Three-dimensional Structure of a Hyperthermophilic 5'-Deoxy-5'-methylthioadenosine Phosphorylase from Sulfolobus solfataricus*

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Todd C. Appleby†, Irimpan I. Mathews‡, Marina Porcelli§, Giovanna Cacciapuoti§, and Steven E. Ealick¶‡
From the †Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853 and the §Dipartimento di Biochimica e Biofisica, Seconda Universita di Napoli, Via Costantinopoli, 16, 80138 Naples, Italy

The structure of 5'-deoxy-5'-methylthioadenosine phosphorylase from Sulfolobus solfataricus (SsMTAP) has been determined alone, as ternary complexes with sulfate plus substrates 5'-deoxy-5'-methylthioadenosine, adenosine, or guanosine, or with the noncleavable substrate analog Formycin B and as binary complexes with phosphate or sulfate alone. The structure of unliganded SsMTAP was refined at 2.5Å resolution and the structures of the complexes were refined at resolutions ranging from 1.6 to 2.0 Å. SsMTAP is unusual both for its broad substrate specificity and for its extreme thermal stability. The hexameric structure of SsMTAP is similar to that of purine-nucleoside phosphorylase (PNP) from Escherichia coli, however, only SsMTAP accepts 5'-deoxy-5'-methylthioadenosine as a substrate. The active site of SsMTAP is similar to that of E. coli PNP with 13 of 18 nearest residues being identical. The main differences are at Thr389, which corresponds to serine in E. coli PNP, and Glu165, which corresponds to proline in E. coli PNP. In addition, a water molecule is found near the purine N7 position in the guanosine complex of SsMTAP. Thr389 is near the 5’-position of the nucleoside and may account for the ability of SsMTAP to accept either hydrophobic or hydrophilic substituents in that position. Unlike E. coli PNP, the structures of SsMTAP reveal a substrate-induced conformational change involving Glu165. This residue is located at the interface between subunits and swings in toward the active site upon nucleoside binding. The high-resolution structures of SsMTAP suggest that the transition state is stabilized in different ways for 6-aminouracil, 6-oxo substrates. SsMTAP has optimal activity at 120 °C and retains full activity after 2 h at 100 °C. Examination of the three-dimensional structure of SsMTAP suggests that unlike most thermophilic enzymes, disulfide linkages play a key role in its thermal stability.

5'-Deoxy-5'-methylthioadenosine phosphorylase (EC 2.4.2.28)

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The atomic coordinates and structure factors (code 1JDU (native SsMTAP), 1IE1 (complex with guanosine and sulfate), 1JDV (complex with adenosine and sulfate), 1JDJ (complex with MTA and sulfate), 1JDS (complex with phosphate (space group: P21)), 1JJP (complex with sulfate), 1JDD (complex with Formycin B and sulfate), and 1JDE (complex with phosphate (space group C221)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† To whom correspondence should be addressed. Tel.: 607-255-7961; Fax: 607-255-1227; E-mail: see@cornell.edu.

‡ The abbreviations used are: SsMTAP, Sulfolobus solfataricus 5'-deoxy-5'-methylthioadenosine phosphorylase; MTA, 5'-deoxy-5'-methylthioadenosine phosphorylase; PNP, purine nucleoside phosphorylase; MTA, 5'-deoxy-5'-methylthioadenosine; FMB, Formycin B.
nucleoside phosphorylase shown to utilize MTA as a substrate. In contrast, human MTAP is a member of the trimeric class of enzymes. Recently, the complete genome sequence revealed that S. solfataricus contains a second enzyme that is homologous to trimeric human MTAP (GenBank™ accession number AE006641). However, no obvious PNP is encoded in the S. solfataricus genome.

Many of the enzymes in the PNP family have been well characterized, and crystal structures have been obtained for both trimeric and hexameric PNP s. Atomic resolution structures of human (8), bovine (9–11), and Cellulomonas sp. (12) PNP s have been determined, and a human MTAP structure has recently been reported (13). Each of these structures reveals a similar trimeric arrangement of subunits. The crystal structure of E. coli PNP has also been described and represents an example from the hexameric class of enzymes (7, 14). The structure of uridine phosphorylase from E. coli (15) represents a second example of the hexameric class. The structures of thymidine phosphorylase from E. coli (16, 17) and pyrimidine-nucleoside phosphorylase from Bacillus steareothermophilus (18) show that these enzymes belong to a separate structural family.

Here, we report the crystal structures of unliganded SsMTAP and binary and ternary complexes of SsMTAP bound to substrates and substrate analogs. Our studies on MTAP from S. solfataricus are aimed toward understanding the structural basis for its broad substrate specificity and toward understanding the factors that contribute to its extreme thermostability.

