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Engineering salt bridge networks between transmembrane helices confers thermostability in G-protein Coupled Receptors

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Abstract

Introduction of specific point mutations has been an effective strategy in enhancing the thermal stability in detergents that aid the purification of mono-dispersed G-protein coupled receptors (GPCRs). Our previous work showed that a specific residue position on transmembrane helix 6 (TM6) in class A GPCRs consistently yields thermostable mutants. The crystal structure of human chemokine receptor CCR5 also showed increased thermostability at two positions, A233D^{6.33} and K303E^{7.59}. With the goal of testing the transferability of these two thermostabilizing mutations in the other chemokine receptors, we tested the mutations A237D^{6.33} and R307E^{7.59} in human CCR3 for thermostability and aggregation properties in DDM detergent solution. Interestingly, the double mutant exhibited a 6–10 fold decrease in the aggregation propensity of the wild type protein. This is in stark contrast to the two single mutants whose aggregation properties resemble more to the wild type (WT). Moreover, Unlike in CCR5, the two single mutants separately showed no increase in thermostability compared to the wild type CCR3, while the double mutant A237D^{6.33}/R307E^{7.59} confers an increase of 2.6°C in the melting temperature compared to the WT. Extensive

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Supporting Information

Sequence alignment between the template CCR5 and the target CCR3 (excluding N and C-termini), Initial conformation of the receptor-micelle complex built using CHARMM-GUI, RMSD of the TM C α regions of the protein for the three replicate simulations performed on each CCR3 mutants, average non-bonded protein and protein-DDM interaction energy along with standard error bars, fraction of variance covered by different PCs, dot products for the first two principal components for each system combinations separately, RMS deviation of other clusters from the most populated cluster 1 obtained from the PC based FESs, persistence of polar network obtained from the distribution of salt bridge distance between specific donors and acceptors, conformational clustering between interhelical packing and helicity, visual representation of water penetration, hydrophobic coverage provided by DDM to the TM6-TM7 regions of the protein for each CCR3 mutants, comparison of strength of polar network between TM3, TM6 and TM7 of CCR5, CCR9, CCR2, the double mutant CCR3 structures, the comparison of the population distribution of the donor-acceptor hydrogen bond distances between key residues forming the extensive salt bridge network in the two conformation clusters obtained from the free energy surface of the double mutant A237D^{6.33}/R307E^{7.59} and the sequence alignment of C-C chemokine receptors CCR1-CCR10 showing the viability of introducing the mutation at 6.33 and 7.59 positions for C-C chemokines lacking crystal structures and This information is available free of charge via the internet at <http://pubs.acs.org>.

Disclosure Statement

The authors declare no competing financial interest

all-atom molecular dynamics (MD) simulations in detergent micelles show that a salt bridge network between transmembrane helices TM3, TM6 and TM7 that is absent in the two single mutants confers stability in the double mutant. Free energy surface of the double mutant shows conformational homogeneity compared to the single mutants. An annular n-dodecyl maltoside (DDM) detergent layer packs tighter to the hydrophobic surface of the double mutant CCR3 compared to the single mutants providing additional stability. The purification of other C-C chemokine receptors lacking such stabilizing residues may benefit from the incorporation of these two point mutations in appropriate TM regions.

Graphical Abstract

The free energy surface of thermostable mutant of the chemokine receptor CCR3, a class A GPCR, shows conformational homogeneity and less prone to aggregation.

Keywords

G protein-coupled receptor (GPCR); chemokine; mutagenesis; micelle; aggregation; molecular dynamics

Introduction

G-protein coupled receptors (GPCRs), the largest class of integral membrane proteins, are key players in the complex and diverse regulation of the cellular signaling and represent major target for widely marketed drugs.^{1,2} Structure and function studies using purified GPCRs that are pivotal for structure-based drug discovery, are however challenging due to the inherent flexibility and instability of GPCRs in detergents.^{3,4} Typically, GPCRs denature and aggregate during its extraction from cell membranes into aqueous detergent solutions due to its instability in detergents. Strategies such as the truncation of flexible regions of the GPCRs for making fusion constructs with stabilizing protein like T4-lysozyme and BRIL^{5,6} and deriving thermostable mutations with systematic alanine scanning of the entire receptor have been used successfully to stabilize GPCRs in detergent solutions.^{7,8}

Chemokine receptors belong to class A GPCRs and modulate multiple immune functions. They activate various signaling pathways in the cell depending on the chemokines that

