Extremophiles (2014) 18:179–187
DOI 10.1007/s00792-013-0595-8

ORIGINAL PAPER

Specificities and pH profiles of adenine and hypoxanthine–guanine–xanthine phosphoribosyltransferases (nucleotide synthases) of the thermoacidophile archaeon *Sulfolobus solfataricus*

Michael Riis Hansen · Kristine Steen Jensen · Mads Skytte Rasmussen · Stig Christoffersen · Anders Kadziola · Kaj Frank Jensen

Received: 2 August 2013 / Accepted: 10 October 2013 / Published online: 25 October 2013

Abstract Two open reading frames in the genome of *Sulfolobus solfataricus* (SSO2341 and SSO2424) were cloned and expressed in *E. coli*. The protein products were purified and their enzymatic activity characterized. Although SSO2341 was annotated as a gene (*gpT-1*) encoding a 6-oxopurine phosphoribosyltransferase (PRTase), the protein product turned out to be a PRTase highly specific for adenine and we suggest that the reading frame should be renamed *apT*. The other reading frame SSO2424 (*gpT-2*) proved to be a true 6-oxopurine PRTase active with hypoxanthine, xanthine and guanine as substrates, and we suggest that the gene should be renamed *gpT*. Both enzymes exhibited unusual profiles of activity versus pH. The adenine PRTase showed the highest activity at pH 7.5–8.5, but had a distinct peak of activity also at pH 4.5. The 6-oxo PRTase showed maximal activity with hypoxanthine and guanine around pH 4.5, while maximal activity with xanthine was observed at pH 7.5. We discuss likely reasons why SSO2341 in *S. solfataricus* and similar open reading frames in other Crenarchaeota could not be identified as genes encoding APRTase.

Keywords Nucleotide synthesis in sulfolobales · Phosphoribosyltransferase · Crenarchaeota · Archaea · Substrate specificity of enzymes

Abbreviations

PRPP	5-Phosphoribosyl-α-1-diphosphate
PRTase	Phosphoribosyltransferase
APRTase	Adenine PRTase
HGXPRTase	Hypoxanthine–guanine–xanthine PRTase
P_i	Orthophosphate
PP_i	Pyrophosphate

Communicated by L. Huang.

M. R. Hansen · K. S. Jensen · M. S. Rasmussen · K. F. Jensen (✉)
Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, 2200 Copenhagen N, Denmark
e-mail: kajfrankjensen@gmail.com

Present Address:
M. R. Hansen
Department of Biochemistry, Temple University School of Medicine, 3307 N Broad St., Philadelphia, PA 19140, USA
e-mail: mrh@temple.edu

Present Address:
K. S. Jensen
Chemistry Department, TU München, Lichtenbergstrasse 4, 85747 Garching, Germany
e-mail: ksjensen@tum.de

Present Address:
M. S. Rasmussen
Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway
e-mail: mads.s.rasmussen@uit.no

S. Christoffersen · A. Kadziola
Department of Chemistry, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen Ø, Denmark

Introduction

The phosphoribosyltransferases (PRTases) are responsible for the formation of all N-glycosidic bonds in nucleotides, both by salvage and de novo biosynthetic pathways, and are thus essential for all synthesis of RNA and DNA in living organisms. They catalyze an Mg^{2+}-dependent reaction between a nitrogenous base and 5-phosphoribosyl-α-1-pyrophosphate (PRPP) leading to formation of a nucleotide and pyrophosphate (PP_i) according to Scheme 1 (top). In the pyrimidine bases, orotate and uracil, the reactive group is N1 and in the purine bases, shown in Scheme 1 (bottom), the reactive group is N9.

For the salvage of purine bases, organisms generally contain one PRTase specific for adenine and one or more 6-oxo PRTases responsible for the salvage of hypoxanthine, xanthine and guanine (Jensen et al. 2008). However, in the genome of *Sulfolobus solfataricus*, which we became interested in because the annotated enzymes of nucleotide metabolism showed only little sequence similarity to similar well-characterized enzymes from other organisms, two open reading frames (SSO2341 and SSO2424) were both annotated as genes encoding 6-oxopurine PRTases (*gpT-1* and *gpT-2*, respectively), while no putative gene for an adenine PRTase (APRTase) was found (She et al. 2001). Likewise, no gene encoding APRTase has been identified in other Crenarchaeota species.

To resolve the differences in specificity we cloned the two open reading frames (SSO2341 and SSO2424), expressed them in *E. coli* and purified the protein products. Enzymatic analysis revealed that SSO2341 encodes an APRTase and should be named *apT*, while SSO2424 encodes a true 6-oxopurine PRTase active with hypoxanthine, guanine and xanthine (named HGXPRTase), but not with adenine. Furthermore, we found that both enzymes exhibited unprecedented profiles of activity as a function of pH.

