Thermal Unfolding of the Human Serotonin Transporter: Differential Effect by Stabilizing and Destabilizing Mutations and Cholesterol on Thermodynamic and Kinetic Stability

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Received September 20, 2021; accepted November 21, 2021

ABSTRACT

Folding-deficient mutants of solute carrier 6 (SLC6) family members have been linked to human diseases. The serotonin transporter ([SERT]/SLC6A4) is an important drug target in the treatment of depression, anxiety, and obsessive-compulsive disorders and—with structural information in several conformational states—one of the best understood transporters. Here, we surmised that thermal unfolding offered a glimpse on the folding energy landscape of SLC6 transporters. We carried out molecular dynamic (MD) simulations to understand the mechanistic basis for enhanced and reduced stability, respectively, of the thermostabilized variant SERT-Y110A/I291A/T439S, which had previously been used for crystallization of human SERT in the outward-facing state, and of the folding-deficient SERT-P601A/G602A. We also examined the hydrophobic mismatch caused by the absence of cholesterol to explore the contribution of cholesterol to protein stability. When compared with wild type SERT, the thermodynamic and kinetic stability of SERT-Y110A/I291A/T439S was enhanced. In the other instances, changes in these two components were not correlated: the mutations in SERT-P601A/G602A led to a drop in thermodynamic but an increase in kinetic stability. The divergence was even more pronounced after cholesterol depletion, which reduced thermodynamic stability but increased the kinetic stability of wild type SERT to a level comparable to that of SERT-Y110A/I291A/T439S. We conclude that the low cholesterol content of the endoplasmic reticulum facilitates progression of the folding trajectory by reducing the energy difference between folding intermediates and the native state.

SIGNIFICANCE STATEMENT

Point mutations in solute carrier 6 (SLC6) family members cause folding diseases. The serotonin transporter ([SERT]/SLC6A4) is a target for antidepressants and the best understood SLC6. This study produced molecular dynamics simulations and examined thermal unfolding of wild type and mutant SERT variants to understand their folding energy landscape. In the folding-deficient SERT-P012A/G602A, changes in kinetic and thermodynamic stability were not correlated. Similarly, cholesterol depletion lowered thermodynamic but enhanced kinetic stability. These observations allow for rationalizing the action of pharmacochaperones.

Introduction

With more than 400 members, the solute carrier family is the second largest membrane protein family in the human genome (Perland and Fredriksson, 2017). The genetic variation in SLC transporters is large and more than 100 SLC transporters have been linked to a human disease (Schaller et al., 2016). In the past 20 years, there has been a steady increase in the number of point mutations of SLC transporters, which give rise to folding-deficient proteins (Bhat et al., 2021a). Based on the number of publications, the serotonin transporter ([SERT], solute carrier 6 (SLC6)A4) can be argued to be the most extensively studied transporter (César-Razquin et al., 2015): it has a rich pharmacology (Sitte and Freissmuth, 2015), which comprises both typical and atypical inhibitors, partial and full releasers (Bhat et al., 2019; Hasenhütl et al., 2019); its structure has been solved in several conformations (Coleman et al., 2016, Coleman and Freissmuth, 2018; Coleman et al., 2019) and its transport cycle has been analyzed with high precision (Schicker et al., 2012; Hasenhütl et al., 2016; Bhat et al., 2021b).

The folding trajectory of SLC transporters is of interest, because folding-deficient mutants are amenable to rescue by...
pharmacochaperoning (El-Kasaby et al., 2010; Koban et al., 2015; Beerepoot et al., 2016; Kasture et al., 2016; Asjad et al., 2017; Bhat et al., 2017; Bhat et al., 2020) or by interfering with the endogenous proteinaceous chaperones (Fujiiwara et al., 2013; El-Kasaby et al., 2014; El-Kasaby et al., 2019). Conceptually, the action of pharmacochaperones can be accounted for by four mechanisms of action (Marinko et al., 2019): binding to and stabilization of the (i) native (= folded) state or (ii) folding intermediate(s); (iii) suppression of aggregate formation; and (iv) dissolution of aggregates. In the vast majority of cases, the pharmacochaperoning action is thought to arise from the first mechanism, i.e., thermodynamic stabilization of the native state (Marinko et al., 2019). Circumstantial evidence, however, suggests that pharmacochaperones may rescue folding-deficient variants of SERT and of the dopamine transporter (DAT) (SLC6A3) by an action on (a) folding intermediate(s) (Bhat et al., 2020). Progress is hampered by the elusive nature of the folding intermediates. The folding trajectory can be conceptualized as a conformational search, where the protein moves through a funnel-like energy landscape to reach a minimum energy conformation (Dill and Chan, 1997). However, the surface of this landscape is rugged, i.e., energy barriers must be overcome during the folding trajectory (Hartl et al., 2011). Mutations increase the propensity for trapping of folding intermediates; these traps can be overcome by pharmacochaperones (Freissmuth et al., 2018). In addition, the native state is not necessarily the minimum energy conformation (Hartl et al., 2011). In fact, because SLC transporters operate by an alternating access mechanism, the transporter has to visit several distinct conformations during the transport cycle, most importantly, the outward, occluded, and inward facing states. Hence, the required conformational flexibility imposes an additional limit on the shape of the energy landscape. Here we surmised that thermal unfolding of SERT provided information on the barriers, which separated the folded from unfolded states. We compared wild type human SERT to a thermostabilized version of human SERT, which was instrumental for crystalization of the protein (SERT-Y110A/I291A/T439S) mutants after serial mutagenesis. We denote the variants T5* and T5*, which were employed in this study, by asterisk to indicate the full-length protein containing the specified mutations, as opposed to the crystallized and N- and C-terminally truncated SERT-TS2 and SERT-TS3 (Coleman et al., 2016). The presence of the mutation(s) was verified by sequencing the entire reading frame of each construct.

