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Purification and characterization of 5'-methylthioadenosine phosphorylase from the hyperthermophilic archaeon *Pyrococcus furiosus*

Substrate specificity and primary structure analysis

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Abstract 5'-Methylthioadenosine phosphorylase (MTAP) was purified to homogeneity from the hyperthermophilic archaeon *Pyrococcus furiosus*. The protein is a homo-examer of 180 kDa. The enzyme is highly thermoactive, with an optimum temperature of 125 °C, and extremely thermostable, retaining 98% residual activity after 5 h at 100 °C and showing a half-life of 43 min at 130 °C. In the presence of 100 mM phosphate, the apparent T_m (137 °C) increases to 139 °C. The enzyme is extremely stable to proteolytic cleavage and after incubation with protein denaturants, detergents, organic solvents, and salts even at high temperature. Thiol groups are not involved in the catalytic process, whereas disulfide bond(s) are present, since incubation with 0.8 M dithiothreitol significantly reduces the thermostability of the enzyme. N-Terminal sequence analysis of the purified enzyme is 100% identical to the predicted amino acid sequence of the gene *PF0016* from the partially sequenced *P. furiosus* genome. The deduced amino acid sequence of the gene revealed a high degree of identity (52%) with human MTAP. Nevertheless, unlike human MTAP, MTAP from *P. furiosus* is not specific for 5'-methylthioadenosine, since it phosphorytically cleaves adenosine, inosine, and guanosine. The calculated k_{cat}/K_m values for 5'-methylthioadenosine and adenosine, about 20-fold higher than for inosine and guanosine, indicate that 6-amino purine nucleosides

are preferred substrates of MTAP from *P. furiosus*. The structural features and the substrate specificity of MTAP from *P. furiosus* document that it represents a 5'-methylthioadenosine-metabolizing enzyme different from those previously characterized among Archaea, Bacteria, and Eukarya. The functional and structural relationships among MTAP from *P. furiosus*, human MTAP, and two putative MTAPs from *P. furiosus* and *Sulfolobus solfataricus* are discussed here for the first time.

Keywords 5'-Methylthioadenosine phosphorylase · Purine nucleoside phosphorylase · *Pyrococcus furiosus* · Protein hyperthermostability · Disulfide bridges

Introduction

Hyperthermophilic microorganisms thrive at temperatures near or above 100 °C (Adams and Kelly 1988; Sterner and Liebl 2001). Therefore, their enzymes have developed unique structure-function properties of high thermostability and optimal activity at these temperatures. These features make hyperthermophilic proteins useful models for understanding the molecular mechanisms of structural and functional adaptation of proteins to extreme temperatures and for designing stable proteins of biotechnological significance.

Comparative studies on mesophilic, thermophilic, and hyperthermophilic proteins have concluded that protein thermostability cannot be achieved by a single mechanism; rather, each protein evolves individually, through a limited number of basic rules or principles in varying combinations, which occur at different levels, also involving the amino acid sequence and the quaternary structure of the proteins (Kumar and Nussinov 2001; Vieille and Zeikus 2001; Vogt et al. 1997). Among the numerous factors that have been evaluated, those cited most often include increased hydrogen bonding, better hydrophobic packing, enhanced secondary

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structure propensity, helix-dipole stabilization, removal of residues sensitive to oxidation or deamination, and improved electrostatic interactions (salt bridge networks) (Kumar and Nussinov 2001; Sterner and Liebl 2001; Vieille and Zeikus 2001; Vogt et al. 1997). However, because the crystallographic structures of hyperthermophilic proteins available at present are still limited, it is possible that other factors, such as disulfide bonds, could play a key role in the adaptation of enzymes to extreme temperatures. Current studies are generating a wealth of information on genome sequences from mesophilic and hyperthermophilic organisms. This increased information, and the rapidity with which X-ray structures are obtained, will certainly shed light on the problem of protein thermostability.

5'-Methylthioadenosine phosphorylase (MTAP) catalyzes the phosphorylation of 5'-methylthioadenosine (MTA), a sulfur-containing nucleoside formed from S-adenosylmethionine by several independent pathways, of which polyamine biosynthesis is quantitatively the most important (Williams-Ashman et al. 1982). The products of the MTA cleavage reaction are adenine and 5-methylthioribose-1-phosphate. MTA phosphorylase was first characterized in rat ventral prostate (Pegg and Williams-Ashman 1969). The enzyme was purified to homogeneity from mammalian tissues (Della Ragione et al. 1986, 1990; Toorchon and Miller 1991) and from *Sulfolobus solfataricus* (Cacciapuoti et al. 1994). Moreover, crystal structures have been obtained for human MTAP (hMTAP) (Appleby et al. 1999) and for 5'-methylthioadenosine phosphorylase from *S. solfataricus* (SsMTAP) (Appleby et al. 2001). On the basis of the quaternary structure, these two MTAPs may be classified as belonging to the low- and high-molecular-mass classes of purine nucleoside phosphorylases (PNP), respectively (Bzowska et al. 2000). Moreover, hMTAP and SsMTAP show negligible sequence homology and are also different in their substrate specificities since hMTAP is specific for MTA (Della Ragione et al. 1986, 1990; Toorchon and Miller 1991), while SsMTAP is able to recognize as substrates inosine, guanosine, and adenosine in addition to MTA (Cacciapuoti et al. 1994).

The results on MTAP from *S. solfataricus*, an extreme thermoacidophilic archaeon (De Rosa et al. 1975), showed that this enzyme is highly thermophilic and is characterized by features of extreme resistance to chemical, enzymatic, and thermal inactivation processes (Cacciapuoti et al. 1994). Moreover, the elucidation of the three-dimensional structure revealed that SsMTAP is a hexamer with three intersubunit disulfide bonds that might account for the extreme enzyme thermostability (Appleby et al. 2001).