**EXPERIMENTAL PROCEDURES**

**Protein Production and Crystallization**—Recombinant SsMTAP utilized for these structural studies was expressed and purified according to the methods of Cacciapuoti et al. (19). The recombinant enzyme is similar to the wild-type enzyme regarding molecular weight, hexameric structure, presence of intersubunit disulfide bonds, substrate specificity, and specific activity. However, the recombinant SsMTAP demonstrates both lower thermostability and thermostability (19).

The protein was concentrated to 7–10 mg/ml using ultrafiltration. All SsMTAP crystals were grown at either room temperature or 18 °C using the hanging drop vapor diffusion technique. The drops contained 1.2 μl of protein solution and 1.0 μl of reservoir solution suspended over 850 μl of reservoir solution. For the native SsMTAP crystals, the reservoir solution contained 28–30% dioxane, 12% 2-methyl-2,4-pentanediol, 0.12 M MgCl₂, 0.04 M NaCl, and 0.1 M Tris-HCl, pH 7.4. Microseeding with previously grown crystals improved the size and diffraction quality of the crystals. Native crystals of SsMTAP are plate-like with typical dimensions of 0.25 mm × 0.25 mm × 0.1 mm. The 12% 2-methyl-2,4-pentanediol in the mother liquor acts as a suitable cryoprotectant when freezing the crystals.

**Preparation of Protein-Ligand Complexes**—Crystals containing SsMTAP complexed with guanosine, adenosine, MTA, or Formycin B (FMB) plus sulfate ion were generated by co-crystallization. The conditions were identical to those given above for the native SsMTAP crystals except that the reservoir solution contained 28–30% dioxane, 12% 2-methyl-2,4-pentanediol, 0.12 M MgCl₂, 0.03 M MgSO₄, and 0.1 M Tris-HCl, pH 7.4. In order to obtain the structure of the enzyme bound to phosphate, magnesium sulfate was replaced by ammonium chloride and potassium phosphate was added to the reservoir solution. The final reservoir solution contained 28–30% dioxane, 12% 2-methyl-2,4-pentanediol, 0.12 M NH₄Cl, 0.02 M KH₂PO₄, and 0.1 M Tris-HCl, pH 7.4. We also attempted to prepare SsMTAP crystals containing FMB and phosphate using these conditions but only phosphate was observed in the active site (see below).

**X-ray Data Collection and Structure Determination**—Data for unliganded SsMTAP were collected using a single frozen native SsMTAP crystal (0.25 mm thick) at the National Synchrotron Light Source (CHESS) (Cornell University, Ithaca, NY). Data for the SsMTAP crystals grown in the presence of phosphate with and without FMB were also collected at CHESS. All data for SsMTAP complexes were collected using frozen crystals. The CHESS data were processed with MOSFLM (21) and scaled using SCALA (22) from the CCP4 suite of programs (23).

The native SsMTAP crystals and SsMTAP crystals containing MTA, sulfate, FMB/sulfate, FMB/phosphate, and sulfate alone all crystallized in the orthorhombic space group C222₁. The SsMTAP crystals contain guanosine/sulfate, adenosine/sulfate, and phosphate alone all crystallized in the monoclinic space group P2₁. The unit cell parameters for the two space groups are related with a₀ ~ a₁, b₁ ~ 2b₀, and c₀ ~ c₁sinβ₀. There are three subunits per asymmetric unit in the orthorhombic form and the hexamer is formed using a crystallographic 2-fold axis. There is an entire hexamer in the asymmetric unit of the monoclinic form. The Matthews number (V_m) (24) is 2.5 Å³Da for both crystal forms, which corresponds to a solvent content of about 52%. Both forms grow under similar conditions and have similar crystal morphologies. The actual crystal form can only be determined by examining the x-ray intensity data. Data collection statistics for all data sets are given in Table I.

The structure of orthorhombic SsMTAP was determined using molecular replacement with E. coli PNP (PDB entry code: 1ECP) (7) as the search model (sequence identity is 32%). Data between 10- and 4-Å resolution were included in the crossrotation and translation functions, which were carried out using the CNS software package (25). The rotated and translated search model was carried through a round of rigid-body refinement. Electron density maps were calculated using the observed amplitudes measured from the native SsMTAP crystal and phases calculated from the refined search model. The interactive computer graphics program O (26) was then used to replace residues in the E. coli search model with corresponding residues in the SsMTAP sequence. Several rounds of torsion angle simulated annealing using CNS followed by manual refitting of the model in O were performed. Water molecules were included in the model during subsequent rounds of refinement. This refined model was then used as a search model in molecular replacement to determine the structure of the monoclinic form of SsMTAP, which contains an entire hexamer in the asymmetric unit. Refinements of all structures were performed with iterative cycles of torsion angle simulated annealing using CNS and manual refitting of the models in O.

