Ribose-5-phosphate isomerase (Rpi) acts as a key enzyme in the oxidative and reductive pentose-phosphate pathways for the conversion of ribose-5-phosphate (R5P) to ribulose-5-phosphate and vice versa. We have determined the crystal structures of Rpi from Thermus thermophilus HB8 in complex with the open chain form of the substrate R5P and the open chain form of the C2 epimeric inhibitor arabino-5-phosphate as well as the apo form at high resolution. The crystal structures of both complexes revealed that these ring-opened epimers are bound in the active site in a mirror symmetry binding mode. The O1 atoms are stabilized by an oxanion hole composed of the backbone amide nitrogens in the conserved motif. In the structure of the Rpi-R5P complex, the conversion moiety O1-C1-C2-O2 in cis-configuration interacts with the carboxyl oxygens of Gly-108 in a water-excluded environment. Furthermore, the C2 hydroxyl group is presumed to be highly polarized by short hydrogen bonding with the side chain of Lys-99. R5P bound as the ring-opened reaction intermediate clarified the high stereoselectivity of the catalysis and is consistent with an aldose-ketose conversion by Rpi that proceeds via a cis-enediolate intermediate.

Ribose-5-phosphate isomerase (Rpi); EC 5.3.1.6) is ubiquitous throughout all living cells and highly conserved in amino acid sequences. Rpi acts as a key enzyme in the oxidative pentose-phosphate cycle where it catalyzes the reversible conversion of ribose-5-phosphate (R5P) to ribulose-5-phosphate (Ru5P). Rpi additionally plays a central role in the reductive pentose-phosphate cycle (Calvin cycle) of photosynthetic organisms. Rpi catalyzes the final step of the conversion of glucose-6-phosphate into ribose-5-phosphate, which is required for the synthesis of nucleotides. It also converts R5P to Ru5P in the final step to regenerate ribulose-1,5-bisphosphate as the acceptor of CO2 in the Calvin cycle. In the non-oxidative pathway of the pentose-phosphate cycle, R5P is the precursor of 5-phosphoribosylpyrophosphate, which is utilized for syntheses of amino acids such as histidine and tryptophan, purine and pyrimidine nucleotides, and NAD (1), whereas Ru5P is the riboflavin precursor (2). In the Calvin cycle, it has been shown that Rpi forms a functional multienzyme complex with five other enzymes, including ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), to catalyze the consecutive reactions on chloroplast thylakoid membranes in situ (3). The direction of the reaction catalyzed by Rpi is essentially driven by the R5P and Ru5P concentrations.

The catalytic mechanism of Rpi (Fig. 1A) is believed to initiate with a ring opening of the substrate followed by isomerization of the open chain form. Isotope exchange studies have suggested that the isomerization between ketose and aldose by triosephosphate isomerase (TIM), phosphoglucose isomerase (PGI), and Rpi involves a proton transfer between the C1 and C2 positions of the substrate via the cis-enediolate intermediate (a, b). The transferred proton in catalysis remains on the same side of the plane in the enediolate structure (6). Then, the proton abstracted by a single base is given back to the C1 or C2 position, resulting in the conversion of Ru5P to R5P and vice versa (Fig. 1A).

Recently, the crystal structures of Escherichia coli Rpi in both the apo form and in complex with the β-anomer furanose form of arabino-5-phosphate (A5P) have been determined (7, 8). Based on this structure, a ring-opening mechanism was proposed. In addition, the crystal structures of a tetrameric Rpi from the hyperthermophilic bacterium Pyrococcus horikoshii in the apo form and in complex with the inhibitor 4-phosphorylpyronic acid have also been reported and have elucidated aspects of the thermostability of this protein (25). However, to date, there is little structural information on the aldose-ketose isomerization mechanism of Rpi.

