Crystal structure of 5-methylthioribose 1-phosphate isomerase product complex from *Bacillus subtilis*: Implications for catalytic mechanism

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Abstract

The methionine salvage pathway (MSP) plays a crucial role in recycling a sulphahydryl derivative of the nucleoside. Recently, the genes and reactions in MSP from *Bacillus subtilis* have been identified, where 5-methylthioribose 1-phosphate isomerase (M1Pi) catalyzes a conversion of 5-methylthioribose 1-phosphate (MTR-1-P) to 5-methylthioribulose 1-phosphate (MTRu-1-P). Herein, we report the crystal structures of *B. subtilis* M1Pi (Bs-M1Pi) in complex with its product MTRu-1-P, and a sulfate at 2.4 and 2.7 Å resolution, respectively. The electron density clearly shows the presence of each compound in the active site. The structural comparison with other homologous proteins explains how the substrate uptake of Bs-M1Pi may be induced by an open/closed transition of the active site. The highly conserved residues at the active site, namely, Cys160 and Asp240 are most likely to be involved in catalysis. The structural analysis sheds light on its catalytic mechanism of M1Pi.

Keywords: aldose–ketose isomerase; 5-methylthioribose 1-phosphate isomerase; open/closed transition; crystal structure

Methionine is an essential amino acid for many crucial cellular functions, including the initiation of protein synthesis, methylation of DNA and rRNA, and biosynthesis of cysteine, phospholipids, and polyamines (Rando 1996). The amount of methionine in cells is typically limiting. Additionally, de novo synthesis of methionine is energetically expensive. Therefore, it is essential to salvage methionine

from methylthioadenosine (MTA), the end product of spermidine and spermine anabolism (Winans and Bassler 2002).

The methionine salvage pathway (MSP) is an important metabolic pathway in maintaining the amount of intravital methionine, which is frequent in bacteria (and in eukarya) (Sekowska et al. 2004). MSP has attracted much attention, as one of the genes (MTA phosphorylase) in this pathway acts as a tumor-suppressor gene (Christopher et al. 2002). Recently, we have identified five additional genes in MSP from *Bacillus subtilis* (Ashida et al. 2003), which revealed that MSP in *B. subtilis* was composed of 11 enzymes (Sekowska and Danchin 2002; Ashida et al. 2003).

In MSP from *B. subtilis*, 5-methylthioribose 1-phosphate isomerase (M1Pi; E.C. 5.3.1.23) catalyzes an interconversion of 5-methylthioribose 1-phosphate (MTR-1-P)

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Abbreviations: M1Pi, 5-Methylthioribose 1-phosphate isomerase; MTR-1-P, 5-methylthioribose 1-phosphate; MTRu-1-P, 5-methylthioribulose 1-phosphate; IF, translation initiation factor; IF2B, including initiation factor 2B.

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and 5-methylthioribulose 1-phosphate (MTRu-1-P) (Scheme 1) (Ashida et al. 2003), which is classified as an aldose–ketose isomerase reaction. In general, two possible catalytic mechanisms have been proposed for an aldose–ketose isomerization (Scheme 2) (Rose 1975). One is the *cis*-enediol mechanism that has been observed in triose-phosphate isomerase, ribose 5-phosphate isomerase, and phosphoglucose isomerase (Topper 1957; Rose and O’Connell 1960; Rose 1975). These enzymes do not require any metals to exhibit their activities. The other mechanism proceeds via the hydride transfer as observed with xylose isomerase (XI) (Fenn et al. 2004), which requires two divalent cations such as Mg^{2+} or Mn^{2+} (Blow et al. 1992; Asboth and Naray-Szabo 2000).

Our recent study has shown that M1Pi does not require any metals to exhibit its catalytic activity, which is analogous to the enzymes that proceed via a *cis*-enediol intermediate. On the other hand, NMR and mass spectrometry suggest the isomerase reaction of M1Pi in D_2O proceeds without incorporation of deuterium from solvent into the product (Saito et al. 2007). These spectrometric features resemble those of XI, which adopts the hydride transfer mechanism (Fenn et al. 2004). At present, there is no positive proof whether enzymatic reaction of M1Pi proceeds via a direct hydride transfer or via a *cis*-enediol intermediate. The lack of structure of substrate/product or the inhibitor complexes with M1Pi hinders the better understanding of the catalytic mechanism; the structural information is thus awaited.

