Structural and Mutational Analysis of Substrate Complexation by Anthranilate Phosphoribosyltransferase from Sulfolobus solfataricus

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The metabolic synthesis and degradation of essential nucleotide compounds are primarily carried out by phosphoribosyltransferases (PRT) and nucleoside phosphorylases (NP), respectively. Despite the resemblance of their reactions, five classes of PRTs and NPs exist, where anthranilate PRT (AnPRT) constitutes the only evolutionary link between synthesis and degradation processes. We have characterized the active site of dimeric AnPRT from Sulfolobus solfataricus by elucidating crystal structures of the wild-type enzyme complexed to its two natural substrates anthranilate and 5-phosphoribosyl-1-pyrophosphate/Mg2+. These bind into two different domains within each protomer and are brought together during catalysis by rotational domain motions as shown by small angle x-ray scattering data. Steady-state kinetics of mutated AnPRT variants address the role of active site residues in binding and catalysis. Results allow the comparative analysis of PRT and pyrimidine NP families and expose related structural motifs involved in nucleotide/nucleoside recognition by these enzyme families.

Nucleotide compounds are central to the production of genetic material, the amino acids histidine and tryptophan, and cofactors such as NAD. Their metabolism is largely based on the reversible transfer of a phosphoribosyl group to aromatic bases. Although forward and reverse ribosylation processes share a close resemblance (Fig. 1), they are carried out by different enzymes and in the context of distinct metabolic pathways. Synthesis reactions are performed by phosphoribosyltransferases (PRT).3 These use PRPP as universal phosphoribosyl donor. These reactions, which are dependent on metal ions, involve the displacement of the 1-pyrophosphate group of PRPP and formation of a N1′-glycosidic bond to a nitrogenated base specific for each PRT. Conversely, the release of aromatic bases from nucleotides involves first the production of a nucleoside intermediate by nucleotidases or phosphatases, followed by the cleavage of the glycosidic bond by either nucleoside phosphorylases (NP) or nucleoside hydrolases. NP enzymes are key to nucleotide salvage both in prokaryotes and in eukaryotes. They catalyze the reversible phosphorolysis of the glycosidic bond of nucleosides to yield free bases and ribose-1-phosphate.

Despite the chemical resemblance of the compounds intervening in synthesis and degradation reactions by PRTs and NPs (Fig. 1), respectively, the enzymes involved in these catalyses lack structural similarity. Three diverse PRT classes that act on aromatic bases are known to date, as follows: (i) those with a canonical fold classified as type PRT-I, which includes most of the PRTs for which an atomic structure is available (1); (ii) type PRT-II comprising quinolate PRT (2–3) and nicotinate PRT (4); and (iii) anthranilate PRT (AnPRT) (5–7) (Fig. 1A). NPs are also classified in two groups with distinct folds as follows: (i) NP-I, which act primarily on purine nucleosides but also accept the pyrimidine uridine, and (ii) NP-II, which degrade thymidine and/or uridine depending on the organism of origin (8) (Fig. 1B) (throughout this text NP-II enzymes will be termed pyrimidine NP (PyNP) irrespective of their substrate specificity). One single evolutionary link has been encountered among these five protein families, exemplified by AnPRT and PyNP, enzymes that share little or no sequence homology but display significant architectural similarities. AnPRT synthesizes an intermediate in the biosynthesis pathway of the essential amino acid tryptophan. It ribosylates the aromatic base anthranilate (AA) by using PRPP to convert it into a nucleotide-like product, 5′-phosphoribosyl-1-aminonucleoside phosphorylase; PyNP, pyrimidine NP; AA, anthranilate; sAnPRT, S. solfataricus AnPRT; mtAnPRT, M. tuberculosis AnPRT; PDB, Protein Data Bank; SAXS, small angle x-ray scattering.
become available recently, with the structure from *M. tuberculosis* (mtAnPRT) being complexed to its PRPP substrate and magnesium ions (7). Although the sequence similarity between AnPRT and PyNP is restricted to their N termini and a few confined motifs (5, 9), their architectural correspondence is significant. Both enzymes share a common fold comprising a small N-terminal, α-helical domain made up of six helices and a large α/β-domain formed by a central β-sheet and a cluster of eight α-helices (Fig. 2A). They also exhibit a similar quaternary structure consisting of head-to-head dimers with equivalent interfaces. A topological comparison of their architectures shows that a particular feature of AnPRT is the presence of a short loop within the substrate binding cleft, which carries a DE sequence motif absolutely conserved within this family (5). This motif, which is not present in PyNPs, has been proposed to confer on AnPRT the ability of binding and activating the PRPP substrate central to PRT catalysis (5).

**Experimental Procedures**

**Crystal Structure Elucidation**—ssAnPRT was overexpressed, purified, and crystallized as reported (12). Substrate complexation was in the crystalline state using soaking at 4 °C, where crystals of the apoenzyme were immersed in cryo-solutions containing mother liquor supplemented with 22% (v/v) glycerol (as cryo-protectant) and ligands. AA and PRPP substrates were purchased from Sigma. Specific soaking conditions and x-ray data statistics are given in Table 1. For x-ray data collection, crystals were flash-frozen at 100 K. The APRV interface (13) to XDS (14) and CNS (15) was used for data processing and interpretation, with initial phases derived from apo-ssAnPRT (PDB entry 1O17) (5). A model for anthranilate was obtained from the Cambridge Structural Data base, whereas PRPP was initially derived from PDB entry 1FSG. Manual rebuilding of protein and substrate models was in O (16). For cross-validation, diffraction data were divided into a working and a test set in XSCALE (14). NCS restraints across domains were applied to all models. Water molecules were built using ARP/wARP (17) and validated visually in electron density maps. Coordinates for ssAnPRT in complex with AA, PRPP/Mg<sup>2+</sup>, and AA/PRPP/Mg<sup>2+</sup> have been deposited in the PDB with accession codes 2GVQ, 1ZXY, and 1ZYK, respectively.