The structures of the complexes were determined by refining the SsMTAP structure in the appropriate space group (P2₁ or C222₁). After an initial round of simulated annealing refinement, difference Fourier maps were used to reveal the active site contents (Fig. 1). The ligand atoms were included in the model followed by iterative cycles of model building and refinement. Individual isotropic B-factors and water molecules were included during the later stages of refinement. Crystallographic data and refinement statistics for all structures are reported in Table II.

**RESULTS AND DISCUSSION**

**Overall Structure of SsMTAP**—The crystal structure reveals that SsMTAP is a hexamer containing six identical subunits of ~27 kDa each, confirming the results from previous biochemical studies (1). The enzyme is dimer shaped and is ~40 Å thick with a diameter of about 106 Å across the hexameric face. The molecule displays D₃ symmetry and can be described as a trimer of dimers with three symmetric intersubunit disulfide bonds linking the dimers to one another. Fig. 2 shows the hexameric arrangement of subunits and the location of the disulfide bonds. The observation of only three disulfide bonds contrasts with results from earlier work, which predicted the
presence of six intersubunit disulfide bonds and suggested that 
the hexamer was arranged as a dimer of trimers (1). The 
quaternary structure of SsMTAP is very similar to that of the 
\textit{E. coli} PNP hexamer (7, 14).

Each SsMTAP monomer contains one active site, which is 
located near a dimer interface. The two active sites in the 
dimeric unit are related by a molecular 2-fold axis and are 
separated by about 20 Å. The dimers are formed mainly by 
contacts between residues from helix H9251, helix H92513, and three 
loops. These dimers form hexamers through contacts involving 
residues on strand H92526, helix H92513, helix H92514, strand H92527, and three 
loops. The hexamer forming contacts include disulfide bonds 
between pairs of 2-fold related Cys125 residues. Cys125 is lo-
cated on the loop between helix H92513 and helix H92514 of each subunit 
behind the center of the hexamer. Because the disulfide linkage 
spans a molecular 2-fold axis, only three disulfide bonds form 
per hexamer.

The final models of SsMTAP and its complexes consist of all 
residues except for 1 or 2 residues at the N terminus of each 
chain and 3 or 4 residues in the loop between the last strand, 
\( \beta 9 \), and the C-terminal \( \alpha \) helix. These residues showed weak 
electron density and are believed to be flexible or disordered. 
The overall fold of SsMTAP (Fig. 3) is very similar to \textit{E. coli} 
PNP (7, 14), consisting of a single \( \alpha/\beta \) domain. The central 
portion of the molecule is made up of a large eight-stranded 
mixed \( \beta \) sheet with topology \( \beta 2, \beta 3, \beta 4, \beta 1, \beta 5, \beta 9, \beta 6, \) and \( \beta 7 \).
Strand \( \beta 8 \) is a 5-residue strand that forms hydrogen bonds with 
the ends of strands \( \beta 7 \) and \( \beta 5 \) resulting in a smaller 5-stranded 
\( \beta \) sheet with topology \( \beta 6', \beta 7', \beta 8, \beta 5', \) and \( \beta 9' \), where the prime designates the C-terminal end of that strand. The two

| Complex | Native | Guo/\( \text{SO}_4 \) | Ado/\( \text{SO}_4 \) | MTA/\( \text{SO}_4 \) | PO\(_4\) | SO\(_4\) | FMB/\( \text{SO}_4 \) | PO\(_4^*\) |
|---------|--------|----------------|----------------|----------------|------|------|--------------|------|
| Resolution (Å) | 30–2.5 | 30–1.8 | 30–2.0 | 30–2.0 | 30–1.8 | 30–1.8 | 30–2.0 | 30–1.6 |
| No. observations | 73,267 | 543,683 | 381,093 | 213,837 | 427,925 | 195,119 | 261,441 | 325,394 |
| No. unique | 27,294 | 139,268 | 100,805 | 50,489 | 136,899 | 50,040 | 53,563 | 80,536 |
| Completeness | Overall (%) | 95.5 | 82.0 | 96.4 | 95.2 | 88.7 | 95.7 | 99.4 | 78.1 |
| Outermost shell (%) | 87.4 | 41.7 | 79.2 | 72.0 | 57.1 | 96.5 | 97.8 | 35.2 |
| \( R \) | Overall (%) | 7.5 | 5.7 | 4.8 | 7.4 | 8.9 | 5.3 | 9.6 | 3.7 |
| Outermost shell (%) | 32.7 | 31.6 | 18.0 | 48.7 | 36.0 | 15.6 | 31.5 | 18.3 |
| \( I/\sigma \) | Overall | 11.8 | 7.4 | 9.8 | 7.1 | 5.9 | 9.3 | 5.9 | 13.9 |
| Outermost shell | 3.5 | 2.3 | 3.9 | 1.5 | 1.5 | 4.4 | 2.0 | 3.8 |

* Both PO\(_4\) and FMB and were present in the crystallization solutions.

