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Thermostabilisation of membrane proteins for structural studies

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Abstract

The thermostability of an integral membrane protein in detergent solution is a key parameter that dictates the likelihood of obtaining well-diffracting crystals suitable for structure determination. However, many mammalian membrane proteins are too unstable for crystallisation. We developed a thermostabilisation strategy based on systematic mutagenesis coupled to a radioligand-binding thermostability assay that can be applied to receptors, ion channels and transporters. It takes approximately 6-12 months to thermostabilise a G protein-coupled receptor (GPCR) containing 300 amino acid residues. The resulting thermostabilised membrane proteins are more easily crystallised and result in high-quality structures. This methodology has facilitated structure-based drug design applied to GPCRs, because it is possible to determine multiple structures of the thermostabilised receptors bound to low affinity ligands. Protocols and advice are given on how to develop thermostability assays for membrane proteins and how to combine mutations to make an optimally stable mutant suitable for structural studies.

Introduction

Structure determination of integral membrane proteins (MPs) is not a trivial undertaking, and the number of their structures in the Protein Data Bank are still underrepresented compared to those of soluble proteins. However, there have been many technological developments in the methodology from gene to structure that are contributing to the exponential increase in the number of MP structures determined1. Probably the most important aspect of any structural biology project on MPs that dictates success is the choice

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of the target, with the highest probability of success correlating with high thermal stability in detergent solution, high expression levels and few post-translational modifications. One highly pragmatic approach is to identify those MPs most amenable for structure determination *i.e.* MPs that are readily overexpressed and are stable in short chain detergents. This process is facilitated by using MPs fused at the C-terminus to green fluorescent protein (GFP); this allows a rapid assessment of both expression levels2,3 and stability4 of the detergent-solubilised unpurified MP by fluorescence-detection size exclusion chromatography (FSEC)5. Parallel screening of thousands of genes from different bacteria expressed in *Escherichia coli* has resulted in numerous structures of membrane proteins being determined and is highly amenable to high-throughput structural biology techniques6. Similar methodologies are proving successful for the expression of eukaryotic MPs in yeast7,8, but the success rate for structure determination of eukaryotic MPs are dramatically lower, which is probably due to the poor stability of these proteins in detergent solution compared to their prokaryotic homologues9. This screening approach also does not address the problem faced when the structure of a specific, unstable, MP is required and not the structure of a distantly related homologue.

One solution to improve the stability of any MP is to add a high affinity inhibitor or ligand to membranes and then to solubilise and purify the MP-ligand complex. Improvements in stability can be dramatic and usually improve in relation to increased affinity and decreased off-rate, although the relationship between all these factors is not always clear-cut. This has been particularly successful in the structure determination of GPCRs10 where there is often a large selection of inhibitors (antagonists) or high affinity activators (agonists) that have resulted from decades of drug development by academic research groups and pharmaceutical companies. However, if the ligands available do not increase stability sufficiently for structure determination, then the proteins themselves can be thermostabilised by a systematic programme of mutagenesis and screening11, which has been termed conformational thermostabilisation due to its ability to stabilise a single conformation of a receptor12–14. Thus, if selection of thermostable mutants is performed using an antagonist, then the ultimate thermostabilised receptor is preferentially in the inactive conformation. Conversely, selection with an agonist results in a receptor preferentially in an active-like state. We have determined structures of a number of thermostabilised GPCRs that were either previously challenging or intractable before thermostabilisation, which include the turkey β_1-adrenergic receptor (β_1AR) bound to 12 different ligands (inverse agonist15, agonists16, partial agonists16, weak partial agonists (antagonists)17–20, biased agonists21), the human adenosine A_{2A} receptor (A_{2A}R) bound to three different agonists22,23 and four different antagonists24,25, and the rat neurotensin receptor (NTSR1) bound to a peptide agonist26. These structures have been instrumental in determining the differences in efficacy of ligands to β_1AR and the conformational changes in A_{2A}R upon agonist binding. The structure of NTSR1 bound to the C-terminal portion of the native agonist neurotensin remains the only structure of a GPCR bound to a peptide agonist, although further structures have since been determined of other thermostabilised NTSR1 mutants in different agonist-bound conformations27,28. There is currently no structure of a ligand-free GPCR29, highlighting the importance of ligands in structural biology projects. Conformational thermostabilisation forms the foundation of the StaR™ methodology developed by Heptares

Therapeutics30, which has been used it to determine the structures of multiple receptors including $A_{2A}R$ bound to a range of antagonists25 as well as compounds derived from their structure-based drug design programme24, the corticotrophin-releasing factor receptor31 and the metabotropic glutamate receptor (mGlu5)32. Thermostabilising mutations have also been essential for the structure determination, by other research groups, of the GPCRs CCR533 and GPR4034, the *Drosophila* dopamine transporter35 and an ion channel, the NMDA receptor36.

The thermostabilisation methodology we devised was developed initially between 2005 and 2008 during the thermostabilisation of three different GPCRs, β_1AR13, $A_{2A}R12$ and NTSR114. They were chosen because prior work at the Laboratory of Molecular Biology, Cambridge, by our colleagues Schertler and Grisshammer had already led to their functional expression and purification37–39, but by 2005 no crystals had been obtained for any of the targets, which we ascribed to their poor stability in short-chain detergents. The thermostabilisation methodology was then further refined on the original three receptors40–43 and expanded to include a neurotransmitter transporter, the cocaine-sensitive serotonin transporter (SERT)44,45. The methods described in this paper are therefore based on our experiences with all these receptors, but the method given is that used for the thermostabilisation of SERT44, because this is what we use for any new project.

Thermostability assays for membrane proteins

Before embarking on the lengthy and costly program to thermostabilise a MP, it is important to consider both the desirability and feasibility of the project (Box 1). The key steps in developing a thermostabilisation procedure for a MP are the following: (1) express the MP in a functional form; (2) solubilise the MP in detergent; (3) perform a thermostability assay (Figure 1). Each of these steps needs to be optimised for the wild type MP before site-directed mutagenesis is started and the mutants assessed for thermostability. The discussion in the rest of the paper will describe methodologies and experiences with GPCRs and transporters that are found normally in the plasma membrane of mammalian cells. Slightly different strategies may be required if the MP of interest is found in intracellular organelles such as the mitochondria or endoplasmic reticulum, but the 3 requirements above remain imperative and will be discussed in more detail below.

(1) Functional expression of a MP—In theory, any expression system could be used to produce the MP and we have used both inducible expression from the *lac* promoter in *E. coli*12–14 and also transient transfection of mammalian cells, using either constitutive expression42 or tetracycline inducible-expression44. However, whichever technique is used, the receptor must be expressed at reasonably high levels so that expression of the mutants can be performed in parallel on a small scale (5 ml of *E. coli* culture or a single well of a 6-well plate (10 cm^2) of mammalian cells) so that high-throughput can be achieved. In addition, the expression host must be easy to lyse with detergent, again on a small scale, and on multiple samples in parallel. *E. coli* is more problematic to lyse than mammalian cells, because its cell wall needs to be broken, efficiently and rapidly, by a combination of lysozyme treatment and freeze-thawing. In contrast, the plasma membrane of mammalian cells is readily solubilised upon addition of detergent. We prefer to use mammalian cells to

routinely express mammalian MPs for thermostabilisation, because they are more efficiently expressed in a correctly folded functional state than if they are expressed in *E. coli* or yeast46–48.

In an ideal world, expression would be sufficiently high in transiently transfected mammalian cells that a single well of a 96-well plate would yield sufficient MP for screening each mutant for thermostability. As 300-350 mutants are typically required to find 10-20 thermostabilising mutations in a small GPCR, this would represent excellent throughput, although more frequently cells have to be grown in 6-well plates due to low expression and/or low specific activity radiolabelled ligand. Optimisation of transient transfection (Box 2, Steps 12-17) will ensure that the MP is expressed at the plasma membrane in optimal amounts. If too much plasmid is used in transient transfections, some MPs will aggregate and remain in an inactive form within the cell (Fig. 2). Including a C-terminal or N-terminal GFP tag on the receptor is exceedingly useful in the initial stages of the project, because fluorescence microscopy allows a rapid assessment of how efficient the transfection is (what percentage of cells express the protein) and also whether a significant proportion of the protein is intracellular (probably misfolded) or at the plasma membrane (probably folded and functional)49. For the serotonin transporter, if too much plasmid was added to cells, the expressed protein was predominantly intracellular (Fig. 2) and no functional transporter could be detected. This is consistent with difficulties encountered expressing SERT50 that arise because N-glycosylation is essential for efficient folding51 and for the recruitment of molecular chaperones52. In contrast, other MPs such as CRF1R (Fig. 2) are expressed at the cell surface regardless of how much plasmid is added in a transient transfection.

(2) Detergent-solubilisation of the membrane protein—The choice of detergent is critical for maintaining the MP in a biologically relevant state and therefore for the success of the radioligand binding assay performed on the detergent-solubilised MP53. Detergents have a spectrum of ‘harshness’, which refers to the ability of the detergent to inactivate membrane proteins54. Mild detergents include digitonin, LMNG and DDM (in approximate decreasing order of mildness), while harsh detergents include LDAO, nonylglucoside, SDS and octylglucoside (in approximate increasing order of harshness). The ability of a detergent to inactivate a MP increases as the detergent head-group decreases in size and also as the hydrophobic portion of the detergent decreases in size. This has also been observed when the thermostability of an ultra-stable β_1AR mutant was measured in different detergents41. However, there is also an important ‘chemical’ component, which is probably due to specific interactions between the membrane protein and the detergent, consistent with the observation that ordered detergent and lipid molecules are often observed in high-resolution structures of MPs55,56. Thus thermostabilised β_1AR is very stable in most detergents, but it is rapidly inactivated by polyoxyethylene detergents, such as C12E8, which was used to crystallise the sarcoplasmic reticulum Ca^{2+}-ATPase57. Sometimes, adding lipids or sterols may improve the stability of the MP, such as the stabilisation of DDM-solubilised GPCRs through the addition of cholesteryl hemisuccinate37. It is important to remember that adding a ligand/inhibitor to membranes before solubilisation can significantly improve the amount of functional MP extracted, such that ligand binding is measurable, whereas if the ligand

was added after solubilisation, then no binding may be observed. This reflects the dramatic difference in stability of a ligand-free MP compared to a ligand-bound MP, where ligand in this context could be an inhibitor, substrate, antagonist, agonist or conformation-specific antibody. The choice of detergent to extract a membrane protein in a functional state is entirely empirical and must be determined for each membrane protein separately. However, digitonin (and the chemically similar detergent glycol-diosgenin, GDN58) is probably one of the mildest detergents and should solubilise at least a proportion of most membrane proteins in a functional form suitable for the development of the thermostability assay.

(3) Development of a thermostability assay—The radioligand thermostability assay is at the heart of the conformational thermostabilisation strategy and therefore warrants careful development to ensure the identification of thermostable mutants with a low level of false-positives. There are three main advantages of using a radioligand for measuring thermostability. Firstly, measurements can be performed on detergent-solubilised cells, so each mutant does not have to be purified. Secondly, the assays are very sensitive, so only a few picomoles of MP are required per assay point, thus requiring only small volumes of cell culture to produce a significant signal. Thirdly, radioligands are exquisite indicators of whether a protein is correctly folded, because side chains from multiple different transmembrane α-helices, and sometimes loop regions as well, all participate in ligand binding. This ensures that the thermostabilised MP has a biologically relevant conformation and its structure will therefore be informative. If a radioligand for the target MP is unavailable, other assays could be used provided they can detect whether the MP is correctly folded or not. For example, conformational specific antibodies could be used to detect correctly folded proteins if they were fluorescently labelled. Before starting development of a thermostability assay in detergent, it is essential to define conditions that allow radioligand binding to the MP in whole cells or membranes (Box 3) and this will also allow an estimation of the number of cells required for the assays in detergent described below.