activate these receptors. They form a major drug target class and therefore structure and dynamics of chemokine receptors are very vital to drug design.⁹ Chemokine receptors are thus much sought after GPCRs for stabilization and purification in detergents.¹⁰ Tan et al. stabilized and purified the human chemokine receptor type 5 (CCR5) in detergents by engineering several point mutations of the receptor.¹¹ Most notably, the mutation A233D^{6.33} was introduced to facilitate an ionic-lock formation between A233D^{6.33} and R11^{3.50} thus stabilizing the inactive conformational state that has short TM3-TM6 distance in class A GPCRs. Here we have used the Ballesteros-Weinstein GPCR residue numbering system (referred as BW numbering hereafter).¹² The first number is the transmembrane (TM) helix in which the residue is present and the second number is the residue position with respect to the most conserved residue in that helix which is given a number 50. The mutation K303E^{7.59} was also introduced to engineer a salt bridge with the Y72^{2.42} on TM2. These two single point mutations K303E^{7.59} and A233D^{6.33} showed an increase of 1.7 °C and 4.4 °C increase in the apparent melting temperature compared to the WT respectively.¹¹ In our previous studies on developing a computational method to predict single point mutations¹³ we observed that position 6.33 on TM6 consistently yielded a thermostable mutant in β 1-adrenergic receptor, neurotensin receptor NTSR1, and adenosine receptor A_{2AR}.^{14, 15} With the goal of identifying the transferability of the two thermostabilizing mutations identified in CCR5 to other chemokine receptors, we have used a combination of all-atom Molecular Dynamics (MD) simulations and experimental testing and identified the double mutant A237D^{6.33}/R307E^{7.59} of the human chemokine receptor, CCR3 as thermostable in detergents. CCR3 is an important drug target with no crystal structure available.¹⁶ The double point mutations A237D^{6.33} and R307E^{7.59} in CCR3 (system referred as A237D^{6.33}/R307E^{7.59} hereafter) showed an increase of 2.6 °C in melting temperature of purified CCR3-T4L construct in detergents. The double mutant A237D^{6.33}/R307E^{7.59} also showed a ten-fold improvement in the ratio of peak volume of aggregate to monomer in size exclusion chromatography measurements over the wild type CCR3 (WT CCR3). We performed MD simulations of the double mutant CCR3 in n-dodecyl β -maltoside (DDM) detergent micelles and our simulations show the formation of an extensive salt bridge network between TM helices TM3, TM6 and TM7. Such an extensive salt bridge network could likely be the mechanism behind the synergy of stabilization in the double mutant CCR3 compared to the two single mutants A237D^{6.33} and R307E^{7.59}. Based on the analysis of available crystal structures of chemokine receptors and sequence alignment to other chemokine receptors we predict that such inter-helical salt bridge networks could be extended to other chemokine receptors for which crystal structures are not available in the public domain. The findings of this work show that structure-enhancing mutations can be transferred effectively between GPCRs with high sequence identity and similar functions.¹⁷

Experimental Methods:

CCR3-T4L Cloning and Expression

The WT human CCR3 cDNA was synthesized with sequence optimization for insect cell expression by GeneArt and then cloned into a modified pFastBac1 vector (Invitrogen), which contained an HA signal sequence at the N-terminus prior to the receptor sequence, and a PreScission protease site followed by a FLAG tag and a 10xHis tag at the C-terminus. A

truncated T4 lysozyme was inserted into the third intracellular loop. The mutations were introduced by standard QuickChange PCR. Recombinant baculovirus was generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen) and used to infect H5 insect cells at a density of 1×10^6 cells per ml. Culture flasks were shaken at 27 °C for 72 h, then cells were harvested by centrifugation and stored at –80 °C until use.

Protein Purification

Cells were lysed by thawing frozen cell pellets in a hypotonic buffer containing 10 mM HEPES, pH 7.5, 10 mM MgCl₂, 20 mM KCl, 5 µg/ml Pefabloc SC (Roche) and EDTA-free complete protease inhibitor cocktail (Roche). The cell suspension was filtrated by a 100 µm filter followed by the cell lysis via nitrogen decompression at 50 bar for 30 min (4635 Cell Disruption Vessel, Parr Instrument GmbH). The lysate was centrifuged and the pellet was suspended under usage of a Wheaton Homogenizer in hypotonic buffer (+ 50 µM BMS-639623 antagonist¹⁸ and 10 µg/ml Pefabloc SC). After the addition of glycerol up to 30% (v/v) and the drop wise addition of n-dodecyl-β-maltopyranoside (DDM, Anatrace) up to 1% and cholesteryl hemisuccinate (CHS, Sigma) up to 0.1%, the membranes were solubilized over night at 4 °C. The supernatant solution was isolated by centrifugation at $100,000 \times g$ for 1 h, and supplemented with 10 mM buffered imidazole, pH 7.5, and 150 mM NaCl. The solution was incubated with TALON Superflow Metal Affinity Resin (Clontech, 3.75 ml of resin per 1 l of original culture volume was used) for 2.5 hours at 4 °C. After binding, the resin was poured into a column and washed with 25 column volumes of 25 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM, 0.005% (w/v) CHS, 50 mM imidazole, 8 mM ATP, 10 mM MgCl₂ and 25 µM BMS-639623 antagonist. The CCR3 protein was then eluted by five column volumes of 25 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM, 0.005% (w/v) CHS, 200 mM imidazole and 25 µM BMS-639623 antagonist, 10 mM MgCl₂, and exchanged into 25 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM, 0.005% (w/v) CHS, 25 µM BMS-639623, and 10 mM MgCl₂ using a Sephadex G-25 in PD-10 Desalting Columns. Subsequently, the sample was applied to the Superdex200 Increase column (GE Healthcare) into 25 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM, 0.005% (w/v) CHS, and 25 µM BMS-639623 antagonist. The protein containing fractions were pooled and concentrated to 10 µM using a 50 kDa molecular weight cut-off Amicon concentrator (Merck KGaA).

Size Exclusion Chromatography measurements:

The concentrated solution was centrifuged for 5 min at 16,100 g and subsequently applied on the SEC (Superdex200 Increase column) with the SEC buffer (25 mM HEPES, pH 7.5, 10 % Glycerol, 150 mM NaCl, 0.05 % DDM, 0.005 % CHS; + 5 µg/ml Pefabloc SC, + 25 µM BMS-639623 antagonist). 0.5 ml fractions were collected. The protein containing fractions (A280 signal), were sampled (10 µl 4× Lämmli + 30 µl eluate) in Lämmli buffer and applied to a SDS PAGE. The fractions containing the target protein named first peak / aggregates, transient band and second peak/protein were pooled separately. Afterwards the concentration for each of the 3 pools was determined by NanoDrop measurements. The samples were concentrated separately in Amicons (10 µM). Subsequently the concentration was measured again. The protein was stored for further biophysical analysis at 4 °C.

