

SUPPLEMENTARY INFORMATION

Free energy calculations of A_{2A} adenosine receptor mutation effects on agonist binding

Henrik Keränen^a, Johan Åqvist^a and Hugo Gutiérrez-de-Terán^{*a}

Department of Cell and Molecular Biology, Uppsala University, Biomedical Center, Box 596, SE-751 24 Uppsala, Sweden.

Methods

The initial A_{2A}AR*-NECA complex was obtained by combining structural information from the A_{2A}AR-ZM241385 structure (PDB code 4E1Y¹) and the two active-state A_{2A}AR* structures in complex with the agonists UK432097 (PDB code 3QAK²) and NECA (PDB code 2YDV³), as reported earlier^{4,5}. Briefly, a morphing process was applied between the initial (A_{2A}AR-ZM241385) and target receptor structures (A_{2A}AR*-UK432097), followed by superposition of the A_{2A}AR*-NECA complex where the ligand and key water molecules were retained.³ Note that the latter crystal structure was not suitable as a direct starting point for our calculations because it contains some stabilizing mutations of residues that we aimed to study, and a deformed helix VII backbone due to a *cis*-proline in the NPxxY motif. Standard residue sequence numbering for the human A_{2A}AR is used herein, with superscripts according to the GPCR-specific position numbering based on TM helix conservation⁶. The receptor complex was embedded in a POPC lipid bilayer, solvated and further equilibrated using GROMACS4.0.5⁷ with the protocol implemented in the GPCR-ModSim web-server⁸. For subsequent simulations of the binding site region a 25 Å radius sphere was then extracted from the equilibrated system (including lipids and water molecules), centred on the C2 carbon of the adenine moiety of NECA.

The spherical system was used for MD simulations with the software Q⁹, where the standard OPLS all-atom (OPLS-AA) force field^{10,11} was used. Ionizable residues within 5 Å of the spherical boundaries were neutralized, while those within 20 Å from the centre were assigned their most probable protonation state at pH 7. Protonation states of histidines were given particular attention and they were modelled as neutral with the proton on N δ , except for His250^{6,52}, His155^{ECL2} (protonated on N ϵ) and His264^{ECL3} (positively charged). Atoms outside the simulation sphere were tightly restrained to their initial coordinates with a force constant of 200 kcal mol⁻¹ Å⁻² and excluded from non-bonded interactions. A restraint of 20 kcal mol⁻¹ Å⁻² to the initial coordinates was applied to solute atoms (i.e. protein and lipids) within the outer 3 Å shell of the spherical systems. Water molecules at the sphere surface were subjected to radial and polarization restraints according to the SCAAS model.¹² A 10 Å non-bonded cutoff was used together with the local reaction field method¹³ for treating long-range electrostatic interactions. The particular sidechain undergoing transformations was, however, not subjected to any cutoffs. The SHAKE algorithm¹⁴ was used to constrain the solvent bonds and angles.

Initial models of mutant receptors were created by modelling the structurally most probable rotamer of the mutated residue using PyMol.¹⁵ If more than one rotamer could be modelled, all were subjected to MD simulation and the stability was monitored. Simulations were typically considered stable if heavy atom RMSD of the ligand were below 0.5 Å. An additional 0.61 ns equilibration phase was then applied, which involved stepwise heating of the spherical system to 298 K concomitant with release of heavy atom positional restraints (from an initial force constant of 25 kcal/mol/Å²). The apo structures were produced by removing the ligand and solvating the created cavity with waters, thereafter the same equilibration procedure was applied. All production runs were done at 298 K using a separate thermal bath coupling for solute and solvent. The MD free energy calculation production phase for each mutation involved a number (between 3 and 8) of subperturbations, each accounting for 51 λ steps of 10-30 ps each (more sampling is done on the initial and ending subperturbations), using a 1 fs time step. This leads to a total simulation time of 3.1 – 5.6 ns per transformation, depending on the amino acid substitution explored. Each simulation was repeated seven times with different initial velocities, leading to a simulation time of 21 – 39 ns for each horizontal leg in the thermodynamic cycle (Fig 1). It follows that the total simulation time used for a mutation is 4 times this value (84 – 156 ns, ~120 ns on average) when a position is studied for the first time, or 50% of this value for additional mutations on the same position, since the first part of the thermodynamic cycle can be recycled.

