Crystal Structure of a Ligand-free Stable Thyroid Stimulating Hormone Receptor Leucine-Rich Repeat Domain

Supplementary Information

Thermostabilising mutations

Six thermostabilising mutations in TSHR-JMG55™ are located on the surface of TSHR260 rather than in the core of the molecule (Figure 4C and D) and have side chains clearly visible in the 2Fo-Fc map when contoured at 1σ. Of the six mutated residues, one residue, Asp151, interacts with M22™ and with K1-70™ in their respective complexes with TSHR260 (Sanders et al. 2007, 2011) while five residues are not involved in the interactions of TSHR260 with M22™ or with K1-70™. RMSDs were calculated for the backbone atoms of the residues that have been mutated in TSHR260-JMG55™ (residues 63, 112, 143, 151, 169 and 253) for the pairs of molecules: TSHR-M22 and TSHR-A; TSHR-K1-70 and TSHR-A; and TSHR-M22 and TSHR-K1-70 (Supplementary Table 4). For most mutated residues, the RMSDs were less than 1 Å indicating that the mutations do not greatly alter the backbone structure of TSHR260. Larger RMSDs were observed for superimpositions of TSHR-M22 and TSHR-A for I253R located in the C-terminus, which has more disorder in the structures (Supplementary Table 4). Importantly, there were more significant changes in the side chains’ structure and interactions. These changes, including formation of hydrogen bonds, the hydrophobic effect and conformational entropy, most likely contributed to the thermostability of the whole protein (Pace et al. 1996, 2014) and have led to the increased thermostability of TSHR260-JMG55™.
His63 is in two different conformations in TSHR-M22 and TSHR-K1-70 indicating that this side chain is flexible in wild-type TSHR260 (Figure 6A). Indeed, the electron density in TSHR-K1-70 shows that His63 may exist in both conformations. In TSHR-M22, His63 makes non-covalent interactions with Thr88, a zinc ion and a water molecule, while in TSHR-K1-70 it hydrogen bonds to a water molecule. In TSHR260-JMG55™, this flexible His63 is replaced by the smaller Cys residue resulting in reduced conformational entropy, which could improve the thermostability of TSHR260. Cys63 does not make any intramolecular disulphide bonds, as there are no suitable Cys residues nearby. Mutation of His63 to other small residues such as Ala or Ser may have similar stabilising effects.

Flexible Arg112 is in two different conformations in TSHR-K1-70 and TSHR-M22 with poorly defined electron density in the TSHR-M22 structure (Figure 6B). Mutation to the smaller side chain, Pro, reduces conformational flexibility and could thereby increase thermostability. The $\phi$ torsion angle of the Arg112 backbone ($-67.6^\circ$ and $-71.8^\circ$ for TSHR-K1-70 and TSHR-M22 respectively) is similar to the restricted $\phi$ torsion angle of Pro ($-60.8^\circ$ for TSHR-A), all within the ideal Pro $\phi$ torsion angle of $-65^\circ \pm 20^\circ$. Both the restriction of flexibility of the backbone and the smaller, more rigid side chain of Pro could improve the thermostability of TSHR260.
Asp143 is in the same conformation in TSHR-K1-70 and TSHR-M22 with clear electron density, partly due to a hydrogen bond with Thr145 and a salt bridge with Lys146 (Figure 6C). The backbone φ torsion angles of residue 143 are -81.5°, -77.2° and -82.2° for TSHR-K1-70, TSHR-M22 and TSHR-A respectively, all within the ideal Pro φ torsion angle of -65°±20°. In this case, the restriction of the backbone torsion angle by mutating Asp 143 to Pro is sufficient to increase the thermostability of TSHR260. D143P mutation causes the loss of hydrogen bond to Thr145, which allows the Cα atoms of residues 144-146 to move 1 Å away from where the side chain of Asp143 was, possibly releasing tension in the structure and allowing Lys146 to form a salt bridge with Asp120 in TSHR-B thereby increasing the thermostability.

Asp151 forms a salt bridge with Arg28-HC of M22™ and water-mediated hydrogen bonds to the light chain of K1-70™ (Figure 6D). Asp151 also forms hydrogen bonds to the backbone of Phe153 and Ile152 of TSHR in the TSHR-M22 and TSHR-K1-70 structures respectively. Mutation of Asp151 to Glu in TSHR260-JMG55™ would disrupt these interactions with M22™ and K1-70™. The side chain of Glu151 does not make any alternative polar interactions with the TSHR molecule, which one might expect to explain the increase in thermostability. Instead, the longer side chain of Glu protrudes from the surface of TSHR260 allowing it to make more hydrogen bonds with the solvent than would be possible for an exposed Asp residue, and thereby increasing the thermostability of the TSHR260.