In order to determine whether a detergent-solubilised MP binds a radioligand, a high affinity ligand is incubated with cells and then the radioligand-bound MP is solubilised with detergent. The radioligand in solution is then separated from the receptor-bound radioligand, with the latter amount determined by scintillation counting (Fig. 3). Note that because the assays are performed on unpurified receptors, it is important to use a cell type that does not express endogenously a protein that also binds the radioligand with high-affinity. Although the assay itself is simple in concept, in reality the format of the assay is dictated by the physicochemical characteristics of the radioligand and the properties of the receptor. These two factors will be discussed separately below.

An ideal choice of radioligand is one that binds with high-affinity to the receptor (apparent K_D between 0.1 nM to 100 nM) and has a slow off-rate. Sensitivity is improved if the radioligand is labelled with ^{125}I, but ^{3}H-labelled ligands also work perfectly well. Separation of receptor-bound radioligand (‘bound’ radioligand) from the radioligand remaining in solution (‘free’ radioligand) can be achieved by using a small disposable gel filtration column (0.2-3 ml bed volume) that is processed rapidly using a centrifuge (hence they are commonly referred to as ‘spin columns’). The MP-detergent-radioligand complex has a large molecular mass (typically about 130 kDa for a 35 kDa MP solubilised in

dodecylmaltoside59,60), so this appears in the flow-through and the radioligand (typically less than 1.5 kDa) remains on the column. The choice of resin needs to be determined empirically (Box 3) and this choice is affected by the size and hydrophobicity of the radioligand. A problem that is often encountered when hydrophobic radioligands are used is that they partition into protein-free detergent micelles. This can give rise to very high backgrounds when the separation of MP-bound radioligands is performed using the spin assay that relies on size exclusion for separation of the free radioligand (Box 3). Protein-free micelles of DDM are typically 50-70 kDa in size and so will appear in the flow-through of most spin columns, which typically use resins such as Sephadex G25 (size exclusion limit 25 kD) or Toyopearl HW40F. If the radioligand appears in the flow-through of the spin column then preferentially a different radioligand should be used (Fig 4) or a different method for the determination of the amount of radioligand bound to the MP (Box 3), such as scintillation proximity assays.

Once it has been determined that the radioligand remains in the resin of a spin column, then the next step is to determine whether radioligand bound to the detergent-solubilised receptor can be detected using the spin assay. Typically for a first experiment where nothing is known about the stability of the receptor, radioligand is added to mammalian cells expressing the receptor, allowed to bind, and then detergent is added to a final concentration of 1% wt/vol, which is a vast excess for solubilisation but will ensure that all membrane proteins are solubilised. We usually test four separate detergents first, namely digitonin, DDM, LMNG and DDM containing cholesteryl hemisuccinate (CHS; with a ratio of 1:30 wt/wt CHS:DDM). From our experience, these are amongst the mildest detergents for membrane proteins and are therefore the most likely to maintain the receptor in a native conformation. Once binding has been detected, thermostability trials may be performed.

Thermostability assays12–14 entail heating the detergent-solubilised MP at a variety of different temperatures for a given time, and then determining how much functional MP remains, as determined by the radioligand binding assay. Plotting a graph of the amount of functional detergent-solubilised receptor against the temperature at which the sample was heated should give rise to sigmoidal curve from which the apparent T_m can be readily determined by non-linear curve fitting (Fig 4B). The apparent T_m is then defined as the temperature at which 50% of the functional MP remains after heating for a given length of time (30 minutes in our lab). These data can also be used to determine the absolute amount of functional MP expressed in the cells from the amount of radioligand bound to the unheated control. Three different thermostability assays have been developed, which differ with respect to the order in which the radioligand and detergent are added in relation to the heating step (Fig. 3). For the least stable membrane proteins44, radioligand is added to cells, the receptor-radioligand complex solubilised and the heating step performed (‘super-plus’ format). In the ‘plus’ format, the receptor is solubilised and then the radioligand is added40,43. In the ‘minus’ format, the MP is solubilised and heated in the absence of ligand, quenched on ice, and then the radioligand added12–14. The ‘minus’ format was the methodology initially used, but the ‘plus’ format has now superseded it, because adding the ligand before the heating step defines the conformation of the receptor to be stabilised. In reality, we now use only the ‘super-plus’ format because the targets we are thermostabilising are unstable in detergent solution in the absence of bound ligand44.

Thermostabilisation of a membrane protein

Once a robust thermostability assay has been developed and the apparent T_m for the wild type MP in a given detergent and buffer has been determined, the identification of thermostabilising mutations can proceed. In the simplest methodology, Ala/Leu scanning mutagenesis is performed, where every amino acid is changed to alanine, unless it is already an alanine, when the substitution is made to leucine12–14,40,44. All the mutants are verified by DNA sequencing and then each mutant expressed and its thermostability determined. The simplest way to do this is to perform a two-point assay, where the amount of MP remaining after heating at a temperature slightly above or at the apparent T_m for 30 minutes is compared to an unheated control (Fig. 5). For every batch of measurements it is essential to include the wild-type MP as a control to allow normalisation between different sets of measurements. Even after keeping the unheated detergent-solubilised MP on ice, particularly labile MPs can give variations in their binding between different experiments. Given this inherent instability of many MPs, depending on the day, we find that heating it at its apparent T_m may result in anywhere between 40-60% of the receptor remaining functional. After the data for all 300 mutants have been obtained, the top 30-35 mutants are re-tested using a complete thermostability curve to determine their apparent T_m. This is a more accurate assessment of the thermostability than the two-point assay. For GPCRs, we have found between 5-9 % of the mutations are thermostabilising, which allows the most thermostable 16 mutants to be used for the development of an optimally stable mutant.

The most rapid method for combining the mutations40,43 is to take the most stable mutant and then to add each of the other 15 mutations to it to make 15 double mutants (Fig. 6). This method assumes additivity of the thermostable mutations, which occurs sufficiently often for this approach to work. The apparent T_m of each of the 15 double mutants is determined from a thermostability curve. The most thermostable double mutant is then used as a basis for the construction of a triple mutant. Some thermostable mutations when added together do not result in a more thermostable double mutant, presumably because they are stabilising slightly different conformations of the MP43. These non-additive mutations are not used in the development of triple and quadruple mutants.

How thermostable does a MP have to be? This is dictated according to why the thermostable MP is being produced in the first place, but the more thermostable the MP, the easier it will be to purify and crystallise, or to use it in other assays such as surface plasmon resonance61. However, if time is of the essence, then it may not be necessary to produce MPs that are optimally stabilised. Judicious use of fusion proteins62, high-affinity ligands10, nanobodies63, truncations60 and lipidic cubic phase crystallisation strategies64 may allow the crystallisation of only a moderately stabilised MP. Unfortunately, there has not yet been a systematic benchmarking of the stability of all the GPCRs crystallised using a single assay system as has been performed for transporters9, which would have given some insight into the amount of stabilisation required. Generally we would advocate that purification and crystallisation trials are performed in tandem with thermostabilisation to facilitate the timely determination of the required structure.

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Limitations of the thermostabilisation protocol

For the thermostabilisation procedure to work, it is essential that an assay be developed to detect membrane proteins folded in a native conformation in detergent solution. This may be difficult if there are no high-affinity ligands or conformational-specific antibodies that bind only to the native conformation. Although the protocol below has been developed for the use of small radioligands, it is readily adaptable to the use of antibodies or other binding partners. In addition, the membrane protein must be functionally expressed in sufficient quantities in mammalian cells to be detectable with the assay. With the exception of these caveats, the major limitations are both the cost and time of the procedure, particularly for very large membrane proteins containing over 1000 amino acid residues.

Comparisons to other methods

The first MP to be intentionally thermostabilised was diacylglycerol kinase (DGK)65. In this instance, mutants were screened for their ability to catalyse a reaction that was detected using a coupled enzyme assay with a colorimetric output. This is ideal for kinases, but is not readily applicable to many receptors, transporters or ion channels. Parenthetically, the thermostable mutants of DGK never yielded crystals in vapour-diffusion crystallisation trials that were sufficiently well-ordered for structure determination. The structure of DGK was eventually determined using *in meso* crystallisation methodology where crystals are grown in a monoolein cubic or sponge phase, with the most stable mutants yielding the best-diffracting crystals66.

An alternative strategy to thermostabilising GPCRs was proposed by Plückthun and colleagues, based on the assumption that the more highly expressed a membrane protein was, then the higher its thermostability would be. They reasoned that if this were true, then selecting for mutations that increased expression of a GPCR would also lead to its themostablisation. The neurotensin receptor (NTSR1) expressed in *E. coli*, developed by Grisshammer and colleagues67, was mutated, expression levels determined by binding fluorescently-labelled neurotensin and the highest expressing cells isolated by flow cytometry68. Multiple rounds of mutagenesis and selection led to a highly expressed NTSR1 mutant, but thermostability was only improved marginally by a factor of 3-4. This compares to the thermostabilisation of the β_1AR by over 1000-fold13 using the methodology of systematic mutagenesis and ligand binding assays described here. Improvements in the evolution methodology have now been made so that the selection is performed on detergent-solubilised receptors that are maintained within *E. coli* by an encapsulation process69. This allows the sorting by flow cytometry of detergent-stable receptors bound to fluorescent ligand and multiple rounds of evolution yields highly stable receptors that can be crystallised. Interestingly, the structure of NTSR1 thermostabilised by multiple rounds of evolution in *E. coli* appears to be conformationally thermostabilised in an agonist-bound inactive state27, whereas NTSR1 thermostabilised by Grisshammer and colleagues is in an active-intermediate conformation26. The advantage of the evolution methodology in *E. coli* is that it is cheap to develop a thermostable receptor, although it does require a good flow cytometry facility. The disadvantage is that the target membrane protein has to be able to be expressed in either *E. coli* or yeast, and although some membrane proteins can be expressed in these micro-organisms, many require the optimal folding

environment of a mammalian cell to attain a native structure47. In addition, high-affinity fluorescent ligands are required for this process.

Applications of thermostabilised membrane proteins

The properties of thermostabilised GPCRs make them ideal for structural analyses by X-ray crystallography, NMR or electron cryo-microscopy (cryo-EM), other biophysical analyses and for drug discovery. Thermostabilised receptors are all far more stable in short chain detergents than the wild type receptors (Fig. 7), which allowed the structure determination of β_1AR in octylthioglucoside20 and A_{2A}R in nonylglucoside23. In addition, because the receptors are so stable, they can also be crystallised in the presence of ligands with low affinity in the 1-10 μM range16,25. The detergent-stability of the thermostabilised receptors also makes them ideal for NMR studies on the dynamics of MPs in solution and potentially structure determination. For example, ultra-stable β_1AR41 yields well-resolved NMR spectra that have been utilised to study the global conformation changes of the receptor upon agonist binding70. Applications in the structure determination of thermostabilised MPs by cryo-EM have not yet been explored, but it is anticipated that the flexibility of many MPs may limit the resolution of particularly dynamic regions, which could be resolved after thermostabilisation. For example, the recent structures of both P-type and V-type ATP synthases71,72 determined by single particle cryo-EM found that the a-subunit was highly flexible, resulting in this region being the most poorly resolved part of the structure.