Here we report on the first crystal structures of Rpi from the extreme thermophile, Thermus thermophilus HB8, in complex with the ring-opened form of substrate R5P at 2-Å resolution and the open chain form of the C2 epimer inhibitor A5P (Fig. 1B) at 1.7-Å resolution (hereafter referred to as the ttRpi-R5P and ttRpi-A5P complexes, respectively) as well as the apo form at 1.8-Å resolution. The high resolution crystal structure of ttRpi-R5P complex suggests that the isomerization of Rpi proceeds via a cis-enediolate intermediate with the negatively charged O1 (Fig. 1C) stabilized by the oxanion hole.
MATERIALS AND METHODS

Protein Expression and Purification—Rpi with selenium-substituted methionine (SeMet) was prepared for structural determination by multiwavelength anomalous dispersion (MAD) phasing. The plasmid pET-11a (Novagen), which carries a gene encoding ttRpi under the T7 promoter, was supplied from the RIKEN Structurome Project (Kuramitsu and Yokoyama). The protein was over-expressed in the methionine auxotrophic E. coli strain B834(DE3)plysS (Novagen) grown in LeMaster medium with SeMet. After cell disruption (14 g) and removal of debris by centrifugation, the supernatant was heat-treated at 70 °C in 11.5 min. Then, ttRpi was purified with the SuperQ Toyo-pearl (Tosoh), Resource Q (Amersham Biosciences), and hydroxyapatite column chromatography and followed by a Superdex 75 column (Amersham Biosciences). Twenty-nine milligrams of purified protein was obtained. The sample showed a single band at 24 kDa on a 12.5% SDS-PAGE, and was also verified with N-terminal sequence analysis. Rpi in solution was detected as a homo-dimer with Superose 12 (Amersham Biosciences) gel filtration and dynamic light scattering (Protein Solution) analysis. The native proteins used in the R5P-bound complex were purified and verified in a similar way as the SeMet proteins.

Enzymatic Characterization—Reversible isomerization (R5P to Ru5P) enzymatic activity was observed from the absorption changes at 290 nm as described previously by Wood (9). The reaction mixture

| Table I | Data collection, structure determination, and refinement statistics |
|---|---|
| | Apo form (remote) | Apo form (peak) | Apo form (edge) | R5P complex | ASP complex |
| Data | | | | | |
| Wavelength (Å) | 0.9000 (remote) | 0.9791 (peak) | 0.9795 (edge) | 1 | 1.01 |
| Resolution (Å) | 1.80 | 1.80 | 1.80 | 2 | 1.74 |
| Space group | C2221 | C2221 | C2221 |
| Unit-cell parameters | | | | | |
| a (Å) | 62.10 | 62.01 | 62.54 |
| b (Å) | 61.97 | 62.01 | 62.54 |
| c (Å) | 131.34 | 131.17 | 131.25 |
| No. of reflections | 224,876 | 230,010 | 190,790 | 120,105 | 194,173 |
| Unique | 23,909 | 23,023 | 19,3 (3.4) | 42.7 (30.1) | 24.1 (7.7) |
| Completeness (%) | 99.8 (98.1) | 96.1 (76.4) | 97.6 (82.6) | 97.7 (82.6) | 100 (100) |
| I/σ (H) | 29.5 (7.3) | 23.2 (5.2) | 19.3 (3.4) | 42.7 (30.1) | 24.1 (7.7) |
| Rmerge (% | 29.5 (7.3) | 23.2 (5.2) | 19.3 (3.4) | 42.7 (30.1) | 24.1 (7.7) |
| FOM (SOLVE/RESOLVE) | 0.59/0.65 |
| Structural refinement | | | | | |
| Resolution (Å) | 43.9-1.80 | 50.0-2.00 | 44.2-1.74 |
| No. of residues | 225 (3-227) | 225 (3-227) | 225 (3-227) |
| No. of ions (chloride) | 3 | 2 | 2 |
| No. of waters | 249 | 296 | 310 |
| Rcryst (%) | 19.2 (21.3) | 18.8 (20.4) | 21.5 (23.4) |
| Rfree (%) | 21.5 (23.4) | 23.9 (25.9) | 21.5 (27.5) |
| R.m.s. deviation | 0.005 | 0.005 | 0.005 |
| Bonds (Å) | 1.3 | 1.3 | 1.4 |
| Angles (°) | 11.1 | 12.9 | 14.3 |
| Average B factors (Å²) | 11.1 | 16 (27.8) |
| Protein | 14 | 14.4 | 17.8 |
| Ligand | 26.8 | 29.1 | 31.1 |
| Ion | 94.4 | 94.4 | 94.9 |
| Ramachandran plot | 94.4 | 94.4 | 94.9 |
| Most favored (%) | 5.6 | 5.6 | 5.1 |

* Numbers in parentheses refer to data for the high resolution outer shell. The resolution ranges of their outer shells are 1.88-1.80 Å for the apo form, 2.09-2 Å for the R5P complex, and 1.82-1.74 Å for the ASP complex.

