitates the barrier crossing. The uphill step in the partial agonist salbutamol induced activation is distinct from full agonist norepinephrine, and originates from steric hindrance with the aromatic residues on helix6. Virtual ligand screening with the salbutamol-stabilized conformation shows enrichment of noncatechol agonists over the norepinephrine-stabilized conformation. Our computational method provides an unprecedented opportunity to derive hypotheses for experiments and also understand activation mechanisms in GPCRs.

## Introduction

G-protein coupled receptors (GPCRs) are membrane bound receptors that mediate cell signal transduction governing many critical physiological functions and are therefore implicated as targets in many diseases. Biophysical experiments with the purified human $\beta$2-adrenergic receptor ($\beta$2-AR), as well as with the $\alpha_{2A}$-adrenergic receptor in intact cells, have shown that ligands with different efficacies stabilize different receptor conformations.[1−4] The ligands can remodel the energy landscape of the receptors by perturbing this conformational equilibrium in many ways, depending on the nature of the ligand, and the G-proteins that the receptor couples to, thereby conferring functional specificity.[10,6,7]

The way in which the dynamics between these various conformational states lead to functional selectivity in signaling is a challenging question, yet to be answered. Obtaining structures of these various conformations is tedious using any single experimental tool. Thus this challenging yet important question needs to be addressed using multiple tools such as fluorescence spectroscopy, NMR, and computational methods.

The human $\beta$2-AR has been crystallized with the inverse agonists carazolol and timolol bound to it.[11,12] Fluorescence spectroscopic lifetime studies show that binding of epinephrine, norepinephrine (full agonists), or salbutamol (a strong partial agonist) or dopamine (a weak partial agonist) leads to ligand specific conformational transitions as well as stabilization of structurally distinct conformational states.[1,2] Using a rhodamine tagged fluorescent $\beta$2-AR construct (fluorescent rhodamine attached to the intracellular end of TM6), Kobilka and co-workers[1,2] showed that full and partial agonists activate the receptor at very different rates. While the full agonists epinephrine and norepinephrine showed a biphasic fluorescence increase (fast increase followed by a slow increase), partial agonists

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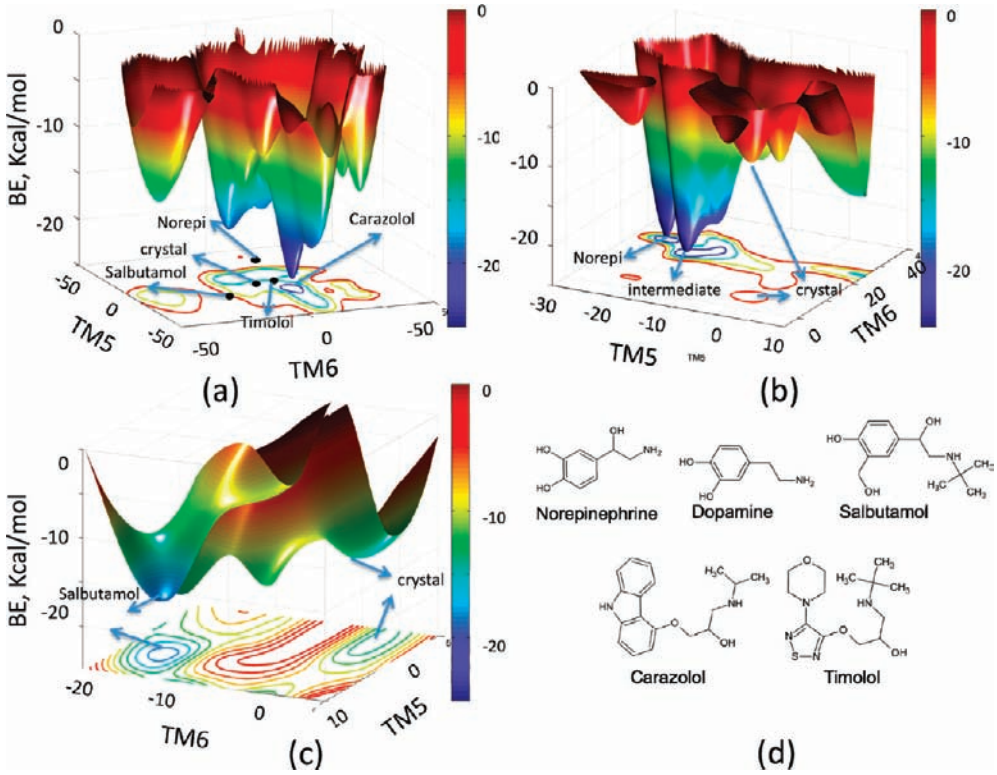


***Figure 1.*** Binding energy surfaces for $\beta$2-AR with (a) inverse agonist carazolol; (b) full agonist norepinephrine; (c) partial agonist salbutamol. The *X* and *Y* axes represent the rotations of transmembrane helices 5 (TM5) and 6 (TM6) in degrees. The various predicted ligand stabilized states are marked on the BE landscapes. Norepinephrine is abbreviated as norepi; (d) structures of $\beta$2-AR ligands.

dopamine and salbutamol showed monophasic fast and slow responses respectively. Bioluminescence Resonance Energy Transfer (BRET) studies pioneered by Bouvier and co-workers, on the kinetics of G protein coupling to $\beta$2-AR and $\beta$1-AR[5,8] in living cells, showed differential activation kinetics by full, partial, and inverse agonists.[5,8] However there is a lack of insight into the different conformations and mechanisms governing the conformational transitions. Computational methods play an important role in providing molecular level insight into these transitions that can be tested by experiments.

Starting from the crystal structure of $\beta$2-AR (pdb code: 2RH1), we have investigated the remodeling of the binding energy landscape of $\beta$2-AR by inverse agonists and agonists of varied efficacies, using a computational method, *LITiCon*, previously developed and validated for rhodopsin activation.[13,14] We have also mapped the ligand-stabilized receptor conformations for various ligands and demonstrated their utility in virtual ligand screening. To explore large-scale conformational changes, we performed conformational sampling in a reduced dimensional space of helical rotations, which allows us to identify the gross features of the active states and the sequence of helical movements leading to activation (Supporting Information section S1). Also some of the atomistic activation events such as breaking and making of interhelical hydrogen bonds (activation switches) could be captured using this procedure. We predicted the minimum energy pathways from the inactive to the active states in this reduced dimensional space and mapped the intermediates and energy barriers along the pathways, comparing them with fluorescence lifetime analysis.[1,2]

## Results

**Binding Energy Landscapes of Full, Partial, and Inverse Agonists in $\beta$2-AR.** We validated the LITiCon method[13,14] starting from the crystal structures of $\beta$2-AR with carazolol and timolol bound, to verify the reproducibility of the crystal structures as detailed in Supporting Information section S1. The carazolol and timolol bound $\beta$2-AR conformations predicted by LITiCon are within 0.5 Å rmsd in coordinates of Cα atoms (CRMSD), to the corresponding crystal structures (Table S1 of the Supporting Information) thus validating the LITiCon procedure. In the binding energy landscape, the inverse agonist carazolol stabilized receptor conformation is the global minimum and is located within the same energy well as the crystal structure. In contrast, the global minima for the full agonist norepinephrine as well as partial agonists dopamine and salbutamol are distinctly different from the crystal structure as shown in Figure 1. A two-dimensional plot of Figure 1 is shown in Figure S2 (Supporting Information).