Cell Culture and Membrane Preparation

HEK293 cells were stably transfected with plasmids encoding YFP-tagged wild type SERT, SERT-T5*, SERT-T5*, SERT-Y367N and the folding-deficient SERT-P601A/G602A. Cells expressing SERT-P601A/G602A were incubated for 24 hours in the presence of 30 μM noribogaine to correct the folding defect by pharmacochaperoning (El-Kasaby et al., 2014). Confluent monolayers of cells were mechanically detached and lysed in hypotonic buffer (20 mM Hepes.NaOH, pH 7.4, 1 mM EDTA, 2 mM MgCl2). The cell suspension was frozen in liquid nitrogen, subjected to freeze-thaw cycles, and sonicated. The homogenate was finally centrifuged at 30,000 g for 30 minutes at 4°C. The pellet was resuspended in buffer at a protein concentration of about 2 mg/mL. Membranes were depleted of cholesterol by incubation in the presence of methyl-β-cyclodextrin (final concentration of 10 mg/mL) for 30 minutes incubation at 37°C with gentle shaking (Scanlon et al., 2001). Thereafter, the membranes were harvested by centrifugation at 16,000 g for 15 minutes, the methyl-β-cyclodextrin containing supernatant was removed, and, after one additional washing step, the pellet was resuspended in buffer.

Thermal Inactivation and Radioligand Binding

Temperature-induced inactivation was determined by incubating duplicate samples (containing 5–40 μg protein) in buffer containing (120 mM NaCl, 20 mM Tris.HCl, 3 mM KCl, 2 mM MgCl2, 1 mM EDTA, pH 7.4) for 15 minutes at temperatures ranging from 43°C to 78°C. In some instances, NaCl was replaced by equimolar choline chloride. Thereafter, the samples were put on ice. For time-dependent denaturation, duplicate samples (containing 5–40 μg of protein per sample) were incubated at the indicated temperature for time intervals ranging from 0 to 80 minutes and subsequently put on ice. After thermal inactivation, the amount of residual, functional SERT was determined by incubating the membranes on ice for 15 minutes in a final volume of 0.2 ml buffer (120 mM NaCl, 20 mM Tris.HCl, 3 mM KCl, 2 mM MgCl2, 1 mM EDTA, pH 7.4) in the presence of [3H]imipramine (3 nM, 6nM for SERT-V367N). Nonspecific binding was defined in parallel incubations in the presence of 10 μM paroxetine. Preliminary experiments verified that binding equilibrium was reached rapidly (i.e., within 5 minutes) on ice (data not shown). The reaction was terminated by rapid filtration through glass-fiber filters precoated in 1% polyethyleneimine with ice-cold buffer (120 mM NaCl, 10 mM Tris-HCl, 1 mM MgCl2, pH 7.4). The radioactivity retained on the filters was determined by liquid scintillation counting.

Materials and Methods

Materials

Cell culture media and G418/geneticine were purchased from SigmaAldrich and Bioechrom, respectively. [3H]imipramine (51.6 Ci/mmol and 40.0 Ci/mmol) was from PerkinElmer, and unlabeled paroxetine from Santa Cruz Biotechnology. Methyl-β-cyclodextrin (M(βCD)) and all other reagents were obtained from SigmaAldrich and were of analytical grade. Mutations were introduced into the plasmid containing the N-terminally-YFP (yellow fluorescent protein)-tagged, full-length human SERT using the QuikChange-Lightning kit for site directed mutagenesis from Agilent Technologies to obtain the SERT-V367N, serotonin transporter variant thermostabilized by two point mutations (SERT-T5*) (SERT-I291A/T439S), and serotonin transporter variant thermostabilized by three point mutations (SERT-T5*) (SERT-Y110A/I291A/T439S) mutants after serial mutagenesis. We denote the variants T5* and T5*, which were employed in this study, by asterisk to indicate the full-length protein containing the specified mutations, as opposed to the crystallized and N- and C-terminally truncated SERT-TS2 and SERT-TS3 (Coleman et al., 2016). The presence of the mutation(s) was verified by sequencing the entire reading frame of each construct.