To investigate whether disulfide bonds could represent a strategy of hyperthermostability, we have chosen as our experimental model system 5'-methylthioadenosine phosphorylase from *Pyrococcus furiosus* (PfMTAP), an anaerobic marine hyperthermophilic archaeon (Fiala and Stetter 1986). Indeed, the higher optimal growth

temperature of *P. furiosus* (100 °C) compared with that of *S. solfataricus* (87 °C) allows us to expect an increase in thermoactivity and thermostability of the pyrococcal enzyme.

This paper reports the purification to homogeneity and the biochemical characterization of PfMTAP, with particular attention to the possible involvement of disulfide bond(s) in extreme enzyme stability. A detailed kinetic investigation has been carried out in order to define the substrate specificity. In this study we will show that PfMTAP represents an MTA-metabolizing enzyme with features not previously found among Archaea, Bacteria, and Eukarya. Furthermore, a careful analysis of the primary structure of the protein has been performed. The alignment of the protein sequence of PfMTAP with those of hMTAP and two recently discovered hypothetical MTAPs from the hyperthermophilic Archaea *S. solfataricus* and *P. furiosus* shows several key residue changes that may account for the differences in substrate specificity of these enzymes.

Materials and methods

Chemicals

[Methyl-¹⁴C]S-adenosylmethionine (50–60 mCi/mmol) was supplied by the Radiochemical Centre (Amersham, UK). MTA and 5'-[methyl-¹⁴C]MTA were prepared from unlabeled and labeled S-adenosylmethionine (Schlenk and Ehninger 1964) and purified by HPLC (Della Ragione et al. 1981); S-adenosyl-L-homocysteine (AdoHcy), O-bromoacetyl-N-hydroxysuccinimide, and standard proteins used in molecular mass studies were obtained from Sigma (St. Louis, Mo., USA). Matrex gel red A was from Amicon Corp. Thermolysin was obtained from Boehringer (Mannheim, Germany). All reagents were of the purest commercial grade.

Enzyme assay

MTA phosphorylase activity was determined by measuring the formation of [methyl-¹⁴C]5-methylthioribose-1-phosphate from 5'-[methyl-¹⁴C]MTA (Cacciapuoti et al. 1994). Unless otherwise stated, the standard incubation mixture contained the following: 20 μmol potassium phosphate buffer, pH 7.4; 80 nmol of [methyl-¹⁴C]MTA (6.5×10⁵ cpm/μmol); and the enzyme protein at a final volume of 200 μl. The incubation was performed in sealed glass vials for 5 min at 80 °C, except where otherwise indicated. Control experiments in the absence of the enzyme were performed in order to correct for MTA hydrolysis. When the assays were carried out at temperatures above 80 °C, the reaction mixture was pre-incubated for 2 min without the enzyme that was added before starting the reaction.

When inosine, guanosine, and adenosine were used as substrates, the formation of purine bases was measured by HPLC using a Beckman System Gold. The amount of purine base formed is determined by measuring the percentage of the absorbance-integrated peak area of purine base formed with respect to the total (nucleoside + purine base) absorbance-integrated peak areas. An Ultrasil-CX column (Beckman) eluted with 0.05 M ammonium formate, pH 3, at a flow rate of 1 ml/min was used when adenosine and/or guanosine were the substrates of the reaction. When the assays were carried out in the presence of inosine as substrate, an Ultrasphere ODS RP-18 column (Beckman) was employed and the elution was carried out with a 5:95 (v/v) mixture of anhydrous methanol and 0.1% TFA in H₂O.

In all of the kinetic and purification studies, the amount of the protein was adjusted so that no more than 10% of the substrate was converted to product, and the reaction rate was strictly linear as a function of time and protein concentration. One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the cleavage of 1 nmol of MTA/min at 80 °C.

Analytical methods for protein

Proteins were assayed by the Bradford method (Bradford 1976) using human γ -globulin as standard. The molecular weight of the native protein was determined by gel filtration on a calibrated Sephacryl S-300 column (2.2×95 cm) and non-denaturing polyacrylamide gel electrophoresis. Non-denaturing polyacrylamide gel electrophoresis was carried out at pH 7.5, as reported in Cacciapuoti et al. (1991). The gels were either stained with Coomassie blue or cut into thin slices that were assayed for MTA phosphorylase activity. The subunit molecular mass was determined by SDS polyacrylamide gel electrophoresis as described by Weber et al. (1972) by using 12.5% or 15% acrylamide resolving gel and 5% acrylamide stacking gel. Samples were heated at 100 °C for 5 min in 2% SDS and 5% 2-mercaptoethanol and run in comparison with molecular weight standards. Isoelectric point was estimated with a Phast System (Pharmacia) apparatus on thin layer polyacrylamide gel. The isoelectric focusing was performed in the range of 3.0–10.0 and 4.0–6.5, following the manufacturer's instructions. N-Terminal sequence analysis of the purified enzyme was performed by Edman degradation on an Applied Biosystem 473A sequencer according to the manufacturer's instructions. The sample was subjected to SDS polyacrylamide gel electrophoresis and electroblotted on a polyvinylidene fluoride membrane prior to analysis. The N-terminal amino acid sequence was used for a BLAST search of the *P. furiosus* database (Utah Genome Center, <http://www.genome.utah.edu>) and for the identification of the *Pfamtap* gene.

Stability and thermostability studies

Stability of PfMTAP in the presence of a given water-miscible organic solvent, denaturant, detergent, and salt was examined by incubating in stoppered glass tubes at 25 °C, 50 °C, 70 °C, and 90 °C a mixture containing the enzyme (about 40 μ g) and the compound to be tested in 20 mM Tris-HCl, pH 7.4, at a final volume of 0.5 ml. Immediately after the addition of the compound, (time-zero control) and at different time intervals, 25- μ l aliquots were removed from each sample and were assayed for enzyme activity. Activity values are expressed as a percentage of the zero-time control (100%). Enzyme thermostability was tested by incubating the protein in sealed glass vials at temperatures between 100 °C and 145 °C. Samples (2 μ g) were taken at time intervals, and residual activity was determined by the standard assay at 80 °C.