The thermostabilised receptors have also proven ideal for studies using surface plasmon resonance61 where the binding and dissociation of small ligands can be assessed and is an essential component of the structure-based drug design as applied to GPCRs73,74. Finally, the thermostable receptors have been used for screening compound libraries by NMR, which identified novel chemotypes that bind to β_1AR differently from other ligands, as observed by the crystal structures17. A receptor thermostabilised in the antagonist-bound state still binds antagonists with similar affinity to the wild-type receptor and the rank order of potency is maintained, but the binding of agonists is often two to three orders of magnitude weaker12,13,30,75. The converse is true for receptors stabilised in the agonist-bound state40. However, these effects on receptor pharmacology are due to the biased conformation of the receptors with the ligand binding pockets being unperturbed11. Similarly, thermostabilised SERT is locked in a single conformation and is incapable of transporting substrate, but it still binds inhibitors with high affinity44.

Materials

Reagents

- ^{125}I-RTI55, 81.4 TBq/mmmol (PerkinElmer, NEX272025UC)
- Bacitracin (Sigma-Aldrich, B0125)
- Blasticidin S HCl (Life technologies, R210-01)
- BSA (Sigma-Aldrich, 05470)
- Complete protease inhibitors, EDTA-free (Roche, 05056489001)

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- Digitonin (Merck Millipore, 300410)
- Dimethyl sulfoxide (DMSO), Hybri-Max™, sterile-filtered (Sigma, D2650)
- Water (Milli-Q)
- Dodecylmaltoside (DDM) (Anatrace, D310)
- DpnI (NEB, R0176S)
- DH5-alpha Competent E. coli (High efficiency) 1x96 well plate (NEB, C2987P)
- Dulbecco's Modified Eagle Medium (DMEM), GLUTMAX™ supplement, pyruvate (life technologies 31966-0210)
- Foetal Bovine Serum (FBS), certified (LifeTechnologies, 16000-044)
- GDN (Anatrace, GDN101)
- GeneJuice (Merck Millipore, 70967)
- Glucose (Sigma, 50-99-7)
- HEK293(TetR) cells (LifeTechnologies, R710-07)
- Lipofectamine 2000 transfection reagent (LifeTechnologies, 11668027)
- Liquid nitrogen (BOC)
- LMNG (Anatrace, NG310)
- KOD hot start DNA polymerase (Millipore, 71086-3)
- MiniPrep plasmid DNA preparation Kit (QIAGEN, 27106)
- NaCl (VWR, 7647-14-5)
- pcDNA™4/TO (Invitrogen, V1020-20)
- Phosphate buffered saline (PBS) solution (Sigma, P5493)
- Polyethyleneimine (PEI) (Sigma, 408727)
- Sephadex G25 medium (GE Healthcare Life Sciences, 17-0033-01)
- SOB medium (Sigma, H8032)
- Tetracycline HCl (Sigma, T77660)
- Toyopearl® HW-40F size exclusion media (Sigma, 807448)
- T-REx™-293 cell line (Life technologies, R710-07)
- UltimaGold Scintillant (PerkinElmer, 6013326)
- XL10 gold competent cells (Stratagene, Agilent Technologies, 200315)
- Zeocin™ (100 mg/ml; Life technologies, R25001)

Equipment

- 96-well non-skirted PCR microplates (Corning Axygen, 14-222-947)

- Benchtop centrifuge (Beckman Coulter or equivalent)
- Cell Culture plate (96-well, round bottom; Corning Costar, 3799)
- Cell Scrapper (Sarstedt, 83.1830)
- Class II biological safety cabinet (Walker)
- CO_2 incubator (Sanyo)
- CoolRack PCR (Biocision, BCS-529)
- Countess™ automated cell counter (Life technologies, C10227)
- Countess™ cell counting chamber slides (Life technologies, C10227)
- CryoTube® vials 1.8 ml (Nunc, cat. no. 377267)
- Dewar flask
- DNA thermocycler (M J Research or equivalent)
- FisherBrand Disposable Filter Columns (Fisher Scientific, 11-387-50)
- Incubator 37 ˚C (LTE Scientific or equivalent)
- Inverted light microscope (K-Tec or equivalent)
- Isoplate-96 microplate (Perkin Elmer, 6005040)
- Lab coat
- Leica DMI LED Fluorescent microscope (Leica Microsystems) coupled to Lumen 200 fluorescence illumination system (Prior Scientific), a QI click camera (QI Imaging) and a computer
- Master block (96-well, Greiner Bio-one, 780285)
- MicroBeta TriLux (Perkin Elmer or equivalent)
- Multichannel pipettes P200 and P20 (Anachem or equivalent)
- $\text{Multiscreen}_{\text{HTS}}$ FB Filter plate (Millipore, MSFBN6B10)
- Nitrile gloves
- Pastettes (Alpha laboratories, LW4811)
- Pipetman P1000, P200, P20
- Pipettes (5 ml, 10 ml, 25 ml sterile, Greiner Bio-one, 606107, 607107 and 760107)
- Pipettes (50 ml sterile, Corning Costar, 4490)
- S1 Pipette filler (Thermo Scientific)
- Safety glasses
- Scintillation vials, 4 ml (Perkin Elmer 566353)

- Steriflip-GP, 50 ml disposable vaccum filter system, 0.22 μm, sterile (Merck Millipore SCG P00525)
- Sterile conical-bottom tubes (15 ml, Sarstedt, 62.547.004)
- Thin-walled 8-strip PCR tube strips (Corning Axygen, AXY-PCR-0208-CP-C)
- Tissue culture dish (6 well, Corning Costar, 3516)
- Tissue culture dish (100 mm, Corning Costar, 353003)
- Tri-Carb Liquid Scintillation Analyzer (PerkinElmer)
- VACUSAFE (Integra)
- Zeba™ 96-well Spin Plates, 40K MWCO (ThermoFisher, 87774)

Reagent Set-Up

2xTY agar plates—Add 1.5 g of bacterial agar per 100 ml of 2xTY nutrient media prior to autoclave. Antibiotics are added, as appropriate, to the cooling agar solution and mixed thoroughly. 15-20 ml of the molten agar mixture is added per 100 mm diameter petri dish and allowed to set at room temperature (21˚C) before storage (4˚C, for up to 1 month).

Ampicillin solution—Dissolve 1 g Ampicillin in 10 ml of autoclaved water. Filter-sterilize with a Steriflip-GP 50 ml. Store 0.5 ml aliquots at -20˚C for up to a year.

Blasticidin solution (5 mg/ml)—Dissolve 50 mg blasticidin S HCl in 10 ml of autoclaved water. Filter-sterilize inside a biological safety cabinet with Steriflip-GP 50 ml. Store 0.5 ml aliquots at -20˚C for up to 6 months. ! CAUTION Toxic. Wear labcoat, gloves and eye protection.

Tetracycline Solution (1mg/ml)—Dissolve 10 mg tetracycline in 10 ml of DMSO. Store 1 ml aliquots at -20˚C for up to 1 year. CRITICAL Tetracycline is light sensitive and should be wrapped in aluminium foil to avoid light exposure.

Complete media—To 500 ml DMEM, add 50 ml certified FBS and 0.5 ml blasticidin stock solution in a biosafety cabinet. Store at 4 ˚C for up to a month.

Selection media—To 500 ml DMEM, add 50 ml certified FBS, 1 ml Zeocin™ (200μg/ml final concentration) and 0.5 ml blasticidin stock solution in a biosafety cabinet. Store at 4 ˚C for up to a month. ! CAUTION Both Zeocin and blasticidin are toxic. Wear labcoat, gloves and eye protection.

Induction media—To 1 ml selection media, add 1 μl Tetracycline stock solution for a 1μg/ml final concentration. CRITICAL Make it fresh for each use and do not store.

Glucose Solution (20% wt/vol)—Dissolve 20 g glucose in 100 ml of autoclaved water. Filter-sterilize with 0.22μm filter. Store 20˚C for up to 6 months.

LB media—Add 10 g of tryptone, 5 g yeast extract and 10 g NaCl to 800 ml distilled water and stir until dissolved. Add further water in a measuring cylinder to make the final volume up to 1 L. Autoclave the media for 20 minutes at 121˚C and store at 4˚C for up to 6 months.

PCR Reaction Mix—2.45 ml PCR grade water, 500 µl 10x KOD buffer, 500 µl 0.2 mM dNTPs, 300 µl 1.5 mM $MgSO_4$, 450 µl 9% (vol/vol) DMSO. Mix well, then add 100 µl KOD enzyme (2.5 units/µl). Mix well and use immediately. 4.3 ml is required per 96-well plate

PEI stock (1 mg/ml)—Dissolve 50 mg of PEI in 50 ml PBS. Filter-sterilize inside a biological safety cabinet with Steriflip-GP 50 ml. Store in 1 ml aliquots in autoclaved tubes at -20ºC for up to 1 year.

SOC medium—Add 20 mM glucose (20% wt/vol) to SOB medium. Filter-sterilize using 0.22µm filter and store at 20˚C for up to 6 months.

Cell buffer—Mix 20 mM Tris pH 7.4 and 150 mM NaCl. Add complete EDTA-free protease inhibitors just according to manufacturer's instruction immediately prior to use. Do not store.

Detergent stock solutions—Dissolve 10 g of DDM, LMNG or GDN in water and make up to a final volume of 100 ml. Store for up to one year at -20˚C in aliquots to avoid repeated freeze-thawing. ! CAUTION The powder is a respiratory sensitiser, so handle powder in a fume hood.

Digitonin solution—Add 5 g of digitonin to 90 ml water, boil for 2 minutes, cool on ice, make up to a final volume of 100 ml and filter through Whatmann 3MM filter paper. Store for up to one year at -20˚C in small aliquots to avoid repeated freeze-thawing. ! CAUTION The powder is a respiratory sensitiser, so handle powder in a fume hood.

Procedure

Ala/Leu scanning mutagenesis • TIMING 1 week

The protocol below is for mutating the rat serotonin transporter (SERT), fused at its C-terminus to mCherry, in pcDNA4/TO (Invitrogen) with a total insert size of 2.5 kb44. The plasmid plus insert is 8 kb in size, of low copy number and it contains an ampicillin resistance gene. Appropriate adjustments will be required in the protocol for the use of plasmids of different sizes, different copy number and different antibiotic resistance. Kits for the PCR reaction can be purchased, but it is cheaper to use individual components bought separately. However, if the user is not already familiar with PCR mutagenesis, it is worth constructing a small sub-set of mutants using the protocol below to become familiar with the process on a small scale. Mistakes in 96-well plate protocols are costly both in time and money.

1. Design the mutagenic primers to change every desired amino acid residue to, for example alanine. If a residue is already an alanine, then change it to a bulkier

amino acid, such as leucine. Forward and reverse primers are designed using an in-house proprietary program (OptimusPrimer 2.0) based on Ref 76, but similar tools are available on the internet (e.g. primerX; http://www.bioinformatics.org/primerx/ or QuikChange protocol from Agilent www.agilent.com/genomics/qcpd). Primers (desalted, but not purified) are ordered in 96-well plates as 100 μM solutions, with the forward and reverse primers in separate plates and primer pairs in identical well positions to facilitate setting up the mutagenic reactions.

2. Primers are diluted to a final concentration of 10 μM, with complimentary forward and reverse primers in the same well of a 96-well plate.

3. Using a multi-channel pipette, add per well 43 μl of PCR Mix and 5 μl of the oligonucleotides primer mix (forward primer and reverse primer, 1:1 vol:vol) and 2 μl (20 ng) template plasmid

4. Place in a 96-well PCR block and run the following cycle:

Cycle number	Denature	Anneal	Extend
1	96°C, 2 min		
2-21	96°C, 20 sec	65°C, 60 sec	72°C, 2.5 min
22			72°C, 7 min

PAUSE POINT The samples can be stored at -20˚C for several days.

5. Add 2 μl (40 units) DpnI to each well and incubate overnight at 37˚C to cleave the template plasmid.

PAUSE POINT The samples can be stored at -20˚C for several days.

