Molecular Basis of Ligand Dissociation from the Adenosine A2A Receptor

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ABSTRACT

How drugs dissociate from their targets is largely unknown. We investigated the molecular basis of this process in the adenosine A2A receptor (A2AR), a prototypical G protein–coupled receptor (GPCR). Through kinetic radioligand binding experiments, we characterized mutant receptors selected based on molecular dynamic simulations of the antagonist ZM241385 dissociating from the A2AR. We discovered mutations that dramatically altered the ligand’s dissociation rate despite only marginally influencing its binding affinity, demonstrating that even receptor features with little contribution to affinity may prove critical to the dissociation process. Our results also suggest that ZM241385 follows a multistep dissociation pathway, consecutively interacting with distinct receptor regions, a mechanism that may also be common to many other GPCRs.

Introduction

G protein–coupled receptors (GPCRs) represent the largest class of drug targets; they are home for one-third of all marketed drugs (Overington et al., 2006). Recent developments in this field have yielded several crystal structures that provide an atomic view on the ligand-receptor interaction (Katritch et al., 2013). This information is vital for molecular understanding of the ligand-receptor interaction; however, the knowledge gained is largely “frozen” in nature and reflects only the final interaction at the ligand-binding site. How a ligand dissociates from its binding pocket and which residues are involved in this dynamic process are still largely unknown. Here, we chose the human adenosine A2A receptor (A2AR), a prototypical GPCR, for such a mechanistic exploration. We applied molecular dynamics (MD) simulations to the crystal structure of the A2AR [Protein Data Bank (PDB) ID: 4EIY] (Liu et al., 2012) to help in the selection of amino acid residues that have potential interactions with the crystallographic ligand, ZM241385, along its dissociation pathway from the receptor. The identified residues, many of which were not in contact with the ligand in the crystal structure, were subsequently mutated, and the resulting mutant receptors were then subjected to experimental determination of the ligand’s affinity and dissociation kinetics. We observed that the E169ECL2Q, H2647.29A, and T2566.58A mutants accelerated ZM241385’s dissociation from the receptors, and the I662.61A, S672.64A, K153ECL2A, and L2677.32A mutants slowed down the process. Interestingly, these mutations only minimally influenced ZM241385’s binding affinity. Our results also suggest that ZM241385 follows a multistep dissociation pathway, consecutively interacting with topographically distinct regions of the receptor. We speculate that such a multistep dissociation process may be common to other GPCRs as well.

Materials and Methods

Chemicals and Reagents. [3H]-ZM241385 (specific activity 47.7 Ci · mmol⁻¹) was purchased from ARC Inc. (St. Louis, MO). ZM241385 was a gift from Dr. S. M. Poucher (Astra Zeneca, Macclesfield, UK). Adenosine deaminase was purchased from Boehringer Mannheim (Manheim, Germany). Bicinchoninic acid (BCA) and BCA protein assay reagent were obtained from Pierce Chemical Company (Rockford, IL). All other chemicals were of analytical grade and obtained from standard commercial sources.

Molecular Dynamics. MD simulations of the ZM241385-A2AR complex were performed on a special-purpose machine, Anton (Shaw et al., 2009). The simulation system was prepared and equilibrated following the protocols detailed in Kruse et al. (2012). Briefly, the system was prepared using the crystallized complex of PDB ID 4EIY.
solvated in approximately 14,000 three-point water model molecules (MacKerell et al., 1998), 28 sodium ions, 38 chloride ions, and 134 dipalmitoylphosphatidylcholine lipids in a 72-Å³ box consisting of approximately 65,000 atoms. The prepared system was minimized and equilibrated as previously described (Kruse et al., 2012).

We then ran 10 temperature-accelerated molecular dynamics (TAMD) simulations (Maragliano and Vanden-Eijnden, 2006) of the ZM241385 ligand dissociating from the A2A receptor, starting from the equilibrated snapshot of the ligand-receptor complex with different random initial velocities. Trajectories ended with the ligand completely dissociated from the receptor. TAMD is a method for enhancing sampling along a chosen set of collective variables (CVs; e.g., the center-of-mass of a group of atoms). The acceleration is achieved by sampling along a chosen set of collective variables (CVs; e.g., the center-of-mass of a group of atoms). The acceleration is achieved by performing Brownian motion at a higher temperature. With a proper choice of fictitious temperature,

\[ T_{\text{eff}} = \frac{T}{2} \]

and nonaccelerated orthogonal degrees of freedom of the real system remain properly distributed at the real temperature. In the TAMD simulations, the center-of-mass of the heavy atoms of the ZM241385 ligand was accelerated to encourage ligand dissociation. The spring constant tethering the center-of-mass of the ligand dissociation. The spring constant tethering the center-of-mass of the ligand was 2 kcal mol\(^{-1}\) Å\(^{-2}\).