The carazolol binding energy surface (Figure 1a) showed a broad and deep potential well centered around the crystal structure where the inverse agonist stabilized conformations were located, while the norepinephrine stabilized conformation was located in an energetically unfavorable region outside this well. The salbutamol specific state was in the periphery of the inactive well and closer to the crystal than norepinephrine state. On the contrary, the carazolol and timolol bound states were less energetically favorable in the norepinephrine landscape compared to the carazolol landscape (Figure 1b). In the salbutamol stabilized binding energy surface (Figure 1c), the

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carazolol and the salbutamol states were parts of two distinct energy wells separated by a barrier, where the salbutamol well was deeper and broader than the carazolol bound well.

**Activation Pathway for the Full Agonist Compared to a Partial Agonist.** Depending on the magnitudes of the energy barriers separating the crystal structure and the various ligand-stabilized states, different agonists will have distinct transition rates going from the inactive to the active states. In this discussion we define the crystal structure as the inactive state and the various ligand stabilized states predicted by LITiCon as the active states. In the reduced dimensional space of rotational coordinates, the activation pathway or the minimum energy pathway (MEP) for various agonists, going from inactive to the active state, has been calculated (see Methods section) using a conformation directed Monte Carlo (MC) protocol, where a slight "nudge" in energy was provided to ensure efficient convergence to the final predicted ligand-stabilized state. The MC trajectories are shown in Figure 2a. We observe that norepinephrine induced a fast conformational change to the receptor, from the inactive to a stable intermediate state (Figure 2b) as marked by a favorable energy well close to the ligand-stabilized state and separated from the latter by an energy barrier. Thus in the intermediate state, the receptor sampled many conformations inside the energy well before changing the conformation to the final state. Due to structural similarities with norepinephrine, the epinephrine landscape and the MEP were qualitatively similar to those of norepinephrine (Figure S3). In contrast, the dopamine pathway did not show any stable intermediate energy well between the inactive and the dopamine specific states (Figure 2b). The conformational changes were energetically downhill all along the reaction pathway. In the salbutamol landscape, the receptor took a tortuous pathway through many short-lived intermediates to avoid the unfavorable energy region shown by the red/yellow area in Figure 2a.

Since the density of MC sampled conformations at each intermediate state is proportional to the residence time of the receptor in that state, the number of MC steps gives an estimate of the time associated with the conformational changes. Using the sampled receptor conformations along the MEPs, the % conformational change was calculated as a function of the MC step (Figure 2b). We also examined the collective behavior of an ensemble of receptors (e.g., in a cell environment) with multiple MC simulations starting with unique random seeds. Depending on the random seed, each MC simulation followed a slightly different trajectory to reach the final state. The residence times at the stable intermediate and the time taken to cross the energy barriers differed among the different MC runs. The average over ~200 MC runs for each ligand is shown in Figure 2c. The resulting population averaged trajectories were compared to fluorescence lifetime measurements reported for $\beta$2-AR agonists.[1,2] The calculated, conformational change with time for norepinephrine and epinephrine show a biphasic response, a fast step followed by a slow step, in good agreement with experiments. Dopamine showed only a fast response, and salbutamol showed only a slow response. To our knowledge this is the first theoretical calculation of lifetime analysis of conformational changes leading to activation for GPCRs. Our calculated results are in remarkable agreement with the experimental results as shown in Figure 2d and e. Although the dynamic trends for norepinephrine and epinephrine are qualitatively similar, we note that in experiments the slow phase for epinephrine is slower compared to norepinephrine, while, in the predicted trends, the slow phase for epinephrine is faster compared to norepinephrine. The activation kinetics could be dependent on the position of the fluorescent tag in the receptor as well as the cell environment. The activation rates measured by Swaminath et al.[1,2] were in seconds, whereas the rate reported by Vilardaga et al.[8] was in the range of milliseconds. The experiments by Vilardaga et al. were conducted in a living cell environment where the activation kinetics was measured by the rate of change of fluorescence between the receptor and G-protein. Binding of G-protein to the receptor could lower the energy barrier for receptor conformational change thus increasing the activation rate. Besides, other factors such as the presence of a lipid bilayer and cholesterol as well as the pH of the system can also affect the activation rate.

From the sampled receptor conformations along the MEP, the change in binding energy along the reaction coordinate was calculated for the agonists (Figure 3). Norepinephrine shows an energetically downhill fast step leading to the stable intermediate, and the final state is separated by an energy barrier that results in the slow step (Figure 3a). The reaction profile for dopamine shows no significant energy barrier, and the entire activation process consists of a single fast step (Figure 3b). For salbutamol, there is only a single energy barrier of similar magnitude as norepinephrine, which explains the monophasic slow step involved in the salbutamol activation pathway (Figure 3c). The conformational change induced by norepinephrine leads to substantial improvement in the ligand binding energy compared to both dopamine and salbutamol. Thus norepinephrine leads to greater stability of the active state compared to dopamine or salbutamol, which is in agreement with the experimental efficacies of the three agonists in increasing cAMP accumulation in cell assays.[16,17] The efficacies of dopamine and salbutamol are 46% and 89% respectively of that of norepinephrine.

Caveats in the calculation of the activation pathway: The conformational search performed in LITiCon is in the reduced space of helical rotations, and hence it is a coarse grain approach to calculating the binding energy surface. Moreover, the helical rotations are sampled over a grid of 5° rotations, and the binding energies are interpolated for conformations between two grid points. This could lead to mislocation of the activation barrier in the pathway. Since the calculation of the binding energy does not include explicit entropy, the magnitude of the barriers provides an upper limit. More details on the solutions to overcome these pitfalls are given in the Methods section.