Cell culture and homogenate preparation

P. furiosus was grown at 95 °C and pH 7.5 as reported in Guagliardi et al. (1995). Sixty grams of bacteria underwent freeze-thawing twice and was then mixed with 50 g of sand and 200 ml of 10 mM Tris-HCl, pH 8, containing 1 mM EDTA (buffer A) and homogenized in Omni Mixer. The homogenate was centrifuged at 4,000 g for 20 min at 4 °C to remove the sand and was ultracentrifuged at 160,000 g for 60 min at 4 °C. The homogenate was then added with streptomycin sulfate 0.5% (w/v), centrifuged at 25,000 g for 30 min and dialyzed extensively in buffer A.

Purification procedure

All the purification steps were performed at room temperature. The enzyme was loaded onto a DEAE-cellulose column (DE52,

Whatman) equilibrated with buffer A. The column was washed with 200 ml equilibration buffer, and bound proteins were eluted at a flow rate of 40 ml/h by a linear gradient 0–0.3 M NaCl in buffer A. The active fractions were pooled, concentrated, and dialyzed overnight against 100 vol of 10 mM Tris/HCl, pH 7.4, containing 1 mM EDTA (buffer B).

AdoHcy-Sepharose chromatography

The DEAE pool was applied at a flow rate of 20 ml/h to a column of AdoHcy-Sepharose (2×12 cm) prepared according to Porcelli et al. (1993) equilibrated in buffer B. The column was washed stepwise with 50 ml of the equilibration buffer and then with the same buffer containing 0.6 M NaCl. PfMTAP activity was eluted with 3 mM MTA in buffer B containing 0.6 M NaCl. Active fractions were pooled, concentrated, and dialyzed.

Phenyl-Sepharose chromatography

A column (2.5×6 cm) was equilibrated with buffer B containing 1 M ammonium sulfate. The sample from the previous step was mixed with ammonium sulfate at a final concentration of 1 M and applied to the column at a flow rate of 20 ml/h. After washing with 50 ml equilibration buffer, a linear descending gradient of 1–0 M ammonium sulfate in 150 ml of buffer B was applied to the column. PfMTAP was eluted by dropping the concentration of ammonium sulfate to 0 with buffer B. The active fractions were pooled, concentrated, and dialyzed.

Matrex gel red A chromatography

The dialyzed pool from the phenyl-Sepharose chromatography was applied at a flow rate of 20 ml/h to a Matrex gel red A column (1×6 cm) equilibrated with buffer B. PfMTAP was eluted from the column by two bed volumes of equilibration buffer and was concentrated.

Mono Q chromatography

To obtain the homogeneous enzyme, the concentrated sample from the Matrex gel red A chromatography was applied to a Mono Q 10/10 column (Amersham Pharmacia Biotech) that had been equilibrated with buffer A. After washing with 5 column volumes with the loading buffer, the protein was eluted with a 150 ml linear gradient of 0–0.3 M NaCl at a flow rate of 1 ml/min. The active fractions were pooled, dialyzed against buffer B, divided into aliquots, and stored at 20 °C.

Proteolytic inactivation

Proteolytic inactivation of PfMTAP was carried out at 37 °C by trypsin and chymotrypsin and at 60 °C with thermolysin. The final mass ratio of substrate protein to protease was 40:1. At different incubation times, the hydrolysis was stopped and the samples were assayed for MTA phosphorylase activity.

Kinetic parameters

All enzyme reactions were performed in triplicate at 80 °C. K_m and V_{max} values were obtained from linear regression analysis of data fitted to the Michaelis–Menten equation. Calculations of k_{cat} were based on an enzyme molecular mass of 180 kDa.

Multiple sequence alignment

Protein similarity searches were performed using the data from Swiss-Prot and Protein Identification Resource (PIR) databanks.

The multiple alignment was constructed using the Clustal method (Higgins and Sharp 1988).

Results and discussion

Enzyme purification and properties

PfMTAP was purified 538-fold to homogeneity with a yield of 6.6%. The result of the large-scale purification is shown in Table 1. The most effective step was the affinity chromatography on Sepharose coupled with AdoHcy, a sulfur-containing nucleoside analogue of MTA. It is interesting to note in this respect that PfMTAP does not recognize MTA-Sepharose, which represented the key step in the purification procedure of SsMTAP (Cacciapuoti et al. 1994), suggesting differences between the two enzymes in their overall structures. The successive chromatographic purification step on Matrex gel red A allowed us to eliminate glutamate dehydrogenase, a protein that represents about 20% of the total proteins of *Pyrococcus furiosus* (Consalvi et al. 1991). Finally, the chromatography on Mono Q enabled us to obtain pure enzyme in significant amounts. The final preparation had a specific activity of 6.35 μmol of MTA cleaved per min per mg of protein at 80 °C.

Several criteria were evaluated to assess the homogeneity of the enzyme. The purified enzyme migrates as a single band on polyacrylamide gel electrophoresis under denaturing and native conditions. When assays are carried out on the material eluted from gel slices under native conditions, the activity corresponds to the protein band. When purified enzyme is analyzed by isoelectric focusing on polyacrylamide gel, a single band is also obtained at pH 5.2. The effect of pH on enzymatic activity was investigated in the pH range 5–10, with the optimum pH of the reaction being found at 7.4.

