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How do mutations thermostabilize G protein-coupled receptors?

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Abstract

Structures of over 30 different G protein-coupled receptors (GPCRs) have advanced our understanding of cell signaling and provided a foundation for structure-guided drug design. This exciting progress required the development of three complimentary methods to facilitate GPCR crystallization, one of which is the thermostabilization of receptors by systematic mutagenesis. However, the reason why a particular mutation, or combination of mutations, stabilizes the receptor is not always evident from a static crystal structure. Molecular dynamics simulations have been used to identify and estimate the energetic factors that affect thermostability through comparing the dynamics of the thermostabilized receptors with structure-based models of the wild-type receptor. The data indicate that receptors are stabilized through a combination of factors, including an increase in receptor rigidity, a decrease in collective motion, reduced stress at specific residues, and the presence of ordered water molecules. Predicting thermostabilizing mutations computationally represents a major challenge for the field.

Keywords

thermostability; GPCR; dynamics; structure

The importance of GPCR thermostability

Over 120 structures of G protein-coupled receptors (GPCRs), with different ligands bound and in a variety of different conformations, have revolutionized our understanding of the architecture of GPCRs, the molecular mechanism of receptor activation and the molecular determinants for ligand efficacy [1–3]. Engineering GPCRs has proven essential to facilitate the formation of well-diffracting crystals and three complementary technologies have been

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developed [4]: (1) fusion of small soluble proteins such as T4 lysozyme (T4L) [5] and thermostabilized cytochrome b562 (BRIL) [6] into intracellular loop 3 (ICL3) or to the amino terminus of the receptors; (2) isolation of conformation-specific camelid or murine antibodies that lock the receptor in a specific state [7]; (3) conformational thermostabilization of the receptors by systematic mutagenesis [8]. In each of these strategies, the stability of the resulting GPCR-ligand complex is a key factor in determining whether crystals can be produced. The stability of receptors with T4L or BRIL fusions is usually dictated by the ligand, and it is often the case that receptor crystals can only be obtained using ligands with very high affinity (10 pM – 10 nM). This is also often the case with antibodies, but in at least one instance the camelid heavy chain antibody (nanobody) imparted sufficient stability to the receptor to allow the structure of the β_2 -adrenergic receptor to be determined bound to a native ligand, adrenaline [9]. In contrast to receptor fusions and antibody strategies, conformational thermostabilization specifically selects for improved thermostability of the detergent-solubilized receptor, and therefore crystallization of the receptor becomes largely independent of the ligand affinity, although it is still the case that no ligand-binding GPCR has had its structure determined in the absence of ligand. Engineering disulfide bridge in the receptor structure was one of the earlier strategies that was used to stabilize the rhodopsin structure [10].

In defining the first structure of a given GPCR, it is largely immaterial which methodology is used. Where structures of identical or similar receptors have been determined using either thermostabilized receptors or receptor-T4L fusions, the structures are virtually identical, within experimental error, and major differences are usually confined to the loop regions due to different crystal contacts [8]. However, if multiple structures of a receptor are required to understand ligand efficacy or for hit-to-lead optimization during drug development, there are significant advantages in the conformational thermostabilization strategy. For example, the thermostabilized β_1 -adrenergic receptor (β_1 AR) has been co-crystallized with 12 different ligands (inverse agonist, weak partial agonist, partial agonist, full agonist) [11–15] that has led to a detailed understanding of the molecular determinants of ligand efficacy and the initial molecular events upon agonist binding that increase the probability of the receptor becoming active and capable of binding a G protein. In the case of the thermostabilized adenosine A_{2A} receptor, multiple structures of lead compounds bound to the receptor led to the development of preclinical candidates for the treatment of Parkinson's disease [16]. The best compounds were based on a novel scaffold that accessed regions of the ligand binding pocket that were previously assumed to be involved only in agonist binding, although it is clear from the pharmacology and the structure that the ligand is an inverse agonist [17].