**Conformational Changes Induced by Full and Partial Agonists:** Computational study of the activation pathway has the distinct advantage of providing insight into the mechanism of how structurally different agonists lead to conformationally distinct receptor active states. We found that structurally analogous norepinephrine and dopamine induced similar conformational changes to the $\beta$2-AR, whereas the conformation stabilized by salbutamol was clearly distinct. The major difference between the salbutamol bound structure and that of norepinephrine and dopamine was in the movement of TM6. For norepinephrine and dopamine, the extracellular (EC) end of TM6 tilted toward the protein core, whereas, for salbutamol, the EC end of TM6 moved away from the protein core. The

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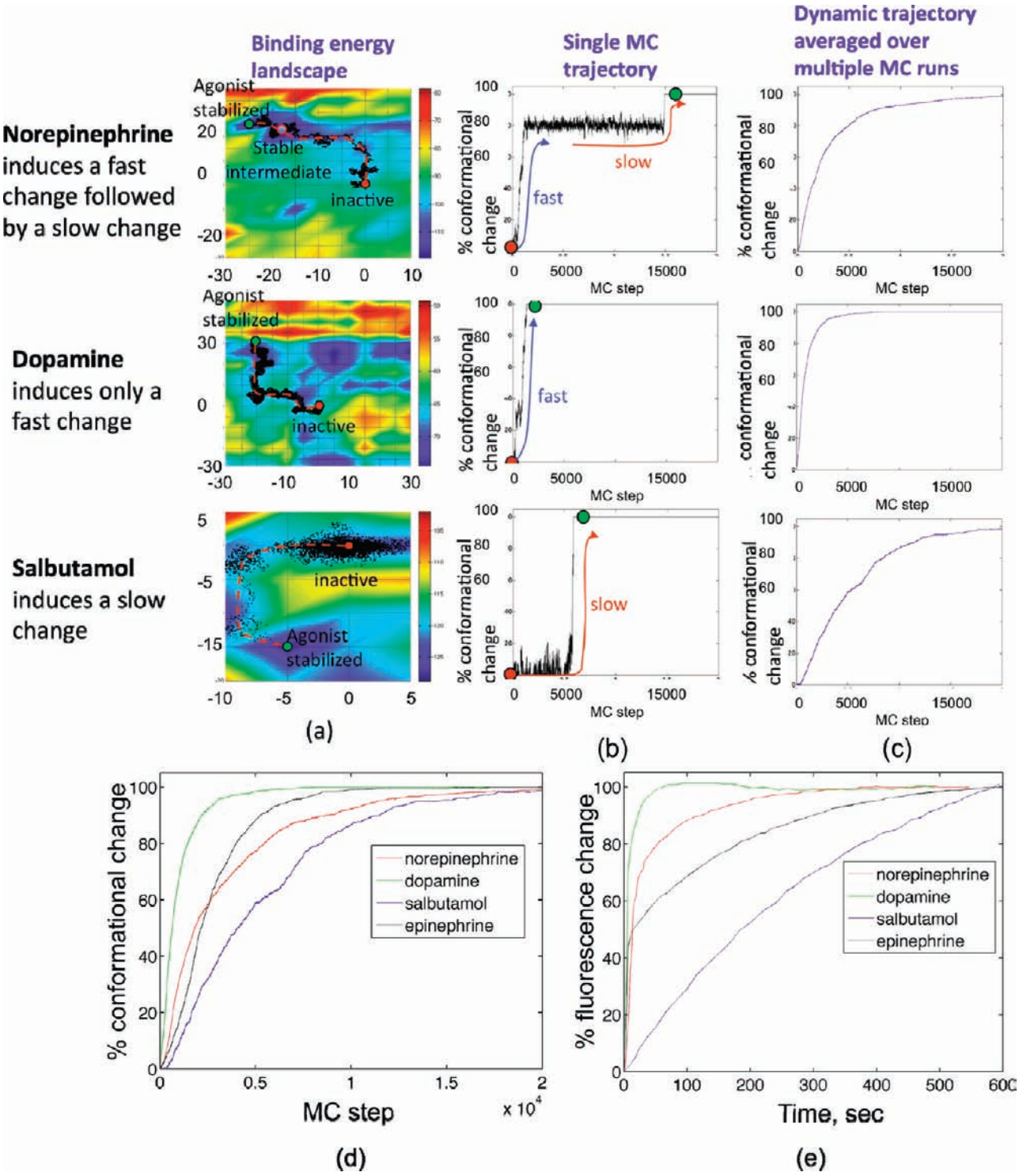


***Figure 2.*** (a) Binding energy landscapes of the agonists, norepinephrine, dopamine, and salbutamol for TM5 and TM6 movements. The energy unit is kcal/mol. The axis coordinates represent the rotation angles of the helices (degrees) relative to the inactive state. The conformations sampled by MC are shown along the calculated minimum energy pathway. The red and the green circles denote the inactive and the agonists stabilized states respectively. (b) The trajectory of sample MC simulations for the agonist bound $\beta$2-AR conformations for a single receptor−ligand complex. The blue and red arrows show the fast and the slow components of the conformational changes respectively. (c) Dynamic trajectory for the conformational changes induced by the agonists to a population of receptors, obtained by averaging the results from multiple MC runs, each starting from a different random seed. (d) Dynamic trend predicted from MC simulations. (e) Change in experimental fluorescent intensity over time for the $\beta$2-AR agonists.[1,2]

binding pocket of norepinephrine in the crystal $\beta$2-AR conformation (Figure S4b) did not make all the experimentally implicated residue contacts for norepinephrine binding[18−20] (Table S2 in the supplementary data), whereas all of these contacts were formed in the predicted norepinephrine-stabilized conformation (Figure S4c) due to the inward tilting of TM6. In comparison with norepinephrine, the conformational changes leading to the salbutamol-stabilized state showed a different set of receptor ligand contacts. As seen in Figure S4d, unlike norepinephrine, N293$^{6.55}$ (the numbering in the superscript is

