A Rationally Designed Monomeric Variant of Anthranilate Phosphoribosyltransferase from *Sulfolobus solfataricus* is as Active as the Dimeric Wild-type Enzyme but Less Thermostable

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A rational design strategy was used to engineer a monomeric variant of the anthranilate phosphoribosyltransferase from *Sulfolobus solfataricus* (ssAnPRT) that is as active as the dimeric wild-type enzyme, but less thermostable. The purified double mutant I36E+M47D formed a monomer with wild-type catalytic activity but reduced thermal stability. The single mutants I36E and M47D were present in a monomer–dimer equilibrium with dissociation constants of about 1 μM and 20 μM, respectively, which were calculated from the concentration-dependence of their heat inactivation kinetics. The monomeric form of M47D, which is populated at low subunit concentrations, was as thermolabile as monomeric I36E+M47D. Likewise, the dimeric form of I36E, which was populated at high subunit concentrations, was as thermostable as dimeric wild-type ssAnPRT. These findings show that the increased stability of wild-type ssAnPRT compared to the I36E+M47D double mutant is not caused by the amino acid exchanges *per se* but by the higher intrinsic stability of the dimer compared to the monomer. In accordance with the negligible effect of the mutations on catalytic activity and stability, the X-ray structure of M47D contains only minor local perturbations at the dimer interface. We conclude that the monomeric double mutant resembles the individual wild-type subunits, and that ssAnPRT is a dimer for stability but not for activity reasons.

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**Keywords:** anthranilate phosphoribosyltransferase; hyperthermophile; rational protein design; *Sulfolobus solfataricus*; thermal stability

**Introduction**

The non-covalent association of polypeptide chains into homo-oligomeric and hetero-oligomeric enzyme complexes has important consequences for function and stability. The interplay between assembled subunits can lead to the formation of complex active sites, facilitate the regulation of catalytic activities, and is the prerequisite for substrate channelling.\(^1,2\) Moreover, the comparison of homologous enzymes from mesophiles and hyperthermophiles suggests that a higher oligomerisation state increases the resistance to thermally induced inactivation and unfolding.\(^3–6\) The role of individual residues for the assembly of subunits and the consequences of a specific oligomerisation state for the function and stability of an enzyme can be tested by protein engineering. Along these lines, amino acid substitutions at the contact interface lead to the dissociation of oligomeric enzymes into subunits with more or less reduced reaction rates and conformational stabilities.\(^7–11\)

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Abbreviations used: PRT, phosphoribosyltransferase; AnPRT, anthranilate phosphoribosyltransferase; ssAnPRT, *Sulfolobus solfataricus* AnPRT.
The members of the phosphoribosyltransferase (PRT) enzyme class catalyze the transfer of a phosphoribosyl group to an aromatic base. PRTs have an important role in the metabolism of nucleotides and amino acids; for example, as key components of the purine salvage and the histidine and tryptophan biosynthetic pathways. PRPP is used as the universal phosphoribosyl donor from which the 1-pyrophosphate group is displaced during the formation of the N-1'-glycosidic bond to the base specific for each PRT. Three structurally unrelated classes of PRT can be distinguished. Most PRTs with known X-ray structure belong to class I, whereas class II comprises quinolate PRT and nicotinate PRT. The only known member of class III is anthranilate phosphoribosyltransferase (AnPRT), which catalyzes the Mg-dependent ribosylation of anthranilate to phosphoribosyl anthranilate within tryptophan biosynthesis (Figure 1). The X-ray structures of AnPRTs from Sulfolobus solfataricus (ssAnPRT), Pectobacterium carotovorum, Mycobacterium tuberculosis, Thermus thermophilus and Nostocaceae (PDB entry 1V8G) and Nostocaceae (PDB entry 1VQU) have been solved at high resolution. Their analysis showed that AnPRTs have a similar fold and homodimeric structure as nucleoside phosphorylases, which catalyse the phosphorylisis of the glycosidic bond of nucleosides to yield free bases and ribose -1-phosphate. Figure 2(a) shows a ribbon diagram of the structure of ssAnPRT, which has been solved in complex with two molecules of anthranilate and one molecule of PRPP. This structure, in combination with mutational analysis, made it possible to identify the role of individual residues for substrate binding at the active site of each subunit.

To test the significance of homo-dimer formation for the function and extreme thermostability of ssAnPRT, we exchanged two residues at the contact interface individually and in combination to loosen the dimer. The double mutant formed a pure monomer with wild-type catalytic activity but reduced thermal stability. The two single mutants were present in equilibrium between the monomer and the dimer, and their resistance to thermal inactivation increased with increasing concentration of protein. These results show that dimerization is dispensable for the catalytic activity of ssAnPRT but important for its high intrinsic stability. The comparison of the X-ray structures of wild-type ssAnPRT and the M47D mutant is in full agreement with these findings.

