Enzymatic Characterization of 5-Methylthioribose 1-Phosphate Isomerase from Bacillus subtilis

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The product of the mtnA gene of Bacillus subtilis catalyzes the isomerization of 5-methylthioribose 1-phosphate (MTR-1-P) to 5-methylthioribulose 1-phosphate (MTRu-1-P). The catalysis of MtnA is a novel isomerization of an aldose phosphate harboring a phosphate group on the hemiacetal group. This enzyme is distributed widely among bacteria through higher eukaryotes. The isomerase reaction analyzed using the recombinant B. subtilis enzyme showed a Michaelis constant for MTR-1-P of 138 μM, and showed that the maximum velocity of the reaction was 20.4 μmol min⁻¹ (mg protein)⁻¹. The optimum reaction temperature and reaction pH were 35°C and 8.1. The activation energy of the reaction was calculated to be 68.7 kJ mol⁻¹. The enzyme, with a molecular mass of 76 kDa, was composed of two subunits. The equilibrium constant in the reversible isomerase reaction [MTRu-1-P]/[MTR-1-P] was 6. We discuss the possible reaction mechanism.

Key words: aldose-ketose isomerase; 5-methylthioribose-1-phosphate isomerase; eukaryotic initiation factor 2B α-like protein; methionine salvage pathway; Bacillus subtilis

The major forms of sulfur and nitrogen occur in nature as oxidized inorganic compounds. Both elements need 8 electrons per molecule to be reduced to the sulfhydryl group and ammonia, respectively. Since the conversion of CO₂ to glucose uses 4 electrons per molecule of CO₂, the sulfhydryl and amino groups in living entities are high energy-carrying groups.1) Once sulfur and nitrogen are reduced, organisms usually reutilize the reduced forms many times without oxidation.

The methionine salvage pathway (MSP) is a metabolic pathway for recovery of the reduced sulfur in 5-methylthioribose (MTR), which is formed in the synthesis of polyamines, as methionine.2) The pathway is distributed widely among bacteria, yeasts, plants, and animals, and salvages the reduced sulfur into methionine after the synthesis of polyamines and other compounds such as mucineic acid and ethylene that are specific to individual organisms.3) MSP has attracted particular attention, as it is deleted in a variety of human tumor tissues4) and the analogs of MTR are growth inhibitors of the malarial protozoan Plasmodium falciparum.5) The genes and the reactions of the whole MSP from MTR to methionine were deduced initially in Klebsiella pneumoniae,6) and have been identified using Bacillus subtilis,7) where the reaction from MTR-1-phosphate (MTRu-1-P) to 5-methylthioribulose-1-phosphate (MTR-1-P) is catalyzed by MtnA or MTR-1-P isomerase (Fig. 1).

MtnA has 20% similarity in amino acid sequence to that of the α-subunit of eukaryotic initiation factor (eIF) 2B, and was originally identified as an eIF2Bα-like protein (eIF2Bα-LP) (Fig. 2A).8) eIF2B is composed of five (α to ε) subunits and is involved in the GTP/GDP exchange of eIF2 in the eukaryotic translation step.9,10) The GTP-binding form of eIF2 (eIF2-GTP) transfers initiator methionyl-tRNA (Met-tRNA<sub>Met</sub>) to 40S ribosomes as an eIF2-GTP/Met-tRNA<sub>Met</sub> complex. GTP is hydrolyzed to GDP, and then eIF2-GDP is released from 40S ribosomes. The GDP on eIF2 must be changed to GTP by the GTP/GDP exchange reaction of eIF2B to participate in the next translational initiation. eIF2Bα has been found to participate in the regulation of the GTP/GDP exchange reaction of the eIF2B complex, but

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Abbreviations: MSP, methionine salvage pathway; MTR, 5-methylthioribose; MTR-1-P, 5-methylthioribulose 1-phosphate; MTRu-1-P, 5-methylthioribulose 1-phosphate; eIF, eukaryotic initiation factor; eIF2Bα-LP, eukaryotic initiation factor 2B α-like protein; Met-tRNA<sub>Met</sub>, initiator methionyl-tRNA; HK-MT2)-1-P, 2-hydroxy-3-keto-5-methylthiopentenyl-1-phosphate; NMR, nuclear magnetic resonance; R5P, ribose 5-phosphate; PDB, Protein Data Bank (http://www.rcsb.org/pdb/)
its protein structure and exact function in the complex remain unclear. In this context, it is intriguing that eIF2Ba-related MtnA is an MTR-1-P isomerase functioning in MSP.