Molecular Dynamics Simulations

For the MD simulations, the outward open human SERT crystal structure was used (PDB ID: 5J71; Coleman et al., 2016). Missing side chains and bound ions were positioned using MODELER 9.20 (Shen and Sali, 2006; Webb and Sali, 2014), creating 100 structures from which the best five were selected based on the DOPE (discrete
optimized protein energy) score. The resulting structures where then mutated by replacing P601 and G602 to alanine using Pymol, v1.8.4 (The PyMOL Molecular Graphics System, version v1.8.4 Schrödinger, LLC). All proteins contained 2 Na⁺ ions and a Cl⁻ ion at their respective binding sites. All models were converted into coarse-grain representation using the MARTINI force field (Monticelli et al., 2008; de Jong et al., 2013; Wassenaar et al., 2015) and embedded into a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC):cholesterol membrane (70:30 mol%) to obtain an equilibrated membrane lipid environment. The simulation box was filled with water and 150 mM NaCl. The coarse-grain systems were simulated for 1 μs with the protein structure restrained to avoid conformational changes while equilibrating the lipid membrane. For wild type protein POPC only, membranes were also prepared. Membrane lipids, water, and ions of the equilibrated coarse-grain systems were converted to an all-atom representation (Wassenaar et al., 2014). The original protein models were inserted into the membrane using the memed procedure (Wolf et al., 2010) to relax possible local atom overlaps. Protein, ions, and solvent were described using the amber99sb-ildn force field (Lindorff-Larsen et al., 2010), POPC, and cholesterol by Slipid (Jämbeck and Lyubartsev, 2012; 2013).

All simulations used GROMACS version 2018.1 (Abraham et al., 2015). The final assembled systems were energy-minimized and equilibrated in four steps of 2.5 ns each by slowly releasing the position restraints (1000, 100, 10, 1 kJ/mol/nm) active on the Cα atoms and bound ions. The production runs were carried for 500 ns, each system independently simulated five times without applying restraints. Temperature was maintained at 310 K using the v-rescale (τ = 0.5 ps) thermostat (Bussi et al., 2007), while separately coupling protein, membrane, and solvent. Pressure was maintained at 1 bar using the Parrinello-Rahman barostat (Parinello and Rahman, 1981) in a semi-isotropic manner and applied a coupling constant of 0.1 ps. Long-range electrostatic interactions were described using the smooth particle mesh Ewald method (Darden et al., 1993), applying a cutoff of 0.9 nm. The van der Waals interactions were described using the Lennard-Jones method (Darden et al., 1993), applying a cutoff of 0.9 nm. Long-range correction for energy and pressure were applied. Coordinates of all atoms were recorded at every 10 ps.

Biased Simulations

Equilibrated systems of wild type SERT and SERT-P601A/G602A were tested using well-tempered metadynamics (WTM) (Barducci et al., 2008). The technique allows for fast exploration of the free energy landscape, while limiting the search to low energy conformations. Exploration is promoted by the local addition of small Gaussian shaped potentials, which eventually fill the complete free energy landscape, resulting in a flat free energy hypersurface. Inversion of the total added potential equals the free energy. WTM was performed by simultaneous use of GROMACS and PLUMED (Trlappedello et al., 2014; The PLUMED Consortium, 2019). Biased simulations were 200 ns long in five parallel runs. The C-terminal helix unfolded irreversibly in one trajectory, which was discarded from further analysis. Reweighting of the WTM trajectories (Tiwary and Parrinello, 2015; Lindahl et al., 2017) was used to query the free energy landscape along nonbiased degrees of freedom. We used the distances between P156-S611 and L597-R607, which gives a better description of motions of the C-terminus (cf. Fig. 3D). Moreover, reweighting also allows for merging results of parallel simulations providing a complete free energy estimation of the sampled conformational space.

Statistics

The study was exploratory in nature. Accordingly, the number of independent experiments (n = 3 to 5) was chosen to verify the reproducibility of the data, and there wasn’t any preplanned statistical tests. Data obtained by temperature-dependent inactivation of wild type and mutant versions SERT were fitted by curvilinear regression to a three-parameter logistic equation:

\[
B(T) = \frac{B_0 \cdot T_p}{T_{M0} + T_p}
\]

where \(B(T)\) is binding at temperature \(T\), \(B_0\) is control binding in the absence of thermal denaturation (i.e., at 22 °C), \(T_{M0}\) is the temperature resulting in 50% inactivation, and \(p\) is the slope of the curve. Data obtained by time-dependent inactivation at fixed temperatures were fitted by curvilinear regression to the equation for a mono-

\[
\frac{SS_{ME}}{DF_{ME}} - \frac{SS_{DE}}{DF_{DE}} = \frac{SS_{ME} + SS_{DE}}{DF_{ME} + DF_{DE}}
\]

where \(SS_{ME}\) and \(SS_{DE}\) are the sum of squares of the deviation from the fitted lines for the mono- and the double-exponential decay, respectively; \(DF_{ME}\) and \(DF_{DE}\) are the corresponding degrees of freedom (i.e., the number of data points minus the number of parameters in each equation).

