ABSTRACT: Thiopurine S-methyltransferase (TPMT) is a polymorphic enzyme involved in the metabolism and inactivation of thiopurine substances administered as immunosuppressants in the treatment of malignancies and autoimmune diseases. In this study, the naturally occurring variants, TPMT*6 (Y180F) and TPMT*8 (R215H), have been biophysically characterized. Despite being classified as low and intermediate in vivo enzyme activity variants, respectively, our results demonstrate a discrepancy because both TPMT*6 and TPMT*8 were found to exhibit normal functionality in vitro. While TPMT*8 exhibited biophysical properties almost indistinguishable from those of TPMTwt, the TPMT*6 variant was found to be destabilized. Furthermore, the contributions of the cofactor S-adenosylmethionine (SAM) to the thermodynamic stability of TPMT were investigated, but only a modest stabilizing effect was observed. Also presented herein is a new method for studies of the biophysical characteristics of TPMT and its variants using the extrinsic fluorescent probe 8-anilinonaphthalene-1-sulfonic acid (ANS). ANS was found to bind strongly to all investigated TPMT variants with a $K_d$ of approximately 0.2 μM and a 1:1 binding ratio as determined by isothermal titration calorimetry (ITC). Circular dichroism and fluorescence measurements showed that ANS binds exclusively to the native state of TPMT, and binding to the active site was confirmed by molecular modeling and simulated docking as well as ITC measurements. The strong binding of the probe to native TPMT and the conformity of the obtained results demonstrate the advantages of using ANS binding characteristics in studies of this protein and its variants.

INTRODUCTION

Thiopurine S-methyltransferase (TPMT) (E.C. 2.1.1.67) is an S-adenosylmethionine (SAM)-dependent cytosolic enzyme primarily known for its key role in the metabolism of thiopurines, such as 6-mercaptopurine (6-MP), used as immunosuppressants administered in the treatment of malignancies and autoimmune diseases. Thiopurines are prodrugs that require metabolic activation through a series of intracellular reactions to generate cytotoxic metabolites, thioguanine nucleotides (TGNs). TGNs are nucleotide analogues that are incorporated into the DNA and RNA of the cell, which ultimately cause apoptosis. While the natural substrate of TPMT is not known, the enzyme complicates the metabolism of thiopurines by methylating their metabolic intermediates. As the methylated product can no longer be incorporated into DNA and RNA, the administered dosage of thiopurine drugs is adjusted to account for partial TPMT inactivation. However, TPMT is a polymorphic enzyme with more than 40 naturally occurring variants identified so far, most of which exhibit low or intermediate enzyme activity toward thiopurine substrates; 1 out of 300 in a population is homozygous with two nonfunctional TPMT alleles, resulting in no detectable enzyme activity; 10% are heterozygous and exhibit intermediate enzyme activity; and approximately 90% exhibit normal to high enzyme activity.2,3 Administering standard dosage to patients carrying nonfunctional alleles puts them at the risk of developing life-threatening toxicity by accumulation of high concentrations of active TGNs (which could potentially cause myelosuppression). On the other hand, high TPMT enzyme activity causes accumulation of the methylated product, which could result in hepatotoxicity. Because of the complex metabolism of thiopurines, their therapeutic index is very narrow. To avoid severe side effects in patients, the individual genotype and phenotype (TPMT enzyme activity) are routinely determined prior to thiopurine administration to establish proper dosage. As a consequence of routine genotyping and phenotyping, novel TPMT protein variants are frequently discovered. Even though most of the variants exhibit reduced enzyme activity toward thiopurine substrates, few studies have investigated the molecular causes of decreased functionality. In some cases, the reason for low enzyme activity has been shown to be caused by decreased protein stability, faster proteasomal degradation, autophagy, and aggregation, causing cellular protein levels to decrease.

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However, discrepancies between clinical and in vitro measured activities have been reported. For example, the TPMT*31 variant (originally denoted TPMT*28) was shown to exhibit normal enzymatic function in purified samples, although the protein was found to be destabilized, causing in vivo degradation and reduced enzyme activity owing to lowered protein levels.

Generally, detailed biophysical studies on purified samples of TPMT variants are scarce, and very few studies have determined the molecular causes of decreased enzyme activity.