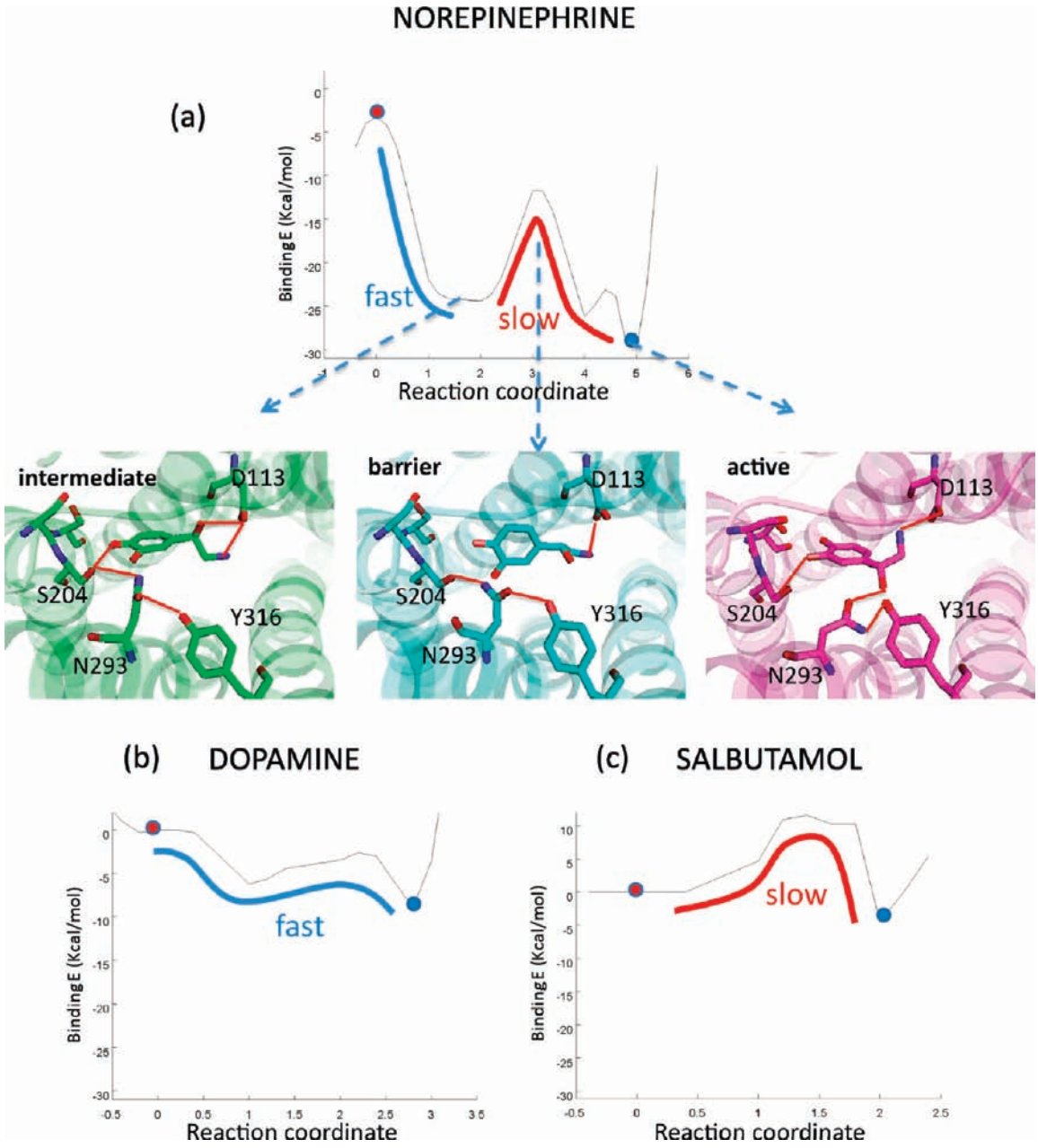


***Figure 3.*** Binding energy reaction profiles for the three agonists along the activation pathways. (a) Reaction profile for norepinephrine. The receptor−ligand conformations corresponding to the intermediates and barrier along the pathway are shown below the reaction profile; (b) reaction profile for dopamine; (c) reaction profile for salbutamol.

according to the class A GPCR residue numbering system by Ballesteros and Weinstein[21]) did not form a hydrogen bond (HB) with the $\beta$-OH group of salbutamol. This is in agreement with mutagenesis studies, where mutating N293$^{6.55}$ to Ala had a marginal effect on the binding of noncatechol agonists, clenbuterol and terbutaline.[20] Instead the $\beta$-OH group of salbutamol formed an HB with D113$^{3.32}$ on TM3, and N293$^{6.55}$ formed an HB with S204$^{5.43}$ on TM5. The meta-OH group on the catechol ring in norepinephrine formed HBs with both S203$^{5.42}$ and S204$^{5.43}$. In salbutamol, the $CH_2OH$ group hydrogen bonded with S204$^{5.43}$ (distance 3 Å), while S203$^{5.42}$ lacked a clear HB partner. Both S204$^{5.43}$ and S207$^{5.46}$ are experimentally implicated in salbutamol binding through site directed mutagenesis,[22] while the influence of S203$^{5.42}$ on salbutamol binding is not reported in mutagenesis studies. More importantly N293$^{6.55}$ formed an HB with S204$^{5.43}$ that is not seen in the norepinephrine bound $\beta$2-AR conformation. This observation is directly testable by solid state NMR or fluorescence studies.

**Virtual Ligand Screening in Agonist Stabilized $\beta$2-AR Conformations.** To assess the utility of the agonist stabilized receptor conformations in drug design, we performed virtual

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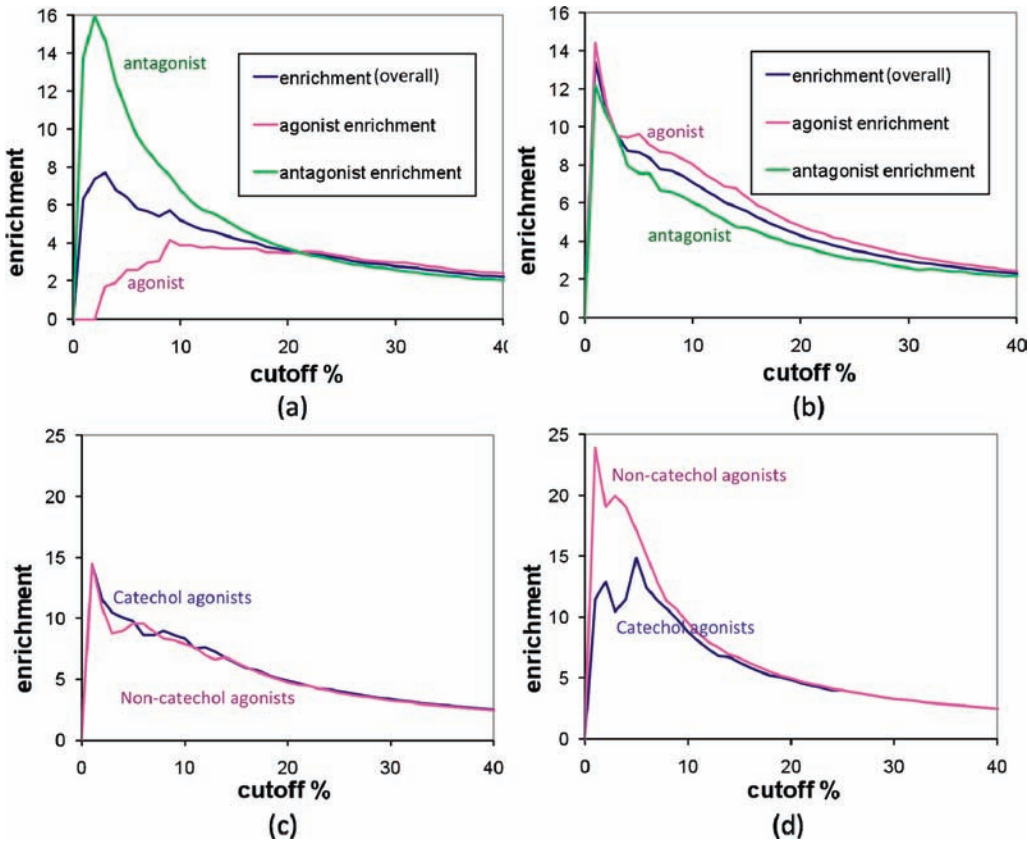