6. Transform 2 μl of each PCR reaction into 30 μl highly competent *E. coli* cells (*e.g.* DH5α, XL1 or equivalent strains) pre-aliquoted into a 96-well plate. It is best to use commercial suppliers that provide cells with competencies of ~10^9 transformants per μg of DNA. Incubate competent cells and PCR reaction mix on ice for 30 min. Heat shock in a water bath at 42˚C for 30 s and transfer back onto ice.

7. After transformation, transfer the mix to 1 ml of pre-warmed SOC medium (37˚C) in a 96-well block and grow shaking at 37˚C for 1 hour.

8. Centrifuge the cells in the 96-well block, remove ~900 μl of the media supernatant, resuspend the cells by gently pipetting up and down using a multi-channel pipette, then plate out the cells on a 2xTY agar 90 mm plate containing 100 μg/ml ampicillin.

9. The following day, pick two colonies from each plate and place into 7 ml of LB media containing 100 μg/ml ampicillin in a 10 ml 24-well deep well plate. Grow overnight culture at 37˚C with shaking.

10. Prepare miniprep DNA using a commercial kit (*e.g.* QIAGEN miniprep kit) and the manufacturer's protocol.

    PAUSE POINT The samples can be stored at -20˚C for several days.

11. Sequence the whole region of the mutated cDNA to ensure that only the single desired mutation is present. We find that the sequencing is necessary, because of a reasonably high frequency of additional mutations in the primer site, particularly in regions with a high GC content.

?TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

Transient transfection of mutants into mammalian cells • TIMING 4 days

Prior optimisation of this step is essential for each target (see Box 2), which may mean varying parameters in the protocol below. The protocol below is for expressing the rat serotonin transporter (SERT) under the control of a tetracycline-inducible CMV promoter in plasmid pcDNA4/TO. This system has proven to be the most useful for expressing a wide variety of mammalian membrane proteins in mammalian cells in a fully functional form46. Parameters that need to be optimised include transfection reagent (PEI, GeneJuice or Lipofectamine), the amount of plasmid per transfection (0.1-3 μg), time before harvesting (12-48 hours) and the amount of tetracycline to induce expression (0.1-3 μg/ml)

12. Prepare a plasmid maxi-prep for each plasmid to be transfected using a commercial kit *e.g.* QIAGEN using the manufacturer's protocol.

13. Add 2 x 10^5 HEK293 cells to each well of a 6-well plate in a final volume of 1 ml DMEM plus 5% vol/vol FBS, using sufficient plates for the number of mutants to be analysed (one well per mutant). Grow at 37˚C in a humidified 5% vol/vol CO_2 atmosphere overnight or until the cells are 70%-90% confluent. Always include a positive control of the wild type membrane protein.

14. Mix 1 μg of plasmid DNA with 3 μg of PEI and add to the cells. Mix gently by tilting the plate from side to side. Incubate for 24 hours at 37˚C.

15. Add tetracycline to give a final concentration of 1 μg per ml (1 μl of a 1 mg/ml tetracycline stock). Incubate for 24 hours at 37˚C.

16. Advisable: image the cells using a fluorescent microscope to determine whether the transfection has worked (positive control) and whether the mutants show defects in expression or cell trafficking. Trafficking defects could be a sign that the mutant is misfolded and inactive (see Box 2).

17. Harvest the cells by using a cell scraper, pipette into a 15 ml centrifuge tube, pellet the cells (500 x*g*, 5 min, 4˚C), and resuspend in PBS (4˚C) containing Complete Protease Inhibitors to give a final concentration of 10^6 cells per ml. If the specific activity of the radioligand is low, it may be necessary to store cells at a higher concentration *e.g.* 10^7 cells/ml to ensure sufficient cells are used in assays.

PAUSE POINT The samples can be stored at -20˚C for several days or at -80˚C for several months. Store the cells in small aliquots to limit the number of freeze-thaw cycles to about 3.

?TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

2-Point thermostability assay of detergent-solubilised mutants • TIMING 1 day

The protocol below is for ^{125}I-RTI55-bound SERT solubilised in 0.1% wt/vol DDM performed in a 96 well plate (super-plus protocol)44. This step will require extensive optimisation to be performed on other membrane proteins, with the parameters to be varied being the number of cells per assay point, the concentration of radioligand, the buffer composition, the concentration of detergent, the type of detergent and the type of resin used (see Box 3).

18. Add ^{125}I-RTI55 (1 nM final concentration *i.e.* 10x K_D) to 10^5 cells in PBS containing protease inhibitors in a final volume of 50 µl, with one mutant per well of a 96-well plate. Ensure 2 positive controls (wild type SERT) are included per 96-well plate in positions A1 and H11. Ensure 2 negative controls (untransfected HEK293 cells) are included per 96-well plate in positions A2 and H12. Prepare duplicate plates. Incubate for 1 hour on ice.

CRITICAL STEP Cells must be resuspended homogenously, otherwise clumps of cells can cause irreproducible assays. Passage of the cells through a 26-gauge syringe containing two 90˚ bends helps to break up clumps or, alternatively, the cells can be sonicated with a narrow-tipped sonicator probe for 2 seconds.

19. Add 5 µl of 1% wt/vol DDM to each well using a multi-channel pipette. Incubate on ice for 1 hour.

CRITICAL STEP The final concentration of detergent needs to be optimised and may vary between 0.1-1% wt/vol DDM, or another detergent may be required (see Box 3)

20. Incubate the 96-well plates for 30 minutes in a PCR block at 32˚C. After exactly 30 minutes, place the tubes in a metal block pre-cooled to ice temperature. A metal block is not essential, but maximises the rate of cooling.

CRITICAL STEP For reproducibility between assays, the heating step must be exactly 30 minutes. If handling multiple plates, ensure plate 1 is put at 32˚C at t=0, and then subsequent plates at 15 second intervals; plates are then removed in exactly the same order, with 15 sec intervals between the plates. Steps 18-20 must be performed without any waiting between the steps. All pipetting must be performed accurately to ensure equal volumes of solubilised SERT are analysed.

21. During the above incubation times, prepare a 96-well spin-plate by adding 300 µl of Toyopearl® HW-40F SEC media to each well (see Box 3).

22. After the heating step is completed (Step 20), centrifuge the spin-plates containing the SEC media at 70 x*g* for 1 minute with a 96-well plate underneath to collect the flow-through (discard). Replace the spin-plate over a new 96-well plate. The SEC media should appear dry (opaque white).

CRITICAL STEP A swing-out rotor MUST be used and the timings and speed MUST be consistent for all assays

23. Load immediately 50 µl of solubilised SERT from Step 20 using a multi-channel pipette. As soon as both plates are fully loaded, centrifuge at 70 x*g* for 1 minute.

CRITICAL STEP A swing-out rotor MUST be used and the timings and speed MUST be consistent for all assays

24. Immediately add 20 µl of solubilisation buffer to each well using a multi-channel pipette. As soon as both plates are fully loaded, centrifuge at 765 x*g* for 5 minutes.

CRITICAL STEP A swing-out rotor MUST be used and the timings and speed MUST be consistent for all assays

25. Carefully remove the spin-plate containing the SEC media and the 'free' ^{125}I-RTI55 and discard (radioactive waste).

26. Transfer the flow-through containing the ^{125}I-RTI55–bound SERT to a 5 ml scintillation vial and add 4 ml of UltimaGold scintillant; count for 1 minute in a scintillation counter.

27. Analyse the data (Box 4).

?TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

Determining the apparent T_m of the most thermostable mutants • TIMING (1 day)

All the mutants that appear to be significantly more thermostable than the wild-type SERT should be re-analysed to determine their stability with respect to one another by determining their apparent T_m. This is more accurate than the 2-point thermostability assay in Steps 18-27.

28. Add ^{125}I-RTI55 (1 nM final concentration *i.e.* 10x K_D) to 10^6 cells in PBS containing protease inhibitors in a final volume of 500 µl, with each mutant in a 1.5 ml microcentrifuge tube. Ensure that a similar tube is prepared of the wild type SERT for each batch of mutants tested. Incubate for 1 hour on ice.

29. Add 5 µl of 10% wt/vol DDM to each tube and mix by gently pipetting up and down (see Box 3 regarding the amount of detergent used). Incubate on ice for 1 hour.

30. Aliquot the samples into 6 different pre-cooled PCR strips (12 tubes per strip), with each strip containing one tube of 60 µl of a detergent-solubilised mutant (11 tubes) and wild type SERT (one tube). The tubes must be pre-cooled to 0-6˚C and this is best performed in a cold room with the pipette tips also pre-cooled.

31. Incubate 5 PCR strips at an appropriate temperature for 30 minutes in a PCR block; *e.g.* 30˚C (apparent T_m), 20˚C, 40˚C, 50˚C, 70˚C. After exactly 30 minutes, place the tubes in a CoolRack pre-cooled to ice temperature.

32. Assay as described in Steps 21-27.

33. Analyse the data and determine the apparent T_m of each mutant by fitting the data to a variable-slope Boltzmann distribution using, for example, the program GraphPad Prism. This will also give an estimate of the error of the curve fitting and the whole experiment should be repeated for accuracy.

34. Optional. The thermostable mutants may be improved upon by trying other amino acid residues at the same position as the thermostabilising alanine residue (Fig 5). For example the mutation I129A thermostabilised β_1AR, but I129V was better; conversely, I55A thermostabilised β_1AR, but I55G was strongly destabilising13. Repeat Steps 1-33 after designing new primers.

?TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

Combining thermostable mutations to make an optimally stable membrane protein • TIMING (4 weeks)

35. Use the most thermostable mutant from Step 33 or Step 34 (mutant A in this example; Fig. 6) as the starting point, unless expression levels are very low, then use the next most thermostable mutant. If two mutations are equally thermostable, use each as a starting point for making the double mutants.

36. Take each of the next 15 thermostable mutations (i.e. mutations B-P) and use the mutagenic primers (Step 1) to construct all the possible double mutants with mutant A. Make sure that the primers do not overlap with mutation A and, if they do, construct new primers as appropriate.

37. Perform mutagenesis (Steps 1-11), transient transfections (Steps 12-17), 2-point thermostability assay (Steps 18-27) and the apparent T_m determination (Steps 28-33) to determine the apparent T_m of each double mutant.

38. Carefully analyse the apparent T_ms and identify those double mutants where the mutation is additive or nearly additive. In this example, the best double mutant was AB and the additive mutants were C, E, F, G, J, L, N and P (Fig. 6).

39. Repeat Steps 35-37 to make triple mutants, but using mutant AB as the starting point and only the additive mutants C, E, F, G, J, L, N and P in the mutagenic process.

40. Analyse the apparent T_ms and identify the most stable triple mutant.

41. Repeat Steps 35-37 to make quadruple mutants, but using the most stable triple mutant as the starting point.

42. Repeat this process until a sufficiently stable thermostable mutant is constructed or adding further mutations from those identified does not improve thermostability further.

?TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

Timing

The timing of the individual steps are given in the protocol above, whilst below the approximate timing is given for the whole process of thermostabilising a MP containing ~300 amino acid residues, *e.g.* a small GPCR. The timings are for a single postdoc, so individual steps can easily be made more rapid if more resources are allocated and/or the step is outsourced to a commercial company if money is no object *e.g.* the creation of Ala/Leu scan mutants. We estimate that in the UK it currently costs £50 in reagents and sequencing costs to make a single sequence-validated mutant.