Usually, the monomer elution from the column is characterized by a higher retention time or volume and a molecular weight (in kDa) in between that of protein aggregate and free micelle.¹⁹

Nano-Differential scanning fluorimetry (nanoDSF) measurements of CCR3 mutant stability and melting temperature

The NT48 high sensitivity capillaries were filled and the measurement was executed with a temperature slope of 1 °C/min from 15 °C to 95 °C. The recorded parameters are the fluorescence ratio of 350/330, the fluorescence at 330 nm and 350 nm and the light scattering. Unfolding transitions were indicated by the software PR ThermControl (NanoTemper version 2.03). Melting points, which were missed by the detection software, were added manually.

Computational methods

Generating CCR3 structural model and MD simulation details

The three dimensional structural model of CCR3 was generated using the homology modeling software MODELLER.²⁰ We used the crystal structure of the inactive state of human CCR5 bound to an antagonist maraviroc (PDB ID: 4MBS)¹¹ as the template for homology model generation. The sequence of CCR3 has 56% identity in the TM region with CCR5 and the sequence alignment of these two receptors done using ClustalW²¹ is shown in Fig.S1 of the Supporting Information. This alignment has only one gap in the ICL3 region of the target CCR3 (Fig. S1) which shows the higher confidence level in the homology models generated for CCR3 in this study. The N and C-termini residues which show low sequence similarity between the target CCR3 and the template CCR5 have been omitted (residue number 1 to 21 and 296 to 331 in the N and C termini of the CCR3 sequence).²² We generated 10 homology models of CCR3 and the best model was chosen based on the DOPE score as implemented in MODELLER. The structural models of the single point mutants of CCR3, A237D^{6,33} and R307E^{7,59}, and the double mutant A237D^{6,33}/R307E^{7,59} were generated from the WT structural model and the mutations were incorporated using VMD.²³ After obtaining the single and double mutants of CCR3 these structures were subjected to 5000 steps of conjugant gradient vacuum energy minimization each in NAMD²⁴ for ensuring side chain relaxation and removal of steric clashes in the starting structures. Thus, we generated three CCR3 structural models, referred as A237D^{6,33}, R307E^{7,59} (two single mutants) and A237D^{6,33}/R307E^{7,59} (the double mutant) hereafter. These four structural models (WT and three CCR3 mutants) were embedded separately in preformed DDM micelles composed of 192 DDM monomers each using the micelle builder utility in CFLARMM-GUI.²⁵ The number of DDM monomers in protein – detergent complex (Fig. S2, Supporting Information) was about 30% higher than the aggregation number of detergents in pure micelle and reflects the total number of detergent molecules bound to membrane proteins in biochemical assays.^{26,27} Each of these systems was solvated by explicit TIP3P water molecules in a triclinic box (approximate dimension of 10.5 nm X 10.2 nm X 9.7 nm) separately and chloride counterions were added for charge neutrality. We used the software GROMACS²⁸ (version 2016.4) in combination with the all-atom CHARMM36²⁹ force field for carrying out all the MD simulations in this study. MD simulations were

performed at 300 K in this study. Solvent and the receptor-detergent micellar complex were independently coupled to a temperature bath with a relaxation time of 0.1 ps.³⁰ Pressure was calculated using molecular virial and held constantly by weak coupling to a pressure bath with a relaxation time of 0.5 ps. Each system was first subjected to a 5000 step steepest descent energy minimization for removing bad contacts.³¹ Then, the systems were heated for 100 ps for attaining the desired temperature under constant temperature-volume ensemble (NVT). Equilibrium bond length and geometry of water molecules were constrained using the SHAKE algorithm.³² The classical equations of motion were integrated using the leap-frog algorithm³³ with a time step of 2 fs. Center of mass motion was removed every 20 fs. The short range electrostatic and van der Waals (VDW) interactions were estimated per time step using a charge group pair list with cut-off radius of 8 Å between the centers of geometry of the charged groups. Long range VDW interactions were calculated using a cut-off of 14 Å and long range electrostatic interactions were treated using the particle mesh Ewald (PME) method.³⁴ Temperature was kept constant by applying the Nose-Hoover thermostat.³⁵ Parrinello-Rahman barostat³⁶ with a pressure relaxation time of 2 ps was used for the attainment of desired pressure for all simulations. The simulation trajectories were saved each 200 ps for analysis. The protein atoms were position restrained using a harmonic force constant of 1000 kJ mol⁻¹ nm⁻² during the NVT equilibration stage while the detergent and water molecules were allowed to repack around the protein. The system was further equilibrated at NPT by reducing the force constant on protein atoms from 5 kJ mol⁻¹ nm⁻² to zero in a stepwise manner for 5 ns each while having the pressure coupling on. We also performed an additional 15 ns of unrestrained simulation before starting the actual production run. This accounts for a total 40 ns of NPT equilibration prior to the production run. We performed three production runs each 600 ns long starting from three independent sets of initial velocities for each system. Three independent simulations (each 600 ns long) were performed for the WT CCR3 and three mutants (two single mutants and one double mutant).

MD Analysis Methods

To test for convergence of each simulation, we calculated the root mean square deviation (RMSD) in coordinates of the Cα atoms of the residues in the transmembrane region with respect to the starting structure, as a function of time, shown in Fig. S3. The moving average of the RMSD showed convergence at about 2.5 Å. To compare the differences in the protein packing in the two single mutants and the double mutant of CCR3, we calculated the total non-bond energy (van der Waals +Coulombic energy) of the double mutant and the two single mutants. As shown in Fig. S4 the interhelical packing energy is more favorable in the double mutant compared to the two single mutants in qualitative agreement with the melting temperature measurements.