Results

Strategy of rational design

The monomerisation of ssAnPRT was planned on the basis of the high-resolution X-ray structure of the dimeric wild-type enzyme (Figure 2(a)). Each subunit of the dimer consists of two domains: a large α/β domain, formed by a central β-sheet and a C-terminal cluster of eight helices, and a small α-helical domain comprising six helices. The substrate-binding cavity for the coordination of AA and PRPP is at the domain interface, while dimer formation is mediated by the small α-helical domains that associate in a head-to-head fashion following an approximate 2-fold symmetry. Details of the intersubunit interactions, which are formed mainly by residues from helices α1, α3 and α8, are given in Figure 2(b). The subunit interface of dimeric ssAnPRT is mainly hydrophobic. From the 24 residues (corresponding to a surface area of 939 Å²) buried per monomer, 20 residues (corresponding to 706 Å²) are apolar (PISA server). A computer-based analysis (the "prot-prot" option of the program PDBsum) showed that the hydrophobic residues Ile36 and Met47, which are located at the N and C-termini of helix α3, form the most numerous and intimate reciprocal intersubunit interactions. The main-chain carbonyl oxygen atom of Ile36 forms a hydrogen bond with the side-chain oxygen atom of Ser39 (the prime designates the residue from the other subunit), and its side-chain forms hydrophobic interactions with residues Glu35, Asn162, Val163, and Thr166. The main-chain carbonyl oxygen atom of Met47 is hydrogen bonded to the Nε atom of Lys13, while the sulphur atom and the Cα atom of its side-chain form hydrophobic interactions with Leu110, Ile111, and Ala144. Based on this analysis, site-directed mutagenesis was used to replace Ile36 and Met47 by the acidic residues glutamate and aspartate, both individually (single mutants I36E and M47D) and in combination (double mutant I36E+M47D). We reasoned that the introduction of the negatively charged side-chains would weaken the stabilizing interactions of Ile36 and Met47 with residues of the other subunit. Moreover, the relatively low distances between the Cα atoms of the two symmetry-related Ile36 residues (6.8 Å) and the two symmetry-related Met47 residues (4.4 Å) indicated that the introduced negative charges further weaken intersubunit interactions by electrostatic repulsion (Figure 2(c)). Furthermore, negatively charged residues at the protein surface increase the solubility of a protein, which could help to stabilize the monomer. Met47 was replaced by aspartate instead of glutamate, because its shorter side-chain has a lower probability of forming a stabilizing hydrogen bond with Lys13. Wild-type ssAnPRT and its mutants I36E, M47D, and I36E+M47D were generated with an N-terminal His6 tag by heterologous gene expression in Escherichia coli. The recombinant enzymes were enriched from the soluble fraction of the cell extract by heat denaturation of the host proteins, followed by metal chelate affinity chromatography. All enzymes had a purity >95%, as judged by SDS-PAGE.

† http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html
The oligomerisation state of the recombinant ssAnPRT proteins was tested by analytical gel filtration chromatography. Figure 3 shows that wild-type ssAnPRT and the single mutants I36E and M47D elute at a similar retention time, while the double mutant I36E+M47D elutes significantly later. The elution volumes of wild-type ssAnPRT, as well as the single mutants I36E and M47D correspond to molecular masses of 64.5 kDa, 63.4 kDa and 61.7 kDa, respectively, which is somewhat smaller than the molecular mass of 77.7 kDa calculated for the dimer. Likewise, the elution volume of the double mutant I36E+M47D corresponds to a molecular mass of 32.4 kDa, which is smaller than the molecular mass expected for the monomer of 38.8 kDa (Table 1).

To test whether these deviations are due to unspecific interactions of ssAnPRT with the gel matrix, the association state of the proteins was determined also by sedimentation equilibrium runs in the analytical ultracentrifuge. The results confirmed that the wild-type enzyme forms a dimer and that the double mutant I36E+M47D forms a monomer under the given experimental conditions. The apparent molecular mass for the single mutants I36E and M47D suggested that they are present in a rapid equilibrium between a minor monomeric and a major dimeric fraction (Table 1).