Investigating and explaining the molecular reasons for varying enzyme activities are important aspects in understanding the consequences of single amino acid substitutions and their impact on treatment efficacy and patient safety. Furthermore, studying variant proteins not only generates information on the molecular causes of altered functionality but also contributes knowledge of the stability, function, and interactions of the TPMT protein and its role in clinical treatment.

The publication of the high-resolution crystal structure of human TPMT in complex with its coproduct S-adenosylhomocysteine (SAH) in 2007 has contributed greatly to further studies of this enzyme and its variants. The molecular structure revealed a plausible reaction mechanism, where the methyl group is transferred from the cofactor SAM to the substrate through an $S_N^2$-type reaction. This mechanism was confirmed by cocrystallization of the murine orthologue with SAH coproduct and 6-MP substrate, as well as QM/MM and molecular dynamics simulation.

Although the mode of action toward thiopurines is well-established, little is known about the natural function and substrate of TPMT. It has been shown to bind a variety of substances and seems to have a predisposition for binding heterocyclic compounds with carboxyl and amine group substituents, and some of these substances could interfere with the enzymatic function. For instance, methotrexate has recently been shown to bind to wild-type TPMT, affecting its enzyme activity toward 6-MP during clinical treatment.

The drug furosemide has also been confirmed as a clinically relevant inhibitor of TPMT. The two naturally occurring protein variants investigated in this study, TPMT*6 (Y180F) and TPMT*8 (R215H), were discovered as variants with low and intermediate in vivo enzyme activity, respectively. Kinetic measurements in mammalian cells (using 6-MP or 6-TG as thiopurine substrates) have been reported, but studies on these protein variants are otherwise scarce. Both mutation sites are situated in the central $\beta$-sheet of the protein (Figure 1), relatively far away from the active site, with Y180 completely buried within the protein core. In the TPMT*6 variant protein, tyrosine 180 is substituted to phenylalanine, a textbook example demonstrating that both TPMT*6 and TPMT*8 are able to contribute favorably to the cellular stability of TPMT. Cells starved of methionine (a precursor of SAM) exhibited reduced TPMT enzyme activity and increased proteasomal degradation, effectively lowering cellular protein levels. To elucidate if these effects are caused solely by the metabolic status of the cell and the availability of SAM, or thermodynamic stabilization of the protein—cofactor complex, we investigated the unfolding under our experimental conditions. Furthermore, we have investigated the use of the extrinsic fluorescent probe 8-anilinonaphthalene-1-sulfonic acid (ANS) as a tool for studying the tertiary structure and stability of TPMT.

### RESULTS AND DISCUSSION

#### Functional Characterization of TPMT*6 and TPMT*8

TPMT*6 (Y180F) and TPMT*8 (R215H) have been reported as low and intermediate activity variants, respectively. Our measurements on purified recombinant protein samples reveal that both of the investigated variants are fully functional in vitro using 6-MP as the thiopurine substrate. These results were confirmed in experiments involving mammalian cells and were interpreted as indicating that the TPMT*31 variant (originally denoted *28) was enzymatically functional but structurally destabilized and prone to aggregation. It has been demonstrated that for some TPMT variants, the main cause of decreased protein levels is proteasomal degradation through the ubiquitylation pathway.

Experiments using mammalian cells have shown a correlation between enzyme activity and protein levels for a number of TPMT variants (including TPMT*6 and TPMT*8), suggesting that nonfunctional or destabilized variant proteins are recognized as defective by the quality control functions of the cells and targeted for degradation. Because the TPMT variants in our study proved to be functional in vitro, we investigated the structural properties of these proteins to elucidate the possible reasons for decreased in vivo enzyme activity.