According to the Pfam database (Bateman et al. 2004), M1Pi belongs to a large protein family related to translation initiation factor 2B α (PFAM family: PF01008). This family is composed of M1Pi and several translation initiation factors (IF) including initiation factor 2B (IF2B) α , β , and δ subunits from eukaryotes, and

Scheme 1. 5-Methylthioribose 1-phosphate isomerase (M1Pi) reaction. The enzyme catalyzes the interconversion of 5-methylthioribose 1-phosphate (MTR-1-P) to 5-methylthioribulose 1-phosphate (MTRu-1-P). Each hydrogen transferred as a hydride between MTR-1-P and MTRu-1-P is shown in boldface. Numbers near carbons stand for the carbon numbers of the sugar phosphates.

Scheme 2. Mechanisms for isomerization of an aldose to a ketose.

initiation factors 2B1 and 2B2 from archaea (Price and Proud 1994; Kyripides and Woese 1998). Generally speaking, IF2B interacts with the IF2–GDP complex, which promotes guanine nucleotide exchange, while M1Pi catalyzes a specific isomerization.

Recently, high-resolution crystal structures have become available for several M1Pi homologous proteins in the Protein Data Bank (PDB) (Berman et al. 2000). Although yeast Ypr118w (PDB code: 1W2W; 38% identity) has been characterized as M1Pi (Bumann et al. 2004), M1Pi-related proteins of *Thermotoga maritima* (PDB code: 1T9K; 50% identity), *Pyrococcus horikoshii* OT3 (PDB code: 1VB5; 32% identity) (Kakuta et al. 2004), and *Archaeoglobus fulgidus* (PDB code: 1T5O; 43% identity) have been annotated as putative proteins of unknown function. At present, M1Pis from *B. subtilis* and yeast are the only enzymes whose activities have been directly measured (Ashida et al. 2003; Bumann et al. 2004). Herein, we describe the crystal structures of M1Pi from *B. subtilis* (Bs-M1Pi) in complex with its product and a sulfate, respectively. The structural analysis explains how the substrate binding may be associated with an open/closed transition of the active site and also sheds light on its catalytic mechanism.

Results

Overall structure

The crystal structure of Bs-M1Pi cocrystallized with the substrate MTR-1-P appears to be a product MTRu-1-P

complex as explained below. Firstly, a $F_o - F_c$ omit map apparently revealed that the MTRu-1-P was bound to the active site (Fig. 1C). Secondly, the equilibrium constant, the concentration ratio $[MTRu-1-P]/[MTR-1-P]$ at the equilibrium, was 6.0 (Saito et al. 2007). The crystallization took 2 wk under low salt conditions, which is long enough to reach equilibrium. The bound species was therefore considered to be the product MTRu-1-P, not the substrate. On the other hand, the structure of sulfate-bound Bs-M1Pi shows tight binding of a sulfate at the active site, judged by the electron density (Fig. 1D).

The asymmetric unit (a.u.) contains two dimers for the MTRu-1-P-bound form, while one dimer is found in the a.u. for the sulfate-bound form. Each complex dimer in the a.u. is related by noncrystallographic twofold symmetry. In the MTRu-1-P-complex, four subunits were structurally similar, and their superposition gives a root mean square deviation (RMSD) ranging from 0.42 Å to 0.47 Å. Whereas the RMSDs of the two polypeptides of the sulfate-bound dimer are relatively large (with an RMSD of 0.96 Å for 339 C_α atoms), because of a conformational difference in the solvent-exposed loop