***Figure 4.*** Enrichment of adrenergic ligands in VLS of inactive and agonist stabilized $\beta$2-AR conformations; (a) antagonist and agonist enrichments in inactive crystal; (b) antagonist and agonist enrichments in predicted norepinephrine stabilized conformation; (c) enrichments for catechol and noncatechol agonists in norepinephrine stabilized conformation; (d) enrichments for catechol and noncatechol agonists in salbutamol stabilized conformation.

ligand screening (VLS) of the agonist stabilized $\beta$2-AR conformations using a test data set seeded with 143 $\beta$2-AR potent compounds (77 agonists, 66 antagonists/inverse agonists) and 10 000 random compounds from the NCI database. For convenience, both inverse agonists and antagonists will be collectively referred to as antagonists for the rest of the discussion.

We first applied the VLS procedure on the $\beta$2-AR crystal structure by Cherezov et al. As shown in Figure 4a, the highest overall enrichment of adrenergic ligands in the crystal structure was 8 (at 3% cutoff) and most of this enrichment came from the antagonists (16 at 2% cutoff), with a low enrichment of 4 (at 10% cutoff) for the agonists. This was an expected outcome since the inactive conformation is optimized for the inverse agonist carazolol. Compared to the crystal structure, the norepinephrine stabilized conformation showed a substantially higher enrichment of agonists at a relatively lower cutoff (14 at 1% cutoff) and a slightly reduced enrichment for antagonists (12 at 1% cutoff), as shown in Figure 4b. This improvement in enrichment of agonists over antagonists in the norepinephrine-stabilized conformation is of enormous value in drug design for GPCRs with the agonist-stabilized conformation showing selectivity toward agonists.

Our goal is to investigate if agonist stabilized receptor conformations may be used in identifying pathway specific agonists, in addition to being able to distinguish agonists from antagonists. We used a diverse data set consisting of 35 catechol agonists (agonists having the catechol ring similar to norepinephrine) and 42 noncatechol agonists. Although salbutamol is a noncatechol agonist, the structures of other noncatechol agonists in the data set were different from salbutamol. Structures of some of the noncatechol agonists in our data set are shown in supplementary Figure S5. The norepinephrine stabilized conformation showed similar enrichment for both catechol and noncatechol agonists (15 at 1% cutoff), while the salbutamol stabilized conformation showed significant improvement in the enrichment of noncatechol agonists (24 at 1% cutoff). Visualizing the structures of the noncatechol compounds in the top 5% of the VLS hits (Figure S5) showed that the salbutamol stabilized $\beta$2-AR model identified noncatechol compounds with diverse chemical structures, rather than the compounds that are similar to salbutamol.

## Discussion

The dynamics between inactive and active states of GPCRs and the fact that ligands of varied efficacies and chemical structure could stabilize any of these active or inactive conformations pose significant hurdles to structural studies. Therefore a confluence of computational methods and biophysical techniques are required to understand the relationship between the ligand structure and its efficacy and the receptor conformation that is stabilized.

Analysis of the ligand binding energy landscapes provides insight into the relationship between ligand structure and its efficacy. The binding energy landscape for the inverse agonist carazolol shows the ligand-stabilized state to be located in a deep energy well with high barriers to access the agonist-stabilized states (Figure 1a). On the other hand agonist bound binding energy landscapes, such as that of norepinephrine and epinephrine, are highly flexible with a broad potential well of energetically favored states, showing a favorable energy channel

connecting the carazolol bound conformational state to the norepinephrine or the epinephrine bound state (Figure 1b). This implies that the receptor is flexible and able to sample the inverse agonist states while bound to norepinephrine. On the contrary, the inverse agonist carazolol trapped the receptor in the inactive conformation, making the agonist bound states inaccessible, thus reducing the basal activity of the receptor. This reduction in basal activity could be due to the reduced affinity of the inactive receptor toward the G-protein. However if the G-protein is already precoupled to the receptor in the inactive state, the reduction in basal activity could be due to the inefficiency of the inactive $\beta$2-AR−G-protein complex for GDP/GTP exchange. Using BRET Bouvier and co-workers showed that the inverse agonist ICI-118551 stabilized $\beta$2-AR showed reduced affinity for coupling with the G-protein.[5] Thus the lowering of the basal activity by carazolol could be due to the reduced coupling of the receptor with the G-protein in the carazolol stabilized conformation.

Next we focus on the differences between the binding energy landscapes of the full agonist norepinephrine and that of the partial agonist salbutamol (Figure 1c). Here efficacy is defined with respect to cAMP accumulation via the $G_s$ pathway for which norepinephrine is a full agonist, dopamine is a weak partial agonist, and salbutamol is a strong partial agonist. The salbutamol stabilized $\beta$2-AR conformation was markedly different than that for the norepinephrine stabilized conformation, and this activated receptor state could have reduced affinity to the G-protein compared to the norepinephrine state. In addition, the change in binding energy caused by the receptor conformational changes is less for salbutamol than that of norepinephrine. These two factors could result in the lower efficacy of salbutamol in stimulating cAMP response.

Dopamine, a weak partial agonist, stabilized an active state that was very similar to that of norepinephrine. However, the binding energy difference between the inactive and active states for dopamine was less than that for norepinephrine. Thus the lower efficacy for dopamine compared to norepinephrine is mainly due to the reduced stability of the active state, while for salbutamol the lower efficacy results from both the lower stability of the active state and presumably from the reduced affinity for G-protein in the salbutamol stabilized conformation.