**Small Angle X-ray Scattering**—SAXS data were collected on the EMBL camera X33 (DORIS III, DESY) with a linear gas detector (18). Samples were in 10 mM HEPES, pH 8.0, at substrate-binding conditions as for crystal soaking (Table 1). Data were recorded at multiple protein concentrations (1.8–15.0
mg/ml) at a temperature of 4.0 ± 0.2 °C in the range of momentum transfer 0.15 < s < 3.2 nm⁻¹ (s = 4πsin(θ)/λ, where 2θ is the scattering angle and λ = 0.15 nm is the x-ray wavelength). The data, collected in 15 successive 1-min frames, showed no significant variation as a function of time, indicating the absence of both radiation damage and catalysis on the substrates. Data were processed using the program PRIMUS (19). The forward scattering I(0), radius of gyration Rg, maximum dimension Dmax, and the distance distribution function p(r) were evaluated with the program GNOM (20). The molecular masses of the solutes were derived by comparison of I(0) with that from reference solutions of bovine serum albumin (molecular mass = 66 kDa).

Multiple models of ssAnPRT in open and closed conformations were constructed by systematic rigid-body rotation of the C-terminal α/β-domain relative to its fixed N-terminal counterpart. The interdomain hinge region in ssAnPRT was taken as loops α4-ββ, α8'-α8, and α9-β4 (definition as in Ref. 5). The rotation axis was calculated from noncrystallographic differences in apo crystal models (1O17) using DYNDOM (21). With hinge and rotation axis so defined, the α/β-domain was swung in 5° increments from extremely open to maximally closed conformations. The scattering patterns of 225 generated symmetric and asymmetric dimers, all keeping the dimerization interface as in apo-ssAnPRT, were compared with the experimental data using CRYSOl (22).

Site-directed Mutagenesis—Mutants K106Q, R164A, R170A, and D223N were produced by overlap extension PCR using the plasmid pQE40 ss bufferSizeD (12) as template. Mutants H107A and E224Q were produced with the QuikChange method (Stratagene), again using pQE40 ss bufferSizeD as the template. Double mutants R164A/H154A and P178A/H107A were generated by overlap extension PCR, using the templates pQE40 ss bufferSizeD-R164A and pQE40 ss bufferSizeD-H107A. The details of the PCRs, including the DNA sequences of the oligonucleotides used, are provided as Supplemental Material. 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 entirely sequenced.

Heterologous Expression and Purification of ssAnPRT Mutants—ssAnPRT variants were heterologously expressed at 37 °C in Escherichia coli strain W3110 trp EA2, containing the repressor plasmid pDM (23). 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. N-terminal His₆ tags were removed by proteolysis. Details of gene expression and protein purification are provided as Supplemental Material. As judged by SDS-PAGE, all samples were at least 95% pure. Yields were 0.2–0.8 mg of protein per g wet cell mass. The proteins were dripped into liquid nitrogen and stored at −80 °C.

Steady-state Kinetics—The ssAnPRT reaction was followed at 60 °C by a fluorimetric assay (CARY Eclipse fluorescence spectrophotometer, Varian) (12) performed in 50 mM Tris-HCl, pH 7.5, at various concentrations of MgCl₂. The Michaelis constants kₐₐ and kₗₗ 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 kₜₜ was obtained by dividing the maximum catalytic rate by the total concentration of active sites.

RESULTS

Crystal Structures of Wild-type ssAnPRT in Complex with Its Natural Substrates—Atomic models of ssAnPRT in complex with (i) AA, (ii) PRPP/Mg²⁺, and (iii) AA/PRPP/Mg²⁺, the latter showing the active site at full occupancy, have been obtained by crystal soaking at 4 °C where catalysis by this thermophilic enzyme is not detectable (12). The crystal form employed in this study contained two AnPRT dimers in its asymmetric unit. X-ray data and model refinement statistics are given in Table 1.

Recognition of Anthranilic Acid—ssAnPRT binds two molecules of AA per protomer (Fig. 2, A, C, and E). The two AA molecules are housed within the N-terminal, α-helical domain in consecutive binding pockets (sites AA-I and AA-II). Site I is defined by enzyme residues Asn-109, Ala-150, His-154, Met-157, and Arg-164, located at the N-terminal end of the binding cleft. Site II, positioned at the middle point of the binding groove, is close to a hinge region at the center of the enzyme architecture. It is formed by protein residues Ala-78, Gly-79, His-107, Gly-108, Asn-109, Gly-177, and Pro-178. Protein-AA interactions both at sites I and II are mostly of hydrophobic character. The charged, lateral groups of AA are involved only in few specific interactions, so their contribution to the productive orientation of this compound in either pocket appears to be moderate. AA-I is oriented primarily via its carboxyl group by protein residues Arg-164 and Asn-109. AA-II interacts with Asn-109 via its carboxyl group, whereas its amino group is oriented toward the PRPP binding cavity via a hydrogen bond to the backbone carbonyl group of Gly-79 (Fig. 2E). As a further contribution to substrate orientation, the imidazole rings of His-154 and His-107 at sites I and II, respectively, act as π-acceptors of the aromatic CH groups of anthranilate. These contacts are better classified as weak hydrogen bonds than as unspecific hydrophobic interactions (24–25). Both histidine residues are similarly arranged with respect to their corresponding AA ligands. Besides protein-ligand contacts, the two AA molecules interact directly with each other via an electrostatic interaction between the amino group of molecule AA-I and the carboxyl group of AA-II. This is a prominent contribution to the orientation of these molecules in the binding groove of ssAnPRT.