After our identification of the MtnA function, E2B2, the eIF2Ba-LP in Thermococcus kodakaraensis, was identified as ribose 1,5-bisphosphate (RBP) isomerase (Fig. 2). This enzyme supplies form III ribulose 1,5-bisphosphate carboxylase/oxygenase with ribulose 1,5-bisphosphate for CO₂ fixation. It also catalyses the isomerization reaction of a cyclic sugar harboring the phosphate group on C1, as does MtnA of B. subtilis, but there have been no previous reports of the isomerase reaction of cyclic sugars with the phosphate group on the C1 aldehyde carbon.

MTR-1-P has the phosphate group on C1, which consequently is not able to rise to a free aldehyde structure. Thus its isomerase reaction should be different from those of ordinary sugars that have no modification on the C1 aldehyde carbon. We were interested in the reaction mechanism of this new enzyme MTR-1-P isomerase, and in this study analyzed the general enzymatic properties of the protein. We propose a mechanism for the isomerase reaction of this enzyme.

**Materials and Methods**

**Preparation of MTR-1-P isomerase.** MTR-1-P isomerase was expressed in Escherichia coli as (His)_6-tagged recombinant protein and purified using Ni-NTA His-Bind resin (Novagen, Madison, WI), as described previously. After purification by the His-tag affinity system, MTR-1-P isomerase fraction was passed through a PD-10 column (Amersham Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl and 2.5 mM CaCl₂, and the protein fractions were collected. The (His)_6-tag was excised with 1.3 μg thrombin per mg of MTR-1-P isomerase for 12 h at 22 °C. MTR-1-P isomerase was further purified on a HiloQ Sephadex 200 16/60 column (Amersham Pharmacia) equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl. The column was developed with a fast protein liquid chromatography system (Amersham Pharmacia). The dye-binding method of Bradford was used to determine protein concentration. The purity of the prepared recombinant enzyme was checked on SDS–polyacrylamide gel electrophoresis gel by staining with Coomassie Brilliant Blue R-250.

**Preparation of MTR-1-P and quantification of MTR-1-P and MTRu-1-P.** MTR-1-P was synthesized from S-adenosylmethionine through hydrolysis of the adenine base by HCl, and C1 of the ribose moiety was phosphorylated by ATP with MtnK, an MTR kinase. MTR-1-P was desalted using a column (2.6 × 65 cm) of Sephadex G-10 (Amersham Pharmacia).

MTR-1-P and MTRu-1-P in the reaction mixture were applied to a CarboPac PA1 column ( Dionex, Osaka, Japan) equilibrated with 100 mM NaOH. The column was developed using a linear gradient of 0 to 0.5 M potassium acetate in 100 mM NaOH. The sugar phosphates thus separated were detected with a pulsed amperometry system and quantified as to phosphate content using a phosphate assay kit (Phosphor C, Wako, Osaka, Japan). The flow rate (1 ml min⁻¹) and elution gradients were controlled with a gradient pump GP40 (Dionex).

**Assay of MTR-1-P isomerase.** MTR-1-P isomerase was assayed in a coupling reaction with MtnB and MtnW, previously identified as MTRu-1-P dehydratase and 2,3-diketo-5-methylthiopentyl-1-phosphate enolase respectively. In this assay, the reaction product MTRu-1-P is converted to 2-hydroxy-3-keto-5-methylthiopentenyl-1-phosphate (HK-MTPenyl-1-P) by sequential reactions of MtnB and MtnW. The reaction was started by adding 2 mM MTR-1-P to a mixture containing 50 mM Tris–HCl buffer (pH 8.1), 1 mM MgCl₂, 0.5 μg of MTR-1-P isomerase, 5 μg of MtnB, and 15 μg of MtnW. The final reaction volume was 1 ml and the reaction temperature 35 °C, unless otherwise stated. H-nuclear magnetic resonance (NMR) analysis of the reactions from MTR-1-P to HK-MTPenyl-1-P indicated that virtually all of the MTR-1-P added to the reaction mixture was converted into HK-MTPenyl-1-P. This suggests that the chain of reactions from MtnA to MtnW is strongly inclined toward the formation of HK-MTPenyl-1-P; thus, from the light absorbance of HK-MTPenyl-1-P at 280 nm it was determined to be 9,500 m⁻¹ cm⁻¹. The responses of the activity to temperature and pH were determined between 20 and 50 °C and pH 7 to 9. Activation energy was calculated using activities measured below 35 °C. The amounts of coupling enzymes were not limiting under any assay conditions used in this study. MTR-1-P isomerase was also assayed by measuring the amount of MTRu-1-P
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**Fig. 2.** Homology between MTR-1-P Isomerase and Related Proteins.