† FOM, figure of merit.

‡ Rcryst and Rfree = Σ||Fo| - |Fc|/Σ|Fo|, where the free reflections (10% of the total used) were held aside for Rfree throughout refinement.
Haemophilus influenzae (Swiss-Prot number P44725), spinach (Protein Research Foundation number 2219421A), human (Swiss-Prot number P49247), mouse (Swiss-Prot number P47968), and yeast (Swiss-Prot number Q12189) together with the secondary structure elements, i.e. motifs of ttRpi are aligned with the related enzymes from P. horikoshii C space-filled model. – (residues 85–91; violet rectangles) and /H9252 (anion (yellow magenta) and is highlighted in a monomer of Rpi. The three conserved ligand-binding motifs, is unique to the apo structure.

**FIG. 2. Overall structure of ttRpi.** A, the dimer of the apo form of Rpi shown in ribbon representation viewed along the 2-fold crystallographic axis of symmetry. One subunit is shown in sky blue, and the other is colored in red. Four chloride ions (light green) mediate a monomer-monomer interaction at the dimer interface, whereas the fifth chloride ion (magenta) is unique to the apo structure. B, overall structure of the apo form of a monomer of Rpi. The three conserved ligand-binding motifs, i.e. the phosphate binding P-motif (residues 27–34; green), the sugar binding S-motif (residues 85–91; violet), and the catalytic C-motif (residues 98–110; red), are highlighted. The oxyanion hole within the C-motif binds a chloride anion (magenta) and is highlighted in yellow. The conserved residues between the related proteins are highlighted in lemon yellow in the space-filled model. C, sequence alignments of the conserved ligand-binding motifs of Rpi with secondary structure elements. The ligand-binding motifs of ttRpi are aligned with the related enzymes from P. horikoshii (Swiss-Prot number O50083), E. coli (Swiss-Prot number P27352), Haemophilus influenzae (Swiss-Prot number P44726), spinach (Protein Research Foundation number 2219421A), human (Swiss-Prot number P49247), mouse (Swiss-Prot number P47968), and yeast (Swiss-Prot number Q12189) together with the secondary structure elements, i.e. α-helices (rectangles) and β-strands (arrows). The completely conserved residues between them are highlighted in yellow, and residues interacting directly with R5P and A5P are shown by red letters. The sequences of the P-, S-, and C-motifs are enclosed with green, violet, and red boxes, respectively. The residues involved in binding the chloride ion are indicated by magenta circles above the sequences. The oxyanion hole (residues 100–105) is represented by the yellow waved string.

contains 5 mM Rpi, 0.1 M NaCl, and 50 mM Hepes (pH 7.5). The increase of absorption was measured using R5P (Fluka) ranging from 0.5 to 30 mM at 50 °C. The kinetic parameters were calculated from Lineweaver-Burk plots and obtained by averaging three independent measurements. The inhibition by A5P was assayed at 50 °C in assay solution containing the various concentrations of A5P. The K<sub>i</sub> value was calculated from Dixon plots.