#### Probing the Stability of the TPMT Secondary Structure Using Circular Dichroism

The far-UV CD spectra (Figure 3A) show similar shape and amplitude, demonstrating that both TPMT*6 and TPMT*8 are able to assume the proper fold identical to that of the wild-type protein at room temperature. The interactions between TPMT and its cofactor SAM were also investigated using circular dichroism (CD) spectroscopy. SAM has recently been shown to contribute favorably to the cellular stability of TPMT. Cells starved of methionine (a precursor of SAM) exhibited reduced TPMT enzyme activity and increased proteasomal degradation, effectively lowering cellular protein levels. To elucidate if these effects are caused solely by the metabolic status of the cell and the availability of SAM, or thermodynamic stabilization of the protein—cofactor complex, we investigated the unfolding of
Figure 2. Detailed view of the local interactions of (A) Y180 (TPMT*6) and (B) R215 (TPMT*8) (PDB ID: 2BZG).

Figure 3. Spectroscopic properties of the TPMT variants, shown as filled circles (TPMTwt), open triangles (TPMT*6), and open squares (TPMT*8) (unless otherwise specified), in (A) native far-UV CD spectra, (B) thermal denaturation monitored by CD spectroscopy at 222 nm in the presence of cofactor SAM, inset showing TPMTwt in the presence of SAM (filled circles) and in the absence of SAM (open circles), (C) thermal denaturation monitored by CD spectroscopy at 222 nm in the presence of ANS, and (D) thermal denaturation monitored as ANS fluorescence emission detected at 475 nm.

Table 1. Thermodynamic Characteristics of the TPMT Variants

| Protein Variant | Thermal Melting Temperature (°C) | Circular Dichroism | Fluorescence | Dissociation Constant, $K_d$ | Cofactor | ANS (nM) |
|-----------------|---------------------------------|--------------------|--------------|------------------------------|----------|----------|
|                 |                                 | No Ligand | +SAM | +ANS | +ANS | SAM (μM) | ANS (nM) |
| TPMTwt          |                                 | 46.4 ± 0.6 | 46.8 ± 0.5 | 53.8 ± 0.5 | 54.1 ± 0.5 | 2.2 ± 0.7 | 219 ± 58 |
| TPMT*6          |                                 | 41.4 ± 1.1 | 40.5 ± 0.5 | 48.6 ± 0.7 | 49.6 ± 0.4 | 1.8 ± 0.5 | 187 ± 32 |
| TPMT*8          |                                 | 46.5 ± 0.4 | 47.3 ± 0.3 | 51.5 ± 0.4 | 54.1 ± 0.3 | 1.5 ± 0.1 | 202 ± 11 |

$^a$Thermal melting points, $T_m$, were obtained using CD spectroscopy monitored at 222 nm or ANS fluorescence emission detected at 475 nm. 
$^b$Dissociation constants, $K_d$, were obtained using ITC. 
$^c$Cofactor SAM was added in 10-fold molar excess. 
$^d$The extrinsic fluorescent probe ANS was added in 20-fold molar excess.
the TPMT secondary structure by monitoring the change in ellipticity at 222 nm as a function of increased temperature. The resulting stability curves displayed multiphasic transitions (Figure 3B). However, the transitions were treated as a two-state transition model as previously done for TPMTwt as well as other TPMT variants.4,8,26

Results show only minor contributions to the overall stability of TPMT upon SAM binding (Table 1). The unfolding of the protein was also investigated in the presence of ANS and monitored as the change in ellipticity at 222 nm as a function of increased temperature. The resulting stability curves were assumed to obey a two-state transition model (Figure 3C). Addition of ANS substantially stabilizes the secondary structure of the protein variants investigated herein with an increased $T_m$ values of 7.4, 7.2, and 5.0 °C for wild-type TPMT, TPMT*6, and TPMT*8, respectively. The obtained $T_m$ values in the presence of ANS are 53.8, 48.6, and 51.5 °C for wild-type TPMT, TPMT*6, and TPMT*8, respectively (Table 1). Increased thermostability indicates that ANS interacts preferably with the native state of TPMT, stabilizing the protein and shifting the equilibrium toward the folded state. The thermal unfolding of the investigated variants show that the stability of TPMT*8 is comparable with that of the wild-type protein, whereas the stability of TPMT*6 was found to be decreased, indicating loss of stabilizing interactions within the protein.