Thermostabilized GPCRs are highly desirable for both structure determination and drug development, but the thermostabilizing process is time-consuming and costly. Given the number of thermostabilized receptors engineered (Table 1) and our in-depth understanding of receptor structure, is it possible to perform thermostabilization of a receptor computationally? What is the reason why a particular mutation or combination of mutations has a stabilizing effect? The latter question was particularly perplexing, because it was usually not evident from a static crystal structure why mutations were thermostabilizing. Therefore a series of molecular dynamics (MD) simulations have been performed on

thermostabilized GPCRs [18–20] to elucidate the energetic factors that affect the thermostability and dynamics of a mutant receptor. Here we discuss our understanding of how thermostabilization results in a thermostable receptor and the progress that has been made on predicting thermostabilizing mutations computationally. Although our emphasis here is on GPCRs, the methodology is equally applicable to other membrane proteins, and the structures of a thermostabilized transporter [21] and a thermostabilized ion channel [22] have been determined recently.

The effect of thermostabilization on GPCR pharmacology

To understand the results from the MD simulations discussed below, it is important to appreciate how the thermostabilizing mutations have altered the pharmacology of the receptor and also the conformation of the receptor observed in the crystal structure. GPCRs exist in multiple conformations, such as the inactive, active and other intermediate conformational states [2]. Inactive states are non-signaling receptor conformations which do not activate a G protein, for example, a receptor bound to a full inverse agonist, whereas active states are signaling GPCR conformations which, for example, catalyze nucleotide exchange at the $G\alpha$ subunit of a heterotrimeric G protein. The β_2 -adrenergic receptor (β_2AR) bound to the antagonist carazolol is regarded as an inactive state of the receptor [5], whilst β_2AR bound to the full agonist BI167107 and a nucleotide-free heterotrimeric G protein is regarded as being in the fully active state [36]. However, in some structures of GPCRs bound to agonists, the receptor is described as being in an active-like state, because the receptor conformations have the majority of the characteristics of activated GPCRs, but due to the absence of a heterotrimeric G protein, the fully active conformation is not attained. This has been observed for the structures of $A_{2A}R$ and NTSR1 bound to agonists (Table 1). To add further complexity, different receptor parts such as the ligand binding pocket and the G protein interface may adopt different conformational states within the same GPCR [37, 38]. This is observed in the structure of NTSR1 evolved to be thermostable in the presence of agonist, where the intracellular half of the receptor represents the inactive state of the receptor and yet the extracellular portion of the receptor encompassing the ligand binding pocket is similar in conformation to the active-like state of the receptor [31].

Detailed MD simulations have been performed based on only four thermostabilized receptor structures, namely β_1AR -m23, A_{2A} -StaR2, A_{2A} -GL31 and NTSR1-GW5 (Table 1 and Glossary). Each receptor was stabilized by a process of systematic alanine scanning mutagenesis coupled to a radioligand thermostability assay performed on detergent-solubilized receptor mutants. Single thermostabilizing mutations were then combined to make an optimally stable mutant that contained between 4–8 mutations (Table 1). β_1AR -m23 and A_{2A} -StaR2 were stabilized using the antagonists 3H -dihydroalprenolol and 3H -ZM241385, respectively, and it was found that the affinities of antagonists were similar to the wild-type receptor, but the affinities for agonists were considerably reduced [23, 25]. Thus it appeared that the thermostabilization process had altered the equilibrium between the inactive and active states so that the receptors were preferentially in the inactive state, later confirmed upon structure determination [15, 28]. In the case of β_1AR -m23, the mutant can still couple to and activate G proteins although with reduced ability, whereas no coupling to G proteins was observed for A_{2A} -StaR2. In contrast to β_1AR -m23 and A_{2A} -