**Changes in Interhelical HB Induced by Ligand Binding.** We found that the final receptor states stabilized by the agonists norepinephrine and salbutamol gave rise to distinct patterns of receptor−ligand and interhelical contacts that could be correlated to the chemical structures of these agonists. In both the ligands, the driving force for TM5 movement was to improve the HB contact between S204$^{5.43}$ and catechol hydroxyl groups. In norepinephrine, this optimization required significant rearrangement of the ligand and a rotation of TM5 to bring S204$^{5.43}$ inside the binding pocket. In the salbutamol docked inactive conformation, S204$^{5.43}$ forms an HB network with N293$^{6.55}$ and the long $-CH_2OH$ group. Thus a smaller rotation of TM5 compared to norepinephrine was sufficient to strengthen the HB with S204$^{5.43}$. Norepinephrine directed the EC end of TM6 to move toward TM3 placing N293$^{6.55}$ closer to the ligand to enable formation of an HB between N293$^{6.55}$ and $\beta$-OH of norepinephrine. This inward movement of the EC end of TM6 was previously observed in experiments[23,24] and also in molecular dynamics (MD) simulation of epinephrine bound $\beta$2-AR.[25] In the norepinephrine stabilized conformation, the kink in TM6 is reduced and thus the intracellular (IC) end of TM6 moves outward from the protein core, by 2.5 Å compared to the inactive state (supplementary Figure S9). We also compared the water accessible surface area of the IC and EC interfaces of $\beta$2-AR in the inactive and norepinephrine stabilized conformations. Due to the outward movement of TM6, the surface area of the IC interface increases by 300 Å$^2$ compared to the inactive state. Also the surface area of the EC interface is reduced by 81 Å$^2$ due to the inward movement of the EC end of TM6. This expansion of the IC interface allows additional protein surface to be accessible for the binding of the G-protein. Also the shrinking of the EC interface is consistent with the tightening of the binding pocket to optimize the interactions with norepinephrine. In the salbutamol stabilized conformation, the EC end of TM6 moved outward from the protein core to strengthen the HB between S204$^{5.43}$ and N293$^{6.55}$. Thus in summary, we found that activation by norepinephrine involves a conformational switch breaking the interhelical HB between S204$^{5.43}$ and N293$^{6.55}$ while this switch stays intact during activation by salbutamol.

We calculated the probability of occurrence of the ionic lock between R131$^{3.50}$ and E268$^{6.30}$ in the carazolol and the agonist stabilized states respectively, using the side-chain reassignment program SCREAM.[30] Both carazolol and timolol bound $\beta$2-AR showed a finite population of side-chain conformations with the ionic lock. This is in agreement with the recent microsecond MD simulations of carazolol bound $\beta$2-AR, where removing the T4 lysozyme at the IC end of TM6 retained the ionic lock more than 90% of the time.[31] In contrast to this, the final active states of norepinephrine and salbutamol showed a reduction in ionic lock population relative to the inactive state (81% reduction for norepinephrine, 62% reduction for salbutamol) implying destabilization of the ionic lock as has been observed in other class A GPCRs.[4,27−29] The ionic lock between R3.50 and E6.30 is not a universal feature in all class A GPCRs, since the E3.60 is missing (alanine) in some of the receptors such as the Histamine $H_4$ receptor ($H_4$R). In these receptors, R3.50 might form a salt bridge with some other residue in place of E3.60 and thus stabilize the inactive state. Recent work by Seifert and co-workers shows that mutating A3.60 to E (to form the ionic lock) in human $H_4$R does not stabilize the inactive state. On the other hand, mutating R3.50 to alanine reduces the constitutive activity of human $H_4$R and coupling to the G-protein, thus stabilizing the inactive state.[9] This shows that R3.50 is important for receptor activation and salt bridge/HB involving R3.50 may also stabilize the active state of $H_4$R. We also measured the ionic lock population in the $\beta$2-AR conformations along the predicted activation pathways for both agonists. According to

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our calculations, norepinephrine induced ionic lock destabilization occurred early in the activation process as a result of the tilting movement of TM6, whereas for salbutamol the ionic lock disruption occurred gradually along the activation pathway. Thus our study distinguishes the conformational switches involved in activation by full agonist norepinephrine, versus partial agonist salbutamol.

It has been proposed in the literature that binding of agonists to $\beta$2-AR modulates the backbone kink in TM6 by changing the rotamer of W286$^{6.48}$, known as the rotamer toggle switch, leading to receptor activation. To test whether agonist induced conformational changes can modulate the rotamer preference of W286$^{6.48}$, we estimated the distribution of rotamer population of W286$^{6.48}$ in the inactive and agonist stabilized $\beta$2-AR conformations using the side-chain reassignment program SCREAM[30] (supplementary Figure S10). The rotamers are distinguished from each other by the $\chi$1 and $\chi$2 torsion angles of the W286 side chain. While the $\chi$1 torsion did not show any change in preference between the inactive and the agonist induced conformations, the $\chi$2 torsion angle showed a distinctly different distribution in the norepinephrine stabilized conformation compared to the inactive conformation. While the inactive conformation showed a preference for a $\chi$2 angle of 100°, the norepinephrine stabilized conformation preferred a $\chi$2 angle of −30°. These two rotamers are depicted in Figure S10. In the inactive state the W286$^{6.48}$ rotamer is oriented in the direction of the membrane normal. Binding of norepinephrine flipped the rotamer of W286$^{6.48}$ by 120° to place the nitrogen atom in the direction of TM7. Similar rotamer distributions were observed for the other agonists also. Thus we have shown that agonist binding can modulate the rotamer switch in W286$^{6.48}$.

**Comparison of the Activation Pathways.** In the biphasic behavior of norepinephrine, a fast transition leads to a stable intermediate (Figure 3a) where the EC end of TM6 tilts inward and S204$^{5.43}$ on TM5 has partially moved into the binding pocket. In the inactive state, the $\beta$-OH of norepinephrine formed an HB with D113$^{3.32}$ on TM3 and the para-OH of catechol formed an HB with S204$^{5.43}$. Also N293$^{6.55}$ on TM6 forms an HB network involving S204$^{5.43}$ and Y316$^{7.43}$ on TM7. These contacts are maintained in the intermediate state as well. The slow step of the transition from the intermediate to the active state brings S204$^{5.43}$ fully inside, and a simultaneous structural rearrangement of the ligand disrupts the HB between $\beta$-OH and D113$^{3.32}$ forming a new HB between $\beta$-OH and N293$^{6.55}$. Also the interhelical HB between S204$^{5.43}$ and N293$^{6.55}$ has to be broken to facilitate the new HB with $\beta$-OH. The transition from intermediate to final state also strengthens the HB between N293$^{6.55}$ and Y316$^{7.43}$. The disruption of the HB between D113$^{3.32}$ and $\beta$-OH and the one between S204$^{5.43}$ and N293$^{6.55}$ constitute the energy barrier that results in the slow transition from the intermediate to the final state. The analysis of the pathway for epinephrine shows a similar activation mechanism as norepinephrine. However the intermediate state for epinephrine shows a smaller rotation of TM5 from the inactive state compared to norepinephrine.