Figure 1. Structure of *Bacillus subtilis* M1Pi. (A) Stereoview of the MTRu-1-P bound Bs-M1Pi dimer, viewed with the internal twofold axis perpendicular to the paper. Ribbon representation of the dimer in which one subunit is yellow and green and the other subunit is cyan and pink. For the respective subunits, the yellow and cyan regions correspond to the N-terminal domains (residues 1–146), green and pink to the C-terminal domains (residues 147–353), respectively. Loop 70–75 is red, which is observed as a most changeable region between MTRu-1-P- and sulfate-bound M1Pi structures. (B) Schematic representation of the secondary structure of Bs-M1Pi. β -Strands (shaded in gray) are represented as arrows and α -helices as cylinders. Residue numbers are indicated at the start and end of each secondary structural element. The secondary structure elements were defined using the program DSSP (Kabsch and Sander 1983). Stereoview showing (C) MTRu-1-P- and (D) sulfate-binding sites of Bs-M1Pi. The $F_o - F_c$ omit maps of the compounds are countered at 3σ (blue) and 5σ (magenta) levels, respectively. The side chains of those residues surrounding the ligands are shown as sticks; (white and sky blue) carbons (MTRu-1-P- and sulfate-bound forms, respectively); (red) oxygens; (blue) nitrogens; (yellow) sulfurs; (cyan spheres) water molecules; (orange dashed lines) potential hydrogen bonds.

comprising residues 70–75 (referred to as Loop 70–75 hereafter) (see Fig. 1A). Since this region contacts with a neighboring molecule in the crystal, the structural difference is probably due to the molecular packing in the crystal. By comparing MTRu-1-P- and sulfate-bound structures, remarkable structural changes were not observed (RMSD of 0.62 Å for 333 C α atoms) except for this loop. Therefore, we will mainly describe the crystal structure of M1Pi complexed with MTRu-1-P (Fig. 1A).

Similar to yeast M1Pi (Yw-M1Pi), each subunit can be divided into two domains, the N-terminal domain (residues 1–146) and the C-terminal domain (residues 147–353), respectively. The N-terminal domain folds into a three-stranded antiparallel β -sheet (β 1–3) followed by five α -helices (α 1–5). The intermolecular contacts within the dimer involve a part of the C-terminal region comprising residues 187–315 (α 7–9 and β 5–10). The buried solvent-accessible surface is ~ 2300 Å² by calculation with GRASP (Nicholls et al. 1991). The C-terminal domain exhibits a Rossmann fold ($\alpha\beta\alpha$ -sandwich) (Fig. 1B), which participates in the binding of MTRu-1-P.

Active-site structure

The residues involved in MTRu-1-P binding can be divided into three groups: those involved in forming hydrogen bonds with (1) the phosphate group of MTRu-1-P, and (2) the backbone oxygens or hydrogens of ribulose, and hydrophobic interactions with (3) the methylthio group (Fig. 2A). Superimposed structures of

M1Pi complexed with MTRu-1-P and sulfate show that the phosphate group of MTRu-1-P is well-fitted onto the sulfate-binding position. The side chains of Arg51, Arg94, Gln199, and Lys251 and the backbone amide group of Gly52 form hydrogen bonds with the phosphate group (Fig. 2A). The residues involved in hydrogen bonds to carbonyl and hydroxyl oxygens of ribulose are listed as follows: The side chains of Asn250 and Asp240 form hydrogen bonds to the C2 carbonyl oxygen; the side chain of Cys160 and a water molecule interact with the C3 hydroxyl group; the side chain of Asp240, the backbone carbonyl group of Ala239; and the backbone amide group of Ala53 are hydrogen-bonded to the C4 hydroxyl group. These residues are absolutely conserved in the M1Pi family (Fig. 3). Of these residues, the side chain of Asp240 only interacts with both C2 carbonyl and C4 hydroxyl oxygens (Fig. 2A).

The hydrophobic interaction of the methylthio group includes the side chains of Pro54, Ala162, Ala166, and Thr167 (Fig. 2A). These residues are almost conserved in all M1Pis structures except for Ala162, which is changed to threonine in Yw-M1Pi (Fig. 3). Since the hydrophobic interaction occurs in C α and C β carbons of Ala162 of Bs-M1Pi, Thr183 of Yw-M1Pi will probably share similar interactions to those of Ala162 in Bs-M1Pi.