**Crystallization and Data Collections**—The screening of the crystallization conditions of SeMet Rpi was performed with the oil batch method (10) using the recently developed fully automatic protein crystallization and observation system “TERA” installed at the Highthroughput Factory at RIKEN Harima institute (11). The crystals were grown within 1 week at 18 °C. Crystals were flash-cooled in the cryoprotectant 20% (v/v) glycerol. X-ray diffraction data of the crystals was collected at 100 K up to 1.8 Å resolution using a charged coupled area detector (Rigaku Jupiter 210) at the beam line BL4-XU-1 (12) at SPring-8. The wavelength was set to 0.900 Å (remote), 0.9791 Å (peak), and 0.9795 Å (edge) with a crystal-detector distance of 200 mm. The crystals belonged to the space group C222, with the unit-cell parameters <i>a</i> = 62.10 Å, <i>b</i> = 61.97 Å, and <i>c</i> = 131.34 Å and one molecule in the asymmetric unit with a V<sub>N</sub> value (13) of 2.6 Å³/Da.

Crystals of R5P- or A5P-bound Rpi were obtained after soaking native or SeMet derivative crystals in solution containing 10 mM R5P (Fluka) or 10 mM A5P (Sigma), for 14 h at 15 °C. Data collections of two complexes were performed at a wavelength of 1 Å. The crystals of ttRpi-R5P and ttRpi-A5P complexes were isomorphous compared with uns soaked crystal and diffracted to 2- and 1.7-Å resolution, respectively. All diffraction images were processed, integrated, and scaled using the program HKL2000 (14)."
AMORe (20) using the apo form structure as the search model. The ligand models generated using the program Quanta (Accelrys) were fitted initially to electron density maps with coefficients $F_{o} - F_{c}$. Refinement of the ttRpiR5P complex was performed at 2-Å resolution with a working $R$ factor of 18.8% and a free $R$ factor of 23.9%. The ttRpiA5P complex was finally refined at a resolution of 1.7 Å to a working $R$ factor of 20.0% and a free $R$ factor of 21.5%.

The model quality for the three structures was verified by the program PROCHECK (21), which showed that all the main chain torsion angles were within the allowed regions. A summary of the statistics for structural determination is given in Table I. Fig. 2, A and B as well as Fig. 4, A and C and Figs. 5 and 6 were made using the programs MOLSCRIPT (22) and Raster3D (23).

RESULTS

The Characterization of ttRpi—The ttRpi sample used for crystalization was shown to exhibit normal enzyme activity. The kinetic parameters of ttRpi in the forward reaction using R5P as the substrate at 50 °C were $k_{cat}/K_{m}$ of 1072 ± 78 s$^{-1}$. $K_{m}$ of 1.63 ± 0.24 m, and $k_{cat}/K_{m}$ of (6.64 ± 0.91) × 10$^3$ M$^{-1}$ s$^{-1}$. The $K_{m}$ value was 0.89 ± 0.14 mM. The enzyme characteristics are consistent with those reported previously for E. coli and spinach Rpis.

Overall Structure of ttRpi—The crystal structure demonstrated that ttRpi exists as a homo-dimer (Fig. 2A), which is consistent with the molecular weights determined for the protein in solution by both gel filtration ($M_w$ = 47,300) and dynamic light scattering measurements ($M_w = 50,000$). The two monomers are related by a crystallographic 2-fold symmetry. The close interaction between the two monomers is stabilized by two chloride ions (Fig. 2A). There are also many hydrophobic interactions, four side chain salt bridges between the side chains of Arg-193 and Glu-197 and between Asp-75 and Arg-146, and a water molecule-mediated hydrogen bond network. The buried solvent-accessible surface is 2,547 Å$^2$ as calculated using the program GRASP (24).

Each monomer of ttRpi (Fig. 2B) is composed of two $\alpha/\beta$ domains with dimensions of $–45 \times 35 \times 25$ Å as seen in E. coli and P. horikoshii Rpis (7, 8, 25). The larger domain (residues 3–130 and 206–227) comprises a seven-stranded mixed $\beta$-sheet formed by six parallel (S3, S2, S1, S4, S7, and S13) and one anti-parallel $\beta$-strands (S14), four $\alpha$ helices (H1–4), and a three-stranded anti-parallel $\beta$-sheet (S5, S6, and S12). The active site is located within the larger domain with the architecture formed by loops S1-H2, S4-S5, and S6-H4 and H4. The small domain consists of a four-stranded, anti-parallel $\beta$-sheet (S9, S11, S8, and S12) and two helices (H5 and H6).