Results

Thermostability of SERT Variants in the Lipid Bilayer

The thermostability of SERT and of mutated variants was previously determined in detergent solution (Green et al., 2015). Here we assessed the thermostability of SERT in membranes. We first verified that [3H]imipramine bound to SERT, if the membranes were incubated on ice. This was the case: the binding equilibrium was rapidly reached (k_{off} = 0.54 minute⁻¹ at 3 mM radioligand concentration); binding remained stable for at least 60 minutes (data not shown). Hence, in all experiments, samples were subjected to heat denaturation for defined time intervals at the specified temperature and the remaining active (i.e., binding competent) SERT was subsequently determined by binding of [3H]imipramine at 0 °C. We first compared the temperature stability of wild type SERT with the two thermostable variants identified by Green et al. (2015), namely SERT-TS2* (= SERT-I291A/T439S) and SERT-TS3* (SERT-Y110A/I291A/T439S). When incubated for 15 minutes, wild type SERT was remarkably stable: temperatures exceeding 55 °C were required to cause loss of binding (full circles in Fig. 1). As expected, the denaturation curve of the heat-stable mutants SERT-TS2* (triangles in Fig. 1) and SERT-TS3* (squares in Fig. 1) were shifted to the right. In addition, we also examined SERT-P601A/G602A; this mutation, which is in the loop connecting the transmembrane segment (TM)12 with the C-terminal α-helix (cf. Fig. 3), results in misfolding and endoplasmic reticulum (ER) retention of SERT (El-Kasaby et al., 2010), but the folding deficit can be corrected by pharmacochaperoning with noribogaine (El-Kasaby et al., 2014). Accordingly, we incubated cells...
and $[^3]$Himipramine and $[^3]$Hparoxetine) requires SERT to reside in the outward-facing conformation. SERT-TS3 has been crystallized in the outward-facing state and was shown to be binding competent but transport-deficient (Green et al., 2015). SERT-TS3 is locked in the outward-facing state and fails to undergo the conformational transitions required to support a transport cycle. This phenotype is consistent with the implicit assumption that the three mutations in SERT-TS3 (SERT-Y110A/I291A/T439S) privilege the outward-facing conformation over all other conformations. The increased stability is expected to be detectable by an increased rigidity. We examined this conjecture by carrying out comparative molecular dynamics simulations of wild type SERT and SERT-TS3 with the proteins embedded in a lipid bilayer. These simulations reveal distinct local structural and/or dynamic effects for each of the three mutations (Y110A, I291A, and T439S, illustrated in Fig. 2A) that contribute to structural stabilization (Fig. 2). The mutations lead to a small global change of fluctuations in SERT-TS3 (Fig. 2B). In addition, the conformation of the bundle domain is less open, as TM1b moves toward a more occluded geometry (Fig. 2C and D). The mutation of Y110A changes a large amino acid (tyrosine) to a small amino acid (alanine). The side chain of Y110 is buried between TM1, TM7, TM8, EL3, and EL4 (Fig. 2C). The alanine mutant leaves empty space, which is filled by a closer packing of these helices and loops (Fig. 2E) and by the movement of TM1b (Fig. 2C).

Similarly, the I291A mutation introduces a smaller amino acid, which is located in the interface between TM5 and TM8 (Fig. 2F). Conceptually, this reduction in size of the side chain at position 291 creates a local void that leads to a closer association of TM5 and TM8 (Fig. 2G). This displacement fills a large part of the empty volume created by the mutation, but residual subatom size cavities remain detectable (Fig. 2F). In addition, the rearrangement also translates into a compression of the helix-helix interface of the preceding and following helical turn. Thus, the structural and dynamic changes in the vicinity of A291 stabilize the TM5–TM8 interface through tighter association. It is conceivable that this suppresses the propensity of SERT-TS3 to visit conformations, which allow for transition to the unfolded state(s). Most prominently, the dynamics of the juxtaposed residue I426 are increased by the I291A mutation visible as an altered dihedral distribution (Fig. 2H). This leads to an increase in local conformational entropy (wild type SERT: −24.9 ±2.2 kJ/mol; SERT-TS3: −21.6 ±2.2 kJ/mol relative to the unfolded state, obtained using PDB2ententropy; Fogolari et al., 2018). The effect of the third mutation (T439S) on SERT stability can be accounted for by two effects. (i) Removal of the $\beta$-branched methyl-group of threonine changes the side chain conformation alters the hydrogen bonding pattern of the side chain hydroxyl group. The TM8 destabilizing hydrogen bond between the side chain of residue 439 and the backbone carbonyl oxygen of G435 (Fig. 2I and K) is replaced by the interaction with the buried side chain of N177 (Fig. 2J and L). Both the loss of the interaction between G435 and T439 and the newly acquired H-bond to N177 in SERT-TS3 contribute to stabilizing the outward-open conformation due to the absence of a helix destabilizing effect and the presence of a helix interconnecting interaction. (ii) In addition, the reorientation of the side chain of residue 439 leads to a stabilization by strong interactions of a structural water molecule between TM3 and TM8 (Fig. 2J).
Wild type SERT, the position of this water molecule is diffuse (Fig. 2I), because it lacks the stabilizing interaction, which is only established after the rotation of the hydroxyl group of S439 in SERT-TS3. Taken together, both the mutations Y110A and I291A introduce a much smaller residue, leading to potential voids. These residue changes lead to a local compacting of SERT-TS3. These changes therefore are predicted to raise ΔG* for unfolding (i.e., the energy barrier separating the folded from the unfolded states), because they increase the interaction enthalpy of the tightly packed residues around the mutation sites. Concomitantly, ΔG for unfolding (i.e., the free energy difference between the folded and the unfolded states) declines because the entropy of the closely associated residues is increased. Accordingly, upon unfolding, the free energy gain in entropy is smaller. The T439S mutant changes the hydrogen bonding pattern and thus contributes to stabilizing TM8 and to the interaction with a structural water molecule.