The standard error of the mean (s.e.m.) was evaluated as the addition of the s.e.m. of all (four) simulations involved. This value it is expected to increase by a factor of $\sqrt{2}$ as compared to alanine mutations, when only two

MD simulations were needed (left side of the thermodynamic cycle in Fig 1), according to the following equation:

$$s.e.m. = \sqrt{\left(\frac{s_1}{\sqrt{n}}\right)^2 + \left(\frac{s_2}{\sqrt{n}}\right)^2} = \{s = s_1 = s_2\} = \sqrt{2\left(\frac{s}{\sqrt{n}}\right)^2} = \sqrt{2}\frac{s}{\sqrt{n}} \quad (1)$$

where s_1 and s_2 is the variance observed in the wt→Ala and mut→Ala transformations, respectively, which are expected to be of the same magnitude; n is the number of independent replicate simulations and s/\sqrt{n} the expression for the s.e.m.

References

1. V.-P. Jaakola, M. T. Griffith, M. A. Hanson, V. Cherezov, E. Y. T. Chien, J. R. Lane, A. P. IJzerman, and R. C. Stevens, *Science*, 2008, **322**, 1211–1217.
2. F. Xu, H. Wu, V. Katritch, G. W. Han, K. A. Jacobson, Z. G. Gao, V. Cherezov, and R. C. Stevens, *Science*, 2011, **332**, 322–327.
3. G. Lebon, T. Warne, P. C. Edwards, K. Bennett, C. J. Langmead, A. G. W. Leslie, and C. G. Tate, *Nature*, 2011, **474**, 521–525.
4. H. Keränen, H. Gutiérrez-de-Terán, and J. Åqvist, *PLoS ONE*, 2014, **9**, e108492.
5. H. Gutiérrez-de-Terán, A. Massink, D. Rodríguez, W. Liu, G. W. Han, J. S. Joseph, I. Katritch, L. H. Heitman, L. Xia, A. P. IJzerman, V. Cherezov, V. Katritch, and R. C. Stevens, *Structure*, 2013, **21**, 2175–2185.
6. J. A. Ballesteros and H. Weinstein, *Methods Neurosci.*, 1995, **25**, 366–428.
7. B. Hess, C. Kutzner, D. van der Spoel, and E. Lindahl, *J. Chem. Theory Comput.*, 2008, **4**, 435–447.
8. H. Gutiérrez-de-Terán, X. Bello, and D. Rodríguez, *Biochem. Soc. Trans.*, 2013, **41**, 205–212.
9. J. Marelius, K. Kolmodin, I. Feierberg, and J. Åqvist, *J. Mol. Graph. Model.*, 1998, **16**, 213–225.
10. W. L. Jorgensen, D. S. Maxwell, and J. Tirado-Rives, *J. Am. Chem. Soc.*, 1996, **118**, 11225–11236.
11. G. A. Kaminski, R. A. Friesner, J. Tirado-Rives, and W. L. Jorgensen, *J. Phys. Chem. B*, 2001, **105**, 6474–6487.
12. G. King and A. Warshel, *J. Chem. Phys.*, 1989, **91**, 3647–3661.
13. F. S. Lee and A. Warshel, *J. Chem. Phys.*, 1992, **97**, 3100.
14. J.-P. Ryckaert, G. Ciccotti, and H. J. Berendsen, *J. Comput. Phys.*, 1977, **23**, 327–341.
15. The PyMOL Molecular Graphics System, version 1.4 Schrödinger, LLC.