The friction coefficient for the fictitious particle was 100 ps kcal mol\(^{-1}\) Å\(^{-2}\), and the fictitious temperature, \( k_{B,\text{eff}} \), was 2 kcal mol\(^{-1}\) to fix the position and orientation of \( A_{G,\text{eff}} \), the receptor was weakly restrained with harmonic restraints on the x-y-z-positions of Ca atoms in the intracellular region (residues 22–29, 43–48, 122–128, 97–102, 193–200, 223–238, and 284–290) with a force constant of 0.5 kcal mol\(^{-1}\) Å\(^{-2}\). These simulations were run in the NPT ensemble at 37°C and 1 bar. A flat-bottom harmonic distance restraint between the center-of-mass of the ligand, and the initial position of the ligand in the binding pocket was maintained throughout the simulations with the harmonic region beginning 30 Å away from the binding pocket with a force constant of 100 kcal mol\(^{-1}\) Å\(^{-2}\).

**Site-Directed Mutagenesis.** Site-directed mutants were constructed by polymerase chain reaction mutagenesis using pcDNA3.1-hourA2AR with N-terminal HA and FLAG tags and C-terminal His tag as a template. The mutants E169A and E169Q were generated by Baseclear (Leiden, The Netherlands), and the other mutants were created in house as follows. Mutant primers for directional polymerase chain reaction product cloning were designed using the online Quickchange primer design program (Agilent Technologies, Santa Clara, CA), and primers were obtained from Eurogentec (Maastricht, The Netherlands). All DNA sequences were verified by Sanger sequencing at LGTC (Leiden, The Netherlands).

**Cell Culture, Transfection, and Enzyme-Linked Immunosorbent Assay.** We followed procedures as described previously (Lane et al., 2012). Briefly, human embryonic kidney (HEK) 293 cells were grown as monolayers in Dulbecco’s modified Eagle’s medium supplemented with stable glutamine, 10% newborn calf serum, streptomycin, and penicillin at 37°C in a moist, 7% CO2 atmosphere. The cells were transfected with plasmid DNA using a calcium phosphate method followed by 48-hour incubation before membrane preparation. For enzyme-linked immunosorbent assay, 24 hours after transfection, cells were split into 96-well poly-d-lysine-coated plates at a density of 1 × 10⁶ cells/well. After an additional 24 hours, the cells were fixed with 4% formaldehyde and then washed with phosphate-buffered saline (PBS) before adding the primary antibody, monoclonal M2-anti-FLAG antibody (1:1000) and incubating for 30 minutes at 37°C. Next, the antibody was removed and the cells were washed with Dulbecco’s modified Eagle’s medium/25 mM HEPES before adding the second antibody, monoclonal anti-Mouse-HRP 1:5000 and incubating for 30 minutes at 37°C. After removing the second antibody and washing the cells with warm PBS, 3, 3’, 5, 5’-tetramethylbenzidine was added and incubated for 5 minutes in the dark. The reaction was stopped with 1 M H₃PO₄, and absorbance was read at 450 nm using a Victor² plate reader (PerkinElmer Life and Analytical Sciences).

**Membrane Preparation.** Cells were detached from the plates by scraping into PBS. Cells were collected and centrifuged at 700g (3000 rpm) for 5 minutes. Pellets from 10 plates (10 cm ø) were pooled and resuspended in 8 ml ice-cold buffer containing 50 mM Tris-HCl, pH 7.4. Cell suspension was homogenized with an UltraTurrax homogenizer (Heidolph Instruments, Schwabach, Germany). Cell suspension was centrifuged at 100,000g (31,000 rpm) in a Beckman Optima LE-80K ultracentrifuge at 4°C for 20 minutes. The pellet was resuspended in 4 ml of Tris buffer, and the homogenization and centrifugation step was repeated. After this, Tris buffer (2 ml) was used to resuspend the pellet, and adenosine deaminase was added (0.8 IU/ml) to break down endogenous adenosine. Membranes