Gel filtration on an analytical column of Sephacryl S-300 suggests that, under native conditions, the isolated PfMTAP forms a hexameric structure with a molecular mass of 180 ± 9 kDa. The apparent monomer mass of PfMTAP, as determined by SDS-PAGE, is 30 ± 1 kDa. On the basis of these results, PfMTAP is a hexameric

Table 1 Purification of 5'-methylthioadenosine phosphorylase from *Pyrococcus furiosus*. A typical purification from 60 g of wet cells is shown

Purification	Total protein (mg)	Total activity (units)	Specific activity (units/mg) ^a	Yield (%)	<i>n</i> -fold
Crude extract	3937	44100	11.2	100	
DEAE-cellulose	543	35280	65	80	5.8
AdoHcy-Sepharose	27.2	11466	422	26	38
Phenyl-Sepharose	5.1	7718	1519	17.5	136.6
Matrex gel red A	1.96	5336	2719	12.1	234
Mono Q	0.46	2934	6353	6.6	538

^a Specific activity is expressed as nmol of MTA cleaved per min per mg of protein at 80 °C

enzyme like MTAP from *Sulfolobus solfataricus* (Cacciapuoti et al. 1994), able to cleave MTA as substrate. Human MTAP, indeed, is a homotrimer (Appleby et al. 1999). It is interesting to note that the enzymes responsible in various sources for the catabolism of MTA are characterized by different quaternary structures, from the monomeric structure of *Escherichia coli* MTA nucleosidase (Della Ragione et al. 1985), to the trimeric organization of mammalian MTAP (Appleby et al. 1999), to the more complex hexameric structure of MTAPs from *S. solfataricus* (Appleby et al. 2001) and *P. furiosus*. However, all these enzymes are characterized by a similar molecular mass subunit, ranging from 26.5 kDa to 32 kDa.

Thermoactivity, thermostability, and stability

The temperature dependence of enzyme activity of PfMTAP and its optimum temperature were determined by carrying out enzymatic assays at differing temperatures from 70 °C to 160 °C (Fig. 1). The enzyme is highly thermoactive, with an optimum temperature of 125 °C, which is 25 °C above the organism's optimum growth temperature (Fiala and Stetter 1986). To the best of our knowledge, this value represents the highest optimum temperature reported for a hyperthermophilic enzyme.

Two different activation energy-dependent processes occurred, below and above 100 °C, as suggested by the biphasic Arrhenius plot (see inset in Fig. 1). The protein is thought to adjust itself to changes in thermic environment, generating enzymatic forms with different catalytic properties. This hypothesis has been verified by spectroscopic methods for propylamine transferase from

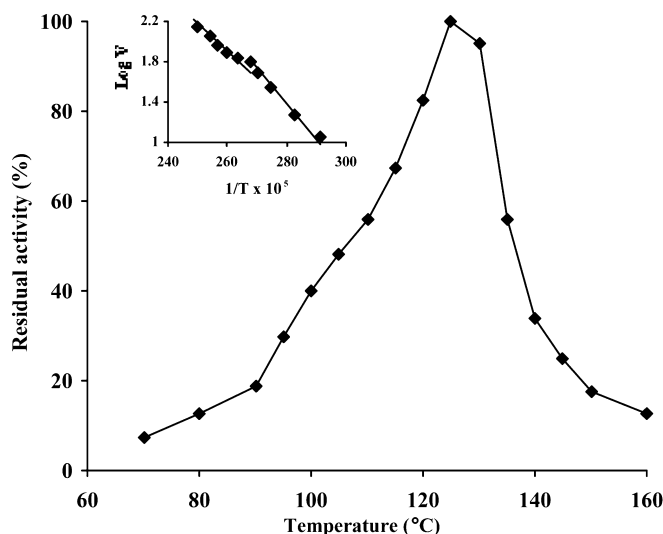


Fig. 1 The effect of temperature on PfMTAP activity. The activity observed at 125 °C is expressed as 100%. The assay was performed as indicated under Experimental procedures. Arrhenius plot is reported in the inset. *T* is measured in K

S. solfataricus (Ragone et al. 1992) and for poly(ADP-ribose) polymerase-like enzyme from *S. solfataricus* (Faraone Mennella et al. 1998).

The stability of PfMTAP to reversible denaturation was investigated by carrying out short-time kinetics of thermal denaturation. The diagram of the residual activity after 10 min of pre-incubation as a function of temperature, reported in Fig. 2a, is characterized by

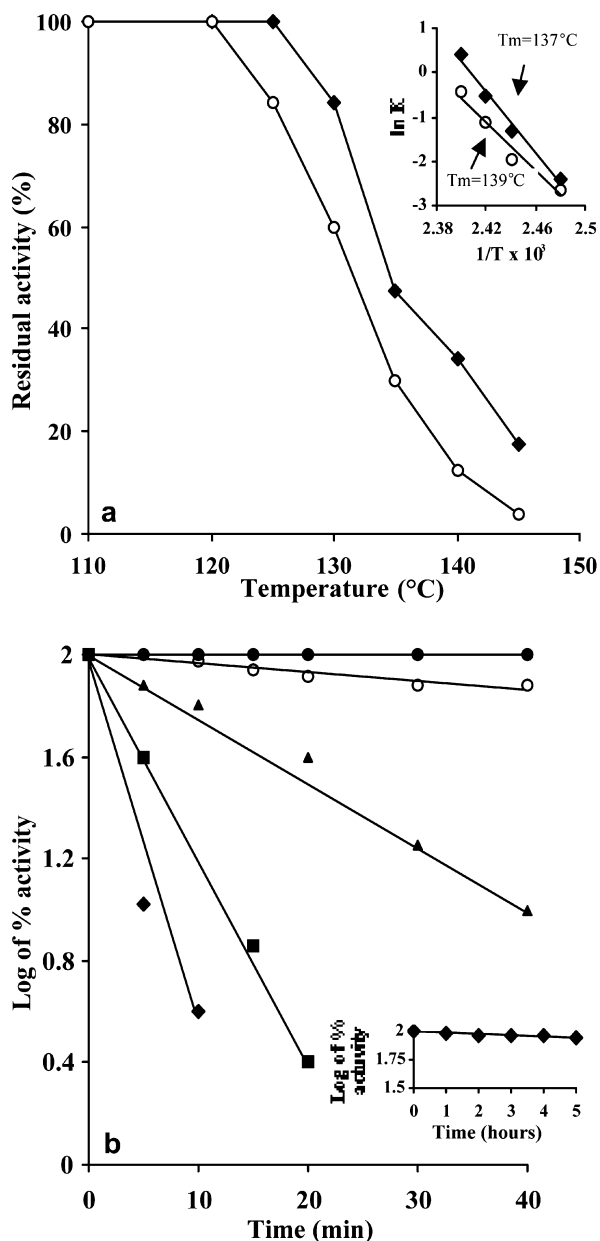


Fig. 2a, b Hermostability of PfMTAP. **a** Residual PfMTAP activity after 10 min of incubation at temperatures shown in the absence (square) or in the presence (circle) of 100 mM phosphate. Apparent T_m is reported in the inset. **b** Kinetics of thermal inactivation of PfMTAP as a function of incubation time. The enzyme was incubated at 100 °C (inset), 110 °C (filled circle), 120 °C (unfilled circle), 130 °C (triangle), 140 °C (square), and 145 °C (diamond) for the times indicated. Aliquots were then withdrawn and assayed for the activity as described under Experimental procedures