A. The deduced amino acid sequence of *B. subtilis* MTR-1-P isomerase (MtnA, NP_389238) was compared with sequences for several MtnA homologs, *Homo sapiens* (H. sapiens1, NP_001026897), *Arabidopsis thaliana* (A. thaliana1, A2g05830), *Hordeum vulgare* subsp. *vulgare* (BAB21393), *Caenorhabditis elegans* (C. elegans1, NP_056714), *Saccharomyces cerevisiae* (Ypr118wp, NP_015443), *Leishmania major* (CAJ09465), *Thermotoga maritima* MSB8 (NP_228719), *Archeoglobus fulgidus* DSM 4304 (NP_069206) and *Thermococcus kodakarensis* KOD1 (YP_182598), and those of the alpha subunits of the eukaryotic initiation factor 2B in *H. sapiens* (H. sapiens2, NP_001405), *A. thaliana* (A. thaliana2, At1g72340), *C. elegans* (C. elegans2, NP_499106), and *S. cerevisiae* (GCN3). The phylogenetic tree was produced by CLUSTALW (http://www.ddbj.nig.ac.jp/search/clustalw-j.html) and TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treview.html) program.

B. Multiple alignments of sequences underlined in A. Identical and similar amino acid residues are shaded in black and gray, respectively. The sequences are numbered according to that for the *B. subtilis* sequence. Dimer-forming residues are indicated by open triangles, putative proton-subtracting residue by filled triangle. Alignment was displayed with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) program.
product formed using the ferricyanide reducing sugar assay and cysteine-carbazole methods.

**Gel filtration for molecular mass determination.** A Supercos 6 10/300 GL column (Amersham Pharmacia) equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM MgCl₂ and 150 mM NaCl was used for molecular mass determination. The same buffer was used for development of the column. The following proteins from the Gel Filtration Calibration kit (Amersham Pharmacia) were used for calibration of the column: ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa).

**NMR analysis of the MtnA reaction product.** The NMR spectrum was recorded at 298 K in a Bruker DRX-800 spectrometer operated at 800 MHz. MTR-1-P was lyophilized for 16 h, dissolved in phosphate buffer made with unopened 99.9% D₂O solution (Wako) and used as the starting substrate. H₂O in the buffer used for purification of recombinant enzyme was replaced with D₂O by passage through a NAP-5 column (Amersham Pharmacia) equilibrated with the D₂O-phosphate buffer.

**Mass spectrometry of the reaction product of MTR-1-P isomerase.** Ribose 5-phosphate (RSP, Sigma-Aldrich, St. Louis, MO) or lyophilized MTR-1-P was dissolved in 50 mM Tris–HCl buffer (pH 8.0) prepared with 99.9% D₂O (Wako) and used as the substrate. RSP isomerase (Sigma-Aldrich) was dissolved in the Tris–HCl buffer (pH 8.0) containing 1 mM MgCl₂. MTR-1-P isomerase purified as above was passed through an NAP-5 column (Amersham Pharmacia, USA) equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The isomerase reaction of RSP and MTR-1-P isomerases, both at 10 μM in 100-μl reaction mixture, proceeded in the presence of 1 mM RSP and MTR-1-P respectively for 1 h at 37 °C, and was stopped by cooling to 4 °C and removal of proteins with Centricon YM-3 vial (Millipore, Billerica, MA) at 4 °C. The protein-free reaction mixture was lyophilized and dissolved in Milli-Q water to analyze the molecular mass of the reaction product with an ESI ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany).

**Results**

There have been no previous reports of MTR-1-P isomerase from any source been analyzed enzymologically. Here we determined the general physicochemical and enzymological properties of the *B. subtilis* protein before analyzing its reaction mechanism (Table 1). The molecular mass of the purified active enzyme was found to be 76 kDa by gel filtration. Since the molecular mass of the subunit was deduced to be 38.9 kDa from its gene sequence (Fig. 2B), the enzyme is probably composed of two subunits.

The activity of the forward reaction from MTR-1-P to MTRu-1-P was maximal at pH 8.1 and 35 °C, and decreased sharply above or below this pH and temperature. The activation energy, E_a, of the isomerase reaction was calculated from the Arrhenius plot to be 68.7 kJ mol⁻¹ at pH 8.1. Isomerase activity increased in a MTR-1-P concentration-dependent manner, and V_max and K_m were 20.4 μmol min⁻¹ mg protein⁻¹ and 138 μM for MTR-1-P, respectively. The maximum activity corresponded to 13 turnovers s⁻¹ site⁻¹. The specificity of the enzymatic reaction (k_cat/K_m) was about 9.3 × 10⁴ M⁻¹ s⁻¹.