The thiol group of Cys160 interacts with the C1 (3.6 Å) and C2 (3.5 Å) of MTRu-1-P through van der Waals interactions, and also makes hydrogen bonds to the amide NH group of Asn161 and the ϵ -amino group of Arg51. According to a previous computational study (Naor and Jensen 2004), the hydrogen bonds between the thiol group of cysteine and the amide NH group of the main chain lower the pK_a of the thiol group by >1.0 pH unit. The pK_a value of Cys160 would be estimated at ~ 7 , so that the thiol group of Cys160 may be deprotonated at the optimal pH of M1Pi (pH 8.1) (Saito et al. 2007). Accordingly, Cys160 is likely to stabilize δ^+ formed transiently during catalysis.

The side chain of Asp240 is surrounded by the hydrophobic pocket formed by residues Thr96, Ala97, and Phe317 (Fig. 2B; Tables 1 and 2 below), which are absolutely conserved in all M1Pis (Fig. 3). This hydrophobic pocket appears to restrict the rotation of the Asp240 side chain, which may permit a favorable interaction with the substrate (or product). In addition, a hydrophobic environment generally allows the pK_a of the carboxyl group of aspartate to increase (Langsetmo et al. 1991; Inoue et al. 1992; Mehler et al. 2002). For instance, the pK_a of Asp26 buried in the hydrophobic cavity in *Escherichia coli* thioredoxin was changed from 4.4 to 7.5 (Langsetmo et al. 1991). The pK_a of Asp240 is likely to be increased, which might enable Asp240 to play a dual role as a proton donor/acceptor.

Figure 2. Active-site structure of Bs-M1Pi. (A) Schematic drawing of the active-site structure, showing the atoms and interactions around MTRu-1-P. (Dashed lines) Hydrogen bonds with the distance in angstroms. (Cyan semicircles) The hydrophobic interactions. The residues involved in (orange) the binding of the phosphate group and (green) the backbone oxygens of MTRu-1-P. (B) CPK representation of nonpolar residues (Thr96, Ala97, and Phe317) around conserved Asp240 in M1Pi. MTRu-1-P is shown as a stick model.

Figure 3. Sequence alignment of M1Pi-related proteins. The sequences are M1Pi from *B. subtilis* (Bs-M1Pi), yeast Ypr118w, M1Pi-related proteins of *T. maritima* (Tm-M1PiRP), *A. fulgidus* (af-M1PiRP), *P. horikoshii* OT3 (Ph-M1PiRP), and human eIF-2B α (GenBank accession no. NP_001405) and yeast eIF-2B α (Gcn3, GenBank accession no. NP_012951). Numbers above the sequences as well as the secondary structure assignment refer to the Bs-M1Pi sequence. (Black boxes on the sequence) Indicate the position of six conserved sequence motifs. The product MTRu-1-P ligands are labeled with a red asterisk above the sequence. Residues involved in (orange) the binding of the phosphate group and (green) the backbones of MTRu-1-P. (Cyan) The residues involved in the hydrophobic interaction. The color code is the same as in Figure 2A. The residues with different hydrogen-bonding networks between open (PDB code: 1T50) and closed (MTRu-1-P-bound Bs-M1Pi) forms are surrounded by yellow boxes. The secondary structures, which are defined by DSSP (Kabsch and Sander 1983), are (red) α -helices and (blue) β -sheets. The sequence alignment was made with CLUSTALX (Thompson et al. 1994).

Structural comparison with other M1Pi-related proteins

To date, several crystal structures of M1Pi-related proteins have been identified in PDB. However, most of these proteins were annotated as putative translation

initiation factor 2Bs (IF2Bs) or putative M1Pis despite the fact that there is no biochemical evidence. Although Yw-M1Pi (PDB code: 1W2W) has been characterized as M1Pi, M1Pi-related proteins of *T. maritima* (Tm-M1PiRP), *P. horikoshii* OT3 (Ph-M1PiRP), and *A. fulgidus*