To clarify whether ssAnPRT binds AA preferentially at a given pocket, a titration series was carried out in the crystal at AA soaking concentrations of 0.2, 0.5, 1, and 2 mM over 30 min to ensure equilibration. Binding became interpretable only at the highest concentration of 2 mM and in two of the four ssAnPRT copies in the asymmetric unit. The analysis of electron density maps indicated that the enzyme within the crystal lattice favors binding of AA at pocket II and that the AA binding mode is the same at high and low substrate concentrations. A visual inspection of the lattice suggested that this result is unlikely to be due to impaired accessibility of pocket I.
Protein groups involved in AA coordination at sites I and II are highly conserved (>85%) within the AnPRT family (Fig. 3). Glycine residues at positions 79 and 177 are required to prevent steric blockage of substrate binding to pocket II. Of all residues involved, only Arg-164 at site I is absolutely conserved across other PRTs (Fig. 2F). This is likely to correspond to the high affinity metal-binding site present in the PRPP compound, which forms in solution a predominant mono-magnesium complex thought to be the true substrate of PRTs (26, 27). Accordingly, crystal structures of the unrelated PRT-I and -II in complex with PRPP reveal an equivalent metal ion at this site, also lacking direct protein contacts (Fig. 2B) (1, 3). In ssAnPRT, the coordinated action of Mg-1 and a hydrogen bond between the amino group of Gly-79 and a phosphate oxygen induce an eclipsed conformation in the pyrophosphate group (Fig. 2, D and F). Eclipsed arrangements are also common in PRPP coordination by the canonical PRT-I, and it probably provides electrostatic assistance to catalysis (1). In ssAnPRT, a second metal site (MG-2) reconciles binding of the 5-phosphate group of PRPP to a cluster of negatively charged protein residues comprising Asp-223 and Glu-224 (Fig. 2, D and F), which constitute an absolutely conserved motif in the AnPRT family (Fig. 3). Eclipsed arrangements are also common in PRPP coordination by the canonical PRT-I, and it probably provides electrostatic assistance to catalysis (1). In ssAnPRT, a second metal site (MG-2) reconciles binding of the 5-phosphate group of PRPP to a cluster of negatively charged protein residues comprising Asp-223 and Glu-224 (Fig. 2, D and F), which constitute an absolutely conserved motif in the AnPRT family (Fig. 3). Eclipsed arrangements are also common in PRPP coordination by the canonical PRT-I, and it probably provides electrostatic assistance to catalysis (1). In ssAnPRT, a second metal site (MG-2) reconciles binding of the 5-phosphate group of PRPP to a cluster of negatively charged protein residues comprising Asp-223 and Glu-224 (Fig. 2, D and F), which constitute an absolutely conserved motif in the AnPRT family (Fig. 3). Eclipsed arrangements are also common in PRPP coordination by the canonical PRT-I, and it probably provides electrostatic assistance to catalysis (1). In ssAnPRT, a second metal site (MG-2) reconciles binding of the 5-phosphate group of PRPP to a cluster of negatively charged protein residues comprising Asp-223 and Glu-224 (Fig. 2, D and F), which constitute an absolutely conserved motif in the AnPRT family (Fig. 3). Eclipsed arrangements are also common in PRPP coordination by the canonical PRT-I, and it probably provides electrostatic assistance to catalysis (1). In ssAnPRT, a second metal site (MG-2) reconciles binding of the 5-phosphate group of PRPP to a cluster of negatively charged protein residues comprising Asp-223 and Glu-224 (Fig. 2, D and F), which constitute an absolutely conserved motif in the AnPRT family (Fig. 3). Eclipsed arrangements are also common in PRPP coordination by the canonical PRT-I, and it probably provides electrostatic assistance to catalysis (1). In ssAnPRT, a second metal site (MG-2) reconciles binding of the 5-phosphate group of PRPP to a cluster of negatively charged protein residues comprising Asp-223 and Glu-224 (Fig. 2, D and F), which constitute an absolutely conserved motif in the AnPRT family (Fig. 3). Eclipsed arrangements are also common in PRPP coordination by the canonical PRT-I, and it probably provides electrostatic assistance to catalysis (1). In ssAnPRT, a second metal site (MG-2) reconciles binding of the 5-phosphate group of PRPP to a cluster of negatively charged protein residues comprising Asp-223 and Glu-224 (Fig. 2, D and F), which constitute an absolutely conserved motif in the AnPRT family (Fig. 3). Eclipsed arrangements are also common in PRPP coordination by the canonical PRT-I, and it probably provides electrostatic assistance to catalysis (1).
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**FIGURE 2. Substrate coordination by ssAnPRT.** A, overview (only one protomer of the homodimer is shown). Protein domains are color-coded where the N-terminal α-helical domain is gold and the larger C-terminal α/β-domain is orange and cyan. The active site is shown at full occupancy, and key directional interactions are indicated by dotted lines. Arg-164 (α-helical domain) and Lys-106 (α/β domain), mediating interactions conserved across AnPRTs and PyNPs, are displayed in dark blue. Gly-79 (in green) orients AA-II with respect to PRPP/Mg2+. Take place in the enzyme upon full substrate binding. Domain displacements are not detectable when comparing substrates in these structures are too far apart to allow catalysis to occur. The nitrogen groups of AA-I and -II are separated by 14.6 and 8.6 Å, respectively, from the C-1 ribose atom of PRPP. For the synthesis reaction to proceed, substrates must be brought together, probably by means of a hinge motion where the N- and C-terminal domains close upon each other. Such domain rearrangements are likely to be hindered within the crystal lattice.