Representative structure calculations from MD simulation trajectories:

Representative snapshots from the most occupied conformation cluster for each system were chosen for structure representation in the figures. The conformation clustering of the snapshots in the MD trajectories was done using RMSD based clustering method using the GROMACS modules gmx rms and gmx cluster with a 1.2 Å cut-off on the concatenated trajectory from three simulations (a total of 1.8 μs simulation time) for each system. The

most representative structure of the most populated cluster was calculated as the frame that has the smallest RMSD to the center of this cluster conformation. Snapshots were rendered using VMD²³ and PYMOL.³⁷

Principal Components Analysis and Free Energy Surface Generation: For each system (WT and three mutant CCR3), we merged the three independent MD runs into one concatenated trajectory. We then performed the principal component analysis using the gmx covar module of GROMACS for each system using covariance matrix of the Ca atoms of all the residues. The gmx sham module of GROMACS was used to compute the probability of the microstates and convert it to free energy. The free energy surface thus generated was projected on the principal component space covered by PC1 and PC2. We then clustered the MD snapshots by the values of PC1 and PC2 using the in-house extractcluster.py script in MATLAB and calculated the population of each of these conformational clusters (TABLE S1, Supporting Information).

Details of other MD Analysis: We calculated the radial density distribution function of DDM head and tail groups from the center of mass of the protein using the gmx density utility of GROMACS. The same protocol was used for computing the distribution of water molecules from the center of mass of the protein. The spatial density distribution function (SDF) was computed using the gmx spatial module of GROMACS and an isovalue of ~10 is chosen to draw the detergent iso surfaces in VMD for all the systems. The distribution of the side chain rotamers of residues (angle X¹ and X²) was calculated from the MD simulation trajectories using the gmx chi utility of GROMACS. The solvent accessible surface area (SASA) in nm² was calculated using the utility gmx sasa in GROMACS. The number of water molecules penetrating into the TM core of the protein was calculated as follows. We defined the region between TM6 and helix8 on the intracellular side as covered by residue range 218 to 295 at the end of helix8 excluding the loop regions. We calculated the average number of water molecules across the MD trajectories that are within 4 Å of these residues.

Calculation of interhelical hydrogen bonds and van der Waals

interactions: The van der Waals packing analysis is performed using the contactFreq.tcl script of VMD. The TM regions were chosen by visually inspecting the protein in VMD by excluding the loop regions. Only the polar and non-polar contacts that were found sustained for above 40% of the entire trajectory were included in this analysis. A hydrogen bond was considered to be formed if the donor-acceptor distance and the donor-hydrogen-acceptor angle was within 3.5 Å and 120 ° respectively.³⁸

Results and Discussion

Melting Temperature and size exclusion chromatography measurements of CCR3 mutants

Fig. 1A to 1C shows the nano-DSF curves to get the melting temperature of the populations of the folded versus the unfolded conformations of the CCR3 single and double mutants as a function of temperature. The double mutant showed an increase of 2.6 °C in the melting temperature compared to the WT while the melting temperature of the CCR3 single mutants was similar to that of the WT. The most notable experimental observation follows Fig. 1D

showing the size exclusion chromatography (SEC) profile for the three CCR3 mutants. The elution corresponding to the first peak was proportional to the amount of the aggregated protein whereas a second peak was indicative of the protein in a pure, monodispersed form. The lower peak intensity of the aggregate/monomer peak ratio indicated the colloidal stability of the protein under consideration. The SEC profiles of the two single mutants were similar to the WT in the sense that they both show similar/worse aggregation propensity to that of WT that rendered the latter protein difficult to extract in the absence of suitable point mutations (Fig. 1D). The quantitative experimental data obtained from nanoDSF and SEC is given in TABLE 1. It can be seen that the double mutant showed a marked improvement in stabilizing a monodispersed solution of CCR3 (Fig. 1D) in the SEC experiments where the aggregate/monomer peak ratio was diminished 6 – 10 fold when compared to that of the WT in three replicate experiments (last entry, TABLE 1). It can be seen that in addition to improving thermal and colloidal stability the incorporation of the double mutant also gave rise to a higher yield of the recovered protein (column 2, TABLE 1) as compared to the single mutants. This might be an added advantage while attempting the purification of CCR3 with these two mutations incorporated simultaneously. The purifications were done thrice for WT, twice for the double mutant and once for each of the single mutant. For the WT we report the average of four different melting points (47.1, 47.9, 47.9 and 47.8 °C). For the two single mutants three different melting points correspond to three different fractions collected from the same purification step (TABLE 1). The double mutant showed two similar melting points (50.2 and 50.3 °C) from two fractions corresponding to the two-separate purification step (peak ratios 0.53 and 0.38, data corresponding to the second purification not listed in TABLE 1). This points at the reproducibility of the colloidal and thermal stability data obtained by independent experiments for the CCR3 double mutant. To confirm that the double mutant CCR3 receptor folds into the inactive state and binds to an antagonist we measured the SEC profile of the double mutant in the presence of 25 μ M (red trace) or absence (black trace) of the antagonist BMS-639623. Purification was done both from 1L expression volume. The shift of the SEC profile to a monomeric peak in the presence of antagonist BMS-639623 shown in Fig. 1E indicates binding of the antagonist to stabilize the double mutant receptor.

To understand why the double mutant exhibits synergy and confers thermal and conformational homogeneity to the receptor while the two single mutants do not we performed all-atom MD simulations (results shown below) to provide insights into the structural and dynamical basis for the thermostability and conformational homogeneity with propensity to aggregate.