StaR2, the thermostabilization procedure for the engineering of A_{2A}-GL31 and NTSR1-GW5 used the radiolabelled agonists ³H-NECA and ³H-neurotensin, respectively [28, 39]. Pharmacological evaluation of these thermostabilized receptors thus found the opposite to that observed for β₁AR-m23 and A_{2A}-StaR2 *i.e.* the affinities of agonists remained similar to the wild-type receptors whilst the affinities of antagonists were considerably weaker. The structures of both A_{2A}-GL31 [28] and NTSR1-GW5 [30] showed that the receptors were in an active-like state, because rotamer changes of key amino acid side chains and helix movements in the core of the receptors had occurred, but the cytoplasmic end of TM6 partially occluded the G protein-binding site, whereas in the fully active state it would be expected to have moved a further 4–8 Å. Coupling to G proteins was impaired, with A_{2A}-GL31 showing very weak ability to activate G proteins [39] whilst NTSR1-GW5 could not activate G proteins [30]. However, in the latter case, reversion of three out of the six of the thermostabilizing mutations restored the ability of the NTSR1 mutant to activate Gαq to almost the levels observed for the wild-type receptor [40].

Insights from Molecular Dynamics simulations of thermostable mutant GPCRs

It is possible to rationally engineer thermostability in a GPCR by, for example, creating a salt bridge in the extracellular loops of β₁AR [41]. Thermostabilization can also be explained for the R68S mutation in thermostabilized β₁AR through the removal of charge repulsion and the creation of a hydrogen bond [13]. The Y227A^{5.58} mutation in β₁AR could also be rationalized, as it appears that the tyrosine is important in the stabilization of the activated state of the receptor [4]. However, many other mutations defy such a simplistic interpretation, which also ignores the fact that it is the free energy of the whole receptor that is important for stability, and a single mutation may have global consequences on the dynamics of the whole receptor, which are impossible to quantify from cursory inspection of a static crystal structure. Therefore MD simulations were performed on the thermostable mutants and the respective wild-type receptors, specifically the antagonist-bound inactive states of β₁AR-m23 and A_{2A}-StaR2, and the agonist-bound active-like states of NTSR1-GW5 and A_{2A}-GL31. It must be noted that transitions from the inactive state to the active state of a GPCR cannot be simulated using brute force MD simulations possibly due to insurmountable energy barriers to go to the active state in the absence of the agonist and the G protein. Thus all the simulations discussed explore receptor conformations within an explicit lipid bilayer of either the inactive state or the active-like state, depending upon the starting structure used for the MD simulations.

Comparison of the energetics of thermostable mutants to the wild-type receptors

Analysis of the enthalpic and entropic contributions (see Glossary) to the free energy of stabilization for the four thermostable mutants (β₁AR-m23, A_{2A}-StaR2, A_{2A}-GL31 and NTSR1-GW5) suggest that a significant proportion of the stabilization comes from the enthalpic component of the free energy [18, 19] except for β₁AR-m23, where entropic contributions stabilize the receptor as much as enthalpic contributions [20]. In the case of β₁AR-m23, the flexibility of the side chains (but not the main chain) of the amino acids in the transmembrane (TM) domain increase in β₁AR-m23 compared to the wild-type receptor.

Analysis of the structural basis for the enthalpic stabilization of the thermostable mutants showed an increase in the number and strength of inter-helical hydrogen bonds and van der Waals packing interactions. MD simulations of the thermostable mutants showed rearrangement of side chain conformations in residues neighboring the mutations leading to formation of new inter-helical hydrogen bonds and favorable van der Waals packing interactions. Thermostable mutations also led to improved protein-lipid interactions, with the lipid forming a tight layer of annular lipids around the surface of the protein contributing to favorable hydrophobic matching interactions [18]. Note that in the thermostability assays performed on detergent-solubilized GPCRs, there will be considerable amounts of lipid present (see discussion in reference 51). MD simulations also revealed that the interhelical packing interactions dominate in the intracellular region of the receptors compared to other regions in the thermostable mutants. The decreased receptor flexibility at the intracellular receptor surface may in part explain why the active-like A_{2A}-GL31 and NTSR1-GW5 mutants show a dramatic reduction in their ability to catalyze nucleotide exchange at the G protein, because flexibility of the intracellular TM regions may be a key prerequisite to activate G proteins or arrestins.