Investigating the Stability of the TPMT Tertiary Structure. Even though TPMT contains five tryptophan residues, intrinsic tryptophan fluorescence is not a suitable tool for monitoring the unfolding of the tertiary structure of this particular protein. Four out of five tryptophan side-chains are solvent accessible, which precludes the monitoring of shift in emission wavelengths as the changes when going from one state to another are very small and cannot be reliably distinguished. However, the fluorescent compound ANS has been shown to bind extensively to the native state of the protein.8 ANS is commonly used to study the conformational changes during protein unfolding, but it is known to also bind to the native states of other proteins.27−29 ANS has been proven useful in other cases where measuring intrinsic tryptophan fluorescence is not feasible.30 To determine whether or not ANS is a suitable tool for studying the TPMT tertiary structure, we investigated the interactions between the protein and the probe. The unfolding of the protein was also monitored as changes in ANS fluorescence as a function of increased temperature (Figure 3D). A distinct thermal denaturation profile was observed for all variants and interpreted as a two-state transition. The $T_m$ value of TPMT*6 (49.6 °C) was found to be lower than the $T_m$ values of wild type and TPMT*8 (54.1 °C for both variants) (Table 1). ANS fluorescence intensity was the same in the samples above denaturing temperature as in the reference buffer, indicating no binding of ANS to the denatured state of the protein, confirming preferential binding to the native state and dissociation of ligand upon unfolding.

Characterization of SAM and ANS Binding to TPMT. Characterization of the binding of SAM and ANS to the
investigated TPMT variants were also studied using isothermal titration calorimetry (ITC). Titration of cofactor SAM to wild-type TPMT yielded an equilibrium dissociation constant, $K_d$, of approximately 2 μM (Figure 4A). Titration of SAM to TPMT*6 and TPMT*8 exhibited similar affinities (Table 1), whereas titration of ANS resulted in a dissociation constant, $K_d$, 10 times lower than that of SAM for TPMT wild-type (Figure 4B) as well as variant proteins (Table 1). For both SAM and ANS, the binding model established that only one ligand molecule was bound per protein molecule.

**Investigating the ANS Binding Site.** ANS has been shown to interact with the adenosine binding sites of other nucleotide-dependent proteins, and the structural similarities between ANS and the cofactor SAM together with the experimental results showing binding of ANS exclusively to the native state of TPMT (with a 1:1 ratio) suggested that the probe might bind to the active site. To support the experimental results, we simulated the docking of ANS to the crystal structure of TPMT (PDB ID: 2BZG) using AutoDock Vina. The best-modeled structures were all found to colocalize with the cofactor binding site. The two best-modeled structures (lowest $\Delta G$) were used for a more detailed view of putative interacting partners of TPMT. From the modeled data, two obvious binding modes were obtained (Figure 5) with residue Trp29 or Ile91 as hydrogen bonding partners of the phosphate moiety of ANS.

**Y180 is Important for Stability.** Our studies of the naturally occurring protein variant TPMT*6 (Y180F) show that substitution of tyrosine 180 to phenylalanine decreases the stability of the TPMT*6 variant protein relative to wild type. The TPMT*6 mutation site is situated in the central $\beta$-sheet of the protein, completely buried by flanking helices. Amino acid substitution causes removal of the tyrosine side-chain OH-group that participates in a hydrogen bonding network within the core of the wild-type protein. Hydrogen-bonded tyrosine OH-groups have been shown to make substantial contributions to protein stability, and burial of polar groups has been demonstrated to contribute more to stability than burial of nonpolar volume equivalents, explaining the dramatic effects of this type of mutation on TPMT*6 protein stability. Furthermore, recent work on the destabilized TPMT*23 (A167G) variant protein showed that substitution of A167 to glycine distorts helix $\alpha G$ (residues 162–173), which causes disruption of the interactions between Y166 and its hydrogen-bonding partners D151 and Y180. Similarly, molecular dynamics simulation suggests that mutation of A154 to threonine (TPMT*3B) also disrupts the Y166–D151–Y180 hydrogen-bonding network, causing rearrangement of helix $\alpha G$ (residues 162–173) and destabilization of the TPMT*3B variant protein. Our studies of the TPMT*6 (Y180F) variant confirm that the Y166–D151–Y180 hydrogen-bonding network is highly sensitive to perturbations, which seem to inevitably cause destabilization of the TPMT protein. Although the TPMT*6 protein assumes the proper fold and exhibits normal functionality in vitro, the decreased stability of this variant would likely lead to increased degradation and shortened half-life in vivo, effectively lowering protein concentrations that decreases the enzymatic activity toward thiopurine substrates. This would explain the discrepancy in functionality observed for the TPMT*6 protein variant.