Steps 1-11, construction of 300 Ala/Leu scan mutants: 2-4 months

Steps 12-17, transient transfection of mammalian cells: 2 weeks

Steps 18-27, 2-point thermostability assay for 300 mutants: 1-2 months

Steps 28-34, determination of apparent T_m of 30 mutants: 1-2 months

Steps 35-42, combining mutations to make an optimally stable mutant: 2-3 months

Box 1, initial considerations: 1-2 months

Box 2, development of transient transfection protocol for membrane protein expression: 2 weeks

Box 3, Development of a thermostability assay: 1-2 months

Box 4, Analysis of data from the 2-point thermostability assay: 1 hour

Anticipated Results

The thermostability assay (Fig. 4) is a good guide to how stable a MP is and how much more thermostabilisation is required to improve the likelihood of obtaining crystals. For example, if the thermostability can be measured only in digitonin with a high-affinity ligand bound, this suggests that the MP is unstable and will need considerable thermostabilisation before the structure can be determined. If the thermostability in 1% wt/vol DDM in the absence of any ligand is > 40˚C, then there is a reasonable probability of getting crystals provided that high-affinity ligands are available. Creating the Ala/Leu scan mutants throughout the receptor is standard molecular biology and is merely tedious with a guaranteed outcome. Normally we find that the first ~70% of the mutants can be obtained from the conditions given in the protocol. The majority of the remaining mutants can be obtained by altering the

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conditions of the primer annealing. The remaining handful of mutants can be more challenging and may require new primers to be designed, although it is now quicker just to get these mutant cDNAs synthesised in their entirety.

Screening the mutants using the 2-point thermostability assay usually produces plenty of potential thermostabilising mutations12–14,30,40,41,43. These need to be re-screened using the 7-point thermostability assay to remove any false positives and to accurately determine an apparent T_m. From this step we find that about 5-9% of the mutants are more stable than the native receptor, although the figure was much lower during the thermostabilisation of SERT (2%)44. Combining the mutations pairwise with the most thermostable mutation rapidly identifies those mutations that are additive and which, upon further combination will produce a thermostable membrane protein40,43. We normally stop recombining mutations when there is no further measurable difference in apparent T_m.

How stable does a membrane protein have to be to get crystals? The original benchmark we used was rhodopsin, which is still the only native GPCR to have its structure determined77 and has an apparent T_m of about 55˚C (30 min heating in DDM)78. Note that this value *cannot* be compared to thermostability assays determined by other techniques *e.g.* the CPM assay79, and can only be compared directly with assays were the heating step is 30 minutes. *i.e.* data from the Pluckthun lab who heat samples for 20 minutes, which therefore give higher apparent T_m values80,81. It is important to remember that rhodopsin contains a covalently bound ligand, and so the thermostability is equivalent to a GPCR assayed in the super [+] format. The benchmark of rhodopsin stability is probably reasonable if crystallisation is to be attempted in detergent solution by vapour diffusion. However, GPCRs with lower apparent T_ms could possibly be crystallised using the lipidic cubic phase technology as T4L or BRIL fusion proteins, because stabilising agents such as lipids, cholesteryl hemisuccinate or cholesterol can be added at high concentrations without inhibiting crystal formation, as is observed in detergent-based vapour diffusion crystallisation trials.

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Box 1

Initial considerations

Before starting to even consider whether a membrane protein should be thermostabilised, there are a number of important factors to consider and information that is needed.

1. Is thermostabilisation really required? The process is time consuming, costly and demands skills and expertise not always found in a structural biology lab. If it is only a matter of determining a single structure, could other techniques be more practicable and be easier to implement?
2. Are suitable ligands available that bind to the membrane protein with high affinity, preferably better than 100 nM? Are they available in a radiolabelled form?
3. Are there detailed protocols for radioligand binding assays in the literature? If there are, this will help in the initial choice of buffer conditions.
4. Is there any information in the literature with respect to expression of the target membrane protein?

Once it is decided that thermostabilisation should go ahead, it is essential to perform initial studies to demonstrate the feasibility of the process.

1. Express the membrane protein in the expression system of choice. We recommend transient transfection of HEK293 cells as the simplest and most effective expression system (Steps 12-17, Box 2). Assess expression levels by western blotting and/or fluorescence microscopy.
2. Check that the binding assay works on membranes or cells containing the membrane protein of interest. There should be good signal to noise, preferably 10:1, and ideally the assay should yield 5000-10000 dpm per assay point performed at a concentration 3-fold higher than the apparent K_D of the ligand. Factors to vary include pH, salt concentration, the type of salt, the concentration of divalent cations (e.g. Mg^{2+} or Ca^{2+}) and the presence of blocking agents such as BSA and/or bacitracin to reduce non-specific binding. Many of these factors may already have been defined during initial pharmacological evaluation of a membrane protein.
3. Ensure that expression levels are sufficient to give good binding from a small volume of cells. Thermostabilisation becomes somewhat onerous if a litre of cells is required to assess the stability of each mutant (see Box 2).

Box 2

Development of a transient transfection protocol for membrane protein expression. (Steps 12-17)

There are many protocols for transient transfection of mammalian cells using either proprietary cationic amphiphiles like GeneJuice and Lipofectamine or off-the-shelf reagents like PEI. The effectiveness of the transient transfection is dependent on the healthiness of the cells (they should be doubling every ~24 hours) and the transfection reagent used. We have noticed that some membrane proteins are expressed better using one transfection reagent compared to another, even when the vectors are identical. It is unclear why this is the case, so for a new target we test all three of the above transfection reagents. For membrane protein functional expression the most crucial factor is the amount of mRNA produced in the cell, because too much may lead to non-functional expression of the membrane protein, as was observed for SERT44. We therefore use an inducible mammalian promoter, which allows us to control the level of expression through altering both the amount of plasmid in the transfection mix and the amount of inducing agent. If MPs are expressed with a C-terminal GFP tag then cells may be observed by fluorescent microscopy 12-48 hours after transfection to determine the optimal time for cell surface expression compared to the accumulation of intracellular aggregates, which are usually composed of misfolded membrane protein49. Some examples are shown in Fig. 2.

The amount of MP expressed per cell needs to be high enough for the high-throughput thermostability assays (Box 3). A typical iodinated radioligand such as ^{125}I-RTI55 has a specific activity of 81.4 TBq/mmol, which means that you will need 2.047×10^{-15} moles of SERT bound to ^{125}I-RTI55 to give 10,000 dpm in a scintillation counter. We frequently use 50,000 cells per assay point, which means that we would need only 25,000 copies of SERT per cell to give 10,000 dpm (equivalent to 2 μg/L of cells assuming 10^6 cells/ml). This is a very low level of expression considering that for structural studies we would be aiming for expression levels in the range of 1 mg/L. A confluent 6-well tissue plate would be expected to yield about 300,000 HEK293 cells upon harvesting. However, if a tritiated ligand was to be used for the thermostability assays, expression levels would need to be higher and/or more cells would have to be used, because the specific activity of a typical tritiated ligand may be only 1.1 GBq/mmol *i.e.* 74-fold lower than for the iodinated ligand.

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Box 3

Development of a thermostability assay

The thermostability assay of detergent-solubilised membrane protein is the key aspect of the whole thermostabilisation process. For reliable results in the high-throughput format, we aim to have a signal-to-noise ratio of about 10:1. There are two main variables in the assay, the physicochemical properties of the radioligand and the stability of the detergent-solubilised receptor. During the development of the thermostability assay each of these needs to be considered carefully. For example, hydrophobic ligands may partition into detergent micelles which may give rise to high backgrounds as lipid detergent micelles may be reasonably large *e.g.* a micelle of dodecylmaltoside has an apparent molecular weight of about 50-70 kDa. Assay development using mini gel filtration columns processed by centrifugation for speed ('spin columns') follows three main steps

1. Development of a radioligand binding assay for the membrane protein in whole cells or in purified membranes.
2. Definition of conditions where the radioligand in detergent-containing buffers remains within a spin column and does not appear in the eluate.
3. Definition of conditions where the detergent-solubilised radioligand-bound membrane protein appears in the eluate.

Development of a radioligand binding assay in membranes

Very often, inspection of the literature will identify appropriate conditions for the binding of a radioligand to a specific membrane protein. Ideally, the ligand should bind with high affinity *i.e.* better than 100 nM. If the off-rate of the ligand is too fast, then dissociation may occur whilst the radioligand-membrane protein complex is loaded onto and passing through the mini SEC column. Factors that affect ligand binding such as salt concentration and pH must be optimised and will be dependent on the target membrane protein. Inclusion of blocking agents such as 0.1 % wt/vol BSA, 0.1 % wt/vol bacitracin may decrease the amount of non-specifically bound radioligand (*e.g.* binding to the membrane surface), thus improving the signal-to-noise ratio.

Development of a spin assay that retains the radioligand in the column

The 'gold-standard' spin assay that we use initially to develop a thermostability assay uses Sephadex G25 (medium) in a mini spin column (FisherBrand Screening Columns) and is ideal for small water-soluble radioligands such as ^{125}I-RTI55. The buffer used for both the assay and the gel filtration media is identical to the buffer that gives good binding of the radioligand to the membrane-bound membrane protein. Initially, a range of mild detergents should be tested such as 0.1% wt/vol digitonin (or GDN), 0.1% wt/vol LMNG and 0.1% wt/vol dodecylmaltoside. Note that the concentrations of all the detergents are well above their respective CMCs and have been found to be sufficient to solubilise membrane proteins from the small amounts of mammalian cells used in these assays42. There is about 0.2 mg of total protein in one million HEK293 cells, as assessed by an amido black protein determination assay82, which will allow readers to correlate our solubilisation conditions with those used in their labs. Higher amounts of detergents

(up to 1% wt/vol) may improve solubilisation efficiencies, particularly if the MP of interest is expressed at low levels in bacteria or yeast.

1. Add 100 g of dry Sephadex G25 (medium) to 1 L of buffer containing 0.1 % wt/vol detergent in a glass bottle and leave to equilibrate overnight at 4°C.
2. Remove excess buffer from the swollen Sephadex to leave a ratio of 2:1 resin:buffer (vol/vol).
3. Prepare 180 µl of Assay Mixture. For these initial tests, this will be buffer + 0.1% wt/vol detergent + radioligand at a final concentration of 5-10x the K_D for binding to the MP of interest. Eventually this will also contain detergent-solubilised MP.
4. Thoroughly resuspend the resin by inverting bottle.
5. Add 3.6 ml of resuspended resin to each spin column, pre-placed in a 10 ml test tube. Prepare 3 columns for each Assay Mixture to be tested as each assay point is determined in triplicate. Use a 5 ml automatic pipettor to pipette the resin into the columns and mix the resin thoroughly by inversion after every 3 columns poured as it starts to settle out. It is crucial to have the same volume of resin in each tube for reproducible results. The columns can be poured a few hours before use and left at 4°C.
6. Pre-spin columns in a swing-out rotor in a bench top centrifuge in a cold room at 275 x*g* for 3 min. Remove spin columns from the test tubes onto scintillation vials. After the pre-spin the columns should be used in a few minutes.
7. Load columns with 50 µl of Assay Mixture slowly, with the pipette tip near to the centre of the resin surface. A maximum of ~16 columns should be loaded in one go and each assay point should be determined in triplicate.
8. Spin columns for 4 min at 375x*g*.
9. Discard columns, add scintillant to the scintillation vials, cap them and then count in a scintillation counter. Also add 5 µl of Assay Mixture in triplicate to determine the total radioligand concentration in the Assay Mixture.

The expected results are that the radioligand is retained on the column. If in 50 µl of assay mixture there was a total of 200,000 dpm ^{125}I-RTI55, then in the flow-through from the column there should be only 100-200 dpm.