**Table I**

\textbf{Summary of x-ray intensity data}

\textbf{Fig. 1. Examples of final 2\( F_o - F_c \) electron density.} The electron density is superimposed on the final models for: \( a \), guanosine and sulfate; \( b \), adenosine and sulfate; \( c \), MTA and sulfate; \( d \), Tris and phosphate; and \( e \), FMB and sulfate. All maps are contoured at 1.0 \( \sigma \).
fused β sheets form a distorted β barrel near the active site. The core β sheet structure is flanked by seven α helices.

Although no sulfate or phosphate was added during crystallization of native SsMTAP, some residual electron density was observed in the predicted phosphate-binding site. Modeling sulfate or phosphate in this region resulted in a poor fit to the electron density and refinement resulted in extremely high B-factors (>70 Å²) for either of these ions when it was included in the model. Based on these results neither sulfate nor phosphate is present in the final model. It is possible that the

| Complex          | Native | Guo/SO₄ | Ado/SO₄ | MTA/SO₄ | PO₄ | SO₄ | FMB/SO₄ | PO₄⁺ |
|------------------|--------|---------|---------|---------|-----|-----|---------|------|
| Space group      | C222₁  | P₂₁     | P₂₁     | C222₁   | P₂₁ | C222₁| C222₁   | C222₁|
| Cell dimensions  |        |         |         |         |     |     |         |      |
| a (Å)            | 102.6  | 101.8   | 101.9   | 101.1   | 101.5| 102.1| 102.6   | 103.01|
| b (Å)            | 175.5  | 87.6    | 87.4    | 176.3   | 86.2 | 174.3| 176.9   | 175.96|
| c (Å)            | 87.4   | 101.8   | 101.9   | 87.1    | 102.1| 87.0 | 87.3    | 86.57 |
| β (*)            | 120.0  | 120.0   | 119.0   |         |     |     |         |      |
| Resolution limits (Å) | 25–2.5 | 20–1.8  | 20–2.0  | 20–1.8  | 20–1.8| 20–1.8| 20–1.8  | 20–1.6|
| No. of reflections | 23,534 | 127,814 | 100,633 | 50,045  | 129,925| 69,619| 53,304  | 80,331|
| No. of protein atoms | 5,394  | 10,784  | 10,588  | 5,372   | 10,724| 5,366 | 5,208   | 5,217 |
| No. of ligand atoms | 150    | 96      | 75      | 75      | 78   | 15   | 72      | 39    |
| No. of water molecules | 132    | 319     | 306     | 142     | 480  | 256  | 145     | 167   |
| R-factor (%)     | 18.4   | 21.7    | 21.8    | 22.2    | 22.0 | 22.4 | 23.1    | 22.9  |
| Free R-factor (%)| 24.1   | 23.7    | 24.6    | 26.1    | 24.9 | 24.9 | 25.7    | 24.6  |
| Root mean square bond (Å) | 0.006  | 0.006   | 0.007   | 0.006   | 0.006| 0.006| 0.007   | 0.006 |
| Root mean square angle (°) | 1.225  | 1.218   | 1.283   | 1.257   | 1.326| 1.283| 1.308   | 1.409 |
| Average B-factors (Å²) |        |         |         |         |     |     |         |      |
| Protein main chain | 29.3   | 26.1    | 32.2    | 32.4    | 20.1 | 28.0 | 22.2    | 16.9  |
| Protein side chain | 30.9   | 28.7    | 35.2    | 34.9    | 21.8 | 30.3 | 25.0    | 19.2  |
| Ligand           | 32.8   | 40.5    | 40.3    | 40.3    | 17.4 | 33.8 | 37.5    | 16.6  |
| Water molecules  | 24.3   | 28.2    | 32.2    | 31.3    | 24.3 | 32.9 | 23.8    | 20.5  |

* Both PO₄ and FMB and were present in the crystallization solutions.

Fig. 2. A stereoview of the SsMTAP hexamer. The active site requires one red subunit and one green subunit. Stick models for guanosine and sulfate are shown in blue to indicate active site locations. The short black bars near the molecular 3-fold axis indicate locations of the three disulfide bonds, linking red and green subunits.

Fig. 3. Overall fold of SsMTAP. *a*, a ribbon diagram of the monomer. Strands are colored green and labeled 1-9. α-Helices are colored magenta and are labeled α1-α7. The three turns of 3_10 helix are shown in cyan. Ball and stick models (yellow, sulfur; black, carbon; blue, nitrogen; red, oxygen) for sulfate and guanosine indicate the active site location. *b*, a topology diagram showing the location of secondary structural elements.
electron density is due to phosphate that is present at very low occupancy or that the phosphate-binding site is occupied by several disordered water molecules.