**FIGURE 3. Sequence conservation in the AnPRT and PyNP families.** Structure-based sequence alignment of ssAnPRT and human thymidine NP (hsTPN; PDB entry 1UOU) (38). Secondary structure elements are displayed and numbered, and their integrating residues are underlined in the individual sequences. Absolutely conserved residues in each family are shown in black, and green indicates ~85% identity conservation. Conservation estimates are based on a sequence alignment of 97 available sequences of AnPRTs and 57 sequences of PyNPs. Residues coordinating anthranilate are indicated by A. P stands for residues binding phosphate groups of PRPP in AnPRT or the lytic phosphate in PyNP. M, R, and N represent residues that coordinate a magnesium ion, the ribose moiety of PRPP or of a nucleoside substrate, and the nitrogenated base, respectively. Residues reported to coordinate PRPP in mtAnPRT (7) are colored orange, and other PRTs as well as in mtAnPRT (Fig. 2B). Curiously, neither of the magnesium sites proposed for mtAnPRT in complex with its PRPP substrate agree with those proposed here for ssAnPRT or with those encountered in any other PRT (Fig. 2B).

**Active Site at Full Occupancy**—Crystal structures of fully complexed ssAnPRT protomers (Fig. 2A) reveal that no significant structural changes other than the ordering of the glycine-rich loop, which is induced by the binding of PRPP/Mg2+, take place in the enzyme upon full substrate binding. Domain displacements are not detectable when comparing models of the apoenzyme, single liganded forms, and the complete complex. The binding pockets for the AA and PRPP/2Mg2+ substrates in these structures are too far apart to allow catalysis to occur. The nitrogen groups of AA-I and -II are separated by 14.6 and 8.6 Å, respectively, from the C-1 ribose atom of PRPP. For the synthesis reaction to proceed, substrates must be brought together, probably by means of a hinge motion where the N- and C-terminal domains close upon each other. Such domain rearrangements are likely to be hindered within the crystal lattice.

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TABLE 2
Experimental and calculated scattering parameters of ssAnPRT and its substrate complexes

| Sample                          | $R_a$ | $R_g$ | $D_{max}$ | $\chi_c$ | $\chi_d$ | $\chi_{sd}$ |
|--------------------------------|-------|-------|-----------|-----------|-----------|-------------|
| (Apo) PDB entry 1O17           | 3.44  | 3.42  | 11.6      | 1.19      | 1.17      | 1.58        |
| Apo AnPRT                      | 3.44  | 3.42  | 11.0      | 1.22      | 1.21      | 1.59        |
| AnPRT/PRPP                     | 3.42  | 3.40  | 11.0      | 1.24      | 1.24      | 1.21        |
| AnPRT/PRPP/AA                  | 3.30  | 3.24  | 10.5      | 1.24      | 1.24      | 1.21        |
| AnPRT/PRPP/AA                  | 3.30  | 3.24  | 10.5      | 1.24      | 1.24      | 1.21        |

$a$ $R_a$ indicates experimental radius of gyration.

$b$ $R_g$ indicates radius of gyration of the model with lowest fitted discrepancy to the experimental data.

$c$ $D_{max}$ indicates maximum molecular dimensions.

$d$ Best fitted discrepancies between experimental data and scattering curves computed from structural models, where $\chi_c$ refers to experimental data versus the crystallographic model of apo ssAnPRT (PDB entry 1O17), and $\chi_d$ accounts for experimental data versus best models obtained by rigid body rotation, which in this case showed an asymmetric hinge closure, and $\chi_{sd}$ corresponds to experimental data versus mixtures of models in symmetrically opened and closed conformations.

$e$ Calculated values for PDB entry 1O17 are given as reference. Value includes plus 6 Å correction to account for the hydration shell.

In complex with AA, the enzyme exhibits a similar $D_{max}$ but slightly reduced $D_{inter}$ and $R_g$ values, hinting at a minor structural rearrangement upon AA binding. In contrast, the fully complexed enzyme shows $D_{max}$ and $D_{inter}$ values reduced by ~1 nm and a significantly smaller $R_g$, indicating that the enzyme adopts here a much more compact conformation. Consequently, the comparison of experimental data with the scattering computed from the crystal structure of apo ssAnPRT (defined by the discrepancy $\chi_c$ in Table 2) shows that the latter is a close representative of the apo-enzyme in solution and its complex with a single substrate but differs from the overall conformation of the fully liganded enzyme. It can be concluded that although coordination of one single substrate type does not induce significant domain motions in ssAnPRT, the enzyme undergoes a noticeable compaction upon full occupancy of its active site. This suggests that the inherent flexibility of the hinge region is central to the catalytic function of ssAnPRT.