We carried out similar calculations for SERT-P601A/G602A (Fig. 3). Root mean square fluctuations (RMSF) calculations provide evidence for structural instability: in SERT-P601A/G602A, the N-terminal half of TM5 and the segment of the C-terminus adjacent to TM12 are much more dynamic and visited several new conformations not sampled by wild type SERT (Fig. 3B). Based on these fluctuations observed in the C-terminus (Fig. 3B), we surmised that the destabilizing effect of the P601A/G602A mutation arose—at least in part—from an altered interaction of the C-terminal helix with the protein core. We explored this hypothesis in biased simulations to assess how strongly the C-terminus is attached to the rest of the protein using well-tempered metadynamics (Barducci et al., 2008). The method enhances the sampling along a preselected reaction coordinate, which in this case was the distance between TM12 and the C-terminal helix (backbone center of mass of residue 576–584 versus 607–613, Fig. 3A). The bias translates to pulling the C-terminal helix parallel to the z-axis, i.e., away from the core of SERT. Then, using a reweighting technique, the observed changes can be translated to a free energy landscape in relation to the distances between P156–S611 and L597–R607 (Fig. 3D). The P156–S611 distance captures the attachment of the helix to the protein core, whereas L597–R607 describes changes in the geometry of the loop between TM12 and the C-terminus. It is evident that the P601A/G602A mutation lowers the energy barrier to visit other, more open conformational states (Fig. 3D), which is a signature of a decreased overall resistance against unfolding.

Two Components in the Heat-Induced Inactivation of Wild Type and Mutant SERT Variants

Assuming that a possible unfolding route is initiated by next to residue T439 in wild type SERT or by SERT-TS3. Bulk water density is 1000 kg/m³. The last 250 ns of each trajectory with 0.1 ns time resolution were used as input data and averaged over all trajectories. (K) The distance between the hydroxyl group of T439 (WT, blue) or S439 (TS3, red) and the backbone oxygen of G435 or (L) the amino group of N177 indicates changes in the local hydrogen bonding pattern. The respective distances are indicated by orange lines on panels I and J. The last 250 ns of each trajectory with 1 ns time resolution were used as input data.
the detachment of the C-terminus, the results summarized in Fig. 3 are consistent with the lower thermostability of SERT-P601A/G602A (Fig. 1). However, it is only possible to obtain relative comparisons if proteins are subjected to heat inactivation for an arbitrarily selected temperature (15 minutes in the experiment in Fig. 1). More information can be extracted by observing the time-dependent denaturation. This allows for estimating the two components of protein stability, i.e., thermodynamic stability and kinetic stability (Sanchez-Ruiz, 2010): kinetic stability results from an energy barrier (ΔG*), which prevents the native state from visiting (partially) unfolded states. Thermodynamic stability is imparted by the lower energy content of the native folded conformations than of the unfolded states. These two population states are not separated by a high-energy barrier and hence can rapidly equilibrate. If thermodynamic stability is high at a given temperature, the Gibbs free energy favors the native state at equilibrium and thus conformations conducive to unfolding are rarely populated. Accordingly, we explored which component of protein stability was affected by the mutations by incubating membranes harboring wild type SERT (Fig. 4A), SERT-TS* (Fig. 4B) and SERT-PG601,602-AA (Fig. 4C) for different time intervals at temperatures close to their individual melting temperatures, i.e., Tm−5°C, Tm−3°C, Tm, Tm +3°C (Tm rounded to integers). When subjected to this thermal challenge, all SERT variants underwent two distinct phases of denaturation provided that the temperature did not exceed Tm by 3°C: a rapid decrease of [3H]imipramine binding was followed by a slower component (Fig. 4). With the notable exception of curves generated at the highest temperature (i.e., Tm+3°C), the data were better described by the equation for double exponential than for a monoexponential decay. The rate of the slow component increased with higher temperatures. Accordingly, it was possible to estimate the energy barrier, which separated the folded from the binding-incompetent, unfolded state(s), from the corresponding Arrhenius plots: it is evident from Fig. 5A that their slopes differ. Slow thermal inactivation required a substantially higher activation energy in SERT-TS* (303 kJ·mol⁻¹) than in wild type SERT (113 kJ·mol⁻¹) or SERT-P601A/G602A (176 kJ·mol⁻¹). In contrast, the fast component of thermal inactivation did not increase to any appreciable extent with increasing temperature; this is most readily evident for SERT-P601A/G602A (cf. full squares in Fig. 5B). We stress that in wild type SERT (closed circles in Fig. 5B) and SERT-TS* (open triangles in Fig. 5B), the fraction, which denatured rapidly at Tm−5°C, Tm−3°C was too small to obtain a reliable estimate of the rate. Taken together, the observations summarized in Fig. 5A and B are consistent with the interpretation that the fast and the slow component of thermal inactivation reflect the thermodynamic and the kinetic stability, respectively, of wild type and mutant SERT variants.

We extracted the ratios of the slowly denaturing component over the rapidly unfolding component (Pf/Pu) from the biexponential decay curves shown in Fig. 5 and plotted these as a function of temperature (1/K) (Fig. 5C). This allows for estimating the thermodynamic stability of the SERT variants from the x-axis intercept. The thus calculated Tm-values (60.3°C, 70.8°C, and 48.1°C for wild type SERT, SERT-TS*, and SERT-P601A/G602A, respectively) were in reasonable agreement with the estimates obtained from Fig. 1. This was to be expected, because the slow component only contributed to a minor extent to thermal inactivation within the first 15 minutes.