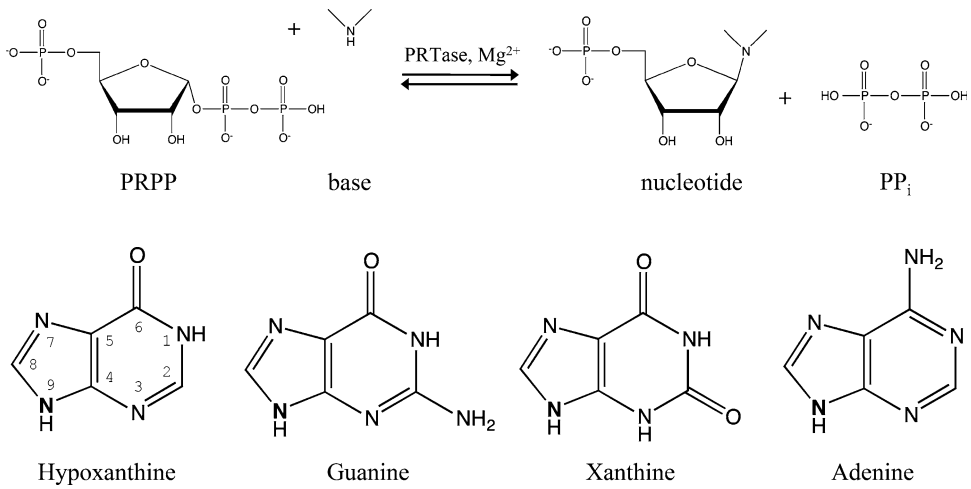
Materials and methods

Materials

[8-^{14}C]-labeled purine bases (50–60 Ci/mol) were bought from Moravek Biochemicals or Sigma-Aldrich. [β-^{32}P]PRPP was synthesized from [γ-^{32}P]ATP (3000 C_i/μmol, Perkin Elmer) using PRPP synthase as previously described (Jensen and Mygind 1996). Unlabeled purine bases and PRPP were from Sigma-Aldrich. Other chemicals were of the purest grade available. The purine requiring *E. coli* strain SØ609 (*ara Δpro-gpt-lac thi hpt deoD purH,J rpsL*) lacking all 6-oxopurine PRTase activity (Jochimsen et al. 1975) was a gift from Dr. P. Nygaard. The cloning strain NF1830 (*araD139 (araABC-leu)7679 galU galK Δ(lac)X74 rpsL thi recA1/F'lacI^{q1} lacZ::Tn5*) that overproduces the *lacI* repressor and the cloning vector pUHE23-2, which drives transcription of cloned genes from the strong LacI repressible $P_{A1/O4/O3}$ promoter (Lanzer and Bujard 1988) were previously described (Andersen et al. 1992). Chromosomal *Sulfolobus solfataricus* P2 DNA was a gift from Dr. Q. She (She et al. 2001).

Cloning and expression

Both open reading frames (SSO2341 and SSO2424) were amplified by standard PCR techniques from chromosomal DNA of *S. solfataricus* using the Vent™ polymerase. We used 29 cycles with a denaturation temperature 95 °C for 30 s, an annealing temperature of 58 °C for 30 s, a polymerization temperature of 72 °C for 1 min and 5 min to complete the last cycle. The oligonucleotides (purchased from TAG Copenhagen) 5′CGCGGATCCGGAGGTA GACAGGATGCAAAAAATACCAGTGAAAG (containing a *Bam*HI site) and 5′GGAAGCTTATCAGACTA TTTTTCTTCTTTTC (containing a *Hind*III site) were used as forward and reverse primers, respectively, for the



Scheme 1 The *upper part* shows the PRTase reaction in which a nitrogenous base displaces the pyrophosphoryl group of PRPP leading to formation of a nucleotide. The *lower part* shows structure formula of four purine bases and the numbering of atoms in the purine ring

amplification of SSO2341 (*gpT-1*), while the oligonucleotides 5′GGGGGAATTCAGGAGAGTTGAATGGTTGAA TATCATATTCCTTCATGGGATGA (carrying an *Eco*RI site) and 5′GGGGGAAGCTTATTTTCTGATCTTTAA TAATTGATTATAC (carrying a *Hind*III site) were used as forward and reverse primers, respectively, for the amplification of SSO2424 (*gpT-2*). The two PCR products (664-bp for SSO2341 and 540-bp for SSO2424) were digested with the mentioned restriction endonucleases and ligated into the vector pUHE23-2 (Lanzer and Bujard 1988) digested with the corresponding endonucleases and treated with alkaline phosphatase to prevent self-ligation. The ligation mixtures were transformed into the *E. coli* strain NF1830 (Andersen et al. 1992) selected for resistance to ampicillin on LB-broth agar plates (Miller 1972). The resulting plasmids were termed pSsGPT-1 and pSsGPT-2 in line with the annotations from the *S. solfataricus* genome, and contained the open reading frames under control of the strongly inducible $P_{A1/04/03}$ promoter. Sequencing revealed that the cloned genes were identical with the published sequences aside of the deliberate change of the original GTG initiation codon of SSO2341 to an ATG codon. The presence of the *F'lacI*^{q1}*lacZ:Tn5* episome in the cloning strain, NF1830 results in overproduction of the lactose operon repressor. This ensures that the cloned genes remain repressed until an inducer is added to the culture medium. To produce the encoded proteins, the transformed cells were grown at 37 °C with vigorous aeration in one liter portions of LB-broth medium (Miller 1972) containing 10 g of glucose and 0.1 g of ampicillin per liter. Transcription was induced at $OD_{436} = 0.8$ by addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG) and growth was continued overnight. The cells were harvested by centrifugation and stored frozen at −20 °C until use.

Purification of APRTase (the SSO2341 gene product)

The frozen cell-paste from one liter of NF1830 transformed with pSsGPT-1 was thawed and suspended in 50 mL of 100 mM Tris–HCl (pH 7.6), 2 mM EDTA. Cells were disrupted by sonication and debris was removed by centrifugation. The cleared extract was heated to 73.5 °C, kept at this temperature for 15 min while gently agitated and subsequently cooled in a melting ice bath. Precipitated material was removed by centrifugation. Solid ammonium sulfate was added to 90 % saturation and stirred at 4 °C for an hour. The precipitated material was collected by centrifugation, dissolved in 12 mL of 25 mM Tris–HCl (pH 7.6), 0.1 mM EDTA (Buffer A). After dialysis against the same buffer, the sample (21 mL) was applied to a 30 mL column of DEAE cellulose (DE52, Whatman) equilibrated with Buffer A. The column was washed with 80 mL of Buffer A and the enzyme was eluted with a linear gradient (200 mL) from 0 to 0.3 M NaCl in Buffer A. The flow rate was 1 mL per min and 5 mL fractions were collected. The enzyme was eluted from the column at around 125 mM NaCl. Four fractions (20 mL) containing most APRTase were pooled, dialyzed for 2 h against 1 L of Buffer A, applied on a Q6 column (BioRad) equilibrated with the same buffer and eluted with a gradient 0–0.3 M NaCl in Buffer A. Four top fractions (8 mL), which appeared more than 95 % homogeneous by SDS-PAGE, were pooled and dialyzed first against 1 L of 10 mM Tris–HCl (pH 8), 0.1 mM EDTA and subsequently against the same buffer containing 50 % glycerol. The enzyme was stored unfrozen at −20 °C. According to UV-absorption measurements using the absorption coefficient $A_{280} = 1.88$ mL mg^{-1} cm^{-1} (Gill and von Hippel 1989), the protein concentration was about 11 mg/mL equivalent to a yield ca. 25 mg per liter culture.