a sharp transition. From the corresponding plot (see inset), it is possible to calculate a transition temperature (apparent T_m) of 137 °C that increases to 139 °C in the presence of 100 mM phosphate. This result indicates that the binding of this substrate raises the noteworthy conformational stability of the enzyme, thus reducing its susceptibility to thermal denaturation. This substrate protection was also observed for SsMTAP, where phosphate binding induces a conformational transition that stabilizes the folded structure of the enzyme (Cacciapuoti et al. 2001).

To study the thermostability properties in terms of resistance to irreversible thermal inactivation, the enzyme was incubated at a defined temperature from 100 °C to 145 °C. As reported in Fig. 2b, the enzyme decay obeys first-order kinetic and shows half-lives of 43 min, 13 min, and 5 min when incubated at 130 °C, 140 °C, and 145 °C, respectively. PfMTAP displayed an extraordinarily strong long-term heat resistance at 100 °C, retaining about 98% activity after a 5h incubation at this temperature (see inset).

The thermoactivity and thermostability of PfMTAP appear exceptionally high and exceed the already extreme values reported for SsMTAP (optimum temperature, 120 °C; T_m , 132 °C). On the one hand, these features can be justified from the higher optimal growth temperature of *P. furiosus* with respect to that of *S. solfataricus*. On the other hand, however, it is just these features that raise doubts on the upper limits of temperature for enzyme stability and activity. Protein stability seems to be limited to temperatures below 120 °C (Daniel et al. 1996), based on universally applied irreversible degradation reactions. In spite of this, the extremely high temperature stability of PfMTAP and the availability of enzymes such as SsMTAP, characterized by half-life at 130 °C of 15 min (Cacciapuoti et al. 1994), or of enzymes that have significant half-lives at 130 °C (Koch et al. 1990; Brown and Kelly 1993), led us to believe that degradation processes at high temperatures may not be as fast as at lower temperatures or may not occur in proteins that are conformationally intact.

The purified enzyme is stable in Tris/HCl 10 mM, pH 7.4, at 20 °C for at least 1 year. PfMTAP shows unusual features of stability in the presence of organic solvents, denaturing agents, detergents, and salts. In fact, at 25 °C it remains fully active after 24 h of pre-incubation in their presence and maintains its catalytic activity, to a different extent, when the temperature increases from 50 °C to 90 °C (Table 2). Due to its rigidity and poor flexibility, the enzyme is also not susceptible to proteolytic cleavage. In fact, after incubation at 37 °C with trypsin and chymotrypsin and at 60 °C with thermolysin for 24 h, the enzyme completely retains its activity. %Triton X-100 1% and NaCl 1 M do not affect enzyme stability after a 1-h incubation at 90 °C (data not shown), indicating that hydrophobic and electrostatic interactions do not play a critical role in the stabilization of the protein.

Table 2 Effect of several compounds on the stability of MTA phosphorylase from *P. furiosus* at 50 °C, 70 °C, and 90 °C. The enzyme was incubated in stoppered glass tubes at 50 °C, 70 °C, and 90 °C in 10 mM Tris-HCl, pH 7.4, with the compounds to be

tested. After 30 and 60 min, aliquots were removed from each incubation mixture and assayed for the residual MTA phosphorylase activity in standard conditions

Compound	Residual activity					
	50 °C		70 °C		90 °C	
	30 min (%)	60 min (%)	30 min (%)	60 min (%)	30 min (%)	60 min (%)
None	100	100	100	100	100	100
50% dimethylformamide	72	48	10	5	ND ^a	ND
50% methanol	100	100	100	100	100	23
50% ethanol	90	90	71	65	37	30
50% propanol-1	92	88	62	58	29	22
50% acetonitrile	100	100	100	100	100	38
50% tetrahydrofuran	77	77	62	50	2	3
6 M urea	100	100	100	100	15	ND
9 M urea	100	100	100	100	ND	ND
4 M guanidine HCl	74	59	44	37	12	4
6 M guanidine HCl	42	37	16	ND	ND	ND
0.5% SDS	81	66	65	51	19	18
1% SDS	77	65	57	46	14	12
2% SDS	69	53	53	30	11	7

^a Not detectable

Reducing agents and disulfide bonds

The catalytic process of PfMTAP does not involve thiol groups, since enzyme activity is not affected by alkylating, mercaptide-forming, or oxidizing thiol reagents even at relatively high concentrations (10 mM). Similar data have been reported for *E. coli* PNP (Jensen and Nygaard 1975) and for MTAP from *S. solfataricus* (Cacciapuoti et al. 1994). Eukaryotic MTAP, in contrast, strictly requires thiol-group-reducing agents and is specifically and rapidly inactivated by thiol-blocking compounds (Della Ragione et al. 1986). Likewise, there are reports that one cysteine residue is directly involved in the catalytic mechanism of several eukaryotic purine and pyrimidine nucleoside phosphorylases (Bose and Yamada 1974; Savarese et al. 1979).