Catalytic activity

Steady-state enzyme kinetic parameters were measured to test whether the introduced amino acid substitutions have an influence on the catalytic activity of ssAnPRT. Table 2 shows that the turnover numbers ($k_{\text{cat}}$) of the mutants and the wild-type enzyme are almost identical. Moreover, the Michaelis constants for both AA ($K_{M,a}$) and PRPP ($K_{M,PRPP}$) differ only slightly between the monomeric I36E + D47M double mutant and the dimeric wild-type enzyme, yielding similar catalytic efficiencies ($k_{\text{cat}}/K_{M}$) of the two enzymes for both substrates.

Thermal stability

To investigate their stability against irreversible inactivation by heat, wild-type ssAnPRT and its mutants were incubated at 80 °C. Samples withdrawn after different time intervals were chilled on ice, and their residual activities were measured at 37 °C. We observed first-order kinetics for the time-dependent inactivation of the double mutant I36E+M47D, but more complicated kinetics for wild-type ssAnPRT and the two single mutants. Nevertheless, the time-courses allowed us to determine apparent half-lives ($t_{1/2}$) as an operational measure of kinetic stability. The $t_{1/2}$ values for wild-type ssAnPRT and the I36E+M47D double mutant were approximately 40 min and 3 min, independently of the applied subunit concentration. In contrast, the $t_{1/2}$ values of I36E and M47D increased from about 4 min and 3 min at the lowest subunit concentrations, to about 40 min and 15 min, respectively, at the highest subunit concentrations (Figure 4).

The concentration-dependence of the $t_{1/2}$ values, together with the results of analytical gel filtration and analytical ultracentrifugation (Table 1), suggested that the single mutants I36E and M47D are present in equilibrium between the highly thermostable dimer and a much more labile monomer. To determine the equilibrium constant ($K_D$) between dimer and monomer at 80 °C, we assumed the following mechanism for the dissociation and inactivation of ssAnPRT:

$$D \rightleftharpoons \text{2M} \rightleftharpoons \text{2U} \rightarrow X$$

(model 1)

$$k_{\text{diss}} \quad k_i \quad k_r$$

The ssAnPRT dimer (D) dissociates with the first-order rate constant $k_{\text{diss}}$ to fully active monomers (M); $k_{\text{diss}}$ is the second-order recombination rate constant, and the equilibrium dissociation constant $K_D = k_{\text{diss}}/k_{\text{ass}}$. The native monomer M then denatures reversibly with the first-order rate constants $k_i$ and $k_r$ to the unfolded state (U), which is inactivated with a first-order rate constant $k_x$.

Since the inactivation of the monomer M is a concentration-independent first-order process, which can be described by the rate constant $k_U$, model 1 simplifies to:

$$D \rightleftharpoons \text{2M} \rightarrow X$$

(model 2)

$$k_{\text{diss}} \quad k_i \quad k_x$$

Equation (5) outlined in Material and Methods was derived on basis of model 2. We used this equation to...
Figure 2. Dimer interface of wild-type ssAnPRT and the M47D mutant. (a) Ribbon diagram of the X-ray structure of ssAnPRT with bound substrates anthranilate (AA I and AA II) and PRPP. Each subunit consists of a large $\alpha/\beta$ and a small $\alpha$-helical domain, which mediates dimerisation in a head-to-head fashion. The side-chains of residues I36 and M47, which are located near the N and C-termini of $\alpha$-helix 3, respectively, are shown as sticks in both subunits. (b) A schematic display of all intersubunit interactions involved in dimerisation as identified by the “prot-prot” option of the program PDBsum. Hydrogen bonds and hydrophobic interactions involving M47 or I36 are in red and blue, respectively. All other interactions are in black. (c) Superposition of the dimer interfaces of wild-type ssAnPRT and the M47D mutant. Residues I36, M47 and D47 are shown as sticks. The distances between the $C_\beta$ atoms of the two I36 residues and the two M47 residues (marked by black stippled lines) are 6.8 Å and 4.4 Å, respectively. The distance between the carboxyl carbon atoms of the two D47 residues (marked by a yellow stippled line) is 3.6 Å.
apply non-linear least squares fitting to the inactivation kinetics, whereby datasets were recorded at different total subunit concentration ($c_{tot}$) (I36E: nine datasets, M47D: four datasets). For each dataset, the fitting analysis yielded an estimate for the $K_D$ of the monomer–dimer equilibrium of a particular single

**Figure 2 (legend on previous page)**
Dimerisation of a Thermostable Homo-dimeric Enzyme

**Figure 3.** Analytical gel filtration chromatography of wild-type ssAnPRT and its mutants. The elution volumes of the corresponding peaks. The experiments were performed on a calibrated Superdex S75 column at 25 °C in 50 mM potassium phosphate (pH 7.5), 300 mM KCl. The proteins were detected by their absorbance at 280 nm. The applied total protein concentration was 0.5 mg/ml in all samples, corresponding to a subunit concentration of 13.3 µM.