The free energy surface from the MD simulations of the double mutant (A237D^{6.33}/R307E^{7.59}) shows conformational homogeneity

We calculated the free energy surface for the single and double CCR3 mutants to investigate the conformational heterogeneity and its effect on the thermostability and aggregation propensity of the mutations studied here. We have shown that the free energy surface projected on the principal components is smoother and conformationally homogenous for the thermostable mutant of β_1 -adrenergic receptor compared to the WT.³⁹ The free energy

surface for the WT CCR3 and the three mutant systems projected on to the first two principal components (PC1 and PC2) are shown in panel A of Fig. 2.

The Principal Component Analysis was done to understand the dominant motions in the receptor sampled during the MD simulations. The fraction of variance in the eigenvalues of the PCs (shown in Fig. S5A) shows that nearly 80% of the variance is captured by the four PCs for each CCR3 mutant. The convergence of MD trajectories was assessed by computing the dot product of the first two principal components (PC1 and PC2 accounting for almost 60–75% of the principal motion of the four systems, Fig. S5A, Supporting Information) obtained from the concatenated trajectories for each system. We obtained the absolute dot product in the range of 0.65–0.9 for PC1 and 0.5–0.8 for PC2 which suggests that the eigenvectors corresponding to PC1 and PC2 are similar in all the four systems (Fig. S5B & C). It follows Fig. 2A that the double mutant is conformationally homogenous (in PC space) with one most prominent free energy minimum (marked as 1 in the figure) with ~80% of the MD snapshots belonging to this conformation cluster. The cluster 2 shown in Fig. 2A for the double mutant is energetically less favorable and less populated than the cluster 1. The free energy surface of the single mutant A237D^{6.33} is rugged and shows conformation heterogeneity implying that it has a broad free energy minimum with multiple microstates. To understand the differences in the conformation of the representative structures from each conformation cluster we compared the RMSD in coordinates between the representatives from various clusters in each system. The RMSD difference in coordinates for each residue between the representative structure for each conformation cluster and the representative structure of cluster 1 of the same system are shown in Fig S6. This was calculated by taking the most populated cluster (cluster 1) as the reference for each system. The RMSD ranges from 0.2 nm to 1.4 nm (Fig. S6, Supporting Information). For the mutant R307E^{7.59}, we observe four free energy wells which are distant from each other in the PC space (Fig. 2A). Thus R307E^{7.59} single mutant is more conformationally heterogeneous even compared to A237D^{6.33} mutant. To verify if the structures in any of these clusters have unraveled we calculated the helicity of the TM regions averaged over the MD snapshots extracted in each cluster. The average helicity for each residue calculated from the MD trajectories is shown as a heat map (red to blue implies low to high helicity) in Fig. 2B. The helicity heat map is shown on the most representative structure of the top conformational cluster with highest occupancy for each system. The single mutants along with the WT showed loss in helicity compared to the double mutant. The loss in helicity comes from the conformations in cluster 2 to cluster 4 that are relatively less populated than the most populated cluster, cluster 1, in every system (TABLE S1, Supporting Information). However, the helicity of the TM regions in the double mutant in cluster 2 is comparable to that in cluster 1 (TABLE S1, Supporting Information). We speculate that the conformations in clusters 2 to 4 in the single mutants and WT are responsible for the structural unraveling and thus show propensity to aggregate. We have shown that the single mutants and the WT CCR3 undergo structural unraveling during the dynamics (Fig. 2B and Table S1 of the Supporting Information) that could trigger aggregation. Although the structural unraveling followed by aggregation is not directly observed from our simulations, there are experimental evidences that support this. Ma et al. have shown that functionally active human Mu opioid receptor in monomeric/dimeric form isolated from the aggregated fraction while purification in detergents have higher helical

content compared to the aggregated fraction using circular dichroism.⁴⁰ Felce et al. have also recently shown that for certain Rhodopsin-family receptors the dimerization interfaces are predominantly located within EL1, TM3, TM4 and IL2 regions using BRET and single molecule spectroscopy. Their findings suggest that the TM regions are expected to retain a well-ordered and more tightly packed conformation to promote the dynamic cross-talk between two monomers and to offset aggregation.⁴¹ These reports support our hypothesis that the single mutants and the WT CCR3 found mostly in aggregated states during experiments is related to their loss of secondary structures in various conformational clusters as evinced by Fig. 2B and Table S1 (Supporting Information).

Increased inter-helical packing interactions through a network of salt bridges between TM3, TM6 and TM7 contribute to the stability of the double mutant compared to the single mutants