Table S1. Calculated and experimental NECA relative binding free energies for A_{2A}AR mutants in kcal/mol.^a

Position	Mutation to "Ala"	ΔG_{holo}	ΔG_{apo}	$\Delta\Delta G_{FEP1}$	$\Delta\Delta G_{FEP2}$	$\Delta\Delta G_{FEP3}$	$\Delta\Delta G_{FEP4}$	$\Delta\Delta G_{FEP5}$	$\Delta\Delta G_{FEP6}$	$\Delta\Delta G_{FEP7}$	$\Delta\Delta G_{FEP8}$	$\Delta\Delta G$	$\Delta\Delta G_{bind}^{calc}$	$\Delta\Delta G_{bind}^{exp}$
V84 ^{3.32}	V	0.8±0.1	-3.2±0.7	0.1±0.0	0.0±0.1	1.1±0.2	2.8±0.5					4.0±0.7		
	L	-0.7±0.5	-3.6±0.4	0.1±0.0	-0.2±0.1	0.6±0.3	1.7±0.5	0.8±0.4				2.9±0.6	1.1±0.9	0.0±0.3
T88 ^{3.36}	T	5.4±0.2	0.5±0.6	3.1±0.2	0.0±0.0	0.2±0.2	1.6±0.4					4.9±0.6		
	D	28.0±0.6	28.9±0.3	-2.8±0.6	-0.4±0.2	0.4±0.4	1.8±0.6					-1.0±0.7	5.9±0.9 (15.2±2.2) ^c	NB
	E	30.1±0.4	30.0±0.5	-1.8±0.6	0.0±0.1	-0.4±0.1	0.8±0.3	1.4±0.5				0.1±0.7	4.8±0.9 (24.4±1.8) ^c	NB
	R	64.5±0.8	65.0±0.6	-3.2±0.9	-0.2±0.1	-0.4±0.2	0.1±0.6	0.1±0.3	1.2±0.3	-0.1±0.3	2.1±0.3	-0.5±1.0	5.5±0.9	2.9±0.4
	S	0.1±0.2	-4.1±0.4	4.7±0.3	-0.3±0.1	-0.3±0.2						4.1±0.5	0.8±0.8	2.2±0.2
Q89 ^{3.37}	Q	41.0±0.9	42.7±0.6	-1.6±0.7	-0.1±0.1	-0.2±0.3	0.3±0.3	0.1±0.2				-1.6±1.1		
	D	29.6±0.4	27.7±1.1	1.1±1.3	-0.2±0.2	0.3±0.2	0.6±0.2					1.9±1.2	-3.5±1.6 (9.6±1.5) ^c	-1.6±0.3
E169 ^{5.30}	E	133.4±1.1	129.2±0.9	4.1±0.7	0.4±0.2	1.4±0.3	-0.6±0.9	-1.0±0.4				4.2±1.4		
	Q	42.9±1.1	42.8±0.8	0.8±0.9	-0.3±0.3	0.3±0.6	-0.9±0.8	0.2±0.5				0.1±1.4	4.1±2.0 (7.8±1.9) ^d	1.7±0.1
N181 ^{5.42}	N	39.6±0.4	41.8±0.3	-3.7±0.3	0.2±0.1	0.2±0.2	1.0±0.3					-2.2±0.5		
	S	-9.1±0.1	-7.7±0.4	-2.7±0.3	0.2±0.1	1.1±0.2						-1.4±0.4	-0.8±0.6	-0.1±0.3
F182 ^{5.43}	F	0.1±0.2	0.4±0.4	0.0±0.0	0.1±0.0	-0.2±0.1	0.0±0.1	-0.1±0.2	-0.1±0.3	-0.1±0.2		-0.3±0.4		
	W	10.6±0.5	10.3±0.6	-0.1±0.0	-0.1±0.1	-0.2±0.1	0.6±0.4	-0.2±0.2	0.0±0.1	0.1±0.2	0.3±0.3	0.3±0.8	-0.6±0.9	1.2±0.3
	Y	7.3±0.5	8.4±0.6	-0.1±0.0	0.0±0.0	-0.4±0.2	-0.9±0.1	0.1±0.2	0.0±0.2	0.2±0.4		-1.1±0.5	0.8±0.7	1.3±0.3
H250 ^{6.52}	H	20.7±0.7	19.7±0.7	1.1±0.2	0.0±0.0	0.0±0.1	0.4±0.5	-0.2±0.5	-0.4±0.3			1.0±1.0		
	F	0.0±0.4	0.2±0.7	0.0±0.1	-0.2±0.1	-0.3±0.2	0.0±0.3	0.5±0.5	0.2±0.3	-0.3±0.3		-0.2±0.8	1.2±1.3	0.4±0.3
	N	38.3±0.6	36.4±1.1	2.8±0.6	0.3±0.1	-0.7±0.3	-0.5±0.7					1.8±1.2	-0.9±1.6	-0.8±0.9
	Y	9.7±0.9	8.9±0.7	1.4±0.5	-0.1±0.2	-1.8±0.5	0.4±0.3	0.7±0.4	0.3±0.5	0.0±0.4		0.9±1.2	0.1±1.5	0.5±0.3
S277 ^{7.42}	S	-5.6±0.2	-4.4±0.6	0.3±0.4	-0.3±0.1	-1.2±0.6						-1.2±0.6		
	C	-1.8±0.2	-2.3±0.7	1.6±0.2	-0.4±0.1	-0.6±0.8						0.6±0.7	-1.7±1.0	0.9±0.1
	E	23.1±1.2	25.5±0.7	-1.3±0.5	-0.2±0.2	-0.4±0.5	-0.1±0.6	-0.4±0.4				-2.4±1.4	1.2±1.5	0.2±0.3
	N	32.8±1.2	36.0±0.7	-1.0±1.1	-0.1±0.2	-1.7±0.2	-0.4±0.4					-3.2±1.4	2.0±1.5	0.5±0.4
	T	-3.4±1.0	-0.9±0.4	0.6±0.4	-0.2±0.1	-0.7±0.5	-2.1±0.6					-2.5±1.1	1.3±1.2	0.4±0.3