Structures of the Binary and Ternary Complexes—Co-crystallization of SsMTAP with the nucleoside substrate guanosine in the presence of sulfate resulted in a ternary complex of SsMTAP-Guo-SO$_4$. Sulfate was chosen as a phosphate substitute on the basis that the two anions share similar chemical characteristics and that sulfate is an inhibitor of the catalytic reaction. There is one hexamer in the asymmetric unit and each of the six active sites contains one molecule of guanosine bound in the nucleoside-binding site and one sulfate ion bound in the phosphate-binding site. The refined guanosine shows a glycosidic torsion angle (O$^4$-C$^1$-N9-C4) of 109° and a C$^4$-endo sugar pucker. This is an unusual conformation compared with nucleosides in solution, which generally have a glycosidic torsion angle near 0° (syn) or 180° (anti) and a C$^2$-endo or C$^3$-exo sugar pucker (27). However, the nucleoside conformation is similar to that found in the mammalian PNP-nucleoside complexes (11).

Co-crystallization of SsMTAP with the nucleoside substrate adenosine in the presence of sulfate resulted in a ternary complex of SsMTAP-Ado-SO$_4$. There is one hexamer in the asymmetric unit and difference electron density maps revealed some variation in the adenosine occupancy among the six active sites. As a result, adenosine was modeled in only four of the six active sites. One sulfate ion was bound in each of the six phosphate-binding sites. Adenosine is observed with a glycosidic torsion angle of 116° and a C$^4$-endo sugar pucker.

Co-crystallization of SsMTAP with the nucleoside substrate MTA in the presence of sulfate resulted in a ternary complex of SsMTAP-MTA-SO$_4$. There is one hexamer in the asymmetric unit. Each of the three active sites contains one molecule of MTA bound in the nucleoside-binding site and one sulfate ion bound in the phosphate-binding site. Both the initial $F_o - F_e$ and the final $2F_o - F_e$ difference maps reveal weak density for the 5′-methylthio group while density for the purine base and the remainder of the ribose moiety is well defined. It is possible that the inability of the 5′-methylthio group to form a hydrogen bond with the side chain of His$_5$ may allow this portion of the molecule to become flexible. Consequently, the weak density for the methylthio group likely results from high thermal motion or disorder. MTA is modeled with a glycosidic torsion angle of 124° and a C$^4$-endo sugar pucker.

Co-crystallization of SsMTAP with either sulfate or phosphate resulted in the binary complexes SsMTAP-SO$_4$ and SsMTAP-PO$_4$, respectively. For each complex there is one hexamer in the asymmetric unit and each of the six active sites contains one sulfate/phosphate ion bound in the phosphate-binding site. Closer inspection of the electron density maps for the SsMTAP-PO$_4$ complex revealed the presence of one well ordered molecule of Tris bound to each phosphate ion and extending into the ribose-binding site. Interestingly, although Tris buffer was used at a concentration of 0.1 M for all co-crystallization experiments, none of the sulfate containing structures revealed the presence of a bound Tris molecule in the active site.

Co-crystallization of SsMTAP with the noncleavable inosine analog FMB and phosphate revealed only phosphate bound in the active site. In this complex, there is one-half of a hexamer in the asymmetric unit and each of the three active sites contains one molecule of phosphate ion. Further inspection of the electron density map revealed that a molecule of Tris was bound instead of FMB. In contrast, co-crystallization with FMB in the presence of sulfate revealed both sulfate and FMB in the active site. The SsMTAP-FMB-SO$_4$ ternary complex has one-half of a hexamer in the asymmetric unit and each of the three active sites contain one molecule of FMB bound in the nucleoside-binding site and one molecule of sulfate ion bound in the phosphate-binding site.

Structure of the Active Site—The active site of SsMTAP was characterized using the binary and ternary complexes described above. The SsMTAP active site is located near the surface of the molecule in a groove formed at the dimer interface (Fig. 2). In contrast to the buried active site of the trimeric PNP's, the SsMTAP active site is relatively open. This exposed active site is similar to that seen in the hexameric E. coli PNP structure (7, 14) (Fig. 4a). The active site of SsMTAP consists of about 18 residues from eight separate polypeptide regions. These include Gly$^{21}$, Arg$^{25}$, Ile$^{64}$, Arg$^{86}$, Thr$^{89}$, Thr$^{90}$, Gly$^{91}$, Phe$^{160}$, Glu$^{163}$, Val$^{179}$, Glu$^{180}$, Met$^{181}$, Glu$^{182}$, Ser$^{204}$, and Asp$^{205}$ from one subunit and Val$_5$, His$_{55}$, and Arg$_{43}$ from an adjacent subunit. The SsMTAP monomer can be divided into two structural domains by cutting at the end of strand $\beta$5. In general, the residues in the N-terminal structural domain are involved primarily in phosphate binding, while the residues in the C-terminal structural domain are involved primarily in nucleoside binding. Of the 18 active site residues 13 are identical in E. coli PNP (Fig. 4b). The active site of SsMTAP can be divided into three main regions: the phosphate-binding site, the purine-base-binding site, and the ribose-base-binding site. This geometric arrangement of bound substrates is very similar to those seen in structures of both trimeric and hexameric PNP's (7, 8, 13, 14, 28). The binding sites are discussed in detail below.