For the A_{2A}-GL31 and NTSR1-GW5 mutants thermostabilized in an active-like conformation, it is essential to have an agonist bound to achieve thermostability [23, 26], presumably because in the absence of agonist the receptor is in the inactive state and the thermostabilizing mutations are specific for the active-like state. Thus, in the absence of agonist, these thermostabilized receptors are far less stable than the wild-type receptor after detergent solubilization. This was investigated by MD simulations which showed that the binding of the agonist, adenosine, stabilizes the active-like mutant A_{2A}-GL31 by 19 kcal/mol compared to the wild-type A_{2A}R [19]. In contrast, receptors stabilized in the inactive state are also extremely stable in the absence of ligand, presumably because the ligand-free receptor is very similar to the inactive state.

Comparison of the dynamics of thermostable mutants to the wild-type receptors

MD simulations suggest that the thermostable mutants are less flexible than their wildtype counterparts. Analysis of the collective motion in the inactive β_1 AR-m23 and A_{2A}-StaR2 without ligands showed that the dominant motions in both receptors are a global pulsating motion of the opening and closing of the extracellular ligand binding site. This movement is restricted when an inverse agonist is bound to the respective thermostable mutants compared to the wild-type receptors [19, 20]. Collective motion analysis of the dynamics of the active-like mutants A_{2A}-GL31 and NTSR1-GW5 shows an outward movement of the intracellular parts of TM5 and TM6 with respect to TM3, as seen for example in the crystal structure of the β_2 AR in complex with the Gs protein [36]. However, A_{2A}-GL31 and NTSR1-GW5 and other thermostable mutants we have simulated show less movement in the intracellular regions of TM5 and TM6 with respect to TM3, compared to their respective wild-type receptors, as shown schematically in Fig. 1. This may explain why these thermostable mutants only poorly activate the G proteins compared to their wild-type receptors and is consistent with the increased rigidity of the intracellular regions as described above [23, 30]. Comparison of the dynamics of the intracellular parts of TM5 and TM6 of the antagonist bound inactive mutant A_{2A}-StaR2 to the active-like mutant A_{2A}-GL31, shows that the

flexibility of TM6 with respect to TM3 is more restricted in the antagonist bound A_{2A}-StaR2 than in the agonist bound A_{2A}-GL31 mutant. This is understandable since the tight interaction between TM3 and TM6 will improve the stabilization of the inactive state of the receptor.

To understand the cause behind the reduced flexibility in the thermostable mutants compared to the wild-type receptors, we calculated the net force on each residue (which we define as stress) in the thermostable mutants β_1 AR-m23, A_{2A}-StaR2, A_{2A}-GL31 and NTSR1-GW5, and compared it to that of the respective wild-type receptors [18–20, 42]. The reasoning behind this calculation is that the net force or stress on any object would lead to movement of that object and the GPCR needs to be flexible to bind and activate the G protein. This flexibility could come from the stress on residues that are required for activation of the receptor. We found that the calculated stress on each residue in the thermostable mutants is always lower than the corresponding stress in the wild-type receptors [18, 19, 42]. This reduced stress per residue in the thermostable mutants leads to lower flexibility of the receptor in a given conformational state stabilized by the bound ligand. However, the reduction of stress also leads to an attenuating effect on the activity of the thermostable receptor mutants in the presence of agonist and G protein. Therefore a balance between reduction of stress and gain in thermostability are important factors in predicting thermostable mutations for GPCRs. Note that the recently described active-like NTSR1-ELF mutant [40] is able to activate G protein, in contrast to the active-like NTSR1-GW5. Thus NTSR1-ELF will offer the unique opportunity to compare the dynamics of those two NTSR1 variants in view of their ability to stimulate nucleotide exchange at the G protein.