Development of a spin assay that measures binding to a detergent-solubilised membrane protein

The assay in Section 2 above is repeated, but now the Assay Mixture loaded onto the column is detergent-solubilised membranes containing the membrane protein of interest. Initially, a range of mild detergents should be tested such as 0.1% wt/vol digitonin (or GDN), 0.1% wt/vol LMNG and 0.1% wt/vol DDM, as these are most likely to maintain the membrane protein in a biologically-relevant conformation that is able to bind ligand. The amount of detergent to be used to solubilise the membrane protein is dependent on

the amount of cells used in the assay, but for highly expressed membrane proteins or when a high-specific activity radioligand is used, then very few cells are required (*e.g.* 2 x 10^5 transiently transfected mammalian cells) and 0.1% (wt/vol) of the detergents above (final concentration) will lead to solubilisation. If detergents of a high CMC are used (*e.g.* CHAPS), the detergent must be used at a concentration well above its CMC (*e.g.* 1% wt/vol CHAPS, which has a CMC of 0.6% wt/vol). Always include a negative control of detergent-solubilised cells that do not contain the protein of interest and, if possible, a negative control containing an unlabelled cold competitor at a 1000-fold molar excess over the radiolabelled ligand.

Object example: the serotonin transporter

For the serotonin transporter, three different inhibitors had been used previously in binding studies of the membrane-bound transporter, ^{125}I-RTI55, ^{3}H-imipramine and ^{3}H-paroxetine. These were all tested for the overexpressed transporter and could be used to give similar binding in membranes. However, when used on the serotonin transporter solubilised in dodecylmaltoside, the results appeared very different (Fig 4). ^{3}H-imipramine gave extremely high binding to the SERT-containing membranes in the presence of the competitive inhibitor cocaine. This commonly occurs when the ligand is very hydrophobic and interacts non-specifically with the detergent micelle. ^{3}H-paroxetine binding was greatest in the sample containing SERT compared to sample containing SERT and excess cocaine, but the ratio between them was 3:1, which is a poor assay and may lead to many false positives during the screening for thermostable mutants. In contrast, the ratio between the SERT and the SERT+cocaine values for ^{125}I-RTI55 was 100:1, which makes an ideal assay that was used to identify thermostable mutants44. Once the assay was developed in the FisherBrand Screening columns, it was further adapted to a 96-well plate format using Toyopearl® HW40F media (Steps 18-27) using a similar rationale to that described above. Commercial spin columns in a 96-well plate format are now available (Zeba™ 96-well Spin Desalting Plates).

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Box 4

Analysis of an idealised data set from the 2-point thermostability assay

Data from a 2-point thermostability assay is represented below for a fictitious protein that demonstrates the type of problems and uncertainties that can arise from real data. Assays are presented for samples analysed on two separate days, but after heating the wild type MP for an apparently identical time, a different proportion remained functional. Therefore, to compare datasets they first have to be normalised.

Separate data sets	Membrane proteins	Binding in samples left at 4°C			Binding after heating (30°C, 30 min)			Binding remaining (%)	Normalised binding remaining WT=50%
		DPM 1	DPM 2	Mean DPM minus background	DPM 3	DPM 4	Mean DPM minus background		
Data set 1	Wild type MP	9150	11050	10000	2750	2400	2475	**25**	**50**
	Mutant S	1475	1625	1450	1445	1375	1310	90	**181**
	Mutant T	5500	6600	5950	4500	3600	3950	66	**133**
	Mutant U	7660	8540	8000	2800	2400	2500	31	**63**
	Untransfected cells	150	50	100	75	125	100	n/a	n/a
Data set 2	Wild type MP	7760	8340	7950	5100	4950	5025	**63**	**50**
	Mutant V	150	1800	875	900	1000	950	109	**86**
	Mutant W	6300	7800	6950	3500	3700	3600	52	**41**
	Mutant X	6200	6900	6450	50	130	90	1	**1**
	Mutant Y	95	45	-30	140	45	92.5	0	**0**
	Mutant Z	120	280	100	200	150	175	175	**139**
	Untransfected cells	45	155	0	80	120	0	n/a	n/a

Consideration of the data suggests that mutations S and T are probably thermostabilising, but that mutations V and Z are probably false positives due to the spuriously low DPM 1 for mutant V and the near-background binding observed for mutant Z. Mutation U could be thermostabilising, but needs a more detailed analysis. Mutant W has similar properties to the wild type protein whereas mutant X has been destabilised by the mutation. Mutant Y does not show significant binding at 4°C, which suggests that either folding was impaired, the protein may be extremely unstable or the mutation is in the binding site and either prevents ligand binding or dramatically alters the apparent K_D for the ligand. Mutants X and Y are interesting in their own right; similar results from the thermostabilisation of β_1AR identified amino acid residues involved in binding the highly-conserved intramembrane Na^+ ion and associated water molecules18.

Two courses of action present themselves from these data. Firstly, mutant V should be re-tested on a two-point thermostability assay whilst mutants S, T and U should be tested on a full 7-point thermostability assay to determine an apparent T_m (Steps 28-33). Secondly, if mutant S is indeed thermostabilising, it may be worth investigating other mutations at this site to improve expression levels. There is no significant correlation between stability and expression levels11, which implies that if one mutation is thermostabiling but dramatically decreases expression levels, substitution with another amino acid residue may maintain the thermostabilising effect and restore expression levels. This was observed for the thermostabilising H103A mutation in NTSR1 that reduced expression levels 5-fold, whilst the substitution H103S restored expression levels and maintained the increase in thermostability14.

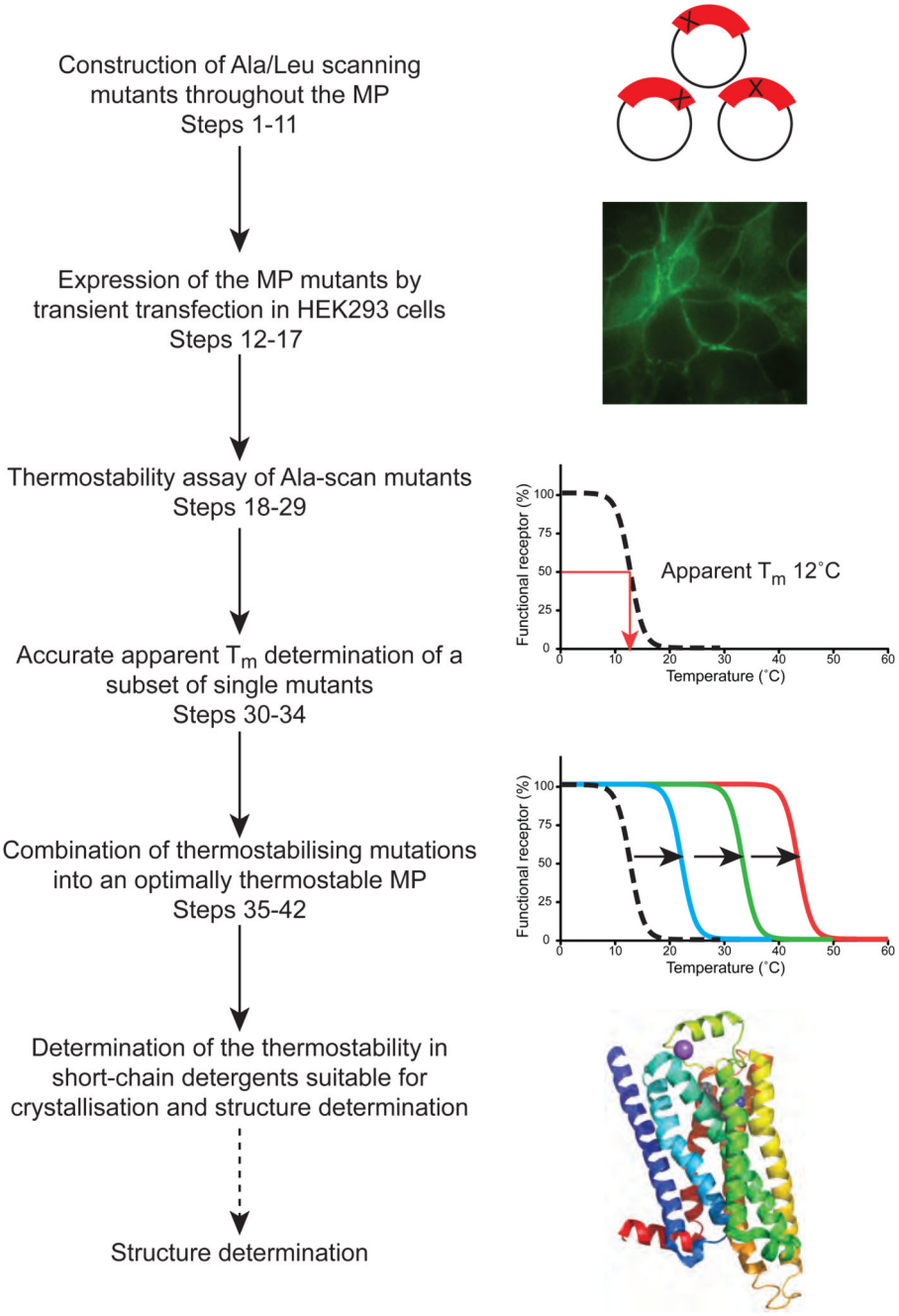

Figure 1.
Flowchart illustrating the thermostabilisation strategy.

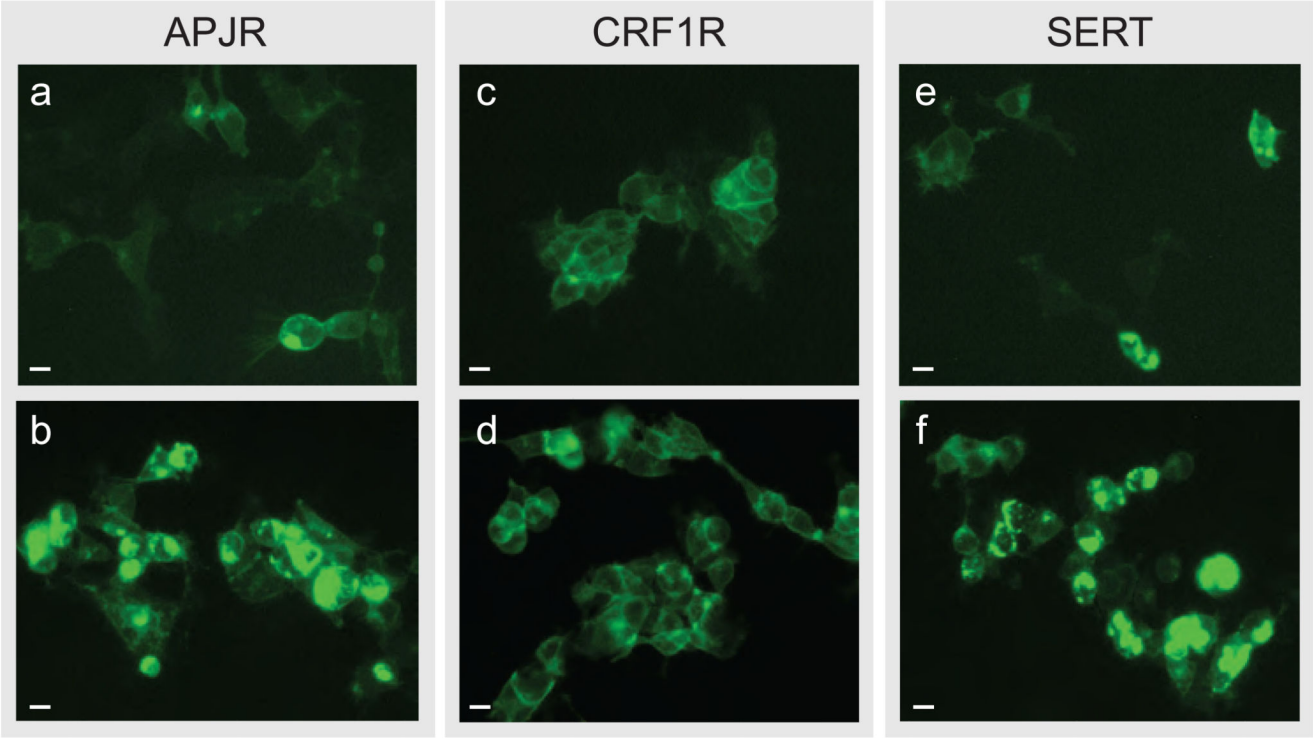

Figure 2.