The structure of the larger domain of ttRpi is overlaid with those of E. coli and P. horikoshii with root mean square deviations of 1.3 Å for 140 Ca and 1.2 Å for 150 Ca, respectively. When the larger domains are superimposed, the small domain of ttRpi moves substantially with respect to the larger domain by a 3-Å shift of the H5 helix.

The Active Site Architecture with an Oxyanion Hole—Three highly conserved motifs (Fig. 2C) form the active site, i.e., the phosphate (P) and sugar (S) recognition motifs as well as the catalytic (C) motif over all the organisms. The P-, S-, and C-motifs are located in the loops of S1-H2, S4-S5, and S6-H4, respectively (Fig. 2, B and C). The active cavity is shallow and binds the dehydrated pentose-phosphates without any water molecules around C1 to C3 in both complex structures (Figs. 3 and 4, B and C). Most of the molecular surfaces of the ligand binding cavity are negatively charged with a noticeable positive patch formed by Lys-9 and Lys-126, which are important for allowing accommodation of the negatively charged phosphate moiety (Fig. 3).

In the apo ttRpi structure, a chloride ion (Cl$^-$ in Fig. 2B) is bound at the bottom of the active site cavity. The side chains of the residues positioned within the active site, including Asp-86, Asp-89, Lys-99, Glu-108, and Glu-112, form hydrogen bond networks with 10 bound water molecules (Fig. 4, A and D). The bound chloride anion interacts with five backbone amide nitrogens (Gly-100 to Leu-105) located at the center of the C-motif, the most conserved stretch of amino acids among the Rpis. Distances between the chloride anion and the amide nitrogens are in the range of 3.3–4.2 Å. Thus, this loop makes an oxyanion hole to stabilize the negatively charged atoms of the reaction intermediate during catalysis (Fig. 1A). Moreover, both the carboxyl oxygen atoms of Glu-108 form a bidentate interaction with the chloride ion (3 Å and 3.7 Å).

Bound R5P Interaction as Reaction Intermediate—In the structure of ttRpi-R5P complex, an extended ring-opened form of R5P provided the best fit to the electron density. This conformation differs from the ring-closed furanose form of A5P observed in the E. coli Rpi-A5P complex structure (Figs. 3 and 4, B and E). The torsion angle of O1-C1-C2-O2 of R5P is almost 0°, and it may represent the cis-enediolate intermediate (Fig. 1A). Contrarily, that of A5P is 93°. However, the possibility cannot be excluded that other forms, including aldose and/or ketose in addition to the enediolate, contribute to the electron density map of R5P, because we could not determine very accurate geometric parameters of the form of R5P from the 2-Å resolution data due to the model bias of the crystallographic refinement. In the best refined current model, the C1-O1, C2-O2, and C1-C2 bond lengths of R5P are 1.37, 1.39, and 1.51 Å, respectively, whereas those of A5P are 1.19, 1.38, and 1.61 Å. The atoms of O1-C1-C2-O2-C3 of R5P are in a plane with the oxyanion hole with distances between backbone amide nitrogens and O1 of 3.5 Å (Gly-100), 3.5 Å (Gly-102), 3.4 Å (Gly-103), 3 Å (Ala-104), and 3.6 Å (Leu-105). A prominent feature of the interaction between ttRpi and R5P is that the O1 of R5P should carry either a full or partial negative charge.

Another notable feature is the interaction between both the carboxyl groups of Glu-108 and O1-C1-C2-O2 of the bound R5P. The Oe2 carboxyl oxygen of Glu-108 is unusually close to both

![Fig. 3. Representation of the R5P binding pocket with surface electrostatic potentials.](http://www.jbc.org/)
C1 (3.3 Å) and C2 (3.2 Å), whereas Oe1 forms hydrogen bond interactions with O1 (3.1 Å) and O2 (2.8 Å) (Fig. 4E). The O2 oxygen of the bound R5P forms the short hydrogen bond with the side chain of Lys-99 (2.5 Å) as well as the backbone amide nitrogen of Gly-100 (3.0 Å). The distances between atoms indicate a tight C–H–O hydrogen bond between C1–C2 and the syn orbital of Oe2, including the putative hydrogen attached to C2 of R5P. This close interaction suggests the idea that Glu-108 acts in the proton transfer from C2 to C1 in the active site.

