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D-Ribose-5-phosphate isomerase B from *Escherichia coli* is also a functional D-allose-6-phosphate isomerase while the *Mycobacterium tuberculosis* enzyme is not

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Summary

Interconversion of D-ribose 5-phosphate and D-ribulose 5-phosphate is an important step in the pentose phosphate pathway. Two unrelated enzymes with D-ribose-5-phosphate isomerase activity were first identified in *Escherichia coli*, RpiA and RpiB. In this organism, the essential 5-carbon sugars were thought to be processed by RpiA, while the primary role of RpiB was suggested instead to be interconversion of the rare 6-carbon sugars, D-allose 6-phosphate and D-allulose 6-phosphate. In *Mycobacterium tuberculosis*, where only an RpiB is found, the 5-carbon sugars are believed to be the enzyme's primary substrates. Here we present kinetic studies examining the D-allose-6-phosphate isomerase activity of the RpiBs from these two organisms, and show that only the *E. coli* enzyme can catalyze the reaction efficiently. D-Allose 6-phosphate instead acts as an inhibitor of the *M. tuberculosis* enzyme in its action on D-ribose 5-phosphate. X-ray studies of the *M. tuberculosis* enzyme co-crystallized with D-allose 6-phosphate and 5-deoxy-5-phospho-D-ribonohydroxamate (an inhibitor designed to mimic the 6-carbon sugar), and comparison to the *E. coli* enzyme's structure, allowed us to identify differences in the active sites that explain the kinetic results. Two other structures, those of a mutant *E. coli* RpiB in which histidine 99 was changed to asparagine, and of wild type *M. tuberculosis* enzyme, both co-crystallized with the substrate ribose 5-phosphate, shed additional light on the reaction mechanism of RpiBs generally.

Keywords: Ribose-5-phosphate isomerase; allose-6-phosphate isomerase; rare sugar; X-ray crystallography; Rv2465c.

Abbreviations

Rpi, D-ribose-5-phosphate isomerase; *Ec*RpiB, RpiB from *Escherichia coli*; *Mt*RpiB, RpiB from *Mycobacterium tuberculosis*; *Sp*RpiB, RpiB from *Streptococcus pneumoniae*; *Tm*RpiB, RpiB from *Thermotoga maritima*; r.m.s., root-mean-square; R5P, D-ribose 5-phosphate; Ru5P, D-ribulose 5-phosphate; All6P, D-allose 6-phosphate; Allu6P, D-allulose 6-phosphate; 5PRH, 5-deoxy-5-phospho-D-ribonohydroxamate; 5PRA, 5-deoxy-5-phospho-D-ribonate; 4PEH, 4-deoxy-4-phospho-D-erythronohydroxamate; PDB, Protein Data Bank (www.rcsb.org/).

Introduction

D-Ribose-5-phosphate isomerase (Rpi, EC 5.3.1.6) is one of the major enzymes in the pentose phosphate pathway, where it catalyses the interconversion of D-ribose 5-phosphate (R5P) and D-ribulose 5-phosphate (Ru5P). Two forms of this isomerase with no amino acid sequence similarity have been characterized in *Escherichia coli*, RpiA and RpiB.^{1,2} *EcRpiB* is not important under most conditions, both because it is not constitutively expressed, and because its rate of catalysis is much slower. It has been proposed that the principal role of this enzyme is instead to catalyze the isomerization of two 6-carbon sugars, D-allose 6-phosphate (All6P) and D-allulose 6-phosphate (Allu6P). The physiological significance has not yet been demonstrated but it is known that *EcRpiB* is associated with an operon that is necessary for survival when D-allose is the sole carbon source.^{3,4} The operon encodes a repressor (*alsR*), three ABC transporter proteins (*alsBAC*), a putative D-allose-6-phosphate 3-epimerase (*alsE*) and a D-allokinase (*alsK*). The structure of the periplasmic D-allose binding protein, AlsB, solved in complex with D-allose supports the idea that the transport system specifically recognizes that sugar.⁵ Activity measurements with AlsK have further shown that D-allose is phosphorylated with a k_{cat} of 17 s^{-1} and a K_m of 0.3 mM, consistent with values obtained for similar enzymes when processing their cognate sugars.⁶ The *rpiB* gene lies just upstream of this operon and is also regulated by the AlsR repressor, also known as RpiR.² However, *EcRpiB*'s ability to catalyze the desired isomerization reaction had not been demonstrated.