The requirement of *B. subtilis* MTR-1-P isomerase for a metal ion was analyzed by incubating the purified enzyme with 100 mM EDTA in the presence of 50 mM Tris–HCl buffer (pH 8.0) for 10 h at 4 °C and measuring the remaining activity by the ferricyanide reducing sugar assay and cysteine-carbazole methods. The activity was not at all influenced by EDTA treatment, indicating that the *Bacillus* MTR-1-P isomerase does not require a metal ion for its catalysis.

To measure the equilibrium constant for the isomerization reaction between MTR-1-P and MTRu-1-P, the reaction of 1 μg of MTR-1-P isomerase protein and 15 μmol of MTR-1-P was monitored for 4 h by ion chromatography equipped with a CarboPac PA1 column. The reaction was completed in 40 min and the reaction products at equilibrium after 4 h were measured by their phosphate contents (Fig. 3). MTR-1-P and MTRu-1-P in the reaction mixture were present at 14.3 ± 0.6 and 85.7 ± 4.8% respectively of total phosphates applied to the column. The ratio was constant over the reaction time for 4 h, indicating that the equilibrium constant [MTRu-1-P]/[MTR-1-P] is 6.0 (Table 1). Indeed, ¹H-NMR of the reaction mixture revealed that most of the proton peaks were from MTRu-1-P (Fig. 4), but not from the substrate MTR-1-P, considered from the measured equilibrium constant of the reaction. The peaks were well defined at 2.09 (m, 3H, 6-CH₃), 2.64 (dd, J_HH = 14.1 Hz, J_HH = 8.3 Hz, 5-CH₃), 2.73 (dd, J_HH = 14.1 Hz, J_HH = 4.5 Hz, 5-CH₃), 4.11 (dt, J_HH = 8.3, 4.8 Hz, 4-CH), 4.47 (d, J_HH = 5.0 Hz, 3-CH), 4.64 (dd, J_HH = 18.9 Hz, J_HH = 8.3 Hz).
$J_{HP} = 6.3 \text{ Hz}, 1-\text{CH}_2$), and 4.70 ppm (dd, $J_{HH} = 18.9 \text{ Hz}, 3J_{HP} = 6.0 \text{ Hz}, 1-\text{CH}_2$).

In the NMR spectrum, two of the C1 peaks were split into double doublets caused by J couplings with hydrogen and phosphorus nuclei in the NMR spectrum (Fig. 4, inset), indicating that C1 has two protons. This suggests that the isomerase reaction of MTR-1-P isomerase proceeds without incorporation of deuterium from the medium into the product. Many sugar and sugar phosphate isomerases show the enediol mechanism, in which enediol is formed between two carbons involved in the isomerization and a proton in the produced sugar is from the medium. These facts indicate the possibility that MTR-1-P isomerase proceeds in its reaction by another mechanism, not by the enediol mechanism. To confirm this, the mass of the reaction product formed from MTR-1-P by B. subtilis MTR-1-P isomerase in D$_2$O buffer was measured (Table 2). The percentage of the intensity of (monoisotopic molecular mass + 1) to that of the monoisotopic molecular mass of MTRu-1-P formed by MTR-1-P isomerase in the presence of 99.9% D$_2$O was 9%. This value is very similar to the theoretical value (7.7%) and to that of the substrate (7%) (Table 2). On the other hand, the percentage of the reaction product of R5P isomerase was 32%, while that of the substrate R5P was 6%. This discrepancy clearly demonstrates that a proton is incorporated from the medium into the product in the R5P isomerase reaction which proceeds in its reaction by the enediol mechanism. It is known that some protons in the product formed through an enediol are from the medium even in isomerases that show the enediol mechanism and that the frequency of the incorporation of the proton from medium changes from enzyme to enzyme. For example, the frequency at which triose-phosphate isomerase transfers the proton on C2 to C1 is only 2% of the total isomerization reaction, while in the phosphoglucoisomerase reaction it is 50%. Considering that an almost negligible level of incorporation of deuterium from the medium is observed (Fig. 4 and Table 2), B. subtilis MTR-1-P isomerase catalyzes the isomerase reaction without adopting its enediol form as a reaction intermediate.