To reconstruct a model of ssAnPRT in its closed, catalytically active conformation, 225 models were generated where the larger $\alpha/\beta$ domains had been systematically rotated in 5° intervals relative to the small $\alpha$-helical domains (see "Experimental Procedures"). The dimerization interface between subunits, which engages the $\alpha$-helical, N-terminal domains of both protomers in a head-to-head fashion, was kept constant. Given that this interface is remote from the active sites of the individual protomers, it is unlikely that it will undergo alterations during catalysis. Resulting models included both symmetric arrangements, where concerted rotations had been applied to both $\alpha/\beta$ domains in the dimer, as well as asymmetric structures, where different rotations had been introduced in the protomers. Scattering curves were computed from all models and compared with the experimental data. The best models and their scattering patterns are displayed in Fig. 4, with $R_a$ and $\chi$ values given in Table 2. Data from solutions of apo ssAnPRT as well as from ssAnPRT in single complex with its PRPP substrate were best fitted by models with domain arrangements similar to those of the apo crystal structure. Best-fitting models for the enzyme complexed to AA displayed a relatively small asymmetric domain closure of the protomers within the homodimer. In contrast, models fitting the fully complexed ssAnPRT were markedly asymmetric (Fig. 4D). To check whether enzyme populations in solution could consist of a mixture of symmetrically open and closed dimers, an attempt was also made to fit the scattering data by linear combinations of symmetric models with various degrees of hinge opening. Such mixtures, however, yielded poor fits (e.g. $\chi = 2.4$ for the fully complexed enzyme). It was thus concluded that binding of AA together with PRPP induces an asymmetric hinge closure in the dimer, where one of the subunits retains a hinge opening similar to that of the apo form.

The reconstructed model of an ssAnPRT protomer in its closed conformation shows how the lower $\alpha/\beta$-lobe swings to bring the PRPP substrate close to the AA molecules, in particular to AA at site II, implying that this site is catalytically relevant (Fig. 4D). The magnitude of the hinge rotation can be estimated as 25–33°. This is in close agreement with crystallographic data on PyNP from Bacillus stearothermophilus, where domain rotations of ~21° are observed upon binding of a nucleoside analog (28). Based on current data, it is difficult to envisage how AA at site I could be brought in contact with PRPP by means of a simple rigid-body rotation of the enzyme domains.

Kinetic Characterization of Wild-type ssAnPRT and Mutated Variants—According to crystallographic data, the active site of an ssAnPRT protomer can host two AA molecules, one PRPP compound and two metal sites in its full occupancy state. The substrates can bind independently to the enzyme, suggesting a random, sequential Bi Bi mechanism. This is in accordance with kinetic data on Saccharomyces cerevisiae AnPRT (29), which show no evidence for ordered sequential or ping-pong mechanisms. To further examine the role of active site residues in catalysis and substrate binding in ssAnPRT, variants with single or double amino acid exchanges were analyzed by steady-state enzyme kinetics. Residues were selected according to their proximity to either one of the two AA molecules or the PRPP/Mg$^{2+}$ substrate. The measured turnover numbers $k_{cat}$ and the Michaelis constants $K_m$ and $K_{PRPP}$ at 60 °C are given in Table 3. In the course of these studies, it was observed that the wild-type enzyme and the variants involving residues unrelated to PRPP binding (R164A, R164A/H154A, H107A, and H107A/P178A) showed unexpected inhibition by high concentrations of Mg$^{2+}$. Their $k_{cat}$ values are decreased 3–10-fold in the presence of 2 mM Mg$^{2+}$ compared with 25–500 μM of added Mg$^{2+}$. Moreover, the PRPP saturation curves of these variants were sigmoidal, with drastically increased $K_{PRPP}$ values (shown in Table 3 for wild-type ssAnPRT). To account for this effect, steady-state measurements on these variants were performed with added 50 μM Mg$^{2+}$, whereas the remaining variants were assayed in 2 mM Mg$^{2+}$.

All ssAnPRT variants designed to test the catalytic relevance of AA-binding sites I and II (R164A, R164A/H154A, H107A, and H107A/P178A) (Fig. 2, C and E) showed increased $K_m$ values, whereas $K_{PRPP}$ values remained similar to those of the wild type, confirming that the effect of these exchanges is local to the AA binding pockets (Table 3). The exchange R164A led to more than a 7000-fold increase in $K_m^{AA}$, although only a mod-
erate 7-fold decrease in $k_{\text{cat}}$, indicating that this residue is crucial for substrate binding but not for catalysis. Similarly, the substitution H107A/P178A, local to site AA-II, resulted in a 300-fold increase of $K_m^{\text{AA}}$ which was mainly caused by the P178A exchange, whereas $k_{\text{cat}}$ remained nearly unchanged. These results suggest that Arg-164 and Pro-178, which are absolutely conserved across the AnPRT family (Fig. 3), are the main determinants of AA recognition by ssAnPRT. Because these residues are local to a different AA binding pocket, ssAnPRT probably ligates AA at both sites I and II.