The transition state at the energy barrier (Figure 3a) shows a norepinephrine conformation where the $\beta$-OH is located halfway between D113$^{3.32}$ and N293$^{6.55}$ implying that the HB between D113$^{3.32}$ and N293$^{6.55}$ is already broken and the new HB between N293$^{6.55}$ and $\beta$-OH is not yet formed. MD simulations on the norepinephrine bound state showed that the breaking of the interhelical HB between $\beta$-OH and D113$^{3.32}$ was facilitated by water polarizing the D113$^{3.32}$ residue as shown in Figure S6d and S6e (Supporting Information). Thus water played an important role in making the slow step in norepinephrine energetically feasible. It is also possible that these energy barriers are lower in the cellular conditions.[32] During MD simulations, the HB between $\beta$-OH and D113$^{3.32}$ was intermittent with the receptor spontaneously switching between a $\beta$-OH and D113$^{3.32}$ HB state and a $\beta$-OH−N293$^{6.55}$ HB state in agreement with previous MD simulations on epinephrine bound $\beta$2-AR.[25] These observations suggest that agonist binding leads to a flexible receptor conformation where the agonist samples many different states rather than a single state. Further downstream events such as G-protein coupling may be required to shift the receptor conformation toward a predominantly active conformation. Thus coupling of the G-protein to the IC interface of the receptor could lead to an increased residence time in the $\beta$-OH−N293$^{6.55}$ HB state.

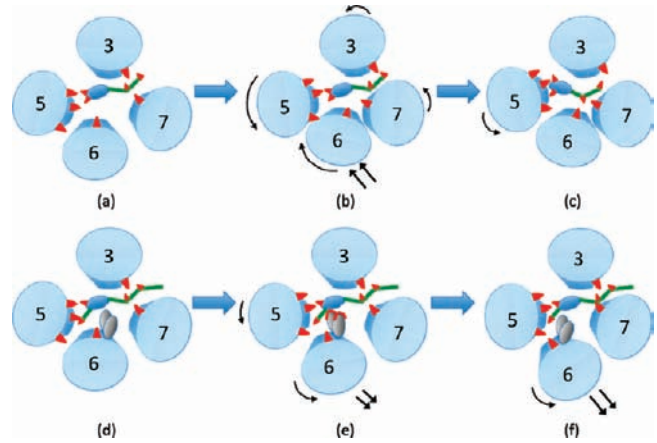


***Figure 5.*** Model for $\beta$2-AR activation induced by norepinephrine (a−c) and salbutamol (d−f). (a) Norepinephrine bound to inactive $\beta$2-AR; (b) stable intermediate where $\beta$-OH in norepinephrine faces D113$^{3.32}$. This state is reached by large rotation/tilt of TM5, TM6 and small rotations of TM3, TM7. (c) Final active state after small rotation of TM5 to bring S204$^{5.43}$ further inward. Rearrangement of ligand breaks the HB between D113$^{3.32}$ and $\beta$-OH and forms new HB with N293$^{6.55}$. This constitutes the slow step, involving water diffusion toward D113$^{3.32}$. (d) Salbutamol bound to inactive $\beta$2-AR. (e) $\beta$2-AR conformation corresponding to the energy barrier; TM6 has rotated counterclockwise and tilted slightly outward, bringing the side chains of F289$^{6.51}$ and W286$^{6.48}$ closer to the aromatic ring of salbutamol. van der Waals clash between the side chains and the ligand is marked in red. (f) Final active state, where the van der Waals clash is relieved by outward tilting of TM6.

Unlike norepinephrine, salbutamol showed only an energy barrier and no stable intermediate. The slow step in salbutamol was initiated by the rotation of TM6 in the counterclockwise direction (viewed from the extracellular end), which brought the side chains of F289$^{6.51}$ and W286$^{6.48}$ close to the aromatic ring of salbutamol causing a steric clash, which was eventually relieved by the tilting of the extracellular end of TM6 away from the protein core. Thus the energy barrier in salbutamol induced activation was due to the rearrangement of the aromatic residues on TM6 with the aromatic ring of the ligand. A proposed model for the activation of norepinephrine and salbutamol is shown in Figure 5.

For drug design, we have shown in this study that (a) a receptor model optimized for agonists can be used to obtain improved enrichment of agonists compared to antagonists in a VLS run; (b) a receptor model optimized for a functionally

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specific partial agonist shows enhanced enrichment for partial agonists compared to full agonists. The norepinephrine-stabilized conformation enriched more agonists than inverse agonists, and the reverse was true for the crystal structure. The reason for the increased enrichment of catecholamine agonists in the norepinephrine stabilized conformation was the optimized placement of residue contacts required for agonist recognition, especially the three serine residues on TM5. Besides being able to distinguish agonists from antagonists, the predicted ligand stabilized models have been successful in distinguishing the catecholamine agonists (agonists containing the catechol group) from the noncatechol ones. The salbutamol stabilized model showed a significantly higher enrichment for noncatechol agonists compared to the norepinephrine stabilized model. We also identified several new hits that were not obtained by the norepinephrine stabilized state. These noncatechol compounds usually possess aromatic groups that are bulkier than the catechol group in full agonists and, thus, need a bigger binding pocket to bind to the receptor compared to the catechol compounds. In the salbutamol stabilized $\beta$2-AR conformation, the outward movement of TM6 opens up the binding pocket thus facilitating the binding of the noncatechol compounds. Since the structures of these compounds (Supporting Information Figure S5) are very different from that of salbutamol, the enrichment of noncatechol agonists obtained from the salbutamol stabilized conformation was not equivalent to a shape based filtering of compounds similar to salbutamol. Thus we have shown that using ligand specific receptor conformations are useful in virtual ligand screening for functional specific drugs.

## Methods

**Ligand Induced Conformational Changes in the Transmembrane Regions of GPCRs (LITiCon).** The LITiCon computational method involves systematic spanning of the receptor conformations involving the helical rotations as a response to the ligand induced conformational changes in GPCRs (discussed in detail in refs 13 and 14). In previous publications,[13,14] movement of the ligand in response to receptor movement was optimized by minimizing the potential energy of the ligand to the nearest minima for every step of LITiCon. Complete flexibility of the ligand is essential for agonist docking, and therefore we refined the LITiCon procedure by allowing redocking the ligand at every step. This was found necessary for calculating accurate binding energy profiles for full agonists such as epinephrine and norepinephrine, which underwent substantial structural rearrangement in going from inactive to active state. We identified that TM helices 3, 5, 6, and 7 are in direct contact with the $\beta$2-AR ligands and performed simultaneous rotations of TM helices 3, 5, 6, and 7 in 5° increments relative to the initial state. The details of the level of optimization used for each receptor conformation spanned are given in the Supporting Information. The local minima in the resulting energy landscapes were identified, clustered, and sorted by total number of interhelical HBs and ligand−receptor HBs and then by binding energy. The final ligand stabilized receptor structural model was selected based on low binding energy and high number of HBs. The LITiCon procedure calculates only the enthalpic component of the binding free energy. In reality, binding of a ligand to the receptor displaces water from the binding cavity increasing the entropy of the released water, whereas the entropy of the bound ligand is reduced. Receptor conformational changes also lead to changes in side-chain and backbone entropy, which are not considered in LITiCon but could be important in determining the ligand optimized state. However an implicit estimate of the entropy from the temperature factor has been included as described in the next section.