**Contribution of Cholesterol to SERT Stability**

Regardless of whether only the component of thermodynamic stability (Fig. 5C) or the global Tm (measured at 15 minutes, Fig. 1) is taken into account, the melting temperature estimates are higher by >10° than those in detergent solution (Green et al., 2015). The difference indicates that the membrane lipids have a strong stabilizing effect. Cholesterol has been visualized in the crystal structure of SERT (Coleman et al., 2016); this is also true for the closely related dopamine transporter/DAT, albeit wedged to the hydrophobic core at a different position (Penmatsa et al., 2013). SERT requires cholesterol for its eponymous action, i.e., serotonin transport (Scanlon et al., 2001), and apparently cannot fold correctly in the absence of cholesterol (Tate et al., 2003). We therefore surmised that cholesterol affected the stability of SERT. Accordingly, we carried out MD simulations with SERT embedded in lipid bilayers containing solely POPC or the combination of POPC and cholesterol (molar ratio 70:30). We first analyzed the thickness of the membrane around the protein by measuring the distance between the phosphate atoms of the POPC head groups in the inner and outer leaflet (Fig. 6A). The average thickness clearly shows that the membrane containing POPC and cholesterol matches the hydrophobic core of SERT (Fig. 6B). In contrast, a bilayer comprised solely of POPC is too thin (Fig. 6B and D). However, it is also visible that the presence of SERT increases the local thickness. The thickness maps (Fig. 6C and D) also indicate that the hydrophobic core of SERT exerts different, nonhomogeneously distributed forces on
the surrounding lipids: in contrast to helix 11, which produces a bulge in the bilayer (seen as the prominent yellow area highlighted by a magenta arrow in Fig. 6D), on the opposite side, the lipid exposed surface in the vicinity of helix 5 allows for membrane thinning (seen as the blue area highlighted by an orange arrow in Fig. 6C). The same is true in the vicinity of helix 2 (seen again as the blue area highlighted by a black arrow in Fig. 6C).

Taken together, these observations suggest that the annular lipid ring surrounding SERT differs in the absence and presence of cholesterol. Without cholesterol, the reduced thickness results in a hydrophobic mismatch, which is predicted to translate into a gain of entropy in both the bilayer and the protein. Accordingly, we explored the consequence of removing cholesterol in comparative molecular dynamics simulations of wild type SERT embedded in a lipid bilayer containing or lacking cholesterol. This analysis revealed that the overall differences in the fluctuations of the backbone (Cα) were modest (Fig. 7A and C): in the hydrophobic core, they were confined to the bottom of helix 8 and the top of helix 11, which increased in the absence of cholesterol. The fluctuations in the side chains were also enhanced in these regions, but they were also increased in other hydrophobic segments, e.g., TM4, TM5, TM6, and TM7 (Fig. 7B and D). Interestingly, in the presence of cholesterol, fluctuations of both the backbone (Fig. 7, A and C) and the side chains (Fig. 7, B and D) were enhanced in EL2 and IL4. This suggests that the absence of cholesterol leads to a gain in entropy in the hydrophobic core but to a drop in entropy in EL2 and IL4.

We verified the effect of cholesterol depletion on the thermostability of SERT by comparing the susceptibility of wild type SERT and on SERT-V367N. This mutation was selected because it reduces the affinity of cholesterol to one of its bona fide binding sites of SERT (Laursen et al., 2018). Thus, it is predicted to be more susceptible to lowering cholesterol levels in the membrane. Membranes harboring wild type SERT and on SERT-V367N were subjected to extraction with methyl-β-cyclodextrin (MβCD) (10 mg/mL) and to a sham procedure. We note that this sham procedure (i.e., incubation of membranes at 37°C followed by centrifugation) per se lowered the melting temperature of wild type SERT (TM = 57.6 ± 0.4°C) by about 3°C (cf. Figs. 1 and 8A). However, removal of cholesterol by exposing the membrane to MβCD shifted the melting curve to the left by an additional 4.1°C (TM = 53.5 ± 0.3°C, full symbols in Fig. 8A). More importantly, SERT-V367N was not only less thermostable (TM = 54.9 ± 0.3°C, open symbols in Fig. 8B) than wild type SERT, it was also more affected by cholesterol depletion. The melting curve shifted by about 7°C (TM = 47.8 ± 0.4°C, open symbols in Fig. 8B). This finding supports the conclusion that cholesterol is one of the lipids that define the thermostability of SERT.

Cholesterol depletion has been proposed to reduce the conformational flexibility of SERT (Laursen et al., 2018) and its close relative DAT (Jones et al., 2012) by trapping the proteins in the inward facing conformation. Accordingly, we
examined the contribution of thermodynamic and kinetic stability to the shift in thermal denaturation after cholesterol depletion (Fig. 8C) and extracted the rates to generate Arrhenius plots (Fig. 8D). After removal of cholesterol, the rate of slow inactivation of SERT increased steeply with temperature (Fig. 8D): thus, after depletion of cholesterol, the kinetic stability of SERT (357 kJ mol$^{-1}$) was substantially higher than in native membranes (113 kJ·mol$^{-1}$; cf. Fig. 5A) and approached that of SERT-TS3*. In contrast, the thermodynamic stability decreased after cholesterol depletion: an x-axis intercept of 52.9°C was calculated from the plot of the fraction of slowly unfolding SERT ($P_f/P_u$) over the reciprocal of temperature (Fig. 8E) and thus consistent with the $T_M$ determined by the approach used in Fig. 8A.