<table>
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**Fig. 1.** MD simulation based on PDB entry 4EIY. A contact was identified when either the oxygen atom in the furan ring (red atom) or the nitrogen atom of the exocyclic amine group (blue atom) of ZM241385 (inset) was within 4 Å of the backbone nitrogen or carbonyl oxygen of a receptor residue. The ZM241385-A2AR binding pathway passes through multiple distinct consecutive steps, represented by three superimposed snapshots: red (initial pose, 0 ns), green (28 ns), and magenta (32 ns). This figure was generated with ICM Browser v9.8 (Molsoft) from snapshots exported from VMD v1.9.1.
Membrane aliquots containing 2.5–5.0 μg of protein were incubated in a total volume of 100 μl of assay buffer (50 mM Tris-HCl, pH 7.4, supplemented with 5 mM MgCl₂) to adjust the assay window to approximately 3000 DPM. Non-specific binding was determined in the presence of 10 μM ZM241385 and represented less than 10% of the total binding. [³H]-ZM241385 did not bind specifically to membranes prepared from parental HEK293 cells. Incubations were terminated by rapid vacuum filtration to separate the bound and free radioligand through 96-well GF/B filter plates using a Perkin Elmer Filtermate-harvester (PerkinElmer, Groningen, Netherlands) after 2-hour incubation to ensure the equilibrium was reached at all concentrations of radioligand. Filters were subsequently washed three times with 2 ml of ice-cold buffer. The filter-bound radioactivity was determined by scintillation spectrometry using a P-E 1450 Microbeta Wallac Trilux scintillation counter (PerkinElmer).

Radioligand Dissociation Assays. Dissociation experiments of [³H]-ZM241385 on the WT or mutant adenosine A₂A receptors were performed by preincubating membrane suspension with [³H]-ZM241385 in 100 μl of assay buffer at 4°C for 2 hours (Guo et al., 2012). After the preincubation, dissociation was initiated by addition of 1 μM of unlabeled ZM241385 in 5 μl. The amount of radioligand still bound to the receptor was measured at various time intervals for a total duration of 2–4 hours at 4°C to ensure that [³H]-ZM241385 was fully dissociated from the WT or mutant adenosine A₂A receptors. Incubations were terminated and samples were obtained as described under Radioligand Homologous Displacement Assays.

Data Analysis. Residue superscripts refer to the Ballesteros-Weinstein numbering (Ballesteros and Weinstein, 1995) in which a single most conserved residue among the class A GPCRs is designated x.50, where x is the transmembrane helix number. All other residues on that helix are numbered relative to this conserved position. All experimental data were analyzed by using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Values obtained are mean ± S.E.M. of at least three independent experiments performed in duplicate for (kinetic) radioligand binding assays and at least three independent experiments performed in quadruplicate for an enzyme-linked immunosorbent assay. IC₅₀ values were obtained from radioligand homologous displacement assays, and pKᵦ values were calculated using a one-site-homologous model. Dissociation data were fitted using a model of one-phase exponential decay to obtain kₑ, where we are accelerating only the ligand, the rest of the system should be minimally affected, and we expect that the observed dissociation pathways should be qualitatively similar—although not necessarily identical—to unbiased dissociation pathways. In particular, protein motions are not enhanced, so we do not expect the conformation of the receptor to be substantially affected. The kinetics of the dissociation process, however, is dramatically accelerated, allowing us to

![Image](https://example.com/image.png)
simulate ligand dissociation on computationally tractable timescales.

Each TMD trajectory began with an equilibrated “snapshot” of the ligand-receptor complex based on the 1.8 Å crystal structure (PDB ID: 4EIY) with different random initial velocities and ended with the ligand completely dissociated from the receptor. Dissociation of ZM241385 from the receptor began between 20 and 300 ns into the TMD trajectories and typically took 20 ns to complete.

To identify residues that may influence the binding kinetics, we searched for contacts between ZM241385 (Fig. 1 inset) and the protein during ligand dissociation. A contact was identified when either the oxygen atom in ZM241385’s furan ring or the nitrogen atom in ZM241385’s exocyclic primary amine group was within 4 Å of the backbone nitrogen or carbonyl oxygen of a receptor residue. In total, 16 residues were identified (Table 1). They are located in the upper part of the receptor, in either the transmembrane helices or the extracellular loops. Many of the identified contacts involved ligand-receptor interactions that were not seen in the crystal structure. We note that we would not necessarily expect all these residues to influence the ligand’s off rate; that rate will depend primarily on the free energy difference between the bound state and the highest energy state along the dissociation pathway (the transition state), and we would not expect that every residue that interacts with the ligand during the dissociation process will influence the energies of these two states. These residues do, however, represent a useful starting point for further experimental investigation.