**Table 1.** Apparent and calculated molecular masses, and deduced oligomerisation states of wild-type ssAnPRT and its mutants

<table>
<thead>
<tr>
<th>ssAnPRT variant</th>
<th>(M^{ag}) (kDa)</th>
<th>(M^{au}) (kDa)</th>
<th>(M^{calc}) (kDa)</th>
<th>Deduced oligomerization state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>64.5</td>
<td>72.2</td>
<td>77.7 (D)</td>
<td>Dimer</td>
</tr>
<tr>
<td>I36E</td>
<td>63.4</td>
<td>66.0</td>
<td>77.7 (D)</td>
<td>Monomer ———— Dimer</td>
</tr>
<tr>
<td>M47D</td>
<td>61.7</td>
<td>60.1</td>
<td>77.7 (D)</td>
<td>Monomer ———— Dimer</td>
</tr>
<tr>
<td>I36E + M47D</td>
<td>32.4</td>
<td>34.1</td>
<td>38.8 (M)</td>
<td>Monomer</td>
</tr>
</tbody>
</table>

\(M^{ag}\), apparent molecular mass as deduced from analytical gel-filtration chromatography (ag) and sedimentation equilibrium runs in the analytical ultracentrifuge (au). Experimental conditions for analytical gel-filtration: 50 mM potassium phosphate (pH 7.5), 300 mM KCl, 25 °C; subunit concentration: 13.3 µM. Experimental conditions for analytical ultracentrifugation: 100 mM potassium phosphate (pH 7.5), 26 °C; subunit concentration: 9.3 µM. \(M^{calc}\), molecular mass calculated from the amino acid sequence for the dimer (D) and the monomer (M).

In order to elucidate the structural consequences of the introduced amino acid exchanges, we tried to crystallize the I36E, M47D and I36E+M47D mutants. However, suitable crystals (diffraction up to 2.85 Å resolution) were obtained only for the M47D

**Table 2.** Steady-state enzyme kinetic constants of wild-type ssAnPRT and its mutants

<table>
<thead>
<tr>
<th>ssAnPRT variant</th>
<th>(k_{cat}) (s⁻¹)</th>
<th>(K_M^{AG}) (µM)</th>
<th>(K_M^{PRPP}) (µM)</th>
<th>(k_{cat}/K_M^{PRPP}) (s⁻¹) (µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.41±0.04</td>
<td>0.085</td>
<td>180</td>
<td>4.8</td>
</tr>
<tr>
<td>I36E + M47D</td>
<td>0.28±0.06³</td>
<td>0.058</td>
<td>150</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Experimental conditions: 50 mM Tris–HCl (pH 7.2), 0.05 mM MgCl₂, 37 °C; various concentrations of AA or PRPP, in the presence of saturating conditions (10× \(K_M\)) of the second substrate; subunit concentration of the enzymes: 0.01 µM.

The \(k_{cat}\) values are the mean and standard deviation from three independent measurements.

The two single mutants show the same \(k_{cat}\) values within experimental error.

**Figure 4.** Differential scanning calorimetry was used to further investigate the thermal stability of ssAnPRT and its mutants. At the applied subunit concentration of 12 µM, wild-type ssAnPRT and I36E are predominantly dimeric, whereas M47D and I36E+M47D are mainly monomeric. The increased apparent melting temperatures of wild-type ssAnPRT (92 °C) and I36E (90 °C) compared to those of M47D (83 °C) and I36E + M47D (82 °C) (Table 3) support the finding that dimerisation stabilizes ssAnPRT against thermal denaturation.

**X-ray structure of the M47D variant**

Temperature (26 °C versus 80 °C), they agree reasonably well with those deduced from the thermal inactivation data. Since the kinetic stability of wild-type ssAnPRT is independent of concentration (Figure 4), it must be a pure dimer even at the lowest applied subunit concentration of 1 µM the lowest concentration of subunit applied. It follows that its \(K_D\) value at 80 °C is smaller than 0.1 µM. Since the kinetic stability of the double mutant I36E+M47D is not increased even at the highest applied subunit concentration of 27 µM, its \(K_D\) value at 80 °C must be larger than 270 µM.
protein (Table 4). The two independent biological homodimers found in the asymmetric unit of this crystal form were structurally equivalent to each other and very similar to wild-type ssAnPRT, with an rms deviation of only 0.7 Å for 339 out of 345 structurally equivalent Cα atoms per subunit. This result demonstrates that the amino acid replacement M47D does not lead to structural perturbations of the polypeptide chain, in accordance with the unchanged turnover number of the mutant (Table 2). The introduced symmetry-related carboxyl groups of Asp47 and Asp47′, which have a distance of 3.6 Å, are located in the plane of the Met47 and Met47′ side-chains buried within the hydrophobic environment of the subunit interface (Figure 2(c)). This finding suggests that the side-chains have elevated pKa values and might therefore be protonated at the pH 6.0 of the crystallization medium. Subunit dissociation will lead to proton release and enable favourable electrostatic interactions of the emerging negative charges with bulk solvent, thus stabilizing the monomer compared to the homo-dimer. The protonation of the Asp47 side-chain could also explain why it does not form a noticeable salt-bridge with the side-chain of Lys13′, whose Nε atom is in its vicinity. Although such an interaction is geometrically possible, it does not appear to be predominant according to crystallographic data, as anticipated in the design process.