### Discussion

MtnA and its related proteins comprise a large family...
in nature and are widely distributed among organisms from bacteria to higher eukaryotes. The family is divided into two clades in the phylogenetic tree (Fig. 2A); one is for eIF2β and the other for eIF2β-α-LP including MtnA. As well as MtnA, Ypr118wp of yeast has been identified as an MTR-1-P isomerase.22) Although eIF2β-α-LPs of *Leishmania major* (PDB ID, 2A0U), *Thermotoga maritima* MSB8 (PDB ID, 1T9K), *Archaeoglobus fulgidus* DSM4303 (Protein Data Bank; PDB ID, 1T5O) have very similar protein structures to that of yeast Ypr118wp (PDB ID, 1W2W), the functions of these proteins and eukaryotic eIF2β-α-LPs in *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Hordeum vulgare*, and *Homo sapiens* have not been analyzed. However, IDI2, an eIF2β-α-LP in *H. vulgare*, involved in the synthesis of mugineic acid together with MtnD (1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase) in MSP, might be an MTR-1-P isomerase.8) E2B2, an eIF2β-α-LP in *T. kodakaraensis*, is RBP isomerase.11) These considerations suggest that eIF2β-α-LPs might be involved in the isomerase reaction of aldose-1-phosphate.

*B. subtilis* MtnA functioned as a dimmer enzyme (Table 1). Ypr118wp of yeast and eIF2β-α-LPs of *L. major*, *T. maritima* and *A. fulgidus* have been identified as homodimers from structural analyses.22) All of these homologues conserve the dimer-forming residues Glu-193 and Arg-195 (the amino acid residues are numbered according to the sequence in the *B. subtilis* enzyme, Fig. 2B).

The equilibrium constant of the isomerase reaction between MTR-1-P and MTRu-1-P was 6, favoring the formation of the latter sugar phosphate (Table 1). The free energy change of the reaction of MTR-1-P to MTRu-1-P at 35 °C was calculated to be 4.6 kJ mol⁻¹, indicating that the free energy of MTR-1-P is slightly smaller than that of MTRu-1-P.

Two mechanisms have been proposed for the aldose-ketose isomerase reaction; the cis-enediol(ate) and hydride transfer mechanisms.18) In the cis-enediol(ate) mechanism, a proton is abstracted from a carbon of the substrate by a base residue to make a cis-enediol(ate), and a proton from another base residue of the enzyme protein is donated to the counterpart carbon of the enediol(ate). Since these protons are in equilibrium with the protons of the water medium and are easily exchanged with deuterium in D₂O, the reaction product must incorporate the deuterium atom from the medium.19,20) The other mechanism is the hydride transfer mechanism, in which a hydride on a carbon is directly transferred to the neighboring cationic carbon.18,23,24) In the isomerase reaction of *B. subtilis* MTR-1-P isomerase, the product of the enzyme reaction in the presence of 99.9% D₂O showed no increase in mass after the
reaction, in contrast to the case with RSP isomerase (Table 2), and was virtually deuterium-free in NMR analysis (Fig. 4, inset). These features of the reaction were similar to those of xylose isomerase, which adopts the hydride transfer mechanism. These results suggest that \( B. subtilis \text{ MTR-1-P isomerase } \) follows the hydride transfer mechanism in its isomerization reaction. An interesting point, unique to \( B. subtilis \text{ MTR-1-P isomerase } \), is that this enzyme catalyzed the reaction without the participation of a metal ion in the reaction (Table 1).

A possible isomerase reaction is depicted in Fig. 5. To start the isomerase reaction, the enzyme must abstract the O2 proton using its base residue, and O2 and C2 form a carbonyl group by transferring O2 electrons to C2. A hydrogen atom in the form of a hydride then shifts from C2 to C1 with \( \delta^+ \). The proton abstracted from O2 initially is backed to O5 to open the ring structure and form MTRu-1-P. The possibly proton-abstracting residue might be Asp240, considering the location of this residue in the crystal of Ypr118wp of yeast. This residue is conserved in \( T. kodakaraensis \) RBP isomerase and other eIF2α-LPs (Fig. 2B). In the MtnA structure predicted by SWISS-MODEL software using eIF2α-Conn, L. \( maritima \) (1T9K) as the template, Asp240 is located in the same position as those in Ypr118wp and eIF2α-LPs from L. major, T. \( maritima \), and \( A. fulgidus \) (data not shown). We must wait for more precise structural analysis of crystals of substrate-binding MTR-1-P isomerase to enable further discussion.

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