It is possible that in PfMTAP, cysteine residues are involved in disulfide bridges. To verify this hypothesis, the thermal stability of purified PfMTAP was investigated by heating in the presence of reducing agents. Up to 80 °C, the enzyme remains stable after 60 min incubation in the presence of 0.8 M dithiothreitol, whereas it becomes susceptible to the effect of the reducing agent as the temperature rises. In fact, a remarkable loss of activity is observed after 30 min incubation at 100 °C (Fig. 3a). The requirements of elevated temperatures and high concentrations of reducing agents to inactivate the enzyme suggest that the disulfide bridge(s) being reduced was quite inaccessible. A similar inaccessibility of disulfide bonds has been postulated for MTAP from *S. solfataricus*, in which a significant loss of activity was observed only after treatment of the protein at 100 °C in the presence of 0.1 M dithiothreitol (Cacciapuoti et al. 1994). Further proof that disulfide bridges can be protected from destruction by their inaccessibility in the protein comes from the observation that native serine-

type protease from *Aquifex pyrophilus* shows a 6-h half-life at 105 °C and at pH 9 (Choi et al. 1999), which is much longer than the half-life calculated for disulfide bridges in unfolded proteins at pH 8 (1 h) (Vieille and Zeikus 2001).

The presence of disulfide bonds in PfMTAP can be detected by the comparison of different mobility levels of the enzyme on SDS polyacrylamide gel electrophoresis run under reducing and non-reducing conditions. When unreduced (Fig. 3b, lane B), PfMTAP migrated more rapidly down the gel with respect to its reduced form (Fig. 3b, lane C). Furthermore, we can hypothesize that the disulfide linkage(s) are positioned intra-subunit because the reduced and non-reduced form of the enzyme migrates as a protein band at about 30 kDa, which corresponds to the monomer of the enzyme.

Although disulfide bonds have not been considered, until now, as a general feature of naturally occurring extremely thermophilic proteins, they have been reported as an important structural mechanism for the thermostability of MTAP from *S. solfataricus* (Cacciapuoti et al. 1994). It is likely that also in the case of PfMTAP, disulfide bridges could contribute to the exceptional thermoactivity and thermostability of the enzyme. In this respect, although more detailed studies are needed in order to check the involvement of other molecular interactions, our results are indicative that PfMTAP can use disulfide bonds as a strategy for thermostability.

Initial velocity studies and substrate specificity

The purified enzyme gave a linear rate of reaction for at least 10 min at 80 °C; thus, an incubation time of 5 min was then employed for kinetic experiments.

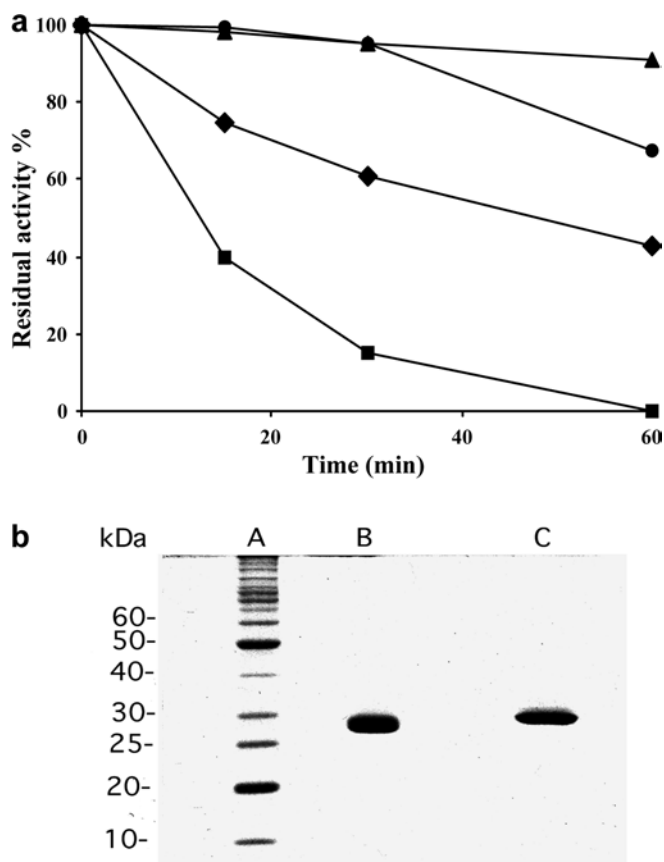


Fig. 3a, b Effect of reducing agents on PfMTAP. **a** Thermostability of PfMTAP in dithiothreitol. The enzyme (2 μg) was incubated for different times in 20 mM Tris/HCl, pH 7.4, containing 0.8 M dithiothreitol at 80 °C (triangle), 90 °C (circle), 95 °C (diamond), and 100 °C (square). Aliquots were withdrawn and assayed for the activity as described under Experimental procedures. **b** SDS polyacrylamide gel electrophoresis of purified PfMTAP. Lane A Molecular weight standards; lane B unreduced PfMTAP; lane C reduced PfMTAP. Samples in lanes A and B were incubated for 5 min at 100 °C in 0.1 M Tris/HCl, pH 6.8 containing 2% SDS, 5% 2-mercaptoethanol, and 0.001% bromophenol blue prior to electrophoresis. Sample in lane C was treated in the same experimental conditions without 2-mercaptoethanol

Like the homologous hexameric MTAP from *S. solfataricus*, the enzyme from *P. furiosus* is characterized by a broad substrate specificity that recognizes as substrates both 6-amino and 6-oxo purine nucleosides. The K_m and V_{max} values for these substrates in the presence of saturating concentrations of phosphate were calculated and typical Michaelis–Menten kinetics were observed (Fig. 4). The relative efficiency of the nucleoside substrates was determined by comparing the respective k_{cat}/K_m ratios, which are the best measure for comparison of the efficiency of product formation and for substrate preference. As reported in Table 3, the enzyme shows a higher affinity for MTA and adenosine compared to inosine and guanosine. Moreover, the comparable $k_{cat}/K_{m,app}$ values for MTA and adenosine are about 20-fold higher than those for inosine and guanosine (Table 3). Therefore, 6-amino-purine