**Biophysical Properties of TPMT*8 Identical to Wild Type.** The biophysical characteristics of the naturally occurring variant TPMT*8 (R215H) are virtually identical to the wild-type protein. R215 is not highly conserved; in fact, histidine occurs in several species (Figure 6), and there seems to be a preference for polar residues at this position equivalent. The $\beta$-strand in which residue 215 is situated sits at the very edge of the central sheet, facing the solvent, surrounded by predominantly polar residues. Combined with the fact that polar residues seem to be preferred at the position 215, it is possible that the very nature of the substitution of TPMT*8 makes it structurally tolerable. Our results clearly show that the structure and stability of this protein are not affected by mutation, and the biophysical characteristics of the TPMT*8 protein variant are identical to those of the wild-type protein. Previous studies using mammalian cells have reported intermediate TPMT*8 enzyme activity toward thiopurine substrates, but the discrepancy cannot be explained by reduced intrinsic stability of the protein as this variant exhibits normal functionality in vitro. We therefore conclude that the decreased TPMT*8 in vivo enzyme activity cannot be explained by molecular causes and must be due to other mechanisms beyond the scope of this study. Interestingly, several novel TPMT variants, TPMT*20 (K238E), TPMT*25 (C212R), TPMT*26 (F208L), and TPMT*31 (I204T, originally denoted TPMT*28) situated in structural elements adjacent to R215 have been reported with decreased in vivo enzyme activity. Of these variants, TPMT*31 (I204T, originally denoted *28) exhibited biophysical characteristics similar to TPMT*8. Additionally, Garat et al. demonstrated that TPMT has an inherent dipole moment and suggested that the TPMT*25 (C212R) amino acid substitution disturbs the electrostatic balance of the protein. It could be that this particular region of the protein is sensitive to perturbations due to amino acid substitution, rather than the nature of the mutations per se. It is possible that TPMT variants exhibiting deviations in this structural region are targeted by the quality control system of the cell, causing increased clearance and shortened half-lives of these proteins. It remains to be discovered if the variants mentioned above exhibit similar structural and biophysical characteristics as TPMT*8.

**SAM Does Not Add Significantly to TPMT Thermodynamic Stability.** The cofactor, SAM, has recently been demonstrated to contribute to the cellular stability of TPMT, protecting the protein from degradation, which was shown to increase in methionine-deficient cells (methionine is a precursor of SAM). We have studied the contributions of SAM and found that the cofactor makes only minor

![Figure 5. AutoDock modeling. Detailed view of the two lowest energy structure models of ANS and interaction partners of TPMT. The coproduct, SAH, is shown in red. The dotted lines illustrate the putative interactions between ANS and Trp29 and Ile91 of TPMT.](image-url)
contributions to the thermodynamic stability of the TPMT secondary structure in vitro, as determined by thermal denaturation monitored by CD spectroscopy. It is worth noting that although the $T_m$ values of the protein with ANS are higher than those with SAM (Table 1), this could be a reflection of the different binding affinities of the two ligands. The $K_d$ of ANS is 10-fold higher than that of SAM, and the $T_m$ values obtained with the respective ligand differ correspondingly, possibly due to coupling of binding and unfolding equilibria during thermal denaturation. Interestingly, molecular dynamics simulation has shown that the cofactor contributes to the ordering of $\alpha E$ (residues 134–141) secondary structure, despite the presence of an otherwise disruptive proline residue (P139) in this helix. In the apoprotein, helix $\alpha E$ was found to be disordered, causing the solvent accessible surface area of the SAM-binding site to increase. This interesting characteristic could be a mechanism to provide increased accessibility to the ordering of helix $\alpha E$ and the $\beta$6 (Y180F) and TPMT $\beta$8 (R215H), respectively. The obtained SIFT scores for TPMT*6 (Y180F) and TPMT*8 (R215H) were 0.13 and 1.00, respectively, indicating the substitutions to be structurally tolerable. Positions 180 and 215 of the human protein sequence are used as reference to describe the homologous positions of the compared species.