Optimisation of transient transfection in HEK293 cells. In the first step of the optimisation procedure, HEK293(TetR) cells were transfected with different amounts of plasmid expressing 3 different MPs fused to GFP, induced with 1 μg/ml tetracycline and visualised by fluorescent microscopy 24 hours after transfection: A & B, apelin receptor (APJR), 0.3 μg and 3 μg plasmid, respectively; C & D, corticotrophin-releasing factor receptor (CRF1R), 0.1 & 3.0 μg plasmid, respectively; E & F, serotonin transporter (SERT), 0.3 & 1 μg plasmid, respectively. Both SERT and APJR were optimised further to minimise the amount of misfolded aggregates inside the cell by testing different amounts of tetracycline and the length of time of induction. All conditions tested for CRF1R resulted in a similar pattern of cell surface expression with no appearance of misfolded protein. The scale bar represents 10 μm.

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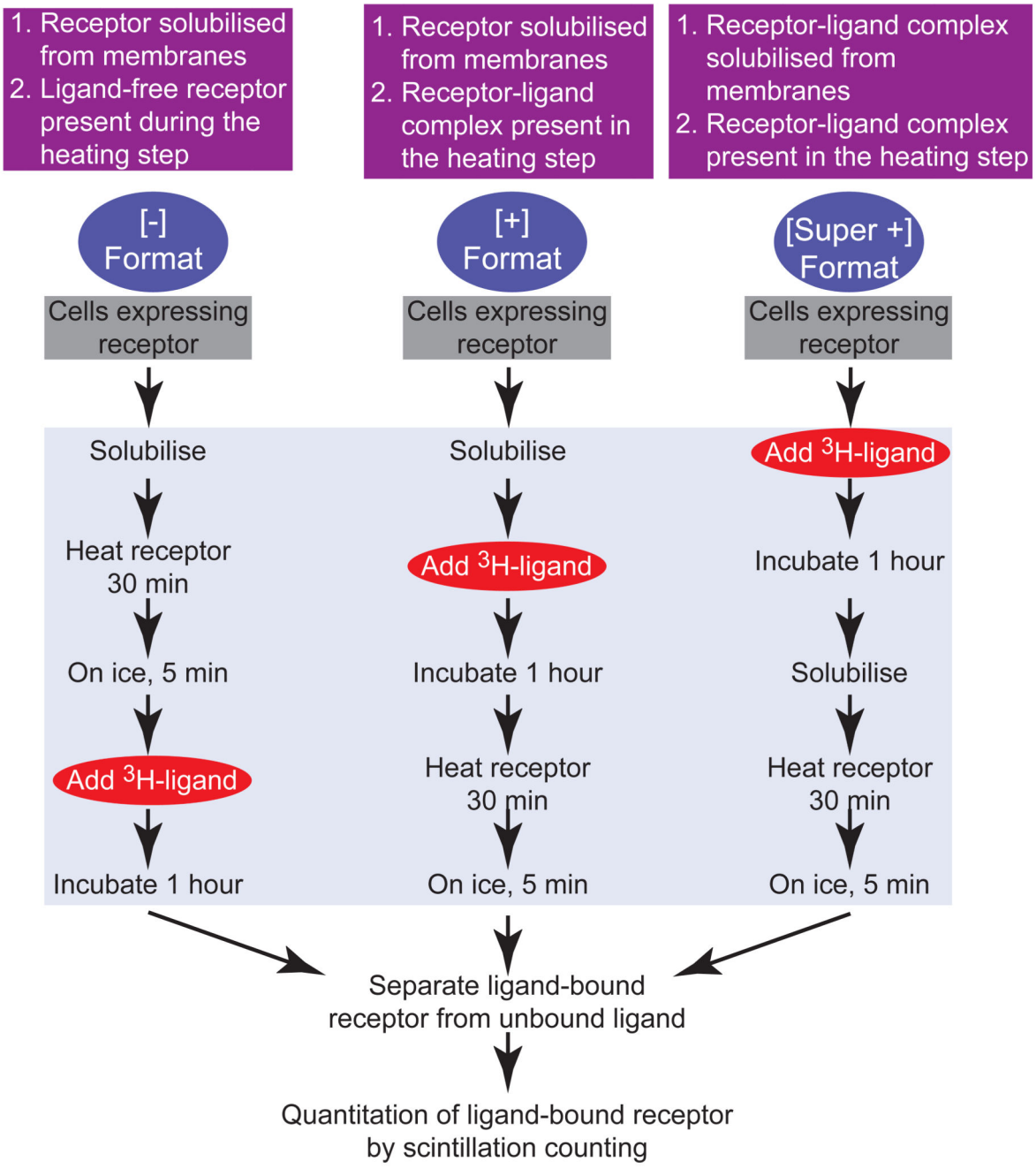

Figure 3.
Different formats of the thermostability assays.

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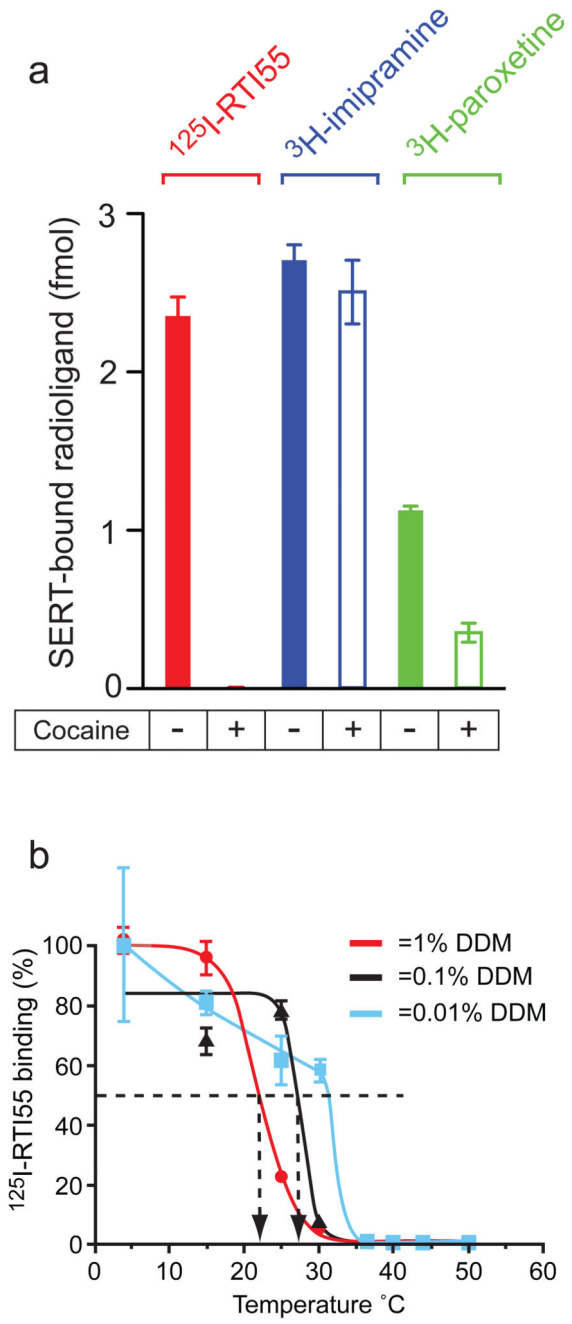

Figure 4.

Development of a thermostability assay for the serotonin transporter. (a) Initial assay to define which radioligand could be used for the thermostability assay. Radioligands were added to cells at a concentration 10-fold above their K_D, either in the presence or absence of a cold competitor (1 mM cocaine) incubated for 1 hour, and then SERT was solubilised in 1% wt/vol DDM at 4ºC. The lysate was passed through a spin column and the amount of radioligand in the flow through (SERT-bound) was determined45. ^{125}I-RTI55 gave the best ratio between the bound radioligand measured in the presence or absence of cocaine. (b)

Aliquots of cells were incubated with ^{125}I-RTI55 and then solubilised with different concentrations of DDM as indicated. After solubilisation, the samples were heated for 30 minutes at different temperatures, quenched on ice, and the amount of bound radioligand determined45. The apparent T_m for SERT varied with the amount of detergent: 1% wt/vol DDM, apparent T_m 25ºC; 0.1% wt/vol DDM, apparent T_m 28ºC; 0.01% wt/vol DDM, apparent T_m 31ºC. Note that 0.01% wt/vol DDM probably failed to completely solubilise the membranes42, which perhaps caused the two-phase curve; these conditions would not be used for thermostabilising a membrane protein.

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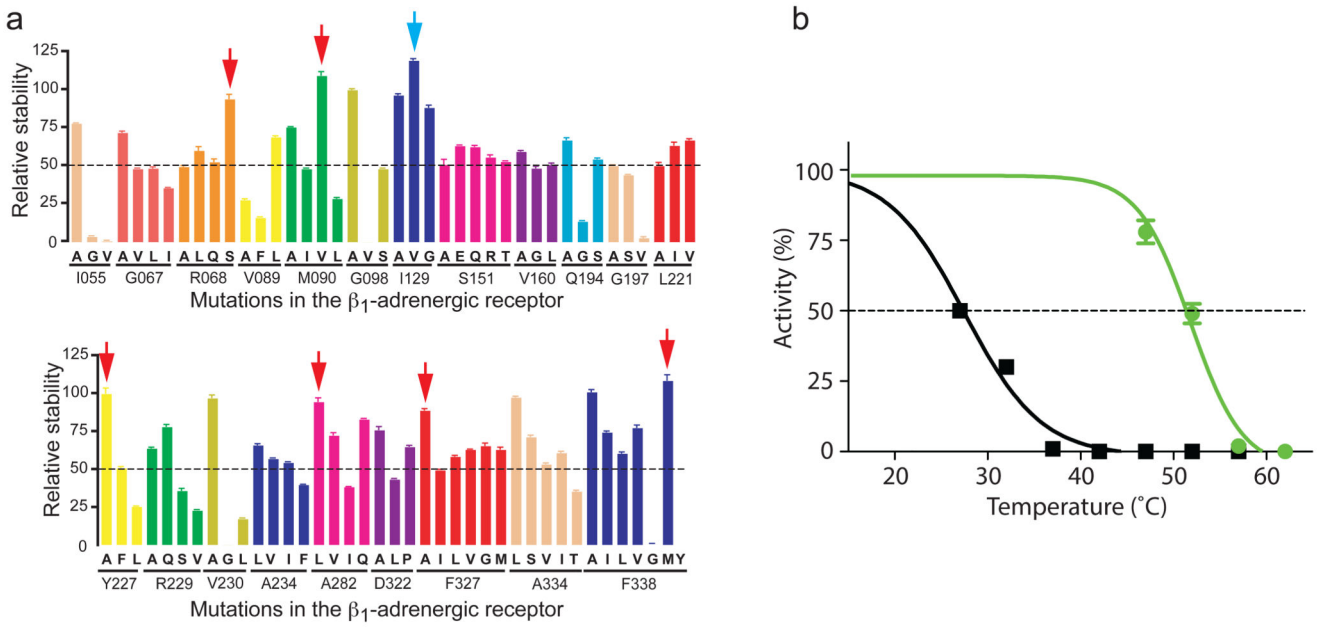

Figure 5.