Table 1. Data collection and refinement statistics

	Sulfate ion complex	MTRu-1-P complex
Crystal system	Tetragonal	Monoclinic
Space group	$P4_1$	$P2_1$
Unit-cell parameters (Å, °)	$a = b = 69.2$ $c = 154.7$	$a = 80.0, b = 84.4$ $c = 95.3, \beta = 92.3$
Resolution range (Å)	41.4–2.70	94.9–2.40
Number of molecules per asymmetric unit	2	4
V_M (Å ³ /Da)	2.8	2.1
V_{solv} (%) ^a	56	41
Number of measured reflections	276,966	145,183
Number of unique reflections	18,648	49,797
$I/\sigma(I)$	24.5 (3.2) ^b	30.1 (3.1) ^c
R_{merge} (%)	6.8 (25.6) ^b	5.5 (29.0) ^c
Completeness (%)	94.1 (76.0) ^b	98.4 (92.7) ^c
R_{cryst} (%)	22.5	21.0
R_{free} (%)	28.4	25.5
RMSD		
Bonds (Å)	0.011	0.007
Angles (°)	2.035	1.391

^a $R_{merge} = \sum |I(k) - \bar{I}| / \sum I(k)$, where $I(k)$ is the value of the k th measurement of the intensity of a reflection, $\bar{I}$ is the mean value of the intensity of that reflection, and the summation is over all measurements.

^b Values in parentheses are for the highest resolution shell (2.80–2.70 Å).

^c Values in parentheses are for the highest resolution shell (2.49–2.40 Å).

(Af-M1PiRP) have been annotated as proteins of unknown function. A careful comparative analysis would help to predict their functions.

Firstly, we compared Bs-M1Pi with Yw-M1Pi as a control model. The structurally superimposed regions are composed of 153 amino acid residues, where the RMSD of the superimposed C $_{\alpha}$ atoms was calculated to be 1.0 Å. Additionally, the 13 residues in the active site of Yw-M1Pi are well superimposed on those of MTRu-1-P-bound Bs-M1Pi (RMSD of 0.5 Å for 33 atoms of side chains) (Fig. 4A). The residues involved in ligand binding are structurally equivalent in both complex structures, although the bound species in the active sites are different (a product for M1Pi and a sulfate for Yw-M1Pi). This analysis confirms that M1Pi shares a structurally conserved active site despite sequence divergence (Yw-M1Pi shows 38% amino acid sequence identity with Bs-M1Pi).

Secondly, we compared Bs-M1Pi with the Tm-M1PiRP, whose structure has been determined by the Midwest Center for Structural Genomics group (MCSG) and identified as a probable M1Pi in PDB. Structural comparison shows that the RMSD of the superimposed C $_{\alpha}$ atoms was markedly low (1.5 Å for 322 C $_{\alpha}$ atoms). The residues involved in MTR-1-P binding are absolutely conserved and superimposed well onto those of Bs-M1Pi (RMSD of 0.7 Å for 33 side chains) (Fig. 4A), suggesting that this protein is probably M1Pi.

On the other hand, Ph-M1PiRP seems to differ from these proteins. Although the structure can be superimposed onto Bs-M1Pi (RMSD of 1.7 Å for 222 C $_{\alpha}$ atoms) and the sequence identity is high (32%), the protein lacks residues involved in MTRu-1-P binding (Fig. 3). In addition, the corresponding residues in the active site are structurally rather different compared to M1Pi (Fig. 4B). These observations suggest that Ph-M1PiRP is not M1Pi, but probably a translational initiation factor.

Finally, we have noticed that the crystal structure of Af-M1PiRP had a remarkable structural difference (RMSD of 2.2 Å for 310 C $_{\alpha}$ atoms) despite the absolute conservation of residues forming the active site (see Fig. 3). This Af-M1PiRP had been annotated by the New York Structural Genomics Research Consortium (NYSGR) as a putative protein of unknown function.

It should be noted that the N-terminal and C-terminal domains of Bs-M1Pi and Af-M1PiRP can fit well separately (RMSD of 1.3 Å for 107 C $_{\alpha}$ atoms of N-terminal domains and 1.2 Å for 216 C $_{\alpha}$ atoms of C-terminal domains, respectively). The active-site residues of each domain are also well superimposed (RMSD values are 0.7 Å for all atoms of the active-site residues of N-terminal domains and 0.4 Å for those of C-terminal domains, respectively) (Fig. 4C,D). These structural congruencies suggest a mechanistic hypothesis, as described below.