Purification of HGXPRTase (the SSO2424 gene product)

Cells (9.9 g) of NF1830 transformed with pSsGPT-2 were suspended in 80 mL of 100 mM Tris–HCl (pH 7.6), 2 mM EDTA and disrupted by sonication. The extract was cleared by centrifugation and the supernatant (77 mL) was heated at 70 °C for 15 min with agitation, cooled in an ice bath and cleared by centrifugation. To the supernatant (65 mL), 7 mL of a 20 % solution of streptomycin sulfate was added, and after gentle stirring for 60 min at 4 °C for an hour, the extract was again cleared by centrifugation. After exhaustive dialysis of the supernatant against 25 mM Tris–HCl (pH 8.8), 0.1 mM EDTA (Buffer B), the volume was 72 mL. Solid ammonium sulfate was added to obtain 50 % saturation. After stirring for 60 min the precipitated material was removed by centrifugation and discarded. The concentration of ammonium sulfate in the supernatant was raised to 80 % saturation and stirred gently at 4 °C overnight. The precipitate was collected by centrifugation, dissolved in 30 mL of Buffer B and dialyzed exhaustively against the same buffer. The dialyzed solution was applied to a column of DE52 (60 mL) equilibrated with Buffer B. After washing the column with 100 mL of Buffer B, a 200 mL linear gradient to Buffer B containing 0.6 M NaCl was applied at a flow rate of 1 mL/min while 5 mL fractions were collected. HGXPRTase appeared as a symmetric peak at a quite low salt concentration (ca. 50 mM NaCl). Four fractions were pooled (20 mL) and dialyzed against 25 mM Tris–HCl (pH 7.3), 0.1 mM EDTA and passed through a column (30 mL) of phosphocellulose equilibrated with the same buffer. The enzyme did not bind to the resin at pH 7.3, so the flow-through fractions were collected, and the enzyme precipitated with ammonium sulfate (90 % saturation). The precipitate was dissolved in

5 mL of Buffer B and loaded on to Sephacryl S-200 column (diameter 2.5 cm, height 85 cm) equilibrated with Buffer B. Flow rate was 1 mL/min while 5 mL fractions were collected. Fractions (25 mL) containing HGXPRTase were pooled and dialyzed against 5 mM sodium phosphate (pH 6.0). Half of the fraction was applied to an UNO S6 column (BIO-RAD) equilibrated with the same buffer. After washing with 10 mL of 5 mM sodium phosphate (pH 6.0), a 40 mL gradient from 5 mM sodium phosphate (pH 6.0) to 5 mM sodium phosphate (pH 6.0) containing 0.5 M NaCl was obtained. The flow rate was 1 mL/min and 1 mL fractions were collected during the wash and the gradient elution. HGXPRTase appeared with a peak at 0.33 M NaCl. The run was repeated with the other half of the enzyme. Fractions (12 mL) from each run were pooled and dialyzed first against Buffer B, then against Buffer B containing 50 % glycerol. The enzyme appeared homogeneous by SDS-PAGE (on 15 % gels). The yield was 6–7 mg per liter and the concentration was 1.02 mg/mL according to the calculated absorption coefficient $A_{280} = 1.935$ mL mg^{-1} cm^{-1} (Gill and von Hippel 1989). The enzyme was stored at −20 °C and has remained stable for over 5 years. Several attempts to improve the expression of SSO2424 from pSsGPT-2 and the yield of enzyme using other *E. coli* strains as host failed.

Mutant D99N APRTase

A plasmid pD99N encoding the mutant D99N APRTase was made by PCR overlap extension using plasmid pSsGPT-1 as template and the two mutagenic primers: 5′ATAGA TGATATAACAAATACCGGAGATAGTATAG and 5′CT ATACTATCTCCGGTATTTGTTATATCATC in combination with two flanking primers: 5′AGTTCTGAG GTCATTACTGG and 5′AATAGGCGTATCACGAGG, respectively. Cloning and expression of the mutant protein were carried out as described for the wild type enzyme.

Activity measurements

Enzyme activity was determined by measuring the conversion of [8-^{14}C]-labeled purine base (5 Ci/mol) and PRPP to the corresponding radioactive nucleotide. The reactions were carried out at 35 and 60 °C. Standard reaction mixtures (total volume of 50 µL) contained 50 mM buffer of specified pH (see below), 100 µM [8-^{14}C]-labeled purine base (5 Ci/mol), 1 mM PRPP, 10 mM $MgCl_2$ and the appropriate amount of enzyme [diluted in 2 mM Tris–HCl (pH 8)]. Reactions were started either by addition of enzyme or PRPP to the pre-warmed mixture of the other components. Samples (10 µL) were withdrawn usually at 1, 2 and 5 min and applied to PEI Cellulose F thin-plates (Merck KGaA, Darmstadt). A reaction without enzyme was used to determine the background (i.e., zero-time) radioactivity. Unreacted purine base was separated from the nucleotide product by chromatography in water, and the radioactivity in each of the two compounds was determined by phosphorimaging using a Packard Cyclone™.

Activities with xanthine were also measured in continuous spectrophotometric assay (1 mL) as previously described (Arent et al. 2006) using the absorbance change $\Delta A_{252} = 5.35$ mM^{-1} cm^{-1}, constant in the pH range 6–9, for the conversion of xanthine to XMP.