Role of water in thermostabilization of GPCRs

Analysis of high-resolution crystal structures of GPCRs along with the waters present in the crystal structures have shown co-localization of water molecules in the vicinity of highly conserved amino acid motifs [43]. In addition, the results from MD simulations on several GPCRs have led to the speculation that water molecules play a functional role in the activation of GPCRs [44–48]. Analysis of the energetics of water molecules in the ligand binding sites has also been used to guide structure-based drug design [49]. We have used the MD simulation trajectories to analyze the role of waters in the thermostabilization effect of GPCRs, but not in receptor activation. As shown for NTSR1, the interhelical hydrogen bonds that are specific to the thermostable mutant and not present in the wild-type receptor are water mediated (Fig. 2). The number of water molecules in the first solvent shell close to the receptor in the inside of the seven TM barrel is higher in the thermostable mutant NTSR1-GW5 compared to the wild-type NTSR1. The water molecules form tight hydrogen bonded water clusters that strengthen the interhelical interactions (Fig. 2). These water clusters resemble the tightly bound “ice-like” water clusters [50, 51] that have been observed in thermophilic proteins. We speculate that these clusters of waters could also be used to absorb the thermal energy when heating the thermostable mutants without disrupting the three-dimensional fold of the protein to preserve its integrity.

Structural conservation of thermostable amino acid residue positions in GPCRs

To determine whether thermostable positions are transferable across receptors, we have analyzed the measured thermostability of all the single point mutants used to initially thermostabilize each receptor [8]. The experimental procedure for the isolation of thermostabilizing mutations started with alanine scanning mutagenesis where every residue was mutated to Ala (except for Ala residues that were mutated to Leu). Each mutant was then expressed and its thermostability was determined using a radiolabeled ligand, relative to the wild-type receptor [23, 25, 39]. Fig. 3 shows the measured thermostability data for single point mutants shown on the respective crystal structures of the thermostable mutants β_1 AR-m23, A_{2A}-StaR2, A_{2A}-GL31, and NTSR1-GW5, bound to their respective ligands. The position of the mutated amino acid residues that show significant thermostability are not conserved either in their positions within the three dimensional structures or the identity of the amino acid residue. Instead they are distributed throughout the receptor structures differently for each receptor. This shows that thermostability may not be transferable and it could stem from subtle changes upon mutation in some global property of the receptor structure. Experimental work supports this contention, as the six thermostabilizing mutations in turkey β_1 AR-m23 could be transferred to human β_1 AR and human β_2 AR [52], but the gain in thermostability was considerably less for human β_2 AR than human β_1 AR.

Predicting thermostable mutations

Computational prediction of thermostabilizing mutations is a challenging process since the thermostability could arise from thermodynamic stability or increasing the kinetic barrier to receptor unfolding, intermeshed with subtle changes in other global properties of the receptor as described above. In one method to improve thermostability, Chen *et al.* exploited the void space present in the structure of β_1 AR-m23 combined with sequence conservation information to computationally design single, double and triple mutants using RosettaMembrane [53] that would fill up the void space and improve packing interactions between amino acid residues in the receptor. These predicted mutants of β_1 AR were later tested using thermostability assays under saturating concentrations of the antagonist and, in the best cases, were found to improve thermostability of the receptor significantly [54]. In a different approach, we developed a systematic rapid screening computational method, *LITiConDesign* for predicting single point alanine mutations to thermostabilize GPCRs [55, 42]. It involves generating an ensemble of conformations using the conformational sampling method called LITiCon [55–57]. The computational thermostability score calculated for each mutant in *LITiConDesign* is based on the enthalpy difference between the mutant and the wild-type, as well as other properties such as stress on each residue and the extent of involvement of each residue in allosteric communication of the ligand binding site to the G protein interaction surface [38, 42]. Mutating residues with high stress or residues that are involved in allosteric communication between the ligand binding region and G protein coupling region leads to poor thermostability. Starting from homology models of GPCRs *LITiConDesign* identified the most stable thermostable mutants in the top 35% of the predicted mutants. This reduces the number of experiments by two-thirds and is a good

starting point especially for orphan GPCRs with very little structural information. These methods lay the groundwork for future advancements as more experimental data emerge on thermostable mutations.