Open/closed conformational transition of M1Pi

The structure of Bs-M1Pi shows that the active site is completely shielded from the solvent region (Fig. 5A). On the other hand, Af-M1PiRP shows that the active-site residues are highly exposed to solvent, which is probably ready to bind the substrate (or product) (Fig. 5B). When the conformations of MTRu-1-P-bound Bs-M1Pi and unliganded Af-M1PiRP are compared by the program DynDom (Hayward and Berendsen 1998), the following results were obtained:

Table 2. Hydrophobic interactions around Asp240

Peptide atom	Contact atom		Distance (Å)
Asp240	O $_{\delta 1}$	Thr96 C $_{\beta}$	4.1
		C $_{\gamma}$	4.1
		C	3.9
	Ala97	N	3.5
		C $_{\alpha}$	3.6
		C $_{\beta}$	3.1
	Phe317	C $_{\delta 1}$	4.1
		C $_{\epsilon 1}$	3.5
		C $_{\zeta}$	3.6

Figure 4. Structural comparison around the active site of M1Pi with M1Pi-related proteins. (A) Comparison of the active sites of (green) Bs-M1Pi, (magenta) yeast Ypr118w, and (yellow) Tm-M1PiRP. The residues are shown as sticks, together with ribbon representation (gray). (Green transparent) MTRu-1-P is shown in ball-and-stick representation. Residue numbers are shown in Bs-M1Pi numbering. (B) Superposition of the active-site residues in (green) Bs-M1Pi onto those of (orange) Ph-M1PiRP. The amino acid residues are shown in Bs-M1Pi numbering/Ph-M1PiRP numbering. (C) Superposition of the active-site residues in the N-terminal domain. The residues of (green) Bs-M1Pi and (cyan) Af-M1PiRP. (D) Superposition of the active-site residues in the C-terminal domain. The color code is the same as in C.

1. The conformational changes can be described as the rigid-body movement of two domains (rotation of 21°) (Fig. 5C).
2. The identified two rigid domains are composed of residues 1–137 and residues 138–343, which roughly correspond to N- and C-terminal domains, respectively.
3. The “bending residues” between the two domains are 11–53, 55–56, 60–134, and 137–137, which include two phosphate-binding residues (Arg51 and Lys94) in the active site. It would suggest the flexibility of the active site composed of the N-terminal domain.

It is thus tempting to speculate that the open/closed domain motion might occur upon substrate binding, since the crystal structures of Af-M1PiRP and M1Pi show apo and holo (MTRu-1-P-bound) forms, respectively.

Structural comparison shows that the remarkable conformational change is observed in loop 93–98 (Fig. 5D), which corresponds to one of the most highly conserved motifs among the M1Pis family (Fig. 3). The side chain of Arg94 on this loop participates directly in binding of the phosphate group of MTRu-1-P. This ion pair seems to lead the rearrangement of the loop 93–98 and the backbone shifts of ~8 Å (red arrow in Fig. 5D). Upon this conformational

change, the side chain of Glu290, which originally interacted with Arg292, is also conformationally changed (cyan arrow in Fig. 5D) to interact with the side chain of Arg94. At the same time, the conformational change of the side chain of Arg292 was also induced to interact with the backbone carbonyl of Val46 (yellow arrow in Fig. 5D). Val46 is located at the C terminus of helix-1; therefore, the side chain of Arg292 is positioned at the negative pole of the helix-1, which seems to stabilize the closed conformation. The residues involved in the rearrangements as described above are absolutely conserved in all M1Pis, except for Val46 (Fig. 3). Thus, the substrate binding is likely to induce the large conformational changes of N- and C-terminal domains as well as the rearrangement of the hydrogen-bond network around the loops 93–98 and 290–294 to stabilize the closed state of the enzyme.

Discussion

M1Pi belongs to the class of aldose–ketose isomerases, which catalyzes the isomerization of its cyclic substrate

Figure 5. Structural comparison of Bs-M1Pi with Af-M1PiRP. Molecular surfaces of (A) Bs-M1Pi (green) and (B) Af-M1PiRP (cyan). (Yellow) The residues involving MTRu-1-P recognition. The arrow in B indicates the modeled MTRu-1-P, which suggests the opening of the catalytic site in the Af-M1PiRP structure. (C) Superposition of the C-terminal domain of Bs-M1Pi onto that of Af-M1PiRP; α -helices are shown in a cylinder representation. The (light green) N- and (green) C-terminal domains of Bs-M1Pi; (light blue) N- and (blue) C-terminal domains of Af-M1PiRP. (D) An enlarged view of the detailed hydrogen-bond network. The color code is the same as C. Dashed lines indicate hydrogen bonds.