When possible the pH-activity data were analyzed by fitting of the parameters in Eq. 1 to the data.

$$\text{Activity} = \text{PA}/\left(1 + 10^{(\text{p}K\text{a}-\text{pH})} + 10^{(\text{pH}-\text{p}K\text{b})}\right) + \text{PB}/\left(1 + 10^{(\text{p}K\text{c}-\text{pH})} + 10^{(\text{pH}-\text{p}K\text{d})}\right) \quad (1)$$

The equation describes the activity of an enzyme with two pH optima, each of which is governed by protonation of a catalytic acid (pK_a and pK_c) and a catalytic base (pK_b and pK_d). The parameters, PA and PB are hypothetical values describing the activities that might be obtained if the catalytic acid and the catalytic base were, respectively, protonated and deprotonated at the same time.

Breakdown and precipitation of PRPP at different pH at 60 °C

[β-^{32}P]PRPP (2.5 mM) in 50 mM buffers supplemented with 20 mM $MgCl_2$ of different pH in a volume of 50 µL was incubated at 60 °C for 5 min, cooled on ice and centrifuged for 10 min at 10,000×*g*. 40 µL of the supernatant was transferred to another tube, and the pellet was re-dissolved in the remaining 10 µL supernatant plus 30 µL of 50 mM sodium succinate (pH 2.5). Aliquots (5 µL) of each sample applied to PEI plates and subjected to chromatography as described (Jensen et al. 1979) to separate phosphorylated compounds. The thin-layer plates were exposed on storage phosphor screens and quantified on a Packard Cyclone. Radioactivity (DLU) in the "spots" corresponding to $^{32}P_i$, $^{32}PP_i$ and [β-^{32}P]PRPP was determined and converted to mM concentration by the equation:

2.5 mM × (DLU in ''spot'')/(Total DLU lane).

The concentrations of the components in the supernatant are shown in Fig. 1c. The residual radioactivity in the three components was found in the pellet following centrifugation.

Molecular size determination

The approximate size of the native enzymes was determined by gel-filtration of the enzymes on Superose S12

column and Superdex 200 10/300 GL column (GE Healthcare) in 50 mM Tris–HCl (pH 7.5) containing 10 mM $MgCl_2$ using *E. coli* orotate PRTase [$M_r = 45000$ (Aghajari et al. 1994)], *L. lactis* dihydroorotate dehydrogenase A [$M_r = 68000$ (Nielsen et al. 1996a, b)] and *L. lactis* dihydroorotate dehydrogenase B [$M_r = 130000$ (Nielsen et al. 1996a, b)] as markers.

Buffers

Buffers of different pH were used. They were prepared at room temperature. In the region of $2.6 \leq pH \leq 6.2$ the buffers were made by mixing 0.25 M succinic acid with 0.25 M Na_2HPO_4 to the desired pH. The buffers' pH 6 and 6.5 were 0.25 M MES adjusted to pH, and buffers in the range $6.4 \leq pH \leq 8.4$ were 0.25 M Tris–HCl. Sodium glycinate (0.25 M) was used for $pH \geq 9$. The actual pH values were determined at 35 °C and at 60 °C in buffers diluted to working strength (50 mM) after addition of 20 mM $MgCl_2$.

Protein concentration determination

Determination of protein concentrations in crude extracts was carried by use of the BCA Protein Assay Reagent (Thermo Scientific).

Results

Specificity analyses

The first insight into the specificity of the two enzymes was gained after transformation of the 6-oxopurine requiring *E. coli* strain SØ609 (*purHJ hpt Δpro-gpt-lac*); with the two plasmids. The strain is unable to use hypoxanthine, xanthine and guanine as source of purine due to the lack of all native 6-oxopurine PRTase activity (Jochimsen et al. 1975). Transformation with pSsGPT-2 enabled the strain to form colonies on minimal glucose and IPTG agar plates supplemented with either of the three purine bases, while transformation with pSsGPT-1 did not, indicating that only pSsGPT-2 encodes an active 6-oxopurine PRTase.

We then measured the activity of the purified proteins using different purine bases as substrates together with PRPP at two different pH values, 4.1–4.3 and 7.5–7.8 (the reason for using two pH values is explained below). The results are shown in Table 1. It is seen that the protein encoded by SSO2341 (*gpT-1*) has a strong preference for adenine as substrate over the 6-oxopurine bases, hypoxanthine and guanine and thus must be the APRTase of *S. solfataricus*. On the other hand, the protein encoded by SSO2424 (*gpT-2*) is the proper 6-oxopurine PRTase with activity towards xanthine, guanine and hypoxanthine.

Molecular size determination

By gel-filtration on Superose 12 and Superdex 200 10/300 GL columns (GE Healthcare) both APRTase (subunit $M_r = 24200$) and HGXPRTase (subunit $M_r = 20600$) eluted approximately like the *E. coli* orotate PRTase ($M_r = 45000$), indicating that both enzymes form dimers in solution.

pH profiles

As the uracil PRTase of *Sulfolobus* species showed maximal activity in the acidic pH range (Linde and Jensen 1996; Jensen et al. 2005), we investigated the activities of the APRTase and HGXPRTase over a wide pH range. The

Table 1 Reactivity of the two *S. solfataricus* PRTases using different purine bases as substrates in buffers containing 100 μM ^{14}C-labeled purine base, 2.5 mM PRPP and 10 mM $MgCl_2$ at 60 °C