The buried surface area per subunit is reduced to 840 Å² for the M47D interface compared to 939 Å² for the wild-type homodimer. The only perceptible deviations are two hydrophobic interactions of the Met47 side-chain with Leu10 and Ala44, which are not formed by Asp47. The crystallographic data

Table 3. Apparent melting temperatures (Tm) of wild-type ssAnPRT and its mutants as deduced from differential scanning calorimetry (DSC)

<table>
<thead>
<tr>
<th>ssAnPRT variant</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>92</td>
</tr>
<tr>
<td>I36E</td>
<td>90</td>
</tr>
<tr>
<td>M47D</td>
<td>83</td>
</tr>
<tr>
<td>I36E+M47D</td>
<td>82</td>
</tr>
</tbody>
</table>

Experimental conditions: 100 mM potassium phosphate (pH 7.5); subunit concentration: 12 μM; scan rate: 1 K/min.

Table 4. X-ray data statistics and model parameters

<table>
<thead>
<tr>
<th>Space group</th>
<th>P2</th>
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<tbody>
<tr>
<td>Unit cell parameters</td>
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<tr>
<td>a (Å)</td>
<td>91.645</td>
</tr>
<tr>
<td>b (Å)</td>
<td>65.852</td>
</tr>
<tr>
<td>c (Å)</td>
<td>115.702</td>
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<tr>
<td>β (deg.)</td>
<td>107.39</td>
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<tr>
<td>Solvent content (%; v/v)</td>
<td>45</td>
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<tr>
<td>X-ray data</td>
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<tr>
<td>X-ray source</td>
<td>X10SA (SLS)</td>
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<tr>
<td>Wavelength (Å)</td>
<td>1.00</td>
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<tr>
<td>Resolution (Å)</td>
<td>15.0–2.85 (2.9–2.85)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>30,238 (1543)</td>
</tr>
<tr>
<td>Rsym (I)</td>
<td>0.11 (0.64)</td>
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<tr>
<td>Multiplicity</td>
<td>2.63 (2.66)</td>
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<td>Completeness (%)</td>
<td>97.9 (98.1)</td>
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<td>I/σ (I)</td>
<td>8.58 (2.25)</td>
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<tr>
<td>Reflections in working set</td>
<td>29,240</td>
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<tr>
<td>Reflections in test set</td>
<td>998</td>
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<tr>
<td>No. protein atomsa</td>
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<td>No. solvent moleculesa</td>
<td>850</td>
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<tr>
<td>R-factor (%)</td>
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<td>Rfree (%)</td>
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<tr>
<td>rmsd from ideal</td>
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<td>Bond lengths (Å)</td>
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<td>Bond angles (deg.)</td>
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<td>Ramachandran plot</td>
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<td>Residues in core regions (%)</td>
<td>85.1</td>
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<tr>
<td>Residues in disallowed regions</td>
<td>A78, K342</td>
</tr>
</tbody>
</table>

*a Four ssAnPRT copies.

Figure 4. Concentration-dependence of the apparent inactivation half-life (t1/2app) of wild-type ssAnPRT and its mutants. The proteins were incubated at 80 °C in 50 mM Tris–HCl (pH 6.7). The time-dependent decay of the residual activities was measured at 37 °C, yielding the t1/2app values shown here. The experiments were performed at the given total subunit concentrations.
validate the design principle of the M47D exchange and explain how it weakens the affinity between the subunits. It could be speculated that the structural consequences of the I36E exchange are similar in nature to those observed in the M47D protein, although its effect on the thermodynamic dissociation constant is less pronounced.