(Ashida et al. 2003). In this case, the reaction mechanism can be proposed that involves the sugar ring opening followed by hydrogen transfer between C1 and C2 of the substrate.

Distinct from the substrates of other aldose–ketose isomerases (that normally possess hydroxyl groups on the cyclic C1), MTR-1-P has a phosphate group on the corresponding C1 position. Owing to the electrophilicity of the phosphate group, the sugar ring of MTR-1-P would be much more chemically stable than the ordinary substrates possessing hydroxyl groups. The sugar ring opening of MTR-1-P probably requires an enzymatic mechanism.

Aldose–ketose isomerase catalyzes the hydrogen transfer via two possible mechanisms: (1) enediol or (2) hydride transfer mechanisms (Rose 1975). The transfer mechanism of M1Pi remains unclear at present. To elucidate the mechanism of M1Pi, we examined the active-site structure in detail; however, the structure does not unambiguously discriminate between two possible mechanisms for the hydrogen transfer. Hence, two possible structure-based mechanisms of M1Pi are discussed here.

At the first step, common in both mechanisms, the positively charged region composed of the side chains of Arg51, Arg94, and Lys251 participates in binding of the phosphate group of the substrate, forming a Michaelis complex. Simultaneously, a remarkable conformational change of the N- and C-terminal domains will be induced upon the substrate binding as described above, so that the pocket would be sequestered from the solvent region.

In the second step, the sugar ring opening is catalyzed by donating a proton to the ring oxygen of the substrate MTR-1-P. As described above, the side chain of Asp240 is expected to play a role as either a donor or an acceptor

(Fig. 6A). Compared to xylose isomerase (XI) (Farber et al. 1989; Collyer et al. 1990), the side chain of Asp240 superimposes well on the catalytic water molecule of XI, which mediates proton transfer between O1 and O2 in XI. In a similar way, Asp240 would donate a proton to the ring oxygen (in the MTR-1-P to MTRu-1-P direction) to promote the ring-opening step of catalysis.

Two possibilities for the hydrogen transfer can be considered. Step 1A to 2A (Fig. 6B) shows the proposed mechanism of M1Pi via *cis*-enediol proton transfer. The deprotonated Cys160 abstracts the proton from C2, and the resulting flow of electrons forms a double bond between C1 and C2, thereby generating the *cis*-enediol intermediate (phosphoenolate, step 2A of Fig. 6B). Asp240 mediates the proton transfer between O2 and O4 simultaneously, and redonation of the abstracted proton by Cys160 forms the product MTRu-1-P. The isolated environment from bulk solvent is expected to prevent the misprotonation to generate nonproductive and inhibitory sugars.

Step 3B (Fig. 6B) shows another mechanism of M1Pi via hydride transfer. The nucleophilic attack by the side chain of Asp240 on the O2 of MTR-1-P initiates the ring opening reaction; then, the deprotonated Cys160 probably stabilizes the positive charge generated on C1. At the end of this step, the hydride on C2 transfers to C1 accompanying the electron flow from O2 toward C2 to form a carbonyl double bond and then yields the product MTRu-1-P.

Previous NMR and MASS spectroscopy of BsM1Pi in D₂O bears some analogy with those of XI, which adopts the hydride transfer mechanism (Collyer and Blow 1990). However, we cannot conclude whether the enzymatic

Figure 6. Proposed reaction mechanisms of M1Pi. (A) Substrate MTR-1-P-binding model of Bs-M1Pi in the active site. (Orange dashed lines) Hydrogen bonds. (White sticks) Residues involved in the recognition of MTR-1-P. (B) Proposed catalytic mechanism of the *cis*-enediol (*upper* site) and 1,2-hydride shift (*underside*) reaction of M1Pi based on the crystal structure of its product complex. The amino acid residues are shown in Bs-M1Pi numbering. D240 donates a proton to the ring oxygen of the substrate MTR-1-P. C1 is likely to become a carbocation intermediate.

reaction of M1Pi proceeds via a direct hydride shift or via a *cis*-enediol intermediate. Further experiments with the deuterium-labeled substrate will elucidate the catalytic mechanism of M1Pi.