Purine base	SSO2341 protein[a]		SSO2424 protein[a]		Crude *E. coli* extract[b,c]	
	pH 4.1	pH 7.8	pH 4.1	pH 7.8	pH 4.3	pH 7.5
Adenine	13.2	12.6	≪0.1	≪0.1	28 (0.48) × 10^{-3}	79 (1.7) × 10^{-3}
Hypoxanthine	0.14	0.24	8.0	3.0	4.8 (≤0.01) × 10^{-3}	36 (≤0.1) × 10^{-3}
Xanthine	0.04	0.07	9.4	9.6	2.1 (≤0.01) × 10^{-3}	79 (≤0.1) × 10^{-3}
Guanine	0.02	0.04	23.1	7.6	15 (≤0.02) × 10^{-3}	74 (≤0.1) × 10^{-3}

The buffers were 50 mM succinic acid/Na_2HPO_4 at pH about 4 and 50 mM Tris–HCl at pH 7.8. The table also shows the activities of a crude *E. coli* NF1830 (*apt*$^+$ *hpt*$^+$ *gpt*$^+$) extract at 35 °C with the different purine bases. Activities are expressed in units equal to μmol min^{-1} mg^{-1}

[a] Assays were performed at 60 °C

[b] Assays were performed at 35 °C

[c] The crude *E. coli* extract was made from a 0.5 L culture of NF1830 transformed with the cloning vector pUHE23-2, without insert, grown and induced with IPTG as described in Materials and Methods. The numbers in parentheses give the activities after heating of the extract for 15 min at 70 °C

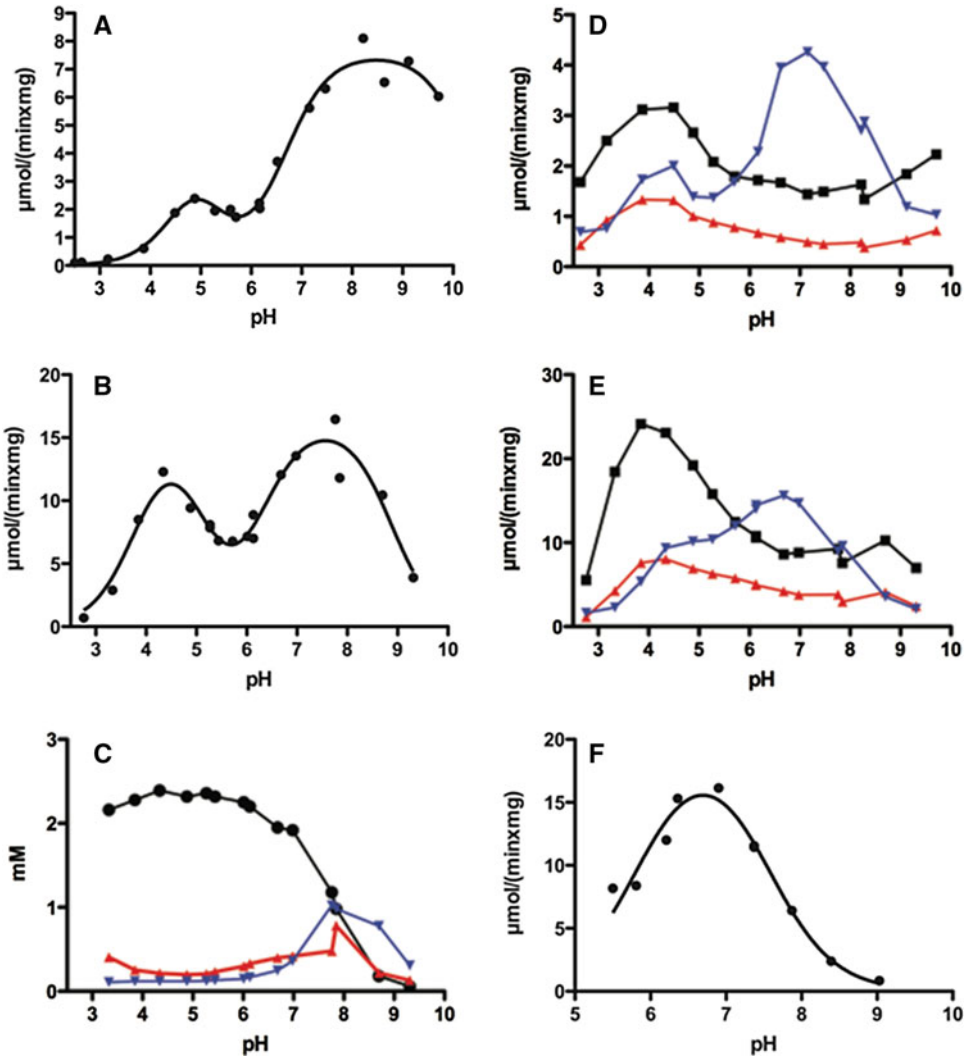


Fig. 1 Purine PRTase activities measured at 35 and 60 °C using a chromatographic assay (**a**, **b**, **d** and **e**). Buffers (50 mM) of different pH contained 2.5 mM PRPP, 20 mM $MgCl_2$ and 100 µM of ^{14}C-labeled purine base. The reactions were started by addition of PRPP at time zero and after 5 min samples (5 µL) were applied to PEI plates. Radioactivity in the nucleotide and in the remaining purine base was determined as described in "Materials and methods". **a** and **b** APRTase at 35 and 60 °C, respectively. They were generated by a fit of Eq. 1 to the pH-activity profiles which resulted in four separated pK values at 35 °C (p$K_a = 4.7 \pm 0.5$, p$K_b = 5.1 \pm 0.5$, p$K_c = 6.7 \pm 0.1$ and p$K_d = 10.3 \pm 0.2$) and at 60 °C (p$K_a = 4.0 \pm 0.2$, p$K_b = 4.9 \pm 0.2$, p$K_c = 6.2 \pm 0.1$ and p$K_d = 8.9 \pm 0.1$). **c** The content of PRPP (*black circle*), PP_i (*red triangles*) and P_i (*blue triangle*) remaining or formed in the same buffers as used in **a**, **b**, but without enzyme after incubation of 2.5 mM [β-^{32}P]PRPP for 2 min at 60 °C. **d, e** HGXPRTase at 35 and 60 °C, respectively; guanine (*black square*), hypoxanthine (*red triangle*), xanthine (*blue triangle*). **f** Shows results of a continuous spectrophotometric assay of xanthine PRTase activity performed at 60 °C. The concentrations were 50 mM buffer, 10 mM $MgCl_2$, 0.65 mM PRPP and 88 µM xanthine. The curve was generated by use of Eq. 1, but leaving out the PB term. The calculated pK values were p$K_a = 5.84 \pm 0.13$ for the rise and p$K_b = 7.55 \pm 0.12$ for the decline at high pH