### Discussion

The simultaneous introduction of the I36E+M47D substitutions resulted in the dissociation of homodimeric ssAnPRT into monomers with unaltered catalytic proficiency. Since the active site of each subunit is located at a distance of about 24 Å from the subunit interface, it is then not unexpected that oligomerisation is dispensable for the enzymatic function of ssAnPRT. Along the same lines, crystallographic analysis showed that the active sites of the wild-type enzyme and the M47D mutant are superimposable within experimental error. Regardless, small-angle X-ray scattering data suggested that in ssAnPRT hinge motions are induced by the binding of the substrates anthranilate and PRPP, which are brought together during catalysis by rotational motions of the large \( \alpha/\beta \) domain versus the small \( \alpha \)-helical domain.\(^{20} \) A similar hinge region and equivalent inter-domain motions induced by substrate binding have been reported for pyrimidine/thymidine nucleoside phosphorylases,\(^{24-26} \) which are closely related to AnPRTs. It has been speculated that such hinge motions might not occur simultaneously in both subunits but that take place in an asymmetric fashion, suggesting a communication across subunits.\(^{20,26} \) Our data show, however, that possible asymmetric motions in ssAnPRT would not have functional consequences and, thus, are unlikely to be derived from inter-subunit communication. Instead, if actually taking place, they might merely reflect steric restrictions within the system. In contrast to ssAnPRT, mutations at the subunit interface of other homodimeric enzymes such as triosephosphate isomerase,\(^{7} \) dihydroorototate dehydrogenase,\(^{9} \) \( \alpha \)-glucoronidase,\(^{10} \) and glutathione transferase\(^{11} \) resulted in the generation of monomers with drastically reduced catalytic activities. Other than in ssAnPRT, in these proteins one subunit stabilizes either directly or indirectly the active site of the neighbouring subunit, which couples enzymatic function to dimer formation.

Compared to wild-type ssAnPRT, the monomeric double mutant I36E+M47D shows a strongly reduced resistance to thermal inactivation (Figure 4). It was unclear \textit{a priori} to what extent this destabilisation is due to the introduced amino acid substitutions \textit{per se} and how much of it is caused by the resulting monomerisation. This problem could be solved by analysing the single mutants I36E and M47D, which are present in a monomer–dimer equilibrium and whose stability is dependent on protein concentration. At the lowest applied concentrations, the apparent inactivation half-lives of both I36E and M47D are almost identical with the (concentration-independent) half-life of I36E+M47D, proving that the single mutant monomers are as labile as the double mutant monomers. Moreover, at the highest concentration applied, the apparent inactivation half-life of I36E is almost identical with the (concentration-independent) half-life of wild type ssAnPRT, proving that the mutant dimer is as stable as the wild-type dimer (Figure 4). These results prove that the amino acid substitutions do not influence the resistance to thermal inactivation significantly, and that the higher kinetic stability of wild-type ssAnPRT compared to I36E+M47D is due to the different oligomeration states of the two proteins.

Our results show that the monomeric forms of the single and double mutants resemble the monomer of wild-type ssAnPRT, which appears to be equally active but less stable than the dimer. A detailed analysis of the inactivation kinetics allowed us to estimate the dissociation constants of the monomer–dimer equilibrium. The mutations induced an increase of the \( K_D \) value by at least three to four orders of magnitude, from less than 0.1 \( \mu \)M in wild-type ssAnPRT to at least 270 \( \mu \)M in the double mutant. Since the value for wild-type ssAnPRT is only an upper limit, and considering a \( K_D \) of approximately \( 10^{-12} \) M for the formation of other homo-dimers,\(^{6,27} \) the weakening of the intersubunit interactions in ssAnPRT caused by the I36E+M47D exchanges is probably even more pronounced. A multiple alignment of 220 AnPRT sequences (L-INS-I strategy of MAFFT version 5.8)\(^{28} \) showed that at the positions corresponding to Ile36 and Met47 in ssAnPRT, about 70% and 80% of all residues are hydrophobic. Whereas at position 36, about 10% of the residues are glutamate or aspartate, at position 47 only 2% of the aligned sequences contain a negatively charged residue. These findings suggest that Met47 is more important for dimer stability than Ile36, in accordance with the higher \( K_D \) value of M47D (about 20 \( \mu \)M) compared to I36E (about 1 \( \mu \)M).