Discussion

Transmembrane transporters operate in a lipid environment and must visit different conformations during their transport cycle. The membrane lipids provide a stabilizing effect by constraining the hydrophobic core, but hydrophobic mismatches must be accommodated during the conformational changes. Here, we compared different versions of SERT to explore the mechanisms underlying changes in their stability and to understand the role of cholesterol in supporting the folding and function of the protein. SERT-TS3 is thermostabilized but transport-deficient because it is trapped in the outward-facing state (Green et al., 2015; Coleman et al., 2016). In contrast, the mutations in SERT-P601A/G602A impair folding of the protein. However, when rescued by pharmacochaperoning, SERT-P601A/G602A translocates substrates and thus is capable of undergoing all conformational transitions required to complete a transport cycle (El-Kasaby et al., 2014; Bhat et al., 2017). The basis for the enhanced and reduced stability of SERT-TS3*-mutant and SERT-P601A/G602A, respectively, can be rationalized from our observations. The MD simulations show that two of the three mutations (i.e., Y110A and I291A) of SERT-TS3 increase the interaction enthalpy and that the third mutation (T439S) provided additional hydrogen bonding. Thus, the mutations were predicted to augment the barrier separating the folded from the unfolded state(s). This prediction was confirmed by determining the time-dependent inactivation of SERT-TS3*: the kinetic stability of the protein was enhanced by about threefold when compared with wild type SERT. In addition, the thermodynamic stability was also substantially increased (by about 10°C). This can be attributed to the enhanced overall rigidity of the protein, but the local gains in entropy, which were detected by the MD simulations, also make a substantial contribution. The link between entropy to thermodynamic stability can be rationalized as follows: increased entropy (e.g., introduction of void by smaller residues as in SERT-TS3*) can lead to increased thermal stability because the $\Delta S$ of unfolding (the energetic gain of the system upon unfolding) decreases (Karshikoff et al., 2015). In addition, the presence of voids, which are too small to be filled by atoms (e.g., water) lead to an increased transition and around $T_M$. Error bars represent the standard error of the parameters extracted from the fitting procedure. The lines were drawn using linear regression.
barrier. Similarly, the MD simulations for SERT-P601A/G602A highlighted the mechanistic basis for the reduced stability of the protein: the substitution by two alanine residues does not per se destabilize the amphipathic helix but allows for many conformations to be visited. Accordingly, the compact structure of SERT, which is supported by a salt bridge between E615 at the end of the amphipathic helix and R152 in intracellular loop 1 (Koban et al., 2015), is not maintained. The population of states, where the C-terminal helix is detached by up to 1 nm from the hydrophobic core, offers possible routes for unfolding of the protein. Thus, the MD simulations predicted a reduced thermodynamic stability for SERT-P601A/G602A, and this prediction was verified by measuring the time-dependent inactivation of the protein.

The melting temperature of wild type SERT SERT-TS2* and SERT-TS3*, which we observed (i.e., about 61 °C, 64 °C, and 69 °C, respectively), were substantially higher than those determined for the solubilized proteins (40 °C, 52 °C, and 59 °C, respectively; Green et al., 2015). Our constructs differ from those used by Green et al. (2015) in two aspects: we examined YFP-tagged, full-length versions rather than the C- and N-terminally truncated versions, which were more suitable for X-ray crystallography. These differences are unlikely to confound the analysis. The truncated portion of the N- and C-termini of SERT are thought to contain large unstructured segments (Fenollar-Ferrer et al., 2014). They are hence unlikely to exert a stabilizing effect on the hydrophobic core. Similarly, the N-terminal YFP-tag is unlikely to affect thermal stability; YFP is a stable protein that can freely float in the cytosol when connected to the flexible N-terminus (Tsien, 1998). It is unlikely to interact with the hydrophobic core of the transporter. In fact, although extensively examined, an N-terminal YFP-tag has not been found to affect any biochemical activity or partial reaction of SERT. Thus, we conclude that it is the membrane lipids that account for the large difference in melting temperature. It is unsurprising that lipids exert a stabilizing effect on SERT. In fact, the melting temperature of the erythrocyte anion exchanger (band 3/SLC4A1) depends on the length of the lipid chains: long chain lipids enhance the stability of the protein, as does cholesterol (Maneri and Low, 1988). Here we focused on cholesterol for several reasons: cholesterol has been visualized in the crystal structures of SERT (Coleman et al., 2016), albeit at a different position of drosophila DAT (Penmatsa et al., 2013). MD simulations indicate up to six putative binding sites for cholesterol in SERT (Ferraro et al., 2016). In the absence of cholesterol, SERT cannot be expressed in a functionally active form (Tate et al., 2003). Our MD simulations of SERT revealed a major hydrophobic mismatch in the bilayer lacking cholesterol. The hydrophobic mismatch introduces a penalty in the free energy of the protein. The predicted impact on protein stability was confirmed by examining the effect of cholesterol depletion using two approaches: (i) depletion of cholesterol lowered the thermodynamic stability of wild type SERT. (ii) We examined the thermostability of the SERT-V367N mutant (Laursen et al., 2018). The mutation disrupts one of the candidate cholesterol binding sites (Ferraro et al., 2016; Laursen et al., 2018). As predicted, SERT-V367N was less stable and more susceptible to cholesterol depletion. The transport cycle of SERT-V367N is impaired, because the protein is trapped in the inward-
Fig. 8. Effect of cholesterol depletion on the melting curve of wild type SERT (A) and SERT-V367N (B) and time-dependent thermal inactivation of wild type SERT after cholesterol depletion (C–E). Binding assays were performed on membranes prepared from HEK293 cells stably expressing wild type SERT (A, SERT-wt) or SERT-V367N (B, SERT-VN). Cholesterol was depleted by incubating membranes for 30 minutes at 37 °C in the presence of 10 mg/ml methyl-β-cyclodextrin (MβCD, open circles). Control samples were subjected to a similar preincubation in the absence of MβCD (black circles). (C) Membranes from HEK293 cells stably expressing wild type SERT were extracted with MβCD as in panel A and incubated at the indicated temperatures for the indicated time periods. Thereafter, the binding reaction was carried out on ice as outlined under Materials and Methods. The rates of slow inactivation (D) and the ratio of slowly to rapidly unfolding (P/P∞, panel E) were estimated by fitting the data in panel C to the equation for a double exponential decay. Data were normalized to control [3H]imipramine binding in the absence of sodium, which stabilizes the outward-facing conformation (Laursen et al., 2018). Similarly, cholesterol depletion is thought to shift the wild type SERT into the inward-facing state (Laursen et al., 2018). We rule out that this conformational switch per se contributes to the reduction in Tm; the thermostability of wild type SERT was comparable in the presence of sodium, which stabilizes the outward-facing conformation, and of choline, which promotes the inward-facing state (Hasenhüttel et al., 2016).