Figure 6. SIFT analysis of the $\beta 7$ and $\beta 8$ strands harboring the mutation sites of TPMT*6 (Y180F) and TPMT*8 (R215H), respectively. The obtained SIFT scores for TPMT*6 (Y180F) and TPMT*8 (R215H) were 0.13 and 1.00, respectively, indicating the substitutions to be structurally tolerable. Positions 180 and 215 of the human protein sequence are used as reference to describe the homologous positions of the compared species.

Probing Stability and Characteristics of the TPMT Tertiary Structure. In this study and in our previous work, we have seen that far-UV CD spectroscopy is useful in detecting variants that are destabilized relative to wild-type TPMT because this method allows for rapid screening of the secondary structure stability and folding pattern of the protein and its variants. However, while this is a useful technique, it is limited to studies of the secondary structure. Because (to our knowledge) no rapid and reliable method of probing the tertiary structure of TPMT is available to date, we have developed a method in which the fluorescent dye ANS is used as a tool to probe the TPMT tertiary structure and active site integrity. Our studies show that ANS binds extensively to the tertiary structure of TPMT, and fluorescence and ITC experiments yielded a dissociation constant, $K_d$, of 0.2 $\mu$M ANS with a 1:1 binding ratio. Fluorescence spectra and thermal unfolding experiments show binding exclusively to the folded state of TPMT, and docking simulations indicate that ANS binds to the active site of the protein, colocalized with the adenine moiety of SAM. The strong binding of ANS to native TPMT and the conformation of the obtained results demonstrate the robustness of this probe and the advantages of this method in studying the biophysical characteristics of this particular protein and its variants. This method in combination with CD spectroscopy is particularly powerful because the results obtained provide comprehensive insight into the properties of the secondary and tertiary structure.
tertiary structures of the protein, and the characteristics of ANS binding are especially interesting in further studies of TPMT and its variants. For example, the increase in stability upon binding of ANS could allow crystallization of the full-length protein as well as reasonably stable variants, which would be tremendously beneficial to future studies of this protein. Also, the extent of ANS binding (compared to wild type) could be used to screen for functionally defective variants that do not exhibit the proper active site tertiary structure. Similarly, ANS binding could be used to rapidly gauge the condition of a TPMT sample prior to binding studies to approximate the number of active protein molecules. Because TPMT (particularly when purified) is not very stable over time and the number of intact active sites could vary between purified batches, this is a potentially very important area of application that could improve the accuracy and reliability of inhibition studies, which could ultimately benefit clinical treatment with thiopurine substances. On a similar note, this method holds great potential as a tool for drug interaction studies using fluorescent displacement techniques. Last, our docking simulations indicated interactions between ANS and Ile91, which is part of a conserved SAM–MT sequence motif. If so, ANS could bind similarly to other SAM–MTs as well, considering the high degree of structural similarity within this protein family and the occurrence of conserved sequence motifs involved in cofactor binding. If so, the method described herein would be beneficial not only to future studies of TPMT and its variants but also to structurally related proteins as well.

## EXPERIMENTAL PROCEDURES

**Enzyme Activity Measurements.** TPMT enzyme activity measurements were performed by measuring the rate of formation of 6-methyl-mercaptopurinoriboside from 6-MP using $^{14}$C-labeled S-adenosyl-L-methionine as a methyl group donor according to the previously published protocol.