The results of this work show similarities to a protein engineering study in which the dodecameric (4 × 3 subunits) ornithine carbamoyl transferase from the hyperthermophilic archaeon *Pyrococcus furiosus* (pOTCase) was dissociated into catalytically active but significantly less stable trimers.\(^{29} \) Moreover, the homodimeric phosphoribosylanthranilate isomerase from the hyperthermophilic bacterium *Thermotoga maritima* (tmPRAI) was loosened by the shortening of loops that protrude reciprocally into the neighbouring subunit and the generation of electrostatic repulsion by replacing a hydrophobic phenylalanine residue by a negatively charged glutamate. The resulting monomer was catalytically as active as the native monomer but drastically less stable.\(^{3} \) The comparatively high oligomerisation state of pOTCase and tmPRAI are characteristic of hyperthermophiles, since the homologous enzymes from mesophiles form trimers and monomers,
respectively. In contrast, all hitherto characterized AnPRT homologues form dimers, independent of the growth temperature of their host organisms and their positions in the phylogenetic tree of life. 18,21,30,31 We conclude that evolution of AnPRT to a dimer occurred in the last common ancestor of all species, which was probably a thermophile or a hyperthermophile. 32 This oligomerisation state of AnPRT apparently has been retained during the evolution of the mesophiles, probably because it allowed the enzyme to increase catalytic activity at low temperatures without compromising stability significantly.

Material and Methods

Site-directed mutagenesis

The single mutants I36E and M47D were generated by overlap extension PCR 33,34 using the plasmid pQE40 ssTrpD 35 as template. In order to produce the 5′ megaprimer, the oligonucleotide CyRI 36 was used as the 5′ primer, and the oligonucleotides 5′-GAA GTT CCA GAG CAA TTA GTA TCA GCA-3′ (for construction of I36E) and 5′-GTA GCA TTA AGA GTA AAA GGT GAA AGT-3′ (for construction of M47D) were used as 3′ primers. In order to produce the 3′ megaprimer, the oligonucleotides 5′-TGG GAA TCA TAA TIG CTC TGG AAC TTG-3′ (for construction of I36E) and 5′-ACT TTC ACC TTT AGC TCT TAA ATG TAC-3′ (for construction of M47D) were used as 5′ primers, and the oligonucleotide CyPstI 35 was used as a 3′ primer (new codons are underlined). The 5′ and 3′ megaprimer were purified by agarose gel electrophoresis, and mixed with CyRI and CyPstI in a third PCR, which yielded the full-length genes. The double mutant I36E+M47D was also generated by overlap extension PCR, using the template pQE40 ssTrpD I36E. Using BamHI and HindIII restriction sites, the mutated genes were cloned into pQE40. Gene expression from pQE40 results in the addition of an N-terminal His6 tag to the produced protein. To confirm the introduced base substitutions and to exclude further inadvertent point mutations, all mutated genes were sequenced entirely.

Heterologous expression and purification of ssAnPRT mutants

Wild-type ssAnPRT and its mutants were expressed heterologously at 37 °C in Escherichia coli strain W3110 trpE2, containing the repressor plasmid pDM. 35 The resulting protein products were purified from the soluble fraction of the crude extract by heat-precipitation of the host proteins and metal chelate affinity chromatography. Details of gene expression and protein purification were similar to those in the literature. 20 Yields were 0.4-0.8 mg of protein per 1 g of wet cell mass. The proteins were dipped into liquid nitrogen and stored at −80 °C.

Analytical methods

Protein concentrations were determined by measuring the absorbance at 280 nm, using molar extinction coefficients that were calculated from the amino acid sequence. 36 Association states of the recombinant ssAnPRT proteins were determined by analytical gel-filtration chromatography and analytical ultracentrifugation. Analytical gel filtration chromatography was performed at 25 °C and a flow-rate of 0.5 ml/min on a calibrated Superdex 75 column (1 cm × 30 cm, Amersham Pharmacia Biotech) that was equilibrated with 50 mM potassium phosphate (pH 7.5), containing 300 mM KCl. Apparent molecular masses were calculated from the corresponding elution volumes, using a calibration curve that was obtained with standard proteins. Sedimentation equilibrium runs were performed at 26 °C in 100 mM potassium phosphate (pH 7.5) in a Beckman analytical ultracentrifuge (Spinco Model E), monitoring the absorbance at 277 nm. The runs were performed at 12,000 rpm (wild-type ssAnPRT) and 16,000 rpm (mutants), and analyzed using the meniscus-depletion method. 37,38 Molecular masses were calculated assuming a partial specific volume of 0.75 ml/g.

Steady-state enzyme kinetics

The ssAnPRT reaction was followed at 37 °C by a fluorimetric assay (CARY Eclipse fluorescence spectrophotometer, Varian) 39 performed in 50 mM Tris–HCl (pH 7.2), in the presence of 0.05 mM MgCl2. The Michaelis constants Ki, AAn and Km, PRPP were determined by analyzing saturation curves that were deduced from initial velocity measurements recorded in the presence of an excess (10×Km) of the second substrate. The turnover number kcat was obtained by dividing the maximum catalytic rate by the concentration of active sites.