We also observed that ZM241385 adopted several metastable poses in the dissociation simulations before completely unbinding (Fig. 1). In most of the trajectories, for example, the ligand paused transiently near H2 and H7, close to I662.63, S672.64, L2677.32, and Y2717.36, before dissociating from the receptor.

Affinity of [3H]-ZM241385 at the WT and Mutant A2ARs. We individually mutated 12 receptor residues of interest from the 16 residues suggested by the MD simulations (Table 1), mostly to alanine, and then assessed the affinity of ZM241385 for these receptor variants (mutations to the (Table 1), mostly to alanine, and then assessed the affinity of ZM241385 for these receptor variants (mutations to the remaining four residues were not considered because these residues were either small side-chain residues [G-1N-term, A2657.30, and G152ECL2] or proline [P2667.31]). Before the binding experiments, we determined the expression level of the transiently transfected receptors at the cell surface. None of the mutant receptors (13 mutants in total) was found to express at a level significantly different from the transiently transfected WT receptor (i.e., P > 0.05 in all cases, one-way analysis of variance) (Fig. 2A).

Next, radioligand homologous displacement experiments were performed to determine the affinity ($pK_i$) values of ZM241385 for the WT and mutant A2ARs. [3H]-ZM241385 was able to bind to 11 of the 13 mutants (Table 2) with relatively high affinity. The two exceptions were E169ECL2A and Y2717.36Å, which showed negligible [3H]-ZM241385 binding. ZM241385 was able to bind to the E169ECL2Q mutant, but with lower affinity than the WT receptor ($pK_i$ = 7.46 for E169ECL2Q compared with $pK_i$ = 7.83 for WT) (Fig. 2B, Table 2). The ligand also bound to the T2566.58Å mutant with lower affinity ($pK_i$ = 7.43) (Fig. 2B, Table 2) and to the Q157ECL2A with a slightly increased affinity ($pK_i$ = 8.03, Table 2) relative to the WT receptor. The remaining mutants displayed affinity similar to that of the WT receptor.

Dissociation of [3H]-ZM241385 from the WT and Mutant A2AR. We then performed radioligand dissociation assays and determined the dissociation rate constant ($k_{off}$) of ZM241385 from the WT and mutant adenosine A2A receptors. The results are detailed in Table 2. Notably, ZM241385 displayed a dramatically decreased RT (1/$k_{off}$) of just a few minutes at these three mutants compared with the WT receptor (84 minutes), as was evident from the significant leftward shift of the dissociation curves to shorter times (Fig. 2C). In stark contrast, on four other mutants—namely, I662.63Å, S672.64Å, K153ECL2Å, and L2677.32Å—significantly increased RTs of ZM241385 were observed (141 ± 2 minutes, 130 ± 4 minutes, 106 ± 3 minutes, and 196 ± 5 minutes, respectively). All other mutants displayed receptor RTs and dissociation rates that were similar to those at the WT (Table 2).

Unlike our MD simulations, which included a physiologic concentration of sodium (∼150 mM), the kinetic radioligand binding experiments described here did not include sodium.