The Phosphate-Binding Site—The phosphate-binding site was characterized using ternary complexes of SsMTAP with nucleosides and sulfate and binary complexes with sulfate or phosphate alone. Residues in the phosphate binding site include Gly$^{21}$, Arg$^{25}$, Thr$^{89}$, and Arg$^{86}$ from one monomer and Arg$_{43}$ from an adjacent monomer. In addition, the 2′- and 3′-hydroxyl groups of the bound nucleoside substrate contribute hydrogen bonds. The arrangement of interactions when sulfate is bound in the active site is the same for all of the SsMTAP ternary complexes as well as for the SsMTAP-SO$_4$ binary complex, and is illustrated in Fig. 5a. The phosphate-binding sites of E. coli PNP (7, 14) and E. coli (15) uridine phosphorylase use the same functional groups and the same binding geometry as SsMTAP, except that Thr$_{89}$ is replaced by serine in these enzymes. In contrast, the phosphate-binding sites of trimeric enzymes in the PNP family are similar to each other (8, 13) but distinctly different from those of the hexameric enzymes.

The phosphate-binding site from the SsMTAP-PO$_4$ binary complex is very similar to that seen in the sulfate containing structures (Fig. 5b). One key difference involves Arg$_{86}^{25}$. This residue seems to be slightly flexible in the sulfate complexes, showing some variation among the crystallographically independent monomers, and in some active sites did not contact the sulfate ion directly. In the phosphate complex, however, Arg$_{86}^{25}$ is well ordered and donates two hydrogen bonds to two different phosphate oxygen atoms. Also in complexes containing phosphate (but not in the ones containing sulfate) the carbonyl oxygen of Ile$_{64}^{4}$ is hydrogen bonded to a water molecule which in turn forms a hydrogen bond with the phosphate.

In complexes containing phosphate a well ordered molecule of Tris was also observed. The Tris-binding site is formed by amino acids that normally participate in ribose binding (Fig. 5c). In addition to donating a hydrogen bond to the phosphate from the amino group, the Tris hydroxyl groups also hydrogen bond to the carboxylate of Glu$_{182}^{4}$ and the side chain of His$_{55}^{5}$ from the neighboring subunit. An additional hydrogen bond is
formed between a Tris hydroxyl group and the backbone amide group of Met$^{181}$. Since these residues are involved in binding the ribose moiety of nucleosides, FMB is excluded from the phosphate containing structures by interactions between SsMTAP and a Tris molecule.

The Purine-binding Site—The purine-binding site was characterized using the ternary complexes guanosine, adenosine, and MTA. Fig. 6a illustrates the active site structure when guanosine is bound. The purine base binding site is composed of residues Phe$^{160}$, Glu$^{163}$, Val$^{179}$, Met$^{181}$, Ser$^{204}$, and Asp$^{205}$. A number of van der Waal contacts are also formed with strands β5 and β8 as they pass by the purine-binding site. Glu$^{163}$ accepts hydrogen bonds from both N-1 and the C-2 amino group of the purine base. In mammalian PNP, Glu$^{203}$ forms similar hydrogen bond with the purine base (11). In the SsMTAP-Guo-SO$_4$ complex the side chain conformation of Asp$^{205}$ is such that

![Structure of SsMTAP](image-url)
its carboxylate group is oriented away from the purine base. Ser204 hydrogen bonds to a water molecule that in turn donates a hydrogen bond to N-7 of the nucleoside. Ser204 and Asp205 are highly conserved in hexameric PNPs. In *E. coli* PNP, the equivalent aspartic acid is believed to be protonated and to stabilize the transition state through a hydrogen bond to N-7 (7). A similar role is proposed for Asp200 in human MTAP (13) and for Asn243 in trimeric PNPs (11, 29). The plane of the aromatic ring of Phe160 is nearly perpendicular to the plane of the purine base generating a herringbone-type stacking interaction between the enzyme and the base. Met181 contacts the purine base, Phe160 and the hydrophobic side of the ribosyl group. A similar interaction, involving an aromatic residue and methionine, is found in all known nucleoside phosphorylase structures (7, 8, 11–14, 28). Strand H9252 and strand H9258, which includes Val179, are located on the opposite side of the purine base.