^a Experimental relative binding free energies ($\Delta\Delta G_{bind}^{exp}$) were calculated from K_i values as $\Delta\Delta G_{bind}^{exp} = RT \ln(K_i^{mut}/K_i^{wt})$. Calculated relative binding free energies ($\Delta\Delta G_{bind}^{calc}$) are obtained from series of small, convergent FEP calculations as $\Delta\Delta G_{bind}^{calc} = (\Delta G_{holo}^{wt} - \Delta G_{apo}^{wt}) - (\Delta G_{holo}^{mut} - \Delta G_{apo}^{mut})$

^b NB = non-detectable radioligand binding. The value corresponding to the experimental detection threshold is indicated with in parentheses.

^c The corresponding result when the mutated residue is considered in its charged state.

^d The corresponding result when H264^{ECL3} is treated as charged in the mutant receptor.

Table S2. Comparison between the mutation schemes going to C β and alanine, and experimental NECA relative binding free energies for A_{2A}AR mutants (kcal/mol).^a

Position	$\Delta\Delta G_{C\beta}^{FEP}$	$\Delta\Delta G_{Ala}^{FEP}$	$\Delta\Delta G_{bind}^{exp}$
V84 ^{3.32}	4.0 ± 0.7	4.7 ± 1.0	NB ^b (>1.4)
T88 ^{3.36}	4.9 ± 0.6	4.7 ± 0.2	2.6 ± 0.2
Q89 ^{3.37}	-1.6 ± 1.1	-0.9 ± 0.8	-1.6 ± 0.1
E169 ^{5.30}	4.2 ± 1.4	4.5 ± 1.8	NB ^b (>2.7)
H250 ^{6.52}	1.0 ± 1.0	1.5 ± 0.7	NB ^b (>2.3)
S277 ^{7.42}	-1.2 ± 0.6	0.5 ± 0.4	3.5 ± 0.2

^a Experimental relative binding free energies ($\Delta\Delta G_{bind}^{exp}$) calculated from K_i values as $\Delta\Delta G_{bind}^{exp} = RT \ln(K_i^{mut}/K_i^{wt})$.

^b NB = non-detectable radioligand binding. The value corresponding to the experimental detection threshold is indicated within parentheses.