results are shown in Fig. 1. Both enzymes showed two pH optima, i.e., around 8 and 4.5. The activity of APRTase was considerably higher at pH 8 than pH 4.5, when assayed at 35 °C (Fig. 1a), while the two activity peaks seemed more equal, when assayed at 60 °C (Fig. 1b). A likely reason for this difference could be the well-known instability of PRPP in alkaline solution 8 (Khorana et al. 1958). At the higher temperature only little PRPP is left for the PRTase reaction when PRPP is broken into orthophosphate (P_i) and 5-phosphoribosyl-cyclic-1,2-phosphate at pH values above 8 (Fig. 1c).

The HGXPRTase showed the highest activity with hypoxanthine and guanine at pH around 4.5, while the activity with xanthine as substrate predominated around pH 7.5 (Fig. 1d, e). The activity profiles for this enzyme were similar at 35 and 60 °C indicating that the

Table 2 Inhibition of the APRTase reaction by adenine nucleotides at 60 °C. The reactions contained 1 mM PRPP, 100 μM ^{14}C-adenine and 10 mM $MgCl_2$. The buffers were 50 mM succinic acid/Na_2HPO_4 (pH 4.1) and Tris–HCl (pH 7.5)

Compound	I_{50} (μM)	
	pH 4.1	pH 7.5
AMP	177 ± 69	300 ± 94
ADP	19.0 ± 1.4	3211 ± 1867
ATP	344 ± 29	574 ± 175

availability of PRPP does not become the limiting factor for activity for this enzyme at high pH and high temperature at the used conditions. We are currently unable to explain the difference in substrate specificity in the acidic pH region, but the decline in activity towards xanthine at pH > 7 ($pK_a = 7.55 \pm 0.12$, Fig. 1f) relative to the activity with hypoxanthine and guanine, is likely caused by HGXPRTase of *S. solfataricus* discriminating against the negatively charged form of xanthine, which dissociates a proton with a $pK_a = 7.5$ (Stoychev et al. 2002; Arent et al. 2006). It is clear that the pH-activity profiles of the two *S. solfataricus* enzymes deviate from the profiles of the corresponding *E. coli* purine PRTases, which only have little activity at pH 4.5 (Table 1), but we cannot state how unusual these profiles are, since—to the best of our knowledge—the activity of purine PRTases has not previously been studied in the acidic pH range.

Feedback inhibition by nucleotides

As the activity of uracil PRTases from *S. solfataricus* and *S. shibatae* is strongly affected by the presence of GTP (an activator) and CTP (an inhibitor) (Linde and Jensen 1996; Arent et al. 2005; Jensen et al. 2005; Christoffersen et al. 2009), we also investigated the effects of the nucleoside triphosphates on the activity of the two purine PRTases. The activity of HGXPRTase was unaffected by the triphosphates ATP, GTP, CTP or UTP, but the activity of APRTase was somewhat reduced by the presence of ATP (Table 2). Further analysis revealed that ADP was a much stronger inhibitor than ATP at pH 4.5, but had virtually no effect on activity at neutral pH.

The catalytic base in APRTase

All type I PRTases and PRPP synthases contain a partly conserved sequence motif called the PRPP motif (Hove-Jensen 1985; Hershey and Taylor 1986), which in crystal structures forms the part of a loop involved in binding of the 5′-phosphoribosyl part of nucleotides and PRPP, see e.g., (Eads et al. 1994; Scapin et al. 1994). The exact sequence of the motif varies in accordance with the specificity towards the nucleobase substrate (Lundegaard and Jensen 1999). The PRPP sequence motifs of the two purine PRTases of *S. solfataricus* aligned with the PRPP motifs of some well-characterized adenine- and 6-oxopurine PRTases are shown in Fig. 2a. Clearly, the PRPP motif of the APRTase of *S. solfataricus* resembles the motif generally seen in the 6-oxopurine PRTases. Note particularly the residue Asp99 of the *S. solfataricus* APRTase, which is generally conserved among 6-oxopurine PRTases, whereas the APRTases mostly have an alanine residue at the corresponding position. In the 6-oxopurine PRTases, this aspartate serves as a catalytic base to stabilize the N7-protonated tautomer of the purine base and liberate N9 for nucleophilic attack on the C1-carbon of PRPP, resulting in formation of the β-N-glycosidic bond, while the corresponding catalytic base function generally is exerted by a glutamate positioned in a flexible loop covering the active site (Schramm and Grubmeyer 2004). An exception to this rule is the highly xanthine specific PRTases of *Bacillus* species (XPRTase in Fig. 2a), which seem to entirely lack a catalytic base, making the reaction dependent upon the spontaneous deprotonation of the purine base at alkaline pH (Arent et al. 2006). To study if Asp99 has a catalytic base function in *S. solfataricus* APRTase, we changed the residue to an asparagine by site-directed mutagenesis. The resulting D99N mutant APRTase had very low activity (≤0.2 μmol min^{-1} mg^{-1}, measured at 60 °C at pH 4.1 and pH 7.6) showing that Asp99 indeed plays a role for the catalytic function of this enzyme.