We study the influence of explicit membrane and water on the receptor−ligand complexes obtained by helical rotational optimization, by equilibrating the ligand stabilized receptor structure in explicit lipid bilayer and water using MD simulations. Besides helical rotations, ligand induced conformational changes also include tilts and kink modulations in the TM helices. Thus the objective of the MD step is to optimize the tilts and kinks in the helices. Details of the MD simulations are given in the Supporting Information.

**Monte Carlo Sampling of Activation Pathway.** We performed Monte Carlo (MC) sampling in the binding energy space to estimate the most probable pathway from the inactive to the active state, where the acceptance of each MC move was slightly biased toward the final active state. Since the binding energy landscapes were very rugged, this step ensured efficient convergence to the final state by facilitating barrier crossing, while closely following the lowest energy pathway. The energy function used in MC was defined as $E_{MC}$ = BE (binding energy) − NF (nudge factor), where NF = $w \times$ (% conformational change toward active state). Adding NF to the energy function favored moves that take the receptor toward the active state and partially suppressed those moves that take the receptor away from the active state. This bias is similar to the steered MD protocol, where a constant force is applied in a particular direction to assist the system to reach the desired configuration. Steered MD is frequently used in mapping pathways for diffusion of ligands into the binding pocket.[33] Here we have designed a modified MC protocol that assists in the convergence of the system to the desired state similar to the steered MD procedure. The weight of NF, *w*, was selected such that the simulation did not diverge away from the desired pathway, while at the same time sampling the low energy conformations in between the start and end states. A value of 1 for *w* was found to be optimum and was kept constant for all agonists studied. The percentage conformational change was defined as

$$\%\ \text{Conformational change} = \frac{\text{Conformational distance between current conformation and inactive state}}{\text{Conformational distance between active and inactive states}} \times 100 \quad (1)$$

where conformational distance was measured using the conformational coordinates of the energy landscape (rotations of the TM helices in degrees relative to the crystal). Multiple MC runs were performed for each ligand, starting with different random seeds that diversify the pathway from the starting inactive conformation. The average conformational change for a given MC step, *t*, which is representative of a population of activated receptors, was calculated as the mean of the conformational changes observed at step *t* in the individual MC runs. For example, if there are *N* MC runs and for run *i* the % conformational change at step *t* is $C_i$, where $i = 1...N$, then the average conformational change at step *t* is given by $C_{avg} = (1/N)\sum_{i=1}^{N} C_i$. Since the binding energy landscape is rugged, the inactive, intermediate, and active states are parts of wide energy wells and are comprised of many degenerate states as shown in Figure 6a. Thus instead of a single MEP, the activation pathway is comprised of many closely related MEPs, all of which follow the blue low energy basin (Figure 6a) connecting the inactive to the active state. Using multiple Monte Carlo runs, we sampled these MEPs as shown in Figure 6b. Density of sampled conformations along the sampled pathways is plotted on the binding energy landscape. The red dotted line represents the boundary of the low energy basin within which the sampled pathways are located. The sampled pathways in Figure 6b closely follow the low energy basin in Figure 6a thus validating our approach.

Pitfalls of the LITiCon method: Since the helical rotations are sampled in increments of 5°, the energies between two consecutive points on the grid are interpolated. Therefore the location and the

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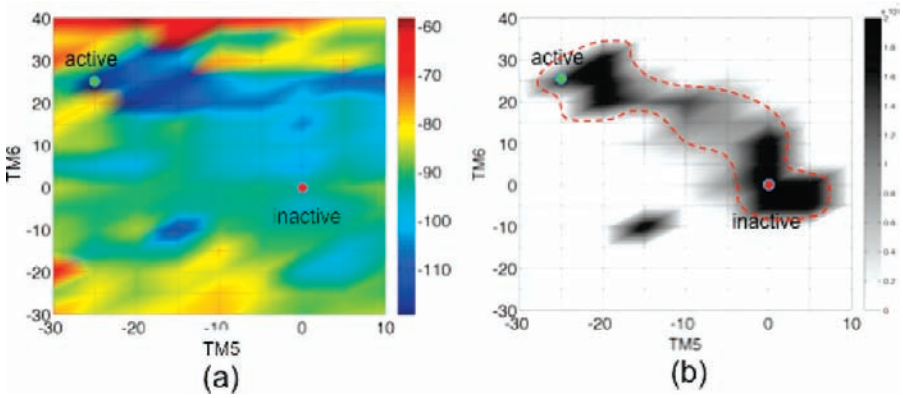


***Figure 6.*** (a) 2D representation of binding energy landscape for norepinephrine bound to $\beta$2-AR. The blue regions are favorable energy wells; red/yellow regions represent the barriers. (b) Density of sampled points along the activation pathways for 200 MC runs. The region bound by the red line represents the zone which includes the majority of the sampled pathways. This region coincides with the low energy well in image a.

magnitude of the activation barriers in the calculated pathway could be misled by these interpolated energies. To overcome this deficiency, we will perform the rotations over a smaller grid angle of 2° to ensure a smoother binding energy surface calculated using LITiCon. We are also pursuing performing MD simulations starting from various conformations along the MC derived pathway to ensure that the MC mapped pathway is indeed a minimum energy pathway. However the minima that have been derived along the pathway including the starting and final structures show meaningful states satisfying the mutation data available for these states.

**Virtual Ligand Screening of Ligand Optimized Receptor Conformations.** The 10 000 ligand test set was picked randomly from 300 000 compounds in the NCI database, and 147 known adrenergic ligands (agonists, antagonists, partial agonists, noncatechol agonists, and inverse agonists) were added to create the final test set of 10 147 compounds. These compounds were then docked to the receptor models using the standard precision mode of GLIDE,[34] using the default parameters and the resulting poses were sorted by glide score. While calculating the enrichment for noncatechol hits, compounds that lacked the catechol motif (aromatic ring with meta and para −OH groups) were defined as noncatecholamine compounds. The % yield of adrenergic ligands for cutoff c is defined as

$$\%\ \text{Yield} = \frac{\text{Number of adrenergic ligands at cutoff c}}{\text{Total number of adrenergic ligands in the test set}} \quad (2)$$

Therefore the yield represents the relative probability of selecting adrenergic ligands at a certain cutoff after filtering compared to randomly selecting ligands from the test set.

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**Supporting Information Available:** Description and validation of LITiCon method and justification of using helical rotations as conformational coordinates, binding energy landscapes of $\beta$2-AR agonists and antagonists, binding pockets for $\beta$2-AR agonists, intermediate receptor conformations along activation pathway, model for $\beta$2-AR activation, error bars in the estimation of conformational change, details of MD simulation. This material is available free of charge via the Internet at http://pubs.acs.org.

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