Although the negatively charged end of R5P requires Rpi to have the most conserved residues, the O3 and O4 atoms of R5P are recognized by Asp-86 and Asp-89 (S-motif) and Lys99 (C-motif) (Fig. 4, B and E). The O3 oxygen atom forms hydrogen bonds with the side chains of Lys-99 (3 Å) and Asp-86 (3.1 Å) and the backbone carbonyl of Thr-30 in the P-motif (2.7 Å) (Fig. 4, B and E). O4 also interacts with Asp-89 (2.7 Å), the backbone amide nitrogen of Gly-102 (3.4 Å) of the C-motif, and the water molecule W1 (2.6 Å).

Binding Mode of the C2 Epimer Inhibitor A5P—As for the ttRpi-R5P structure, it was possible to fit an extended ring-
The phosphate groups of both C2 epimers, R5P and A5P, bind at identical positions in ttRpi and are recognized by residues of the P-motif. The recognition manner of the phosphate group of the ligands is similar and involves hydrogen bonds to ordered water molecules bound at the same positions in both complex structures (Fig. 4, B and C). The phosphate group of R5P or A5P is situated at the N terminus of the H2 helix (Fig. 2, B and C), and a positive dipole moment from this helix may stabilize the negative charge of the phosphate group. The phosphate group of R5P or A5P forms direct hydrogen bonds with the side chains of Asp-86 and Asp-89, respectively.

Recognition of the Phosphate Group of C2 Epimers, R5P and A5P—The phosphate groups of both C2 epimers, R5P and A5P, bind at identical positions in ttRpi and are recognized by residues of the P-motif. The recognition manner of the phosphate group of the ligands is similar and involves hydrogen bonds to ordered water molecules bound at the same positions in both complex structures (Fig. 4, B and C). The phosphate group of R5P or A5P is situated at the N terminus of the H2 helix (Fig. 2, B and C), and a positive dipole moment from this helix may stabilize the negative charge of the phosphate group. The phosphate group of R5P or A5P forms direct hydrogen bonds with the side chains of Asp-86 and the backbone amides of Ser-32 and Thr-33 in the P-motif as well as the side chain of Lys-126. In addition, the amino group of Lys-9 also makes a water-mediated interaction with the phosphate group. The positively charged Lys-9 and Lys-126 may neutralize the negative charge of the phosphate group (Fig. 3). The residues that interact directly with the phosphate group are conserved among Rpis from various organisms (Fig. 2C). Furthermore, the electron density for the phosphate moiety is anisotropic as reflected in the distribution of temperature factors of the four phosphor-linked oxygens. This suggests direction-specific movement coplanar with the O1-C1-C2-O2 plane of the complex structures of ttRpi and are represented by residues of the enzyme.

Interaction Comparisons in the ttRpi-R5P and ttRpi-A5P Complexes, Including the apo Form—Both R5P and its C2 epimeric inhibitor, A5P, interact intimately with the same charged and polar residues such as Ser-32 and Thr-33 in the P-motif, Asp-86 and Asp-89 in the S-motif, and Lys-99 and Glu-108 in the C-motif as well as Lys-126 (Fig. 4, B and C). The configurations of carbon skeleton in R5P and A5P are in mirror symmetry to each other (Fig. 5). Therefore, the positions of O3 and O4 are inversely located in the bound R5P and A5P molecules, whereas the relative orientations of the O1, O2, and O5 atoms of both R5P and A5P can be superimposed. There is almost a counterclockwise round rotation of R5P relative to A5P by −65° for the P-O bond, −120° for the C5-C4 bond, and −135° for the C3-C2 bond in torsion angles. In the apo form of the enzyme, the binding sites of these functional groups in the bound C2 epimers are occupied by solvent molecules, including the chloride anion in the O1 site (Fig. 5).