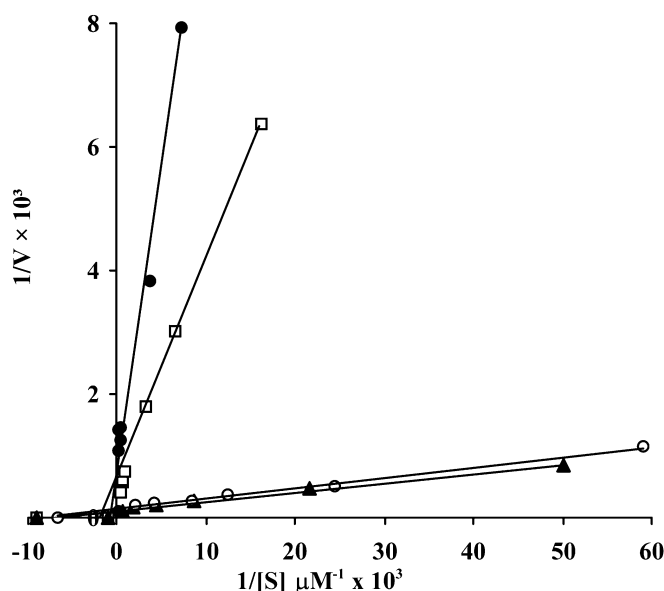


Fig. 4 Determination of K_m for MTA, adenosine, inosine, and guanosine. The enzyme was incubated for 5 min in the presence of 100 mM phosphate and various concentrations of MTA (triangle), adenosine (unfilled circle), inosine (square), and guanosine (filled circle), as shown. The amounts of the purine base produced were determined by the radiochemical or HPLC assay, as described under Experimental procedures. Results are shown as a double-reciprocal plot of the rate of the reaction against the substrate concentration

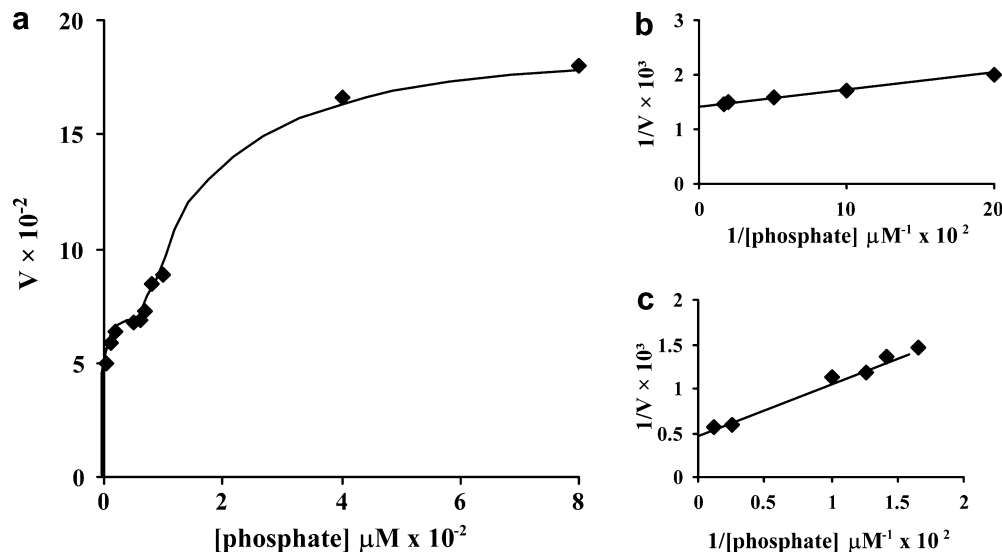
Table 3 Kinetic parameters of MTA phosphorylase from *P. furiosus*

Substrate	$K_{m,app}$ μM	k_{cat} s^{-1}	$k_{cat}/K_{m,app}$ $\text{M}^{-1} \text{s}^{-1}$
MTA	147	24.46	1.66×10^5
Adenosine	109	22.79	2.09×10^5
Inosine	963	9.38	9.74×10^3
Guanosine	916	7.31	7.98×10^3

nucleosides could represent the natural biological substrates for PfMTAP.

When the phosphate concentration was varied at a fixed saturating concentration of MTA, a non-Michaelis kinetics is observed (Fig. 5a). Assuming that Michaelis–Menten kinetics hold within a certain concentration range, straight lines can fit to the linear portions of the Lineweaver–Burk plot to obtain kinetic constants for low and high concentrations of the variable substrate separately. When the phosphate concentration was either above or below 60 μM , the initial velocity data of PfMTAP are linear in a double reciprocal analysis. Consequently, initial velocity data for the phosphorylytic reaction were collected with phosphate concentration between 5 and 60 μM (Fig. 5b) and between 60 and 800 μM (Fig. 5c). The calculated $K_{m,app}$ values for phosphate are, respectively, 2 μM and 132.6 μM . These results are in good agreement with literature data on PNPs reporting that at least two binding constants are necessary to explain the complexity of phosphate binding (Bzowska et al. 2000) and with the recent

Fig. 5a–c Effect of phosphate concentration on the reaction rate. **a** Initial velocity (V) versus phosphate concentration (μM) at fixed level of MTA. **b** Double reciprocal plot in the range of phosphate 5–60 μM . **c** Double reciprocal plot in the range of phosphate 60–800 μM . The enzyme was incubated for 5 min in Tris/HCl 20 mM, pH 7.4 in the presence of 500 μM MTA and various concentrations of phosphate as shown



demonstration by crystallization studies that the hexameric PNP from *E. coli* is a trimer of unsymmetric dimers, which are formed by pairs of monomers with active sites in different conformations (Koellner et al. 2002).