Concluding Remarks

MD simulations have given us insights into the dynamics and energetics of the thermostable mutants in comparison to their wild-type counterparts and have provided us an understanding of how the thermostable mutants are stabilized through the formation of water-mediated interhelical hydrogen bonds and van der Waals interactions. There are many challenges in understanding the pharmacology and conformational dynamics of GPCRs especially in view of the recent findings of biased signaling properties. Computational methods such as molecular dynamics simulations in combination with biochemical and biophysical assays investigating the pharmacological properties and structural dynamics of GPCRs, will provide detailed mechanistic insight into the subtleties of GPCR signaling. The first step towards addressing the signaling specificity of GPCRs is to stabilize them in complex with various G proteins, kinases, or arrestin molecules. However, there are significant experimental challenges arising from the dynamic conformational flexibility exhibited by GPCR-transducer complexes. As a result, the experiments are tedious and time-consuming, and have relied largely on educated “trial and error” experimentation. Progress in understanding signaling properties of GPCRs can be accelerated if we can provide a foundation for experiments that will have a higher probability of success. As enumerated in the Outstanding Questions box, a key challenge is to enhance the probability of predicting thermostabilizing mutations in receptors that have not been crystallized and are unrelated to receptors whose structures are known. Only a single structure of a GPCR bound to a G protein has been obtained, and similarly there is only a single structure of a GPCR bound to arrestin. Ideally many more structures need to be obtained to increase our understanding of G protein and arrestin coupling, but the extreme difficulty in preparing the complexes and obtaining well-diffracting crystals suggests that computational techniques could be well placed to facilitate structure determination by predicting potential avenues for thermostabilization.

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Glossary

Active-like conformation

Agonist-occupied receptor conformations with characteristics of activated GPCRs in the absence of a heterotrimeric G protein. An active-like conformation can be considered as an intermediate state approaching the active state.

Active conformation	Signaling GPCR conformations that, for example, catalyze nucleotide exchange at the G α subunit of a heterotrimeric G protein.
A_{2A}-GL31	Thermostabilized active-like adenosine A _{2A} receptor with the mutations L48A ^{2.46} , A54L ^{2.52} , T65A ^{2.63} , Q89A ^{3.37} [23]
A_{2A}-Star2	Thermostabilized inactive adenosine A _{2A} receptor with the mutations A54L ^{2.52} , T88A ^{3.36} , R107A ^{3.55} , K122A ^{4.43} , L202A ^{5.63} , L235A ^{6.37} , V239A ^{6.41} , S277A ^{7.42} [24].
β_1AR-m23	Thermostabilized inactive β_1 -adrenergic receptor with the mutations R68S ^{1.59} , M90V ^{2.53} , Y227A ^{5.58} , A282L ^{6.27} , F327A ^{7.37} , F338M ^{7.48} [25].
Conformational thermostabilization	Technique that results in a thermostabilized receptor that has the equilibrium between the inactive and active state perturbed by that process, leading to an increase in the stability of the receptor locked into a particular conformation. Thermostabilized receptor mutants can be identified by the systematic alanine scanning mutagenesis approach or by directed evolution.
Directed evolution	High-throughput approach by error prone PCR and FACS-based selection of receptor variants with increased stability in detergent and/or higher expression.
Inactive conformation	Non-signaling receptor conformations, which do not activate a G protein, for example, in the presence of a full inverse agonist.
NTSR1-GW5	Thermostabilized active-like neurotensin receptor with the mutations A86L ^{1.54} , E166A ^{3.49} , G215A ^{ECL2} , L310A ^{6.37} , F358A ^{7.42} , V360A ^{7.44} [26]
NTSR1-ELF	Thermostabilized active-like neurotensin receptor with the mutations A86L ^{1.54} , G215A ^{ECL2} , V360A ^{7.44} [27]
Rational design	Mutations are based on computational predictions using crystal structures, followed by experimental verification of the mutants.
Systematic alanine scanning approach	Receptors containing single point mutations are probed for stability by exposure to heat; receptor stability is further improved by iterative combination of single thermostabilizing mutations. If the heat step is conducted in the presence of a particular ligand such as an inverse agonist or agonist, receptor mutants are expected to be stabilized in inactive states or active-like conformations, respectively.