Discussion

The results herein demonstrate unambiguously that the protein encoded by the open reading frame SSO2341 (*gpT-1*) encodes the synthesis of the APRTase of *S. solfataricus* despite the annotation as a gene encoding a 6-oxopurine PRTase (She et al. 2001), and that the open reading frame SSO2424 (*gpT-2*) of the organism encodes the true 6-oxopurine PRTase active with hypoxanthine, xanthine and guanine as substrates. We, therefore, suggest that SSO2341 should be renamed *apT* and SSO2424 simply *gpT*. Similar change of annotation should also be made of corresponding genes in other Crenarchaeota, as the gene for APRTase could not be identified in any of those on basis of sequence searches. There are three obvious reasons that could explain the failure to find the gene for APRTase in *S. solfataricus*: *First*, the encoded protein shows the highest sequence similarity to the xanthine–guanine PRTase of *E. coli*. *Second*, the sequence similarity to the PRTases includes only the first two-thirds of the encoded protein.

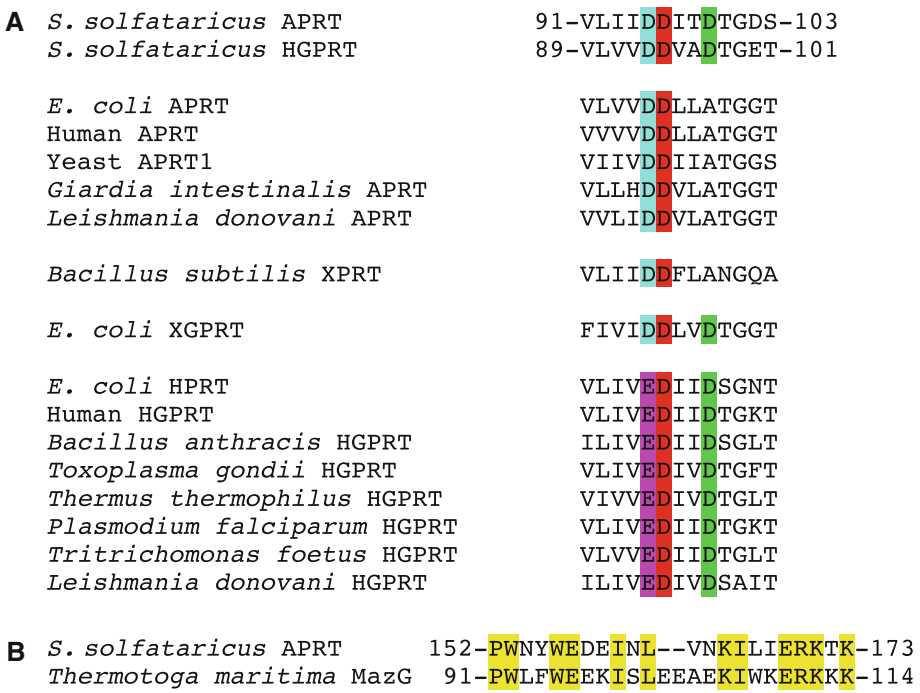


Fig. 2 **a** Sequence alignment of PRPP biding motif of adenine and 6-oxopurine PRTases from selective species as described. *Cyan* Asp (D) is conserved only in APRTases, *B. subtilis* XPRTase and *E. coli* xanthine–guanine PRTase (XGPRTase). *Pink* Glu (E) is conserved only in HGPRTase at this position. *Red* Asp (D) is fully conserved is this position in all purine PRTases. *Green* Asp is a catalytic important residue in HGPRTase. Note that the PRPP binding motif of the *S. solfataricus* enzymes partly diverge to other APRTase and HGPRTase. **b** Alignment of *S. solfataricus* APRTase and MazG-related nucleoside triphosphate pyrophosphohydrolase from *Thermotoga maritima* (PDB code 2YXH, unpublished). Identical residues were shaded *yellow*

The C-terminal part of the protein shows no obvious sequence similarity to any known protein except for a small stretch of 22 amino acid residues with 50 % identity to the MazG protein (Fig. 2b) that may encode a nucleoside triphosphate pyrophosphohydrolase (Zhang and Inouye 2002). *Third*, the PRPP motif of the APRTase resembles that of the 6-oxopurine PRTases rather than the motif of APRTases (Fig. 2A). In the 6-oxopurine PRTases a carboxylate from an aspartate protruding from the PRPP site serves the function as a catalytic base promoting formation of the β-glycosidic bond (Schramm and Grubmeyer 2004), and Asp99 in the PRPP site of *S. solfataricus* APRTase seems to possess similar catalytic functionality as evidenced by the low or vanishing activity of the D99N mutant enzyme.

We do not currently know if the activity of the two purine PRTases in the acidic pH range has any physiologic relevance, but—after all—*S. solfataricus* is a thermoacidophile that grows optimally at pH around 3 (She et al. 2001), or if the strong inhibition of APRTase by ADP at low pH has any relevance (in vivo) in nature.

Our primary purpose of this analysis was to investigate the reaction specificities of the two purine PRTases of *S. solfataricus* (and related Crenarchaeota). The presented results give only a partial characterization of the enzymes, and a more detailed characterization of the individual enzymes, including crystal structures of both enzymes will be published elsewhere.

Acknowledgments We thank Lise Schack for excellent technical assistance, Dr. Qunxin She for the gift of *S. solfataricus* P2 chromosomal DNA and Dr. Per Nygaard for the *E. coli* strain SØ609. We also acknowledge the financial support from the Danish Council for Independent Research | Natural Sciences (FNU).

Conflict of interest The authors declare no conflict of interest and state that the experimental work complies with Danish law.