Fig. 6 shows the nucleoside-binding region of SsMTAP bound to adenosine. Most of the structural features of the active site are similar to the SsMTAP-Guo-SO4 complex. One key difference involves Asp205. In SsMTAP-Ado-SO4, the carboxylate group of Asp205 is near N-7 and the 6-amino group of the purine. This conformation also allows Ser204 to donate a hydrogen bond to the carboxylate group of Asp205, which in turn is positioned to protonate N-7. As observed for the guanosine complex, Glu163 is positioned near the N1-C2 edge of the purine base. In the case of adenosine, N-1 is unprotonated so Glu163 must be protonated to form a hydrogen bond (see discussion below). The structure of the SsMTAP active site containing MTA is shown in Fig. 6c. The active site contacts are very similar to those observed in the adenosine complex with potential hydrogen bonds between the MTA and Asp205 and between MTA and Glu163.

*The Ribose-binding Site—*Potential hydrogen bonds in the ribose-binding site are formed between Glu182 and the 2′- and 3′-hydroxyl groups and between His5 of a neighboring monomer and the 5′-hydroxyl group (Fig. 6). Additional hydrogen bonds are formed between the 2′- and 3′-hydroxyl groups and the sulfate oxygen atoms. Met181 provides another key interaction, packing against the hydrophobic face of the ribose, Phe160 and the purine base. His5, Met181, and Glu163 are highly conserved among the hexameric members of the PNP family (7, 15). A methionine residue equivalent to Met181 has been identified in all known nucleoside phosphorylase structures (7, 8, 11–14, 28). Interestingly, mutation of the equivalent methionine in human PNP had little affect on its activity (29), despite its high degree of conservation. The active site positions the phosphate for nucleophilic attack at the C1′ carbon atom of the nucleoside. In the SsMTAP substrate complexes the distance between C1′ and the closest sulfate oxygen atom is 3.5–4.0.

Since MTA lacks the 5′-hydroxyl group present in guanosine and adenosine, it no longer requires a hydrogen bond donor or acceptor in this vicinity of the active site. Instead, binding would be enhanced by hydrophobic interactions between the 5′-methylthio group and neighboring protein atoms. Even though the active sites of *E. coli* PNP and SsMTAP are similar (13 out of 18 residues are identical) only SsMTAP accepts MTA as a substrate. Residues that are located in the vicinity of the 5′-methylthio group include Ile64, Thr49, Phe160, Met181, and Glu182 from one monomer and Val4, His5, and Arg43 from an adjacent monomer. Of these, only Val4, Ile64, and Thr49 are different than in *E. coli* PNP, in which case these residues are Pro4, Met64, and Ser90, respectively. Although the hydrophobicities of these residues are similar in the two enzymes, the structures of the side chains are different and this may contribute to the formation of a binding pocket for the 5′-methylthio group. One residue that might contribute to a hydrophobic environment near the 5′-position in SsMTAP is Thr49. In the *E. coli* PNP-FMB complex, a water molecule bridges the equiva-
lent Ser90 hydroxyl group and the 5'-hydroxyl group of FMB (14). In SsMTAP Thr89 could serve a similar role for substrates containing a 5'-hydroxyl group and then rotate about the C9251-C9252 bond to provide a more hydrophobic environment for the 5'-methylthio group of MTA. It is interesting to note that human MTAP, which is specific for MTA, contains two active site threonines (13) that are replaced by serines in the structurally homologous human PNP (8), which requires a 5'-hydroxyl group. In addition, human MTAP utilizes a histidine to valine substitution to further increase the hydrophobicity of the 5'-methylthio group environment.

A more subtle difference between the active site structures of the SsMTAP-MTA-SO4 complex and the SsMTAP-Guo-SO4 or SsMTAP-Ado-SO4 complexes involves the side chain conformation of Glu182 in the ribose-binding site and the glycosidic torsion angle of MTA. For MTA, an increase in the glycosidic torsion angle of the MTA (124°) compared with adenosine (116°) and guanosine (109°) causes the sugar to rotate away from His5 of the neighboring subunit. This movement results in the loss of a hydrogen bond between the carboxylate oxygen of the side chain and the 3'-hydroxyl group of MTA and a conformational change in the Glu182 side chain (Fig. 6c). It is possible that the loss of the hydrogen bond between Glu182 and the 3'-hydroxyl of MTA and the change in the glycosidic torsion angle, together with other less obvious structural changes, allows SsMTAP to adjust to the hydrophobic 5'-substituent.