Enthalpy and Entropic contributions

The thermostability score for each single point mutant for a GPCR is calculated as the sum of two terms in the free energy, namely the enthalpy and entropy terms. Enthalpic contribution to the thermostability score comes from the interhelical hydrogen bonds or packing interactions formed or broken in the mutant. The entropic contribution comes from the increased or decreased flexibility in the main chain or side chain atoms of the amino acids.

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- Can we develop a reliable and time-efficient computational method that will provide a foundation for concurrent experiments to stabilize and purify GPCR-transducer complexes?
- Another challenge is to solve the crystal structures of unrelated GPCRs covering more of their phylogenetic tree. Progress towards a complete picture of the breadth of GPCR biology depends crucially on generating highly diffracting crystals. The emerging question facing up to this challenge is: Can we predict accurately, thermostabilizing mutations in GPCRs when the structure has not been determined?
- Is it possible to develop brute force multiscale molecular dynamics methods that do not bias simulations to study the large scale conformation dynamics of GPCRs to provide insight into signaling mechanisms?

Figure 1.

Schematic representation of the dynamics of the thermostable mutant GPCR compared to its wild-type receptor. This schematic is based on the analysis of the extensive MD simulation results on four thermostabilized GPCRs. TM5 and TM6 are more dynamic in the wild-type receptor compared to their thermostable mutant counterpart.

Figure 2.

Role of water in thermostabilization of GPCRs. Clusters of tightly bound water molecules, shown as mesh in the figure, accumulate between the transmembrane helices of the thermostable mutant NTSR1-GW5 to stabilize the receptor, compared to the wild-type.

Figure 3.

Experimentally measured thermostability scores ranging from blue to red (low to high) shown overlaid on the crystal structures of the thermostable mutants β_1 AR-m23, A_{2A} -StaR2, A_{2A} -GL31 and NTSR1-GW5. Thermostability was measured using the following radioligands: the antagonist ^3H -dihydroalprenolol for turkey β_1 AR in the inactive state, the antagonist ^3H -ZM241385 for A_{2A} R in the inactive state, the agonist ^3H -NECA for the A_{2A} R in the active-like state, and the agonist ^3H -neurotensin for NTSR1 in the active-like state. Each structure is shown in two mutually orthogonal views. The residues with high thermostability are marked in spheres and labeled in each receptor.

Table 1

Crystal structures determined of thermostabilized GPCRs

Receptor	Mutant name	Stabilized conformation	Number of mutations	G protein activation	Thermo-stabilization methodology	Ref
β_1 -adrenergic receptor (β_1 AR)	m23	inactive	6	yes	SM*	[15]
Adenosine A_{2A} receptor (A_{2A} R)	StaR2	inactive	8	no	SM	[28]
	GL31	active-like	4	weak	SM	[29]
Neurotensin receptor (NTSR1)	GW5	active-like	6	no	SM	[30]
	ELF	active-like	3	yes	SM	[27]
	TM86V	Agonist-bound, inactive	11	weak	directed evolution	[31]
Chemokine receptor (CCR5)	-	inactive	4	no	Rational design	[32]
Free fatty acid receptor (FFA1R)	-	inactive	4	weak	SM	[33]
Corticotrophin release factor receptor (CRF1R)	-	inactive	12	ND*	SM	[34]
Metabotropic glutamate receptor (mGlu5)	-	inactive	6	ND	SM	[35]

* Scanning mutagenesis; ND, not determined