In the ttRpi-A5P complex structure, the carboxyl of Glu-108 is farther from the C2 atom (3.5 Å for Oe1 and 3.8 Å for Oe2) than in ttRpi-R5P, and the carboxyl group cannot interact with the C2 hydrogen. Elevated temperature factors of the side chain of Glu-108 with respect to the surrounding structure suggests its flexibility within ttRpi. Although the overall structures can be superimposed with a root mean square deviation of 0.2 Å for all atoms, indicating a high level of similarity, the side chain of Glu-108 rotates ~20–30° around χ3 between the ligand complex structures and the apo structure (Fig. 5). The corresponding residue to Glu-108 in the P. horikoshii Rpi also showed the similar conformational changes to the apo form of ttRpi. Conformational variability of residues acting in catalysis and proton transfer is well documented in TIM (26), and the movement of Glu-108 is thus consistent with this side chain having a catalytic role.
FIG. 7. A proposed isomerization mechanism of R5P catalyzed by Rpi after the ring-opening step of the furanose form of R5P. Glu-108 abstracts a proton from the C2 position of the open chain form of R5P, yielding a cis-enediolate intermediate. O1 is likely to become an oxyanion. The oxyanion hole, composed of the backbone amide nitrogens from Gly-100 to Leu-106, would stabilize the negatively charged O1 in the intermediate and assist isomerization. The protonated Glu-108 side chain transfers the same proton to the C1 position of the intermediate. Glu-108 is also likely to abstract the proton from the C2 hydroxyl group, and this would result in a double bond formation between C2 and O2. The proton transfer to O1 may be carried out by Glu-108 concomitant with product release. Lys-99 is usually positively charged and thus plays a role in the promotion of polarization and the stabilization of the O2 in the intermediate.

DISCUSSION

Stereoselectivity for Catalysis—On the basis of the configuration of the bound R5P in the ttRpi-R5P complex structure, the abstracted hydrogen at the C2 of the substrate is expected to point to the carboxyl group of Glu-108 as the catalytic base with a distance of 3.2 Å between the C2 and Oe2 of Rpi. In the isomerization catalysis, the bound R5P in ttRpi is consistent with the reaction intermediate as cis-enediolate stabilized by the oxyanion hole rather than cis-enediol, because O1 should be negatively polarized by the oxyanion hole of the enzyme. It can be assumed that catalysis is followed by the donation of the abstracted proton by Glu-108 to C1 as the general acid with a possible small x3 rotation of Glu-108. Therefore, Glu-108 may act as the general base and acid in proton transfer between C2 and C1 via cis-enediolate as proposed in TIM (27) (Fig. 7).

Conversely, based on the ttRpi-A5P complex structure, A5P is able to bind to Rpi as an inhibitor rather than a substrate (28) because of the stereochemical hindrance of the C2 hydrogen atom. Both Glu-108 and Lys-99 recognize the hydroxyl group of the C2 of A5P forming hydrogen bonds as for R5P. However, the target C2 hydrogen is on the wrong side of the inhibitor plane for interaction with Glu-108.

For proper binding of the C2 epimers R5P and A5P in mirror mode, the ends of both ligands must be tightly associated with the oxygen atom O1 in the oxyanion hole of the C-motif, and the phosphate must be anchored by the P-motif (Figs. 4 and 5). Because the O1 atoms in both of the bound epimers occupy similar positions to the chloride anion in the oxyanion hole, O1 should be negatively charged. The O2 atoms are also suggested as being highly polarized, as evidenced by the short-distance of the hydrogen bond with the amino side chain of Lys-99. The cis-enediolate moiety is isolated from the solvent because of the close interaction with the active site (Fig. 4B) so that the aldose-ketose isomerization is catalyzed only by the carboxyl group of Glu-108 in a highly stereospecific manner.