**Site-Directed Mutagenesis, Protein Expression, and Purification.** TPMT variants (residues 1–245) were constructed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Inc, Santa Clara, CA, USA). The variants were cloned into pET28-LIC vectors, transformed into Escherichia coli (E. coli) BL21-CodonPlus (DE3)-RII cells (Agilent Technologies, Inc, Santa Clara, CA, USA), and incubated in the Luria-Bertani (LB) medium overnight at 37 °C. The overnight cultures were diluted 1:200 in the LB medium and incubated until OD$_{600}$ reached $\approx$1.0. The cells were induced with 1 mM isopropyl-thiogalactoside at 21 °C overnight and harvested by centrifugation at 4 °C and 3000g for 45 min. The cells were resuspended in 20 mM Tris-HCl (pH 8.0), 250 mM NaCl, 5 mM imidazole, 5% glycerol, and 2 mM β-mercaptoethanol ($\beta$-ME). Lysis of the cells was done by sonication, followed by centrifugation at 10 000g at 4 °C for 40 min. The supernatant was applied to preequilibrated Ni-NTA Superflow agarose (Qiagen, Hilden, Germany). Resin with bound protein was washed with binding buffer and wash buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 mM imidazole, 5% glycerol, and 2 mM $\beta$-ME]. Elution of the bound His-tagged TPMT was done with 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 500 mM imidazole, 5% glycerol, and 2 mM $\beta$-ME. The eluted protein was immediately dialyzed against 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM imidazole, 5% glycerol, and 2 mM $\beta$-ME. The His-tag was cleaved off by incubation of the protein solution with biotinylated thrombin (Novagen, Merck Darmstadt, Germany) for 6 h at 4 °C with gentle agitation. The protease was removed from the solution by addition of streptavidin agarose (Novagen, Merck Darmstadt, Germany) and incubated for 1 h with gentle agitation. Streptavidin agarose beads with bound protease were then removed by centrifugation at 4 °C at 1000g. The uncleaved TPMT protein in the supernatant was separated from the cleaved protein by capture on Ni-NTA Superflow agarose. Cleaved TPMT flow-through was subsequently applied to a Superdex 75, S200 column (GE Healthcare, Uppsala, Sweden) and thereafter dialyzed to 20 mM potassium phosphate (pH 7.3), 150 mM NaCl, 10% glycerol, 0.5 mM TCEP, and stored at −80 °C.

**Circular Dichroism Measurements.** Far-UV native CD spectra were collected at 200–260 nm at 21 °C, averaged over three scans with increments of 1 nm on a Chirascan spectrometer (Applied Photophysics, Leatherhead, United Kingdom). Far-UV thermal unfolding was monitored at 22 nm with the temperature increasing from 18 to 80 °C in 2 °C increments, with 10 scans sampled during 60 s per increment. A TPMT sample of 3 μM in 20 mM potassium phosphate (pH 7.3), 75 mM NaCl, 2% glycerol, and 0.5 mM TCEP in a 4 mm path length cuvette was used for measurements. The measurements in the presence of ligand were performed under two different conditions: in the presence of 30 μM cofactor SAM or 60 μM ANS. Determination of $T_m$ values and estimated errors were obtained from three independent measurements using the software CDpal.

**Fluorescence Measurements with Extrinsic Fluorescent Probe ANS.** Fluorescence measurements were recorded on a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon S.A.S., Longjumeau, France) with 3 μM protein solution and 60 μM ANS in 20 mM potassium phosphate (pH 7.3), 75 mM NaCl, 2% glycerol, and 0.5 mM TCEP in a 4 mm path length cuvette, scanned between 18 and 80 °C in 2 °C increments. ANS was excited at 360 nm and its emission was monitored at 400–600 nm. Thermal unfolding was plotted as the relative change in maximum fluorescence intensity at 475 nm as a function of increased temperature. Determination of $T_m$ values and estimated errors were obtained from three independent measurements using the software CDpal.

**Isothermal Titration Calorimetry Measurements.** All experiments were performed on a Malvern MicroCal PEAQ-ITC (Malvern Instruments Ltd, England) instrument. All experiments were performed at 25 °C with a reference power of 7 μcal s$^{-1}$. A protein concentration of approximately 50 μM was used. Ligand concentrations were 250 μM for ANS and 250 μM for SAM dissolved in dialysis buffer. A total of 13 injections were made. All data were analyzed using manufacturers software. At least three separate runs of injection were made for each variant-ligand pair.

**Theoretical Structure Analysis.** SIFT (sorting intolerant from tolerant) program analysis was performed to predict structural effects based upon sequence homology and physical properties of amino acids. Mammalian sequences (16) were aligned. Probability scores of less than 0.05 suggest a deleterious amino acid substitution. Docking of the fluorescent ligand ANS to TPMT was performed using AutoDock Vina with human TPMT (PDB ID: 2BZG) as the template and a search space of 20 × 20 × 20 Å.
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