**Substrate-induced Conformational Change**—In the nucleoside-free native SsMTAP structure and SsMTAP structures containing only sulfate or phosphate, a portion of the loop connecting strand β7 to helix α5 (residues 162–166) is in a relatively extended conformation. With the loop in this conformation, the side chain of Glu163 is pointing away from the active site. Upon nucleoside binding a conformational change takes place and a portion of the β7-α5 loop transforms into a 310 helix. As a consequence of this structural rearrangement, the carboxylate group of Glu163 moves into a position where it can interact with the N1-C2 edge of the purine base. Fig. 7 illustrates the conformational change. This 310 helix occurs in the structures of all three SsMTAP-substrate complexes. In E. coli PNP, Glu163 is replaced by proline (7, 14). In addition to lacking the side chain carboxylate, proline provides rigidity and prevents a similar conformational change from occurring.

**Unusual Binding Geometry in the Formycin B-Sulfate Complex**—Fig. 8a depicts the structure of SsMTAP containing FMB. In SsMTAP, the noncleavable nucleoside analog binds in a dramatically different conformation than the nucleoside substrates. FMB is modeled with a glycosidic torsion angle of –63°, but because the ribose group remains more or less fixed, the purine base is rotated by –180° compared with the guanosine, adenosine, and MTA complexes. As a consequence of this unusual binding geometry, the carboxylate group of Asp205 is oriented down toward the nucleoside-binding site, but instead...
of being near the N-7 and N-6 positions of the purine, it accepts a hydrogen bond from N-1. The 6-oxo group is linked indirectly with the neighboring subunit are designated with an asterisk (*).

Structure of SsMTAP

FIG. 8. Active site drawing of the SsMTAP FMB-sulfate complex. a, the binding geometry for FMB, b, the binding geometry for the E. coli PNP-FMB complex for comparison. The coordinates were taken from PDB entry code 1A69 (14). Hydrogen bonds are shown as dashed lines with the corresponding donor-acceptor distance labeled. Residues belong to the neighboring subunit are designated with an asterisk (*).
ing distance of N-7 and bridging to Ser\textsuperscript{205} and a second water molecule, which are also hydrogen bonded to each other could stabilize the transition state by donating hydrogen bonds to molecules, which are also hydrogen bonded to each other could transition stabilization might be provided by hydrogen bonds to different substrates. In the case of 6-aminopurine nucleosides, likely that SsMTAP utilizes different catalytic strategies for hexamer. In SsMTAP there are two types of 2-fold axes (Fig. 2). The first type of 2-fold axis relates pairs of subunits that when joined generate complete active sites. The contacts between these subunits are extensive. The second type of 2-fold axis joins the closely packed dimeric units. This interface is far away from the active site and has few intersubunit contacts. The three disulfide bonds in SsMTAP link subunits that are related by the second type of 2-fold symmetry axis but not the first and consequently provide overall stability to the SsMTAP hexamer.

Recently there have been reports of single or multiple disulfide bonds in intracellular proteins from hyperthermophilic microorganisms (41–45). In all cases, these disulfide bonds are not present in the homologous mesophilic proteins. The occurrence of three intersubunit disulfide bonds in SsMTAP suggest the possibility that the formation of these covalent links could be a more general strategy for stabilizing hyperthermophilic enzymes.

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Structures of E. coli PNP and E. coli PNP show significant differences, and these differences are likely due to the presence of different amino acid residues. The SsMTAP molecule, which is composed of 132 amino acids, shows a high degree of similarity to the E. coli PNP molecule, with 41% sequence identity. However, the structure of SsMTAP is more compact than that of E. coli PNP, with a lower number of polar and ionic interactions. The differences in structure suggest that SsMTAP may have evolved to function in a thermophilic environment, possibly by gaining a structural feature of SsMTAP that might account for its increased thermal stability in the presence of the three intersubunit disulfide bonds.

Although the presence of disulfide bonds has not been implicated as a general feature of naturally occurring extremely thermophilic proteins, it appears that these might be an important structural mechanism for the thermostability of SsMTAP since SsMTAP lacks any of the common stabilizing features. This is consistent with previous studies of thermostability of SsMTAP in the presence of reducing agents (1) in which a significant loss of activity was observed at 100 °C in the presence of 0.1 M dithiothreitol. The structure of SsMTAP shows that the disulfide bridges are strategically placed to stabilize the hexamer. In SsMTAP there are two types of 2-fold axes (Fig. 2). The first type of 2-fold axis relates pairs of subunits that when joined generate complete active sites. The contacts between these subunits are extensive. The second type of 2-fold axis joins the closely packed dimeric units. This interface is far away from the active site and has few intersubunit contacts. The three disulfide bonds in SsMTAP link subunits that are related by the second type of 2-fold symmetry axis but not the first and consequently provide overall stability to the SsMTAP hexamer.

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