References

Aghajari N, Jensen KF, Gajhede M (1994) Crystallization and preliminary X-ray diffraction studies on the apo form of orotate phosphoribosyltransferase from *Escherichia coli*. J Mol Biol 241:292–294

Andersen JT, Poulsen P, Jensen KF (1992) Attenuation in the *rph-pyrE* operon of *Escherichia coli* and processing of the dicistronic mRNA. Eur J Biochem 206:381–390

Arent S, Harris P, Jensen KF, Larsen S (2005) Allosteric regulation and communication between subunits in uracil phosphoribosyltransferase from *Sulfolobus solfataricus*. Biochemistry 44:883–892

Arent S, Kadziola A, Larsen L, Neuhard J, Jensen KF (2006) The extraordinary specificity of xanthine phosphoribosyltransferase

from *Bacillus subtilis* elucidated by reaction kinetics, ligand binding, and crystallography. Biochemistry 45:6615–6627

Christoffersen S, Kadziola A, Johansson E, Rasmussen M, Willemoës M, Jensen KF (2009) Structural and kinetic studies of the allosteric transition in *Sulfolobus solfataricus* uracil phosphoribosyltransferase: permanent activation by engineering the C-terminus. J Mol Biol 393(3):464–477

Eads JC, Scapin G, Xu Y, Grubmeyer C, Sacchettini JC (1994) The crystal structure of human hypoxanthine–guanine phosphoribosyltransferase with bound GMP. Cell 78:325–334

Gill SC, von Hippel PH (1989) Calculation of protein extinction coefficients from amino acid sequence data. Analyt Biochem 182:319–326

Hershey HV, Taylor MW (1986) Nucleotide sequence and deduced amino acid sequence of *Escherichia coli* adenine phosphoribosyltransferase and comparison with other analogous enzymes. Gene 43:287–293

Hove-Jensen B (1985) Cloning and characterization of the *prs* gene encoding phosphoribosylpyrophosphate synthetase of *Escherichia coli*. Mol Gen Genet 201:5487–5493

Jensen KF, Mygind B (1996) Different oligimeric states are involved in the allosteric behavior of uracil phosphoribosyltransferase from *Escherichia coli*. Eur J Biochem 240:637–645

Jensen KF, Houlberg U, Nygaard P (1979) Thin-layer chromatographic methods to isolate ^{32}P-labeled 5-phosphoribosyl-α-1-pyrophosphate (PRPP): determination of cellular PRPP pools and assay of PRPP synthetase activity. Analyt Biochem 98:254–263

Jensen KF, Arent S, Larsen S, Schack L (2005) Allosteric properties of the GTP activated and CTP inhibited uracil phosphoribosyltransferase from the thermo-acidophilic archaeon *Sulfolobus solfataricus*. FEBS J 272:1440–1453

Jensen KF, Dandanell G, Hove-Jensen B, Willemoës M (2008) Nucleotides, Nucleosides and Nucleobases. EcoSal—*Escherichia coli* and *Salmonella*: cellular and molecular biology. Retrieved 18 August 2008

Jochimsen B, Nygaard P, Vestergaard T (1975) Location on the chromosome of *Escherichia coli* of genes governing purine metabolism. Adenosine deaminase (*add*), guanosine kinase (*gsk*) and hypoxanthine phosphoribosyltransferase (*hpt*). Mol Gen Genet 114:85–91

Khorana HG, Frenandes JF, Kornberg A (1958) Pyrophosphorylation of ribose 5-phosphate in the enzymatic synthesis of 5-phosphorylribose 1-pyrophosphate. J Biol Chem 230(2):941–948

Lanzer M, Bujard H (1988) Promoters largely determine the efficiency of repressor action. Proc Natl Acad USA 85:8973–8977

Linde L, Jensen KF (1996) Uracil phosphoribosyltransferase from the extreme thermophilic archaebacterium *Sulfolobus shibatae* is an allosteric enzyme, activated by GTP and inhibited by CTP. Biochim Biophys Acta 1296:16–22

Lundegaard C, Jensen KF (1999) Kinetic mechanism of uracil phosphoribosyltransferase from *Escherichia coli* and catalytic importance of the conserved proline in the PRPP binding site. Biochemistry 38:3327–3334

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor, Cold Spring Harbor Laboratory, New York

Nielsen FS, Andersen PS, Jensen KF (1996a) The B-form of dihydroorotate dehydrogenase from *Lactococcus lactis* consists of two different subunits, encoded by the *pyrDb* and *pyrK* genes, and contains FMN, FAD, and [FeS] redox centres. J Biol Chem 271:29359–29365

Nielsen FS, Rowland P, Larsen S, Jensen KF (1996b) Purification and characterization of dihydroorotate dehydrogenase A from *Lactococcus lactis*, crystallization and preliminary X-ray diffraction studies of the enzyme. Protein Sci 5:857–861

Scapin G, Grubmeyer C, Sacchettini JC (1994) Crystal structure of orotate phosphoribosyltransferase. Biochemistry 33:1287–1294

Schramm VL, Grubmeyer CT (2004) Phosphoribosyltransferase mechanisms and roles in nucleic acid metabolism. In: Moldave K (ed) Progress in nucleic acids research and molecular biology, vol 78. Academic Press, New York, pp 261–304

She Q, Singh RK, Confalonieri F, Zivanovic Y, Allard G, Awayez MJ, Chan-Weiher CC, Clausen IG, Curtis BA, De Moors A, Erauso G, Fletcher C, Gordon PM, Heikamp-de Jong I, Jeffries AC, Kozera CJ, Medina N, Peng X, Thi-Ngoc HP, Redder P, Schenk ME, Theriault C, Tolstrup N, Charlebois RL, Doolittle WF, Duguet M, Gaasterland T, Garrett RA, Ragan MA, Sensen CW, Van der Oost J (2001) The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. Proc Natl Acad Sci USA 98(14):7835–7840

Stoychev G, Kierdaszuk B, Shugar D (2002) Xanthosine and xanthine. Substrate properties with purine nucleoside phosphorylases, and relevance to other enzyme systems. Eur J Biochem 269:4048–4057

Zhang J, Inouye M (2002) MazG, a nucleoside triphosphate pyrophosphohydrolase, interacts with Era, an essential GTPase in Escherichia coli. J Bacteriol 184(19):5323–5329