The exchanges H154A and H107A had only a limited effect on either $K_m^{\text{AA}}$ or $k_{\text{cat}}$, proving that histidine residues at these positions are dispensable. This is remarkable, given their high conservation within the family (Fig. 3) and that coordination of nitrogenated bases via $\pi-\pi$ interactions involving imidazole groups of histidines is a common structural theme among nucleotide/nucleoside-processing enzymes, including PRTs, NPs, and nucleoside hydrolases. For example, in PyNPs an absolutely conserved histidine residue (His-116 in human thymidine NP) occupies a position structurally equivalent to that of His-107 in ssAnPRT (Fig. 5B). In that family, this residue has been suggested to be involved in catalysis by mediating the transfer of the proton from the lytic phosphate to the O-2 position of thymine (30). Similarly, in quinolinate PRT type II from Salmonella typhimurium (2) and M. tuberculosis (3), residues His-174 and His-161, respectively, coordinate quinolate (Fig. 5B). A related substrate interaction is established by His-82 in the nucleoside hydrolase from Crithidia fasciculata (31). As in AnPRT, these histidine residues are highly conserved within their respective protein families. Given that current data indicate that the contribution of these interactions to substrate coordination can be only minor in AnPRTs, it would be interesting to investigate to which extent they are decisive in other enzyme families.

The role in binding and catalysis of residues mediating PRPP/Mg$^{2+}$ recognition in ssAnPRT was checked by characterizing the variants K106Q, D223N, and E224Q, which involve absolutely conserved residues in this family (Fig. 3). When compared at optimal Mg$^{2+}$ concentra-
Substrate Complexation by AnPRT

### TABLE 3

| Enzyme variant | Ligand | $K_m^{AA}$ | $K_m^{PRPP}$ | $K_m^{cat}$ |
|----------------|--------|------------|--------------|-------------|
| Wild type      |        | 0.05       | 0.04         | 0.16        | 4.2         |
| R164A          | AA-I   | 0.05       | 297          | 0.17        | 0.62        |
| R164A/H154A    | AA-I   | 0.05       | 149          | ND          | 0.73        |
| H107A          | AA-II  | 0.05       | 0.53         | 0.30        | 5.1         |
| H107A/H178A    | AA-II  | 0.05       | 12.5         | ND          | 2.0         |
| K106Q          | PRPP   | 2          | 0.75         | 0.26        | 0.48        |
| D223N          | MG-2   | 2          | 0.16         | 0.07        | 6.0         |
| E224Q          | MG-2   | 2          | 0.91         | 0.26        | 5.6         |

* Ligand complexed by wild-type residue.

### DISCUSSION

**Substrate Coordination by ssAnPRT**—The active site of ssAnPRT accommodates two substrates, AA and PRPP/Mg$^{2+}$, which can bind independently to the enzyme. AA coordination exploits primarily the hydrophobic character of this compound, whereas specific interactions involving its charged, lateral groups are scarce. Site-directed mutagenesis data reveal that a stacking interaction involving Pro-178 and, in particular, electrostatic contact to Arg-164 are decisive for productive AA binding (Fig. 2C; Table 3). Arg-164 establishes a sequential chain of hydrogen bonds along the binding cleft that aligns both AA molecules within the active site (Fig. 2A). It coordinates both the carboxylate and amino groups of AA-I; in turn, the latter forms a salt bridge with the carboxylate group of AA-II; and finally the amino group of AA-II gets oriented toward the PRPP substrate pocket via an interaction of its amino group with the main chain carbonyl group of Gly-79 (Fig. 2E). This residue, absolutely conserved and part of the $^{79}$GTTGGD$^{83}$ signature motif of the AnPRT family, also interacts with the pyrophosphate moiety of PRPP via its main chain amino group (Fig. 2F), so that it appears essential for the relative orientation of PRPP and AA-II substrates and thus for AnPRT activity.

The finding of two AA-binding sites in ssAnPRT agrees well with recent predictions based on the structure of mtAnPRT (7). However, it opens questions about the relative catalytic significance of the sites. Titration of apo ssAnPRT crystals with AA indicated that this compound preferentially occupies pocket II close to the PRPP-binding site, suggesting that AA-II might act as substrate. The catalytic significance of site I is currently unclear. It could be speculated that AA-I plays a structural role in the binding of the “true” substrate, judging by the fact that it constitutes the most prominent orientational anchor for AA-II. Because AA is a small metabolite that can diffuse easily out of the cell, a redundant binding mechanism would maximize AA intake from the cellular medium putatively acting as a “rescue” mechanism.

The coordination of PRPP by ssAnPRT differs from that by other PRTs in its metal binding pattern as well as in the compact conformation imposed on the substrate by the enzyme. Coordination of PRPP via two metal ions is rare in PRTases (Fig. 2B). Commonly, a single metal site is present that interacts with the pyrophosphate and ribose moieties of PRPP, corresponding to the high affinity metal site in the PRPP compound. In those cases where a second metal site is observed, this also involves the pyrophosphate group. In ssAnPRT, however, a second metal site bridges the 5-phosphate group to the negatively charged enzyme residues Asp-223 and Glu-224. This results in a compact arrangement of the bound PRPP, which could either lead to an increased reactivity or simply reflect the flexibility of this compound. This conformation of PRPP is, however, not observed in the complexed enzyme from M. tuberculosis (7). In that case, a different metal binding pattern is present, where two ion sites are encountered but none corresponds to those previously observed in PRTs, including the conserved, high affinity site MG-I thought to be intrinsic to the PRPP substrate (Fig. 2B). The basis for the differences between ssAnPRT and wtAnPRT is currently unknown.

**Loop Structuring and Domain Motions**—In its apo form, ssAnPRT adopts an “open” hinge conformation, where the AA and PRPP substrates bind into distantly located cavities of the N- and C-terminal domains, respectively. According to SAXS data, a structural compaction takes place in ssAnPRT upon binding of both substrates (Fig. 4; Table 2), suggesting that the flexibility of the hinge region is essential for catalysis. The binding pockets for PRPP and AA-II relate to each other via the conserved $^{79}$GTTGGD$^{83}$ loop, where Gly-79 binds both substrates and Asp-83 binds PRPP (Fig. 2). This loop is disordered in the apo crystal structure of ssAnPRT.
in both enzymes as follows: ~25–33° in ssAnPRT and ~21° in B. stearothermophilus PyNP (28), indicating that similar architectural mechanisms underlie hinge motion.