Cholesterol supports the ability of SERT to translocate substrate (Scanlon et al., 2001). This is also true for its close relative DAT (Jones et al., 2012) and the more distantly related GABA-transporter-1 (GAT1/SLC6A1; Shoufani and Kanner, 1990). Cholesterol depletion reduced the thermodynamic stability but enhanced the kinetic stability. In fact, the kinetic stability of wild type SERT in the absence of cholesterol was comparable to that of the transport-deficient SERT-TS3*. We therefore surmise that the enhanced kinetic stability reflects a drop in conformational flexibility required for supporting the transport cycle. Our MD simulations identified a major difference in fluctuations of EL2 and IL4 in the absence and presence of cholesterol. EL2 moves upward during the transition from the outward- to the inward-facing state (Coleman et al., 2019; Esendir et al., 2021). Thus, it is tempting to speculate that the decrease in transport caused by cholesterol depletion is causally related to the reduction in the mobility of EL2 in the absence of cholesterol.

In its simplest version, the Lumry-Eyring model posits a two-step process for protein unfolding, i.e., N ⊃ U ⊃ V ⊃ D, where N, U, and D are the native, reversibly unfolded and irreversibly denatured states, respectively (Lumry and Eyring, 1954). Even brief incubations at elevated temperatures resulted in irreversible loss of binding-competent SERT. Thus, we failed to access the initial transition N ⊃ U, which corresponds to reversible unfolding/refolding. Because of this shortcoming, the energy change ΔG (or ΔH and ΔS), which is associated with the irreversible unfolding/refolding reaction, remains inaccessible. However, based on our data, it is nevertheless possible to separate the two components of protein stability, i.e., thermodynamic and kinetic stability, and to infer the energy barriers ΔG*, which governs the kinetic stability. Thermodynamic and kinetic stability can— but need not—be correlated (Sanchez-Ruiz, 2010). In fact, in SERT-TS3*, there was a concomitant increase in thermodynamic and kinetic stability. In contrast, in SERT-P601A/G602A, the thermodynamic stability was reduced but the kinetic stability was not: it was actually modestly elevated (176 kJ·mol⁻¹) relative to wild type SERT (113 kJ·mol⁻¹). Divergent effects were also seen previously in adenosine A₁-receptor mutants (Nasrollahi-Shirazi et al., 2020). Similarly, cholesterol extraction also reduced thermodynamic stability but enhanced kinetic stability. Folding of nascent membrane proteins must occur in the endoplasmic reticulum. Stabilizing effects of lipids introduce energy barriers in the folding trajectory and may thus impede the conformational search for the stable fold. The membrane of the ER differs from the plasmalemma by its substantially lower cholesterol content. We propose that this lower cholesterol content is a double-edged sword: it facilitates folding by reducing the energy difference between folding intermediates and the native state. The tradeoff is that hydrophobic mismatch can introduce energy barriers, which are difficult to overcome. Under these conditions, point mutations, which impede folding, are more prone to stall the folding trajectory. This is exemplified by SERT-P601A/G602A: when pharmacochaperoned, SERT-P601A/G602A can overcome the conformational trap in the ER and reach the cell surface. There, it is perfectly capable of sustaining the transport cycle, i.e., it can visit many conformations without any apparent rapid inactivation.

Authorship Contributions

Participated in research design: Szőlősi, Koban, Freissmuth, Stockner.
Conducted experiments: Ponleitner, Szőlősi, El-Kasaby.
Performed data analysis: Ponleitner, Szőlősi, El-Kasaby, Koban, Freissmuth, Stockner.
Wrote or contributed to the writing of the manuscript: Szőlősi, Koban, Freissmuth, Stockner.