Analysis of interhelical hydrogen bonds that sustain in more than 40% of the MD snapshots show the presence of extensive salt bridge interactions between TM helices TM3, TM6 and TM7 that strengthen the interhelical packing.^{11, 42} Our simulations suggest that the simultaneous mutation of A237D^{6.33} and R307E^{7.59} generates a network of salt bridges between TM3, TM6 and TM7 as shown in Fig. 3. As seen in Fig. 3A, the seven residues Y301^{7.53}, E307^{7.59}, R329^{6.35}, K236^{6.32}, D237^{6.33}, R109^{3.50}, D108^{3.49} make up the extensive salt bridge/hydrogen bond network in the double mutant. Apart from Y301^{7.53} these salt bridges are not observed in both the single mutants of CCR3. In the single mutant A237D^{6.33}, the residues K236^{6.32} and R109^{3.50} do not come close to the D237^{6.33} due to the electrostatic repulsion between K236^{6.32} and R239^{6.35}. This repulsion is reduced when R307^{3.50} is mutated to Glu. The repulsion between R239^{6.35} and K236^{6.32} leads to differences in the side chain rotamer distributions of residues R239^{6.35} and K236^{6.32} in TM6. In the double mutant compared to the single mutants, the side chains of R307^{3.50} and K236^{6.32} adopt different orientations as shown in Figs. 4 (around 100°), thus facilitating the formation of the salt bridge network shown in Fig. 3A. There is an insignificant population of this side chain orientation of R307^{7.59} and K236^{6.32} in the A237D and R307E single mutants. The distribution of the side chain rotamers of R239^{6.35} (angle X¹) and K236^{6.32} (angle X¹ and X²) calculated from the MD simulation trajectories for the three mutants are shown in Fig. 4. The persistence of these salt bridge interactions during the MD simulations are calculated as the distribution of the distance between the atoms participating in such interactions and compared across three different systems (Fig. S7, Supporting Information). Fig. 5 shows the number of interhelical van der Waals interactions between all the TM helices calculated from the MD simulation trajectories of the single and double CCR3 mutants using the procedure described in the Methods section.

Increased van der Waals packing between the helices contributes to stability of the TM region in the CCR3 double mutant

The total number of interhelical van der Waals interactions (73) is higher in the double mutant compared to the two single mutants A237D^{6.33} and R307E^{7.59} (67 and 63 respectively). This difference mainly stems from the stronger interactions between TM2, TM3 and TM4. We also observe a slightly higher number of hydrophobic contacts between TM6 and TM7 for the double mutant over the two single mutants. This may be attributed to

the rotameric changes of the residues K236^{6,32} and R239^{6,35} on TM6 that improves the hydrophobic packing in the CCR3 double mutant. Interhelical packing interactions and helicity of the TM region helices represent the stability of the receptor in the TM region. Therefore, we clustered the conformations from the MD trajectories of the double and single mutants of CCR3 by the magnitude of interhelical packing interactions and their percentage helicity as shown in Fig. S8 of the Supporting Information. The double mutant shows a single conformational cluster with higher interhelical packing interactions and high percentage helicity compared to the two single mutants (shown inside dotted yellow circles, Fig. S8 of the Supporting Information). This points to the possible correlation between improvement in interhelical packing leading to enhanced secondary structure content of the TM helices. Both these insights drawn from MD simulations seem to contribute to the enhanced thermostability of the double mutant CCR3 compared to the single mutant as observed from experiments. This observation is consistent with our previous work on the origin of thermostability in WT and mutant β i-adrenergic receptor and neurotensin receptor type 1 (NTSR1).^{43,44}

Water penetration through the TM regions

Internal water molecules have been shown to be important in GPCR stability⁴⁵ and signaling.⁴⁶ We have previously shown that the water molecules penetrating the detergent micelle destabilize both the micelle and the GPCR in the adenosine A_{2A} receptor.²⁶ Here, we have analyzed the permeation of water molecules into the TM core of GPCRs originating from the opening of less tightly packed transmembrane helices in the CCR3 single mutants. In the detergent micelle simulations of the two single mutants, we observed considerably more water molecules penetrating into the core of TM helices through the intracellular part of TM6 and helix8 compared to the double mutant. Fig. 6 shows the average number of water molecules entering the intracellular regions between TM6 and helix8 during the MD simulations (see Methods section). Fig. 6 also shows the average solvent accessible surface area (SASA) calculated over the entire MD trajectories. There is significantly less number of water molecules penetrating the core of protein with reduced SASA for the double mutant A237D^{6,33}/R307E^{7,59}. The extensive salt bridge network formed in the double mutant leads to closing up of the TM6-H8 distance. This tight packing between TM6 and H8 is absent in the two single mutants which allows the water molecules to enter the intracellular cavity of the receptor. Fig. S9 of the Supporting Information shows visual representation of the water molecules that enter the intracellular cavity during the MD simulations of the three systems. The water molecules penetrating into the intracellular cavity could weaken the packing interactions and thus destabilizing the single mutants in comparison to the double mutant. This might be another reason behind the experimentally observed low thermal stability and high aggregation propensity in the two CCR3 single mutants.

Role of the detergent micelle in stabilizing the CCR3 double mutants

The choice of a proper detergent is of primordial importance in GPCR stabilization^{47,48} and the thermodynamic/kinetic stability of a well-defined micelle is determined by its efficient coverage of the GPCRs' hydrophobic surface and thereby preventing the influx of water molecules. The distribution of the detergent molecules in the micelle to cover the hydrophobic regions of the receptor was assessed by calculating the number density

distribution of the polar head group and hydrophobic tail group of DDM calculated as shown in Fig. 7A for the double and the two single mutants of CCR3. Fig. 7A shows that the hydrophilic head group of DDM is more widely spread across each receptor whereas the number density of the tail group is the highest near the protein. This is the signature of a stable protein-detergent complex. However, the difference in the average number density of the DDM tail and head group is the lowest for the single mutant A237D^{6.33} (red curves, Fig. 7A). This is indicative of rapid tumbling of the micelle which, in turn, might affect the stability of the A237D^{6.33} mutant adversely owing to inadequate hydrophobic surface coverage to the protein provided by the detergent micelle. The detergent micelle seems to be more well organized around the double mutant CCR3, as evinced by the tail and head density having the highest difference (blue curves, Fig. 7A) in the average values compared to the two single mutants. The mobility of a representative detergent molecule in the vicinity of the receptor calculated as the spatial distribution function is shown in Fig. 7B. The starting conformation of the DDM molecule is shown in sticks in the figure. The representative DDM molecule can be seen to be less mobile in the double mutant compared to the two single mutants. This indicates that the detergent molecules are less labile in the double mutant compared to the single mutants thereby shielding its hydrophobic surface more effectively.