Thermostabilisation of the β_1-adrenergic receptor. (a) Samples of each receptor variant were split into two tubes and were kept at 4ºC or heated at 32ºC for 30 minutes. The thermostability of each mutant is compared to the wild-type receptor (50%, dashed line). The most thermostable mutants (arrows) were used in combination with one another to generate an optimally thermostable mutant. Six thermostable mutations (red arrows) were found to give a highly stable mutant (β_1AR-m23) that has been crystallised bound to many different ligands. An ultra-thermostable mutant β_1AR-JM50 contained an additional three thermostable mutations, including I129V (blue arrow), which crystallised in LCP to yield a 2.1 Å resolution structure bound to cyanopindolol. Figure adapted from Serrano-Vega *et al*13. (b) Comparison of the thermostability of β_1AR (black squares) and β_1AR-m23 (green circles). Both receptors were solubilised in DDM and heated in the absence of radioligand. Figure adapted from Tate & Schertler78.

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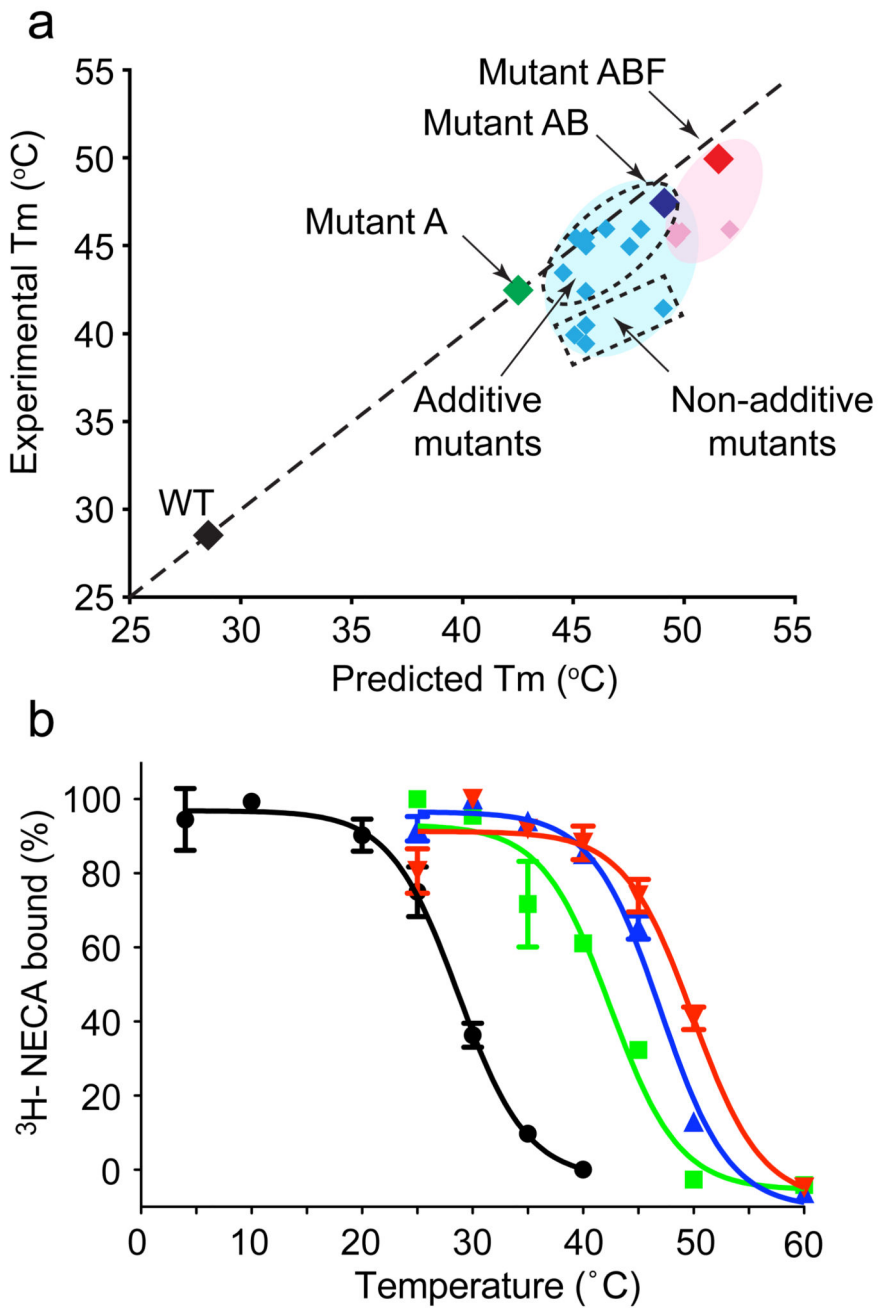

Figure 6.

Thermostabilisation of the adenosine A_{2A} receptor in the agonist-bound conformation. (a) The 16 most thermostable were systematically combined to make the triple mutant A_{2A}R-GL23. The most thermostable single mutation (mutant A, green diamond) was combined with each of the next 15 most stabilising mutations (B-P) and their stabilities were determined (blue diamonds). The most stable double mutant (mutant AB, dark blue diamond) was then combined with additive mutations that also improved the thermostability of mutant A (the predicted T_m was similar to the experimental T_m), to produce triple

mutants (red diamonds), the most stable of which was mutant ABF. In the paper describing this work40, mutant A is GL0, mutant AB is GL10 and mutant ABF is GL23. (b) The thermostabilities of DDM-solubilised $A_{2A}R$ mutants bound to ^{3}H-NECA were compared to the wild type receptor, which gave the following apparent T_ms: WT (black circles), 29ºC; GL0 (green squares), 42ºC; GL10 (blue triangles), 47ºC; GL23 (red inverted triangles), 50ºC. Figures adapted from Lebon et al40.

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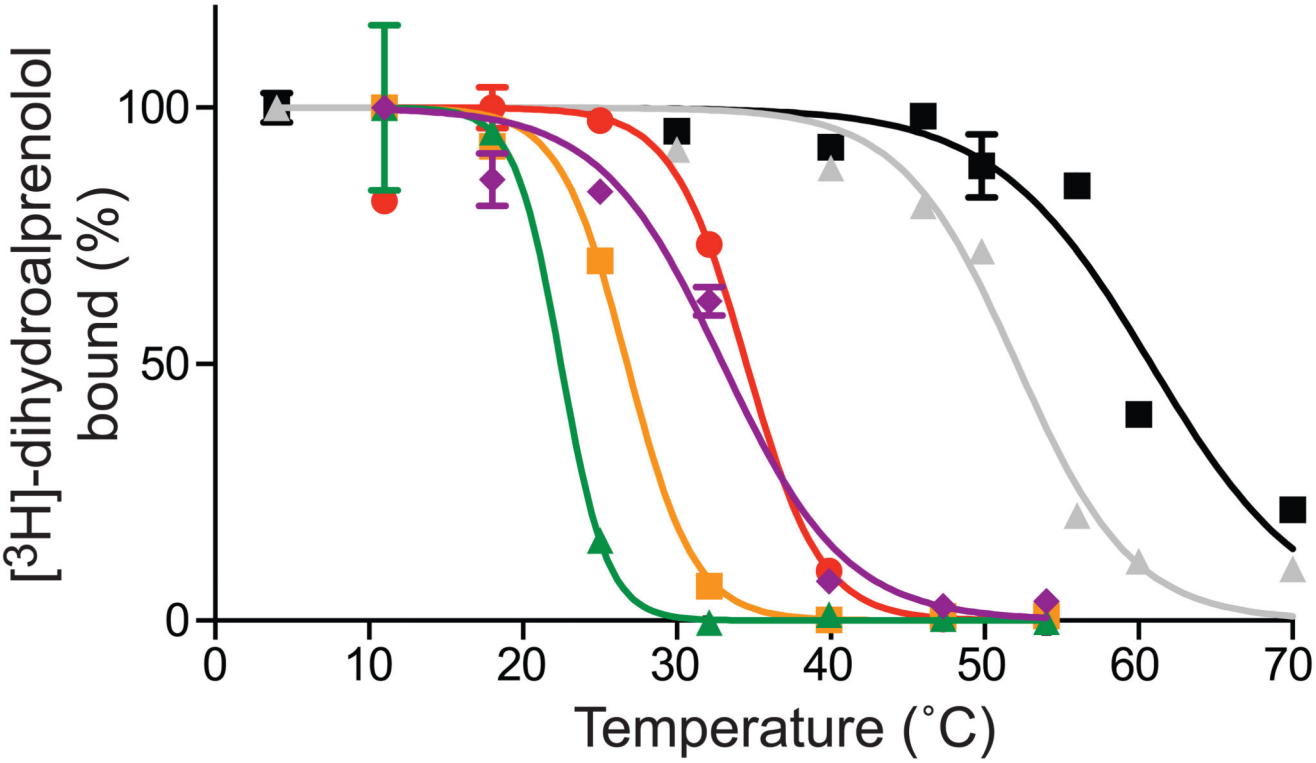

Figure 7.

Thermostability of the ultra-stable β_1AR mutant JM50. Thermostability was measured in a variety of different detergents (apparent T_m in parentheses): black squares, DDM (61ºC); grey triangles, DM (52ºC); red circles, NG (37ºC); purple diamonds, SDS (34ºC); orange squares, OG (27ºC); green triangles, HTG (23ºC). Detergent concentrations were all 2% wt/vol except for SDS that was 1% wt/vol. Assays were performed on detergent-solubilised membranes after expressing the receptor in *Escherichia coli*. Figure adapted from Miller & Tate41.

Table 1

Troubleshooting

	Problem	Possible reason	Solutions
9	No colonies on plate	PCR did not work	Check whether DNA is present by running the PCR reactions on a gel. Try using a different polymerase such as pfu. Change annealing temperature and/or change the primers.
		Transformation did not work	Check competency of cells using positive control. Try different amounts of PCR mix in the transformation.
16	No fluorescence in the plasma membrane of the wild type membrane protein	Cells growing poorly	Re-thaw a new batch of cells and ensure they are doubling every 24 hours.
	Very poor expression of the membrane protein	Complex membrane protein	Engineering membrane proteins to express better in mammalian cells is not easy. Try different host cells, different N-terminal fusions or homologues from different species. Co-expression in the presence of an inhibitor/ antagonist may also be helpful.
27	Very high counts are seen in every sample	Free radioligand is not being retained on the column, probably due to association with the detergent micelle	Try different gel filtration media. Use a different radioligand.
	Very low counts in every sample	The radioligand has not bound to the detergent-solubilised membrane protein	Ensure that the binding assay works for the membrane protein in membranes and optimise buffer conditions. Use the mildest possible detergent e.g. digitonin. Use the super [+] format. Add further stabilising agents e.g. 30% vol/vol glycerol during solubilisation. Use a higher affinity radioligand. Use a homologous membrane protein that is more stable.
	Measurements are extremely variable (> ± 10% of the mean)	Sample is not passing through the gel filtration column properly	Ensure samples are loaded slowly onto the centre of the columns. Make sure columns are uniform and do not contain cracks. Ensure pipetted volumes are all identical. Ensure centrifuge is properly balanced and runs without vibration.
33	All the values are the same at every temperature 20-70˚C	Detergent incorrect	If all the values are high, then use a harsher detergent. If all the values are near background, then use a milder detergent.
38	None of the mutations are additive with the most thermostable single mutation	The single mutation is stabilising a different conformation of the membrane protein compared to all the other mutations	Ignore the most thermostabilising mutation and combine the other mutations. Alternatively, perform scanning mutagenesis of the membrane protein containing the single mutation to thermostabilise that particular conformation.
42	The final thermostabilised mutant is not stable in NG or OG	Insufficient number of thermostabilising mutations	Repeat Steps 1-42, using the thermostabilised mutant as the starting construct rather than the wild type receptor.