Absence of Indispensable Catalytic Residues in AnPRT—Members of the AnPRT family include four primary, highly conserved sequence features as follows: (i) an arginine residue (Arg-164) involved in AA coordination; (ii) a glycine rich-loop host- ing a 79GTGGA83 motif; (iii) a 106KHGN109 β-strand involved in coordination of pyrophosphate and AA groups; and (iv) a 223DE224 motif involved in metal-phosphate binding (numbering according to ssAnPRT; cf. Fig. 3). Of these, we have tested the functional relevance of Arg-164, Lys-106, His-107, Asp-223, and Glu-224 using site-directed mutagenesis. Nonetheless, none of these residues is essential for catalysis (only R164A and K106A exchanges led to a moderate decrease in kcat values) and only Arg-164 is crucial for substrate binding. Given that no active catalysts could be identified, it is reasonable to conclude that the active site of AnPRT might primarily serve as a template for the productive orientation of substrates.

An arginine residue, equivalent to Arg-164 in ssAnPRT, is absolutely conserved also in the PyNP family (Fig. 3 and 5B). There it has been proposed to mediate catalysis by stabilizing the negative charge arising in the base ring upon cleavage of the glycosidic bond (35). Accordingly, its replacement by glutamate resulted in enzyme inactivation (36). However, this did not clarify whether the residue is involved in catalysis or merely substrate binding in that family. Our data show that the conserved arginine in ssAnPRT is of no catalytic relevance, accentuating the need for further comparative studies on the role of conserved residues across AnPRT and PyNP families.

Substrate-binding Motifs across PRTs and PyNPs—Structural data suggest that AnPRTs and PyNPs have evolved from a common ancestor (5), but independently from the unrelated PRT families I and II (Fig. 5A). Nonetheless, AnPRTs, PyNPs, PRTs-I, and -II host related structural motifs at their active sites for nucleotide/nucleoside recognition (Fig. 5B).

Conserved arginine, lysine, and histidine groups as well as glycine-rich loops are frequently found mediating binding of
nitrogenated bases and phosphate moieties (Fig. 5B). This is manifest in AnPRT and PyNP enzymes, which achieve substrate coordination via the following: (i) an absolutely conserved arginine residue, which forms a hydrogen bond with the nitrogenated base; (ii) a “KH” sequence that coordinates a phosphate group and the nitrogenated base, respectively, and (iii) a glycine-rich loop. Glycine-rich loops, termed P-loops because of their binding of phosphate groups, are encountered across the broad spectrum of nucleotide-binding proteins, including kinases, phosphatases, and tri-, di-, and mononucleotide-processing enzymes (Ref. 37 and references therein). A distinct P-loop-like motif involved in phosphate or PRPP binding is found across PyNPs, PRTs-I, and AnPRT (Fig. 5B). It contains a consensus “TGG” motif flanked by residues whose conservation is family-dependent (Fig. 5B). Interestingly, the relative location of the KH and TGG motifs in AnPRTs and PyNPs is not conserved (Fig. 3). Whereas in AnPRT the KH motif is located in strand B2, ~20 residues away from the glycine-rich loop, in PyNPs the KH and TGG sequences are contiguous at the C terminus of B1. This indicates that the KH motif must have developed independently during the divergent evolution of these enzymes, highlighting that even in homologous families recurrent motifs can result from functional needs and not from residual conservation. A related pattern of basic residues is found in quinolinate PRT (type II), whose architecture is unrelated to that of AnPRTs and PyNPs (Fig. 5A). In that family, conserved Arg and His residues (161HR162 in the enzyme from M. tuberculosis) bind the nitrogenated base, whereas a lysine residue (Lys-140) interacts with the 5-phosphate group of PRPP (2, 4) (Fig. 5B). Taken together, these findings indicate a generic significance of these amino acid groups in nucleoside/nucleotide recognition.

Canonical PRT-I do not show a marked conservation of Arg, His, and Lys groups involved in the coordination of their substrates. Instead, the structural hallmark of these enzymes is the presence of two acidic residues that coordinate magnesium and the hydroxyl groups of the ribose fraction of PRPP (1). Similar protein-PRPP interactions are established in the unrelated quinolinate PRT-II by two non-conserved acidic residues, Glu-201 and Asp-222 in the enzyme from M. tuberculosis (3). In ssAnPRT, on the contrary, the conserved doublet 223DE224 primarily coordinates a second metal ion (MG-2), unique to this family, at the 5-phosphate of PRPP (Fig. 2, D and F, and Fig. 5). In summary, acidic doublets frequently recur in enzyme types involved in coordination of phosphoribose moieties, but they do not mediate conserved interactions to the substrate.

This comparative analysis reveals that PyNP and PRT enzyme classes share related strategies for substrate recognition as a result of independent, convergent evolution. Within these enzymes, AnPRT is a unique evolutionary link, which combines the overall architecture of PyNPs with the classical features of a PRT active site. Nevertheless, it has adopted a different mode of substrate binding with the use of a second metal ion, compacting the PRPP substrate and redundant aromatic base coordination.