Annular lipids are known to stabilize membrane protein structures and similarly closely packed detergent molecules could also stabilize the receptor structure. We calculated the number of the DDM molecules that are located within 3 Å of the receptor surface and thus interacting with the hydrophobic surface of the TM region (Fig. 7C). The DDM molecules within 3 Å of the TM region in the double mutant are deeply embedded inside the crevices on the receptor surface (the DDM molecules are circled by black dotted lines for the CCR3 double mutant in Fig. 7C) while in the two single mutants they are not as deeply embedded although some regions of the detergent molecules are located within the 3 Å of the receptor surface. Thus, for the double mutant the annular detergent molecules resemble an annular lipid seen in lipid bilayers.⁴⁹ The average number of DDM molecules within 6 Å and water molecules within 4 Å of TM6 and TM7 are shown in Fig. S10 of the Supporting Information. The double mutant showed more number of DDM molecules in the vicinity of the TM6 and 7 helices, where the two mutations are located, compared to the single mutants of CCR3. This is probably due to the extensive water permeation which disrupts the compaction of the DDM micelle around the receptor in the two single mutants (Fig. S10, Supporting Information).

Discussion

In this study we have shown that the double mutant A237D^{6.33}/R307E^{7.59} CCR3 shows an increase of 2.6 °C in melting temperature and an improved size exclusion profile in the detergent DDM, compared to its wild type. MD simulations of the double mutant (A237D^{6.33}/R307E^{7.59}) CCR3 in DDM detergent micelles showed the formation of an extensive salt bridge network between TM3, TM6 and TM7 by the seven residues Y301^{7.53}, E307^{7.59}, R239^{6.35}, K236^{6.32}, D237^{6.33}, R109^{3.50}, D108^{3.49}. The extensive salt bridge network that tightens TM6 and TM7 interaction with TM3 also brings TM6 closer to helix8. Such salt bridge network is not viable in either of the two single mutants. MD simulations

also reveal tighter interhelical hydrogen bonds and van der Waals interactions that might account for the experimentally observed thermostability of the double mutant compared to the single mutants. The lack of tighter interaction between TM3, TM6 and TM7 in the two single mutants A237D^{6.33} and R307E^{7.59}, leads to water penetration into the TM region through the intracellular loop region that was not observed for the double mutant. The free energy surface of the double mutant shows conformational homogeneity with fewer microstates compared to the two single mutants' that show multiple microstates with conformational heterogeneity. We also calculated the probability of finding the salt bridges between key residues in the two conformation clusters obtained from the free energy surface of the double mutant A237D^{6.33}/R307E^{7.59} (Fig. S11, Supporting Information). The microstates of the single mutants also showed unraveling of helical regions with lower percentage helicity that could be a plausible explanation for the observed aggregation of these mutants in DDM solution. We have previously shown that the detergent micelle contributes significantly to the stability of purified GPCR structures.²⁶ Here we show that the hydrophobic surface of the double mutant A237D^{6.33}/R307E^{7.59} CCR3 is shielded by the hydrophobic tail groups of the detergent DDM while such tight interactions with DDM were not observed in the single mutants.

We performed structural analysis using available crystal structures of all chemokine receptors to predict if the polar network of inter-helical interactions between TM3, TM6 and TM7 that stabilized the double mutant of CCR3 is transferable to stabilize other related chemokine receptors. Such a prediction is valuable to the chemokine receptor structural biology community. Using thermostability measurements we have shown that the two mutations A237D^{6.33} and R307E^{7.59} in CCR3 together thermostabilize the CCR3 receptor. Alignment of CCR3 sequence with other chemokine receptors CCR1 to CCR10 show that the residues at positions 6.33 and 7.59 are conserved (Ala) and conservative replacements (R/K) respectively among the chemokine receptors (see the sequence alignment in Fig. S12). MD simulations on the double mutant CCR3 shows the formation of the salt bridge network between the residues Y301^{7.53}, E307^{7.59}, R239^{6.35}, K236^{6.32}, D237^{6.33}, R109^{3.50}, D108^{3.49} in the intracellular side of TM3, TM6 and TM7 as shown in the Fig. 8 (red color cartoon). We analyzed if the corresponding residues make hydrogen bonds in the crystal structures of CCR5 (PDB ID 4MBS),¹¹ CCR2 (PDB ID 5T1A)⁵⁰ and CCR9 (PDB ID: 5LWE)⁵¹ (Table S2). This analysis showed that not all the interhelical hydrogen bonds observed in the CCR3 MD simulations are present in the crystal structures of other chemokine receptors. However, the MD simulations we performed starting from the crystal structure of CCR5 (PDB ID: 4MBS)¹¹ in detergent micelles, confirm the formation of similar salt-bridges during the simulations. The polar network was formed and sustained during our MD simulations of CCR5 (yellow cartoon, Fig. 8B). The crystal structure of CCR2 (PDB ID 5T1 A)⁵⁰ and CCR9 (PDB ID 5LWE)⁵¹ do not have these double mutations and hence do not show the polar network (Fig. 8) of interactions. We predict that since these residues are conserved in CCR1 to CCR10, that mutation of position 6.33 and 7.59 is likely to stabilize the receptors and this might be a good starting point to purify some of the chemokine receptors that have not yet been crystallized.^{52,53}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CCR3 single and double mutants. **A–C.** The ratio of the fluorescence intensity (T) at 350 and 330 nm respectively is shown in the Y-axes. nano-DSF results for each CCR3 mutant overlaid with the WT. **D.** The size exclusion chromatography (SEC) for each mutant overlaid with the WT CCR3. **E.** SEC profile for the double mutant A237D^{6.33}/R307E^{7.59} in presence (red trace) and in absence (black trace) of the antagonist BMS-639623.