Materials and Methods

Crystallization and data collection

M1Pi from *B. subtilis* was overexpressed in *Escherichia coli* and purified according to a previously reported method (Ashida et al. 2003). M1Pi complexed with sulfate was crystallized using the sitting-drop vapor diffusion method at 20°C, as previously reported (Tamura et al. 2005). The crystallographic and X-ray data statistics are summarized in Table 1.

To prepare the product complex, two difficulties had to be overcome. Firstly, the substrate MTR-1-P had to be synthesized as MTR-1-P is not commercially available. Secondly, the crystallization conditions included neither phosphate nor sulfate as these compounds are generally thought to inhibit the binding of the substrate. In order to solve the first problem, we synthesized MTR-1-P from *S*-adenosylmethionine by using an enzyme MtnK from *B. subtilis*, as reported previously (Ashida et al. 2003). MTR-1-P was then rendered salt-free by purification (Saito et al. 2007). To overcome the second obstacle, crystallization was performed in the absence of sulfate and phosphate, although we had already obtained crystals in the presence of high concentration of sulfate. The crystals of M1Pi complexed with its products were obtained by cocrystallization with the substrate 5-methylthioribose 1-phosphate (MTR-1-P). Detailed crystallization conditions of the product complex are as follows: Drops contained a 1.6:1.4 ratio (in microliters) of a protein solution (10 mg/mL M1Pi in 50 mM Na HEPES at pH 7.4 and 1 mM EDTA, supplemented with 33 μ M MTR-1-P) and a reservoir (0.2 M K formate and 15% [w/v] polyethylene glycol 3350). The crystals were flash-cooled in cryo-protectant 20% (v/v) glycerol. Diffractions of data sets were collected at the beamline BL44XU in SPring-8. Crystals belonged to the space group $P2_1$ with the cell parameters $a = 79.9$, $b = 84.4$, $c = 95.3$, $\beta = 92.4$. Diffraction data were processed with DENZO and SCALEPACK from the HKL program suite (Otwinowski 1993).

Structural determination and refinement

The structure of sulfate-bound M1Pi was determined by the molecular replacement method using the program MOLREP (Vagin and Teplyakov 1997) with a crystal structure of Ypr118w (RCSB PDB ID: 1W2W) as a search model. The structure of MTRu-1-P-bound M1Pi was determined by molecular replacement with the sulfate-bound structure. Refinement procedures for sulfate- and MTRu-1-P-bound structures were carried out using the program CNS (Adams et al. 1997) without non-crystallographic symmetry (NCS) restraints/constraints. The structures were visualized and modified using the programs O (Alwyn Jones, Uppsala University, Sweden) and COOT (Emsley and Cowtan 2004). The final atomic coordinates and structure factor amplitudes have been identified (PDB code: 2YRF for sulfate-bound M1Pi, and 2YVK for MTRu-1-P-bound M1Pi, respectively). The final R_{work} and R_{free} are 22.5% and 28.4% for sulfate-bound M1Pi at 2.7 Å resolution, and 21.0% and 25.5% for MTRu-1-P-bound M1Pi at 2.4 Å resolution, respectively.

The electron density map was clear for almost the entire polypeptide of sulfate-bound M1Pi except for the first nine and the last five residues, while that of MTRu-1-P complex M1Pi was clear except for the last five residues, which were disordered. Pro196 is in the *cis*-conformation in both structures. The stereochemical qualities of the final structures were assessed with the programs PROCHECK (Laskowski et al. 1993) and WHATCHECK (Hoof et al. 1996). Figures were generated by MOLSCRIPT (Kraulis 1991), RASTER3D (Merritt 1994), and PyMOL (DeLano Scientific), and all structural comparisons were carried out using the program LSQKAB (1994).

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