Irreversible thermal inactivation

The kinetics of irreversible heat inactivation were measured as described. 21 The proteins were incubated at 80 °C in 50 mM Tris–HCl (pH 6.7), assuming a ΔpKθ/deg. C for Tris buffer of −0.028. 35 Aliquots were taken at different times, chilled on ice, and centrifuged (Eppendorf 5415R, 13,000 rpm, 3 min, 4 °C). The residual maximum velocity of ssAnPRT in the supernatant was measured at 37 °C in 50 mM Tris–HCl (pH 7.2), 0.05 mM MgCl2.

The dependence of the apparent t1/2 values on the applied subunit concentrations of the single mutants I36E and M47D (Figure 4) prompted us to determine their KD values for the monomer–dimer equilibrium (model 2) as follows. Since monomeric (M) and dimeric (D) ssAnPRT show practically identical turnover numbers (Table 2), the total concentration of active subunits cMtot is given by:

\[
\text{cM}_{\text{tot}} = \text{cM} + 2 \times \text{cD}
\]

Rearrangement to \( \text{cD} = 0.5 \times (\text{cM}_{\text{tot}} - \text{cM}) \) allows one to reformulate the equilibrium constant as:

\[
K_D = \frac{\text{cM}^2}{\text{cD}^2} = 2 \frac{\text{cM}^2}{\text{cD}^2} (\text{cM}_{\text{tot}} - \text{cM})
\]

The solution for the quadratic equation (2) is:

\[
\text{cM} = -0.25 K_D + \left(0.0625 K_D^2 + 0.5 K_D c_{\text{M}_{\text{tot}}} \right)^{1/2}
\]

and the time-dependent loss of active subunits can be described by:

\[
\frac{dc_{\text{M}_{\text{tot}}}}{dt} = -k_d \times \text{cM}
\]
Replacing $c_{\text{tot}}$ in equation (4) by the right-hand side of equation (3) yields:

$$\frac{dc_{\text{tot}}}{dt} = -k_r (-0.25 K_D + 0.0625 K_D^2 + 0.5 K_D c_{\text{tot}})^{1/2}$$

(5)

The differential equation (5) cannot be integrated, but was solved numerically. Using the non-linear least-squares fitting option of the program Maple 10.01, $K_D$ values for the monomer dimer-equilibrium of I36E and M47D were fit from the measured inactivation kinetics at each applied value of $c_{\text{tot}}$, using the $k_r$ value determined at the lowest concentrations of protein where only the monomers are populated.

**Differential scanning calorimetry (DSC)**

DSC experiments were performed with a CSC 5100 Nano differential scanning calorimeter at a subunit concentration of 12 μM in 100 mM potassium phosphate (pH 7.5) using a scan rate of 1K/min. The DSC data were analyzed by applying the programme CpCalc (version 2.1: Calorimetry Sciences Corporation, 1995) to determine the apparent melting temperature ($T_m$) at which half of the protein is unfolded. The irreversibility of thermal denaturation precluded thermodynamic analysis of the data.

**Crystal structure elucidation**

The ssAnPRT variant M47D was crystallized using the hanging-drop method at room temperature from 50 mM MES (pH 6.0), 18% (w/v) PEG 1500, 5% (v/v) glycerol. The protein was dissolved at a final concentration of 5 mg/ml in 10 mM Hepes (pH 7.5), containing 25 mM MES (pH 6.0), 18% (w/v) PEG 1500, 5% (v/v) glycerol. Crystalization drops consisted of equal volumes (1 μl) of protein and reservoir solutions. Crystals grew within 72 h to a maximal size of 200 μm × 100 μm × 20 μm. Before irradiation, crystals in their mother liquor were flash-frozen in liquid nitrogen.

Diffraction data were collected at beamline X10SA, SLS (Villigen) and processed using XDS/XSCALE. Data statistics are given in Table 4. The crystal form used in this study was equivalent to that described for wild-type ssAnPRT. Briefly, crystals belonged to space group $P2_1$ and contained four ssAnPRT molecular copies per asymmetric unit, exhibiting an $A2$ pseudo-symmetry resulting from a screw non-crystallographic symmetry (NCS) axis parallel to the crystallographic 2-fold axis. To account for potential rigid-body motions, phasing was performed by molecular replacement in PHASER using wild-type ssAnPRT (PDB entry 1O17) as the search model. Model building and validation was done with O and COOT, while refinement used CNS (domain definition as described). NCS restraints across N-terminal and C-terminal domains were applied throughout. Solvent molecules were located using ARP/wARP as well as the “water-pick” routine in CNS and validated visually in COOT.

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