Aldose-Ketose Isomerization Mechanism of Rpi—The well studied TIM and PGI mechanisms may help to provide a basis for the aldose-ketose interconversion mechanism of Rpi. Structural studies on eukaryotic TIM complexed with inhibitors such as phosphoglycerolhydroxamate (PGH) and 2-(N-formyl-N-hydroxy)-aminoethylphosphate (IPP) and the substrate dihydroxyacetone phosphate (DHAP) have supported the suggestion that the catalytic mechanism in the conversion of DHAP and glyceraldehyde-3-phosphate is a C1 and C2 proton transfer via a cis-enediolate intermediate (26, 29, 30). Glu-165 in TIM is proposed to be the catalytic base, whereas His-95 and Lys-12 probably facilitate proton transfer between O1 and O2. Based on the recently determined crystal structures of rabbit PGI, a multistep catalytic mechanism has been proposed that includes a ring opening by His-388 followed by isomerization of the glucose-6-phosphate and fructose-6-phosphate with Glu-357 as acid-base catalyst (31–34). Intermediate complexes of ttRpi and TIM were superimposed at the conversion moieties O1-C1-C2-O2-C3 of the ligands (Fig. 6). The putative catalytic residues Glu-108 and Glu-165 showed almost the same geometry in the ttRpi-R5P and TIM-DHAP complex structures, respectively. Thus, the glutamates must be essential for proton transfer between C2 and C1 during isomerization as was reported previously (25).

Prior to the conversion of R5P to Ru5P, the furanose form of R5P undergoes conversion to the open chain form as proposed (8). In solution, the substrate R5P is predominantly in the furanose form (~97%), whereas the open chain form is rare (~0.5%) (28). Based on the structure of E. coli Rpi in complex with the furanose form of A5P, Asp-86 was proposed to abstract the hydroxyl proton from O1 of the furanose form of R5P; then, the hydronium ion (H3O+) in solvent, acting as an acid, was proposed to donate a proton to the ring oxygen equivalent to O4 of the open chain form (8).

We propose the following isomerization mechanism on the basis of our Rpi-R5P complex structure. The carboxylate of Glu-108, acting as a general base, abstracts the hydrogen atom from C2, forming the cis-enediolate intermediate with the negatively charged O1, which is stabilized by the oxyanion hole. Then, the carboxylate of Glu-108, acting as a general acid, donates the proton to C1. However O2 becomes highly polarized by the formation of a hydrogen bond with the positively charged side chain of Lys-99. Therefore, the O2 proton may...
move to O1 via Glu-108 to form Ru5P followed by product release (Fig. 7).

Mutational analysis of Rpi from P. horikoshii showed that the corresponding residue to Glu-108 was essential for catalysis, and another mutational study of spinach Rpi showed that the corresponding residue to Lys-99 played an important role in both catalysis and ligand binding (25, 28). Based on the ttRpi-R5P structure, Lys-99 orients the Glu-108 side chain through salt bridges, contributing to the increase of the basicity of Glu-108, and is likely to be responsible for polarizing the C2 hydroxyl group ready for proton transfer between O2 and O1 via Glu-108.

It was thought that aldose-ketose isomerases, including Rpi, TIM, and PGI, catalyze similar C1 and C2 proton transfer mechanisms in the major sugar metabolic pathway of living cells (4). The oxynion hole-like architectures were reported to play a role in the isomerization of TIM and PGI. The N82 atom of Asn-10 and the N2 of His-95 in TIM were proposed to stabilize the intermediate through the interaction with the negatively charged O1 atom in catalysis (30). It was demonstrated that the side chain of Arg-272 and the backbone nitrogens of Gly-271 and Arg-272 in PGI were located at the proper positions to stabilize the negative charge of the O1 atom of the intermediate (31). In TIM, His-95 is postulated to mediate the proton transfer between the two oxygens on C1 and C2 so that this residue stabilizes the cis-enediolate intermediate (35). In PGI, it was proposed that a water molecule network may act as an acid-base catalyst for proton transfer between O1 and O2 (34). In Rpi, there is no residue corresponding to His-95 of TIM and no bound water molecule as seen in PGI in the vicinity of Glu-108 (Fig. 6). Therefore, we postulate that Glu-108 is the corresponding residue to Lys-99 played an important role to PGI activity (36). It remains to be seen how much secure catalysis proceeds stereospecifically in each isomerase of Rpi, TIM, or PGI with different architectures, although the isomerization should be quite highly stereoselective as described.

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