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**REFERENCES**

1. Sinha, S. C., and Smith, J. L. (2001) *Curr. Opin. Struct. Biol.* 11, 733–739
2. Eads, J. C., Ozturk, D., Wexler, T. B., Grubmeyer, C., and Sacchettini, J. C. (1997) *Structure* 5, 47–58
3. Sharma, V., Grubmeyer, C., and Sacchettini, J. C. (1998) *Structure* 6, 1587–1599
4. Shin, D. H., Onganseyan, N., Janzarik, J., Yokota, H., Kim, R., and Kim, S. H. (2005) *J. Biol. Chem.* 280, 18326–18335
5. Mayans, O., Ivens, A., Nissen, L. J., Kirschner, K., and Wilmanns, M. (2002) *EMBO J.* 21, 3245–3254
6. Kim, C., Xiong, N. H., Edwards, S., Madhusudan, Yee, M. C., Spraggon, G., and Mills, S. E. (2002) *FEBS Lett.* 523, 239–246
7. Lee, C. E., Goodfellow, C., Javid-Majid, F., Baker, E. N., and Shaub Lott, J. (2006) *J. Mol. Biol.* 355, 784–797
8. Pugmire, M. J., and Ealick, S. E. (2002) *Biochem. J.* 361, 1–25
9. Mushegian, A. R., and Koonin, E. V. (1994) *Protein Sci.* 3, 1081–1088
10. Smith, D. A., Parish, T., Stoker, N. G., and Bancroft, G. J. (2001) *Infect. Immun.* 69, 1142–1150
11. Parish, T. (2003) *J. Bacteriol.* 185, 6702–6706
12. Ivens, A., Mayans, O., Szadkowski, H., Wilmanns, M., and Kirschner, K. (2001) * Eur. J. Biochem.* 268, 2246–2252
13. Kroener, M., Dreyer, M. K., and Wendt, K. U. (2004) *Acta Crystallogr.* Sect. D Biol. Crystallogr. 60, 1679–1682
14. Kabach, W. (1993) *J. Appl. Crystallogr.* 26, 795–800
15. Brugger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gross, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J. L., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr.* Sect. D Biol. Crystallogr. 54, 905–921
16. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr.* Sect. A 47, 110–119
17. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) *Nat. Struct. Biol.* 6, 458–463
18. Koch, M. H. J., and Bordas, I. (1983) *Nucl. Instrum. Methods Phys. Res. A* 208, 461–469
19. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J., and Svergun, D. I. (2003) *J. Appl. Crystallogr.* 36, 1277–1282
20. Svergun, D. I. (1992) *J. Appl. Crystallogr.* 25, 495–503
21. Hayward, S., and Berendsen, H. J. (1998) *Proteins* 30, 144–154
22. Svergun, D. I., Barberato, C., and Koch, M. H. J. (1995) *J. Appl. Crystallogr.* 28, 768–773
23. Jurgens, C., Strom, A., Wegener, D., Hettwer, S., Wilmanns, M., and Sterner, R. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97, 9925–9930
24. Brandl, M., Weiss, M. S., Jabs, A., Suhrnel, J., and Hilgenfeld, R. (2001) *J. Mol. Biol.* 307, 357–377
25. Steiner, T., and Koellner, G. (2001) *J. Mol. Biol.* 305, 535–557
26. Thompson, R. E., Li, E. L., Spivey, H. O., Chandler, J. P., Katz, A. J., and Appleman, J. R. (1978) *Bioorg. Chem.* 9, 35–45
27. Meola, M., Yaman, B., Weaver, K., and Sandwick, R. K. (2003) *Inorg. Chem.* 42, 33–40
28. Pugmire, M. J., and Ealick, S. E. (1998) *Structure* 6, 1467–1479
29. Hommel, U., Lustig, A., and Kirschner, K. (1989) *Eur. J. Biochem.* 180, 33–40
30. Mendita, J., Martin-Santamaria, S., Priego, E. M., Balzarini, J., Cama-rasa, M. J., Perez-Perez, M. J., and Gago, F. (2004) *Biochemistry* 43, 405–414
31. Degano, M., Gopaung, D. N., Scapin, G., Schramm, V. L., and Sacchettini,
32. Salerno, C., and Giacomello, A. (1981) J. Biol. Chem. 256, 3671–3673
33. Heroux, A., White, E. L., Ross, L. J., Kuzin, A. P., and Borhani, D. W. (2000) Structure 8, 1309–1318
34. Walter, M. R., Cook, W. J., Cole, L. B., Short, S. A., Koszalka, G. W., Krenitsky, T. A., and Ealick, S. E. (1990) J. Biol. Chem. 265, 14016–14022
35. Rick, S. W., Abashkin, Y. G., Hilderbrandt, R. L., and Burt, S. K. (1999) Proteins 37, 242–252
36. Moghaddam, A., Zhang, H. T., Fan, T. P., Hu, D. E., Lees, V. C., Turley, H., Fox, S. B., Gatter, K. C., Harris, A. L., and Bicknell, R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 998–1002
37. Prasad, G. S. (2001) Curr. Protein Pept. Sci. 2, 301–311
38. Norman, R. A., Barry, S. T., Bate, M., Breed, J., Colls, J. G., Ernill, R. J., Luke, R. W., Minshull, C. A., McAlister, M. S., McCall, E. J., McMiken, H. H., Paterson, D. S., Timms, D., Tucker, J. A., and Pauptit, R. A. (2004) Structure 12, 75–84
39. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Cryst. 26, 283–291