### Table 2

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$pK_i$</th>
<th>Difference from WT</th>
<th>$k_{off}$</th>
<th>Fold over WT</th>
<th>RT</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>M</td>
<td>$pK_i$ units</td>
<td>$min^{-1}$</td>
<td></td>
<td></td>
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<td>WT</td>
<td>7.83 ± 0.04</td>
<td>0.00</td>
<td>0.0119 ± 0.0006</td>
<td>1.00</td>
<td>84 ± 2</td>
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<tr>
<td>I662.63Å</td>
<td>7.84 ± 0.04</td>
<td>0.01</td>
<td>0.0071 ± 0.0002</td>
<td>0.60</td>
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<td>S672.64Å</td>
<td>7.93 ± 0.04</td>
<td>0.10</td>
<td>0.0077 ± 0.0004</td>
<td>0.64</td>
<td>130 ± 4</td>
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<td>0.02</td>
<td>0.0113 ± 0.0004</td>
<td>0.95</td>
<td>89 ± 2</td>
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<tr>
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<td>-0.05</td>
<td>0.0148 ± 0.0009</td>
<td>1.24</td>
<td>68 ± 2</td>
</tr>
<tr>
<td>K153ECL2Å</td>
<td>7.91 ± 0.05</td>
<td>0.08</td>
<td>0.0094 ± 0.0005</td>
<td>0.79</td>
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<td>7.98 ± 0.06</td>
<td>0.15</td>
<td>0.0128 ± 0.0007</td>
<td>1.08</td>
<td>78 ± 3</td>
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<tr>
<td>Q157ECL2Å</td>
<td>8.03 ± 0.05*</td>
<td>0.20</td>
<td>0.0112 ± 0.0006</td>
<td>0.94</td>
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<tr>
<td>T2566.58Å</td>
<td>7.46 ± 0.04****</td>
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<td>L2677.32Å</td>
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<td>0.05</td>
<td>0.0051 ± 0.0002</td>
<td>0.43</td>
<td>196 ± 5</td>
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<tr>
<td>Y2717.36Å</td>
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<td>N/A</td>
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Data are represented as mean ± S.E.M. of three separate experiments each preformed in duplicate. Significantly different from wild-type with *P < 0.05, ****P < 0.0001 (one-way analysis of variance with Dunnett’s test).

*Obtained by fitting data into the model of one-phase exponential decay.

Fold over WT = 1/$k_{off}$/$k_{off}$ WT from one-phase exponential decay; N/A, not available.
As a control, we also measured the dissociation rate of ZM241385 from the WT and the H2647.29A receptors in the presence of 150 mM NaCl. Dissociation from both constructs was slowed by the presence of sodium (data not shown), but the dissociation kinetics from the H2647.29A mutant remained much faster (i.e., 8-fold) than from the WT receptor, as was observed in the absence of sodium ions (i.e., 19-fold). We expect that the presence or absence of sodium will not typically change the qualitative effects of the mutants relative to the WT.

**Discussion**

In this study, we addressed the exit pathway of a drug from its target. We started from a crystal structure of a GPCR, being the most prevalent drug targets (Overington et al., 2006), with its co-crystallized ligand. At the time we conducted our study, one crystal structure stood out due to its high resolution (1.8 Å), the A2AR bound to the antagonist ZM241385 (PDB ID: 4EIY).

Growing computer power, as well as improved software and computer hardware for MD simulations (Shaw et al., 2009; Durrant and McCammon, 2011), have made such simulations increasingly useful (Dror et al., 2012). Such simulations have been used to study both association and dissociation of ligands to various proteins, including GPCRs (Wang and Duan, 2007; Hurst et al., 2010; Dror et al., 2011; Kruse et al., 2012). In this study, MD simulations helped us identify several amino acid residues (Table 2) that upon mutation showed a minimal effect on ZM241385’s equilibrium binding affinity while having a major impact on its dissociation kinetics.

Notably, most of the selected residues would have gone unnoticed in a more classic site-directed mutagenesis study with a primary emphasis on loss- or gain-of-affinity mutations. Indeed, of the 13 mutants generated, only two had been reported previously for loss of affinity. One is E169ECCL2, which was identified to interact through direct H-bonding with ZM241385 (Jaakola et al., 2008). Upon mutation of this residue to alanine (A), ZM241385 displayed no binding at the receptor, as shown previously (Kim et al., 1996); however, when changing it to glutamine (Q) the binding of ZM241385 was restored, most likely due to preserved hydrogen bonding to the ligand. Likewise, Y2717.36A disrupted ZM241385 binding, probably as a result of the loss of its π-π stacking interaction with the antagonist’s phenol group. The importance of Y2717.36A had also been reported for the binding of the A2AR agonist CGS21680 and the antagonist XAC (Kim et al., 1995). Other than at these two mutants, ZM241384 displayed similar binding affinities for the remaining 10 mutant receptors (Table 2). Again, such “similar-function” mutants might be overlooked for further mechanistic investigations if affinity alone were taken into account.