Figure 2.

A. Free energy surfaces (FES) of WT CCR3 (WT) and the three mutants A237D^{6.33}/R307E^{7.59}, A237D^{6.33} and R307E^{7.59}. PC1 and PC2 on the axes are the principal components 1 and 2. The low energy conformation clusters are marked by numerical numbers (1, 2, 3 etc) on the free energy surface. The populations of the conformation clusters for each system and the average helicity are given in Table S1 of the Supporting Information. **B.** Heat map showing the average helicity for the WT and three CCR3 mutant systems. The representative snapshot from the most populated cluster for each system is shown. The color bar in the right ranges from red to blue with red indicating the lowest helicity in the TM regions. Loop regions are excluded from this analysis and have been colored dark gray.

Figure 3.

Salt bridges that stabilize the double mutant CCR3, (A) the double mutant A237D^{6.33}/R307E^{7.59}, (B) single mutant A237D^{6.33} and (C) single mutant R307E^{7.59}. The structures shown here are the representative snapshots from the most occupied conformational cluster in each system. The clustering was based on RMSD in coordinates of the TM regions. Figures have been rendered using PYMOL.

Figure 4. Side chain rotamer distribution of the (A) χ^1 and (B) χ^2 angle of K236^{6.32}, (C) angle χ^1 of R239^{6.35}. They have the same Y axis. Representative structures of the residues K236^{6.32} and R239^{6.35} in the two rotameric states 1 and 2 are shown in the bottom panel.

Figure 5. Number of inter-helical van der Waals interactions for (A) A237D^{6.33}/R307E^{7.59}, (B) A237D^{6.33} and (C) R307E^{7.59}.

Figure 6.

Average number of water molecules penetrating into the intracellular region of TM6 and helix8. In the primary y axis we show the number of water molecules within 4 Å of the TM6-H8 regions. The dotted line bar within each solid bar shows the solvent accessible surface area (SASA) of the protein in the TM6 to helix 8 region in the secondary y axis. The error bars on the histograms with dotted and solid borders represent the standard error associated with the calculation of the SASA and the number of water molecules in TM6 – H8 region respectively.

Figure 7.

(A) Radial number Density Distribution of the head and tail group of DDM micelle from the center of the protein, in the double and two single mutants of CCR3. The solid and dashed lines represent the density of tail and head group respectively for the mutants A237D^{6.33}/R307E^{7.59}, A237D^{6.33} and R307E^{7.59} from left to right. (B) The spatial density distribution function (SDF) of a single representative detergent molecule within 6 Å of the initial protein-detergent complex for the double mutant and the two single mutants in this order A237D^{6.33}/R307E^{7.59}, A237D^{6.33} and R307E^{7.59} respectively from left to right. The initial conformation of the detergent is shown in licorice representation (green for A237D^{6.33}/R307E^{7.59}, red for A237D^{6.33} and blue for R307E^{7.59}) and the corresponding SDFs are shown as isosurfaces around the protein with the same color. (C) DDM molecules within 3 Å of the TM6 and 7 where the mutations are located of CCR3 mutants A237D^{6.33}/R307E^{7.59}, A237D^{6.33} and R307E^{7.59} from left to right. TM6 and 7 of each receptor has been shown in a grey surface representation while the other TMs are shown in white. The representative DDM molecules are shown as vdW spheres with the cyan and the red color

representing the tail and the head part of each detergent. Hydrogen atoms are excluded in the figure for clarity. Detergents resembling annular lipids are circled in black for A237D^{6,33}/R307E^{7,59}

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Figure 8.

Comparison of the polar networks formed between TM3, TM5 TM6 and TM7 residues in (A) double mutant CCR3 (red cartoon), (B) CCR5 (yellow cartoon), (C) CCR9 (magenta cartoon) and (D) CCR2 (green cartoon) structures. Crystal structures of CCR9 and CCR2 along with representative snapshots from MD simulations of the double mutant CCR3 and CCR5 crystal have been chosen for the above figures. Key residues forming polar contacts have been shown as sticks bearing the same color as the cartoon for a certain receptor. Residues in various TM regions have been shown with both their sequence numbering as well as the BW numbering for each receptors. The blue dotted lines indicated a salt bridge interaction between two residues in any of the receptor. Figure were generated using PYMOL.

Table 1.

Melting temperature from nanoDSF and peak ratio from SEC experimental data for the CCR3 WT, single and double mutants. The number of separate purification = 1 for two single mutants, 2 for the double mutant and 3 for the wild type CCR3.

CCR3 mutants	Yield/expression volume mg/l	Aggregate to monomer peak ratio ^a	Mean Melting point ^b °C	T _m °C
Wild type	0.08	3.34	47.6±0.334	-
R307E ^{7.59}	0.09	3.9	46.96±0.152	-0.94±0.182
A237D ^{6.33}	0.08	4.13	47.5±0.3	-0.10±0.034
A237D ^{6.33} /R307E ^{7.59}	0.14	0.53	50.2±0.139	+2.6±0.195

^a is calculated from the intensity of absorption of column elution that corresponds to the aggregated protein with a larger molecular weight followed by the protein in a monodispersed form.

^b is the average melting point (MP) of all three fractions from the same purification step and their standard deviations.

^c is $T_m = T_m(\text{mutant}) - T_m(\text{WT})$