Primary structure comparisons

The homogeneous MTAP from *P. furiosus* was subjected to automatic Edman degradation, and the following 27 residues were positively identified: MPKIGIIGGSGVYGIFEPKETVKVHTP. This sequence was used to search the *P. furiosus* genomic database. A single protein was identified that contained an N-terminus that matched exactly the one determined from the purified enzyme. The structural gene encoding MTAP consists of 774 bp and encodes a protein of 257 residues with a predicted molecular mass of 31 kDa, which is in good agreement with the 30 ± 1 kDa estimated by biochemical analyses. The coding region starts with an ATG triplet, at the position 14581 of the *P. furiosus* genome, in agreement with data from protein amino acid sequence determination, which indicates that the N-terminal methionine is not post-translationally removed.

When the deduced primary structure of *P. furiosus* MTAP was compared with the enzymes present in GenBank, a high identity (61%) was found with a putative MTAP from *Sulfolobus solfataricus* (accession number SS02343) and with a hypothetical MTAP (accession number PF0853) from *P. furiosus*. Direct evidence that this hypothetical PfMTAP is expressed in the microorganism comes from the experimental observation that the phosphorylase activity ratio of PfMTAP toward MTA (or adenosine)/inosine (or guanosine) does not remain constant through the purification steps. In fact, while in the crude extract, this ratio is about 2.5; in the homogeneous enzyme, it increases to about 8. Therefore, in *P. furiosus* more than

one enzyme is responsible for the catabolism of the purine nucleosides.

Among the related proteins isolated from various sources, PfMTAP shows high sequence identity with hMTAP (52%), low identity with human PNP (27%) and with *Cellulomonas* sp. PNP (20%), and no sequence homology with SsMTAP and *E. coli* PNP.

As deduced from the gene, PfMTAP contains 30 cysteine residues (5 per subunit). This feature is unexpected for such a hyperthermostable enzyme, since cysteine is particularly sensitive to oxidation at high temperatures and can contribute to the thermal destabilization of proteins (Mozhaev et al. 1988). On the other hand, the occurrence of numerous cysteine residues in several hyperthermophilic enzymes (Vieille and Zeikus 2001) indicates that they are often involved in specific stabilizing interactions (i.e., disulfide bridges and metal ligands) and/or are inaccessible to the solvent.

Figure 6 reports the multiple sequence alignment among PfMTAP, human MTAP, and the two hypothetical MTAPs from *S. solfataricus* and *P. furiosus*. As shown, all cysteine residues of PfMTAP are completely conserved among the three hyperthermophilic enzymes, and it is probable that, in analogy with PfMTAP also in the hypothetical MTAPs, such cysteine residues are involved in disulfide bonds. This observation and the occurrence of disulfide-bridge-containing hyperthermophilic proteins (Appleby et al. 2001; Choi et al. 1999; Toth et al. 2000; Cort et al. 2001) allow us to hypothesize that these covalent links could represent a general "hyperstabilization" strategy.

Figure 6 also shows the amino acid residues involved in the active site of hMTAP (Appleby et al. 1999) with the corresponding residues of the three hyperthermophilic MTAPs. The active site of hMTAP can be divided into three parts: phosphate-, pentose-, and base-binding sites. While the phosphate- and methylthioribose-binding sites of hMTAP appear conserved among the three hyperthermophilic enzymes with only few conservative



Fig. 6 Multiple sequence alignment of PfMTAP, human MTAP and hypothetical MTAPs from *Sulfolobus solfataricus* and *Pyrococcus furiosus*. Residues conserved in proteins are shaded. Cysteine residues are bold. The positions at the phosphate (asterisk), methylthioribose (triangle), and base (circle) binding sites of hMTAP are indicated. Numbers on the left are the coordinates of each protein

substitutions, the analysis of the base-binding site allows us to speculate on the possible functional role played in vivo by the two hypothetical MTAPs.

We can first consider that these enzymes share a high substrate specificity towards MTA because the base-binding pocket of hMTAP is completely conserved in both PfMTAP and the hypothetical MTAP from *S. solfataricus*. Therefore, in *S. solfataricus* there could be a specific enzyme, i.e., the hypothetical SsMTAP, devoted to MTA catabolism. SsMTAP, indeed, is a typical PNP with a broad substrate specificity. The second observation comes from the comparative analysis of the amino acid sequences of PfMTAP and hypothetical PfMTAP. Despite the high degree of identity, two important substitutions occur in the hypothetical PfMTAP that suggest a different functional role for this enzyme. In the hypothetical PfMTAP, Asn211 replaces Asp204 of PfMTAP. It has to be noted that a similar amino acid substitution is observed when comparing hMTAP and mammalian PNP, where the amino group of Asn243, which is a good hydrogen bond donor for both N7 and O6 of the oxo-purine base (Koellner et al. 1997), replaces Asp220 of hMTAP. The second amino acid substitution is Glu169 of the hypothetical PfMTAP, which replaces Ser167 of PfMTAP. Also in this case an identical event can be observed from a comparison of hMTAP and mammalian PNP. This enzyme, in fact, utilizes Glu201

in place of Ser178 of hMTAP to provide a hydrogen bond acceptor for N1, which is protonated in 6-oxopurines (Koellner et al. 1997). Finally, in the hypothetical PfMTAP, the hydrophobic pocket of PfMTAP is modified by replacing Ala 215 with His 223, as is mammalian PNP by substituting Val 233 of hMTAP with His257. In summary, differences in the active site of PfMTAP and hypothetical PfMTAP reflect the possible differences in substrate specificity between these two enzymes and allow us to hypothesize that *P. furiosus* has developed a specific enzyme, the hypothetical PfMTAP, for the metabolism of 6-oxo-purines.

On the basis of sequence homology, substrate specificity, kinetic parameters, and active site analysis, PfMTAP can be considered an MTAP with unique features. Moreover, the peculiarity of the recognition of purine nucleosides renders PfMTAP significantly different from the MTA-cleaving enzymes found so far in Eukarya as well as in Bacteria and Archaea. The determination of the three-dimensional structure of PfMTAP is needed to elucidate the role played by specific amino acid residues in substrate recognition and binding, as well as in catalysis, and to confirm the hypothesis that hyperthermophilic proteins can utilize disulfide bond formation as a significant strategy to achieve superior levels of thermostability.

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