Kinetic rather than equilibrium radioligand binding studies revealed that several residues are of great importance in determining ZM241385’s dissociation characteristics, particularly three residues (E169ECCL2, T2566.58, and H2647.29) located at the intersection of the binding cavity and the extracellular loops. We observed that ZM241385 dissociated from these mutated receptors much faster than from the WT A2AR (less than 5 minutes vs. 84 minutes; Table 2). Four other mutants—namely, I662.63A, S672.64A, K153ECCL2A, and L2677.32A—significantly decreased the dissociation rate of ZM241385. None of these residues, except E169ECCL2, is located in the binding pocket of ZM241385, which is formed by residues in the upper part of transmembrane helices 5, 6, and 7 (Jaakola and IJzerman, 2010; Liu et al., 2012). These residues may form transient interactions with ZM241385 that contribute to the energetic barriers on the dissociation pathway, although we cannot exclude the possibility that the mutations act instead by influencing the global conformation of the receptor. Interestingly, the mutants that accelerated or slowed down ligand dissociation are located in two topographically different clusters in the A2AR crystal structure, one formed by E169ECCL2, T2566.58, and H2647.29, the other by I662.63, S672.64, and L2677.32 (Fig. 3A). The dissociation of ZM241385 from the A2AR may thus follow a multistep dissociation pathway, with the ligand consecutively moving from one cluster to another.

The MD simulations of ZM241385’s egress from the A2AR further support the experimental observations, and our speculation that the unbinding process of the ligand is multistep. In the 4EIY crystal structure from which our
simulations were initiated, the residues whose mutation accelerates dissociation form a ‘trip’ interacting with ZM241385 through hydrogen bonding together with a structural water molecule (W2517) (Fig. 3A). This intact triad is in agreement with ZM241385’s relatively long RT at the WT A2AR (84 minutes) and short RTs at the E169ECL2, T2566.58, and H2647.29 mutants in which the triad is disturbed (all RTs less than 5 minutes). In the MD simulations, the breaking of the interaction between H2647.29, E169ECL2, and the ligand—a motion loosening the hydrogen bond network formed by the ‘trip’ and enlarging the opening of the binding pocket (Fig. 3B)—precedes dissociation. Mutating residues in this cluster loosens the hydrogen bond network, thus facilitating further movement of ZM241385 toward the extracellular space (Fig. 2C; Table 2). These results are consistent with a previous observation that the interaction between H2647.29 and E169ECL2 leads to low mobility of the extracellular loops. These residues were suggested to act as part of a “lid” closing the binding site and stabilizing the A2AR/ZM241385 complex (Rodríguez et al., 2011). Intriguingly, such a “lid” is reminiscent of that reported at other GPCRs, for example, the M3 muscarinic receptor (M3R). In that receptor, tiotropium, the ococrystallized ligand—is buried within the binding pocket and is shielded by a cluster of tyrosine residues (Y1486.79, Y5066.53, and Y5297.38) from the solvent at the extracellular side (Kruse et al., 2012). Such a lid almost completely prevents the influx of water molecules to hydrate tiotropium, an essential step for ligand dissociation (Schmidtke et al., 2011; Bortolato et al., 2013). This is in accordance with tiotropium’s long RT on the M3R (more than 24 hours) (Casarosa et al., 2009). Not surprisingly, mutation of one of the tyrosine residues significantly influenced tiotropium’s dissociation half-life (reducing it to less than 10 minutes) (Tautermann et al., 2013). We speculate that the triad in the A2AR similarly retains ZM241385 in the binding pocket; a concerted movement of the triad residues into different rotamer configurations is necessary to loosen the ligand from its tightly bound state.

An alternative binding intermediate along the dissociation pathway in the MD simulations involved ZM241385 interacting with I662.63, S672.64, and L2677.32. This forces ZM241385 to assume a pose similar to the one observed in another A2AR-ZM241385 crystal structure (PDB ID: 3PHW) (Dore et al., 2011), where the antagonist’s phenol group projected into the domain aforementioned domain (I662.63, S672.64, and L2677.32). Mutation of these residues into much smaller alanine reduces steric hindrance and increases the ligand’s freedom of rotation. As a result, ZM241385 displayed significantly increased RTs at these three mutant receptors (Fig. 2C; Table 2).

Taken together, our biochemical and computational results provide a molecular description of the dissociation of ZM241385 from the A2AR. The ligand appears to follow a multistep pathway, first breaking the hydrogen bond network formed by the triad of E169ECL2, T2566.58, and H2647.29 and transiently contacting the quite hydrophobic pocket above Y2717.36 consisting of I662.63, S672.64, and L2677.32 before moving further away from the binding pocket into the extracellular domain and bulk solvent. We believe atomic-level descriptions of the kinetic process as in this study will deepen our understanding of ligand-GPCR interactions and will lay the structural foundation for future rational design of drugs with optimized binding kinetics.

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