The mutation of Val169 to the polar residue Arg allows the formation of new hydrogen bonds with the glycan attached to Asn198 in TSHR-A (Figure 6E). This reduces the flexibility of both the glycan and Arg and improves the thermostability of the TSHR260. Additionally, removal of the hydrophobic Val residue from the surface and the introduction of the exposed polar side chain of Arg that can make hydrogen bonds to the solvent, further contributes to the stability of TSHR260.
Similarly to V169R, mutation of Ile253 to Arg allows the formation of stabilising new salt bridge interactions with Glu251 and Asp232 (Figure 6F). These residues both make water-mediated contacts with M22™. Therefore, the mutation I253R, allows Glu251 and Asp232 to form polar interactions in the absence of antibody, thereby stabilising the antibody-free structure of TSHR260. Additionally, the exposed polar side chain of Arg makes hydrogen bonds to the solvent that are not possible for hydrophobic Ile, further contributing to the stability of TSHR.

**Comparison of the ligand-free and autoantibody-bound TSHR260 structures.**

The backbones of ligand-free and autoantibody (M22™ or K1-70™) bound TSHR260 structures are essentially unchanged (Figure 4, Supplementary Table 3) except for disordered N-termini. However, many of the side chains of TSHR260 residues interact with the antibodies in the structures TSHR-M22 and TSHR-K1-70. Some of these residues do not change conformation when the antibodies bind to TSHR260, while others rearrange their non-covalent interactions to form new non-covalent interactions with the antibodies.

In the TSHR-M22 structure, 25 TSHR260 residues interact with M22™ (Sanders et al. 2007) and in the TSHR-K1-70 structure, 28 TSHR260 residues interact with K1-70™ (Sanders et al. 2011) with some overlap between interacting residues (17 TSHR260 residues make non-covalent interactions with both M22™ and K1-70™). Of the 36 residues that form interactions with M22™, K1-70™, or both antibodies, 17 residues do not change conformation when either M22™ or K1-70™ binds: i.e. Gln55, Thr56, Lys58, Arg80, Tyr82, Lys102, Thr104, Glu107, Arg109, Asn110, Lys129, Thr181, Lys183, Asp203, Tyr206, Glu251 and Asn256. Some flexible side chains with poorly resolved electron density in TSHR-A (Glu35, Asp36, Arg38, Lys42, Lys57, Arg80, Lys129, Ile155, Lys183, Lys209 and Arg255) have clearer electron density in TSHR-M22 and/or TSHR-K1-70 structures.
suggesting they are stabilised by their interactions with M22™ and/or K1-70™ (Supplementary Figure 4). If further thermostability was required, mutation of these residues to amino acids with smaller side chains, may improve the thermostability of ligand-free TSHR260 however some of these mutations may affect binding of M22™ and K1-70™ to the receptor.

When M22™ binds, a number of small structural rearrangements of the side chains of TSHR260 occur (Supplementary Figure 4A and B). The flexible side chain of Arg38 is stabilised by interaction with Thr57-HC of the heavy chain of M22™-Fab (M22-HC). The side chain of His105 rotates 84° to make water-mediated hydrogen bonds with M22-HC residues, Thr30-HC, Asp52-HC and Thr53-HC. Asp151 (which is mutated to Glu in TSHR260-JMG55™) changes conformation to interact with Arg28-HC of M22-HC, distorting the positions of Ile152 and Phe153 of TSHR260. LC residues 49 to 58 of M22™ form a network of interactions with Glu157, Tyr185, Asn208, Lys209, Gln235, Glu251 and Arg255 of TSHR260 causing changes in conformation and moving the backbone of the C-terminus of TSHR260 up to 1.5 Å away from M22™. When there is no antibody bound, Glu157 and Tyr185 form a non-covalent interaction with each other that stabilises the ligand-free TSHR260-JMG55™.

In addition, when K1-70™ binds, structural rearrangements of the side chains take place (Supplementary Figure 4C and D). Glu35, Asp36 and Arg38, which have weak, disordered electron density in TSHR260-JMG55™ are stabilised by interactions with Arg54-LC, Ser56-LC, Asp96-HC, Arg101-HC and Tyr102-HC of K1-70™ and the backbone moves up to 2.3 Å towards the N-terminus. The side chain of Lys42 moves 1.7-2.3 Å to bind to Asp31-HC and Ser28-HC of K1-70™. His105 and Glu107 change conformation to bind Asn98-HC and Trp97-HC of K1-70™. The side chain of Phe130 rotates 115° to form π-stacking interactions
with Tyr99-HC of K1-70\textsuperscript{TM}. Asp151 (which is mutated to Glu in TSHR260-JMG55\textsuperscript{TM}) makes water-mediated hydrogen bonds with Lys66-LC and Ser93-LC of K1-70\textsuperscript{TM}. Finally, Phe153 rotates 107° to avoid steric clashes with Ser93-LC of K1-70\textsuperscript{TM}.

These series of side chain changes illustrate how the binding of antibodies makes small changes to the TSHR260 structure and improve its stability by making non-covalent interactions with predominantly charged or polar residues on the surface of the TSHR260.