One particularly puzzling aspect of this theory, however, is the fact that D-allose and D-allulose (also known as D-psicose) are so-called “rare sugars”, not generally considered in cellular metabolism. Naturally occurring D-allose has been found in small amounts in ester glucosides, flavonoids and iridoids in various plant species.⁷⁻⁹ Humans cannot utilize D-allose, and other than *E. coli*, only certain archaea have been reported to use the sugar *in vivo*.¹⁰ Like *E. coli*, the archeons are proposed to incorporate D-allose

into metabolism by converting it to D-fructose 6-phosphate. *In vitro* experiments have shown that L-rhamnose isomerase from *Pseudomonas stutzeri* and RpiB from *Clostridium thermocellum* can convert D-allulose into D-allose.^{11,12} These studies were aimed at finding a cost-efficient means of producing D-allose, due to its reported medical applications.¹³ We do not know of any evidence that the organisms themselves utilize the enzymes in the metabolism of D-allose.

Like many bacteria, *Mycobacterium tuberculosis* lacks an RpiA sequence, having only an RpiB gene; in the genome, the sequence coding for *MtRpiB* has been annotated as *rv2465c*.¹⁴ It is presumed that the major role of *MtRpiB* lies within the pentose phosphate pathway, since ribose is of central importance in any living organism. We have previously solved the three dimensional structure of *MtRpiB* (PDB entry code 1USL)¹⁵ and demonstrated that it catalyzes the R5P isomerization with kinetic constants very similar to those reported for *EcRpiB*.¹⁶ Comparison with the *EcRpiB* structure (1NN4)¹⁶ indicated that while the two proteins are very similar, some key active site residues are different. X-ray structures of *MtRpiB* in complex with two substrate analogues (2BES and 2BET)¹⁷ allowed us to assign roles for most of the active site residues in the 5-carbon sugar reaction (Fig. 1). Kinetic studies with a compound designed to mimic the enediolate intermediate of the proposed All6P reaction, 5-deoxy-5-phospho-D-ribonohydroxamate (5PRH), have shown that a 6-carbon sugar analogue can inhibit the R5P isomerization in *MtRpiB* with a K_i of 0.4 mM.¹⁸

Here we present kinetic studies showing that *EcRpiB* can catalyze the conversion of All6P to Allu6P with reasonable efficiency, but *MtRpiB* cannot. All6P instead acts as an inhibitor of *MtRpiB* in the isomerization of R5P. X-ray structures of *MtRpiB* co-crystallised with All6P and 5PRH reveal differences in the active sites that explain the kinetic results. Two other structures co-crystallized with R5P, an *EcRpiB* H99N mutant, and wild type *MtRpiB*, shed additional light on the reaction mechanism of RpiBs.

Results

Activity of *EcRpiB* and *MtRpiB*

EcRpiB and *MtRpiB* were tested for activity on R5P and All6P. Confirming earlier results,^{15,16} the two enzymes have very similar k_{cat} and K_{m} for R5P. Allu6P production was followed using a novel assay based on the thiobarbituric acid (TBA) colorimetric method previously designed for monitoring D-fructose 6-phosphate production.¹⁹ Fig. 2a-d shows the experimental data, and Table 1 summarizes the catalytic constants derived. *EcRpiB* has a lower K_{m} for All6P than for R5P, although the latter is processed at a 10-fold faster rate. The calculated $k_{\text{cat}}/K_{\text{m}}$ differs only 4-fold when R5P and All6P are compared. *MtRpiB* is less efficient at isomerizing All6P, having a much higher K_{m} , as well as a drastically lower k_{cat} . Further, All6P acts as an inhibitor of the reaction when R5P is used as substrate, with a measured IC_{50} of 2 mM; Allu6P is a slightly worse inhibitor with an IC_{50} of 6.3 mM (Fig. 2e,f).

MtRpiB Complex Structures

X-ray data were collected for wild type *MtRpiB* co-crystallized with the substrate R5P, and two inhibitors, 5PRH and All6P. The three models were refined to resolutions of 2 Å or better (Table 2). Each asymmetric unit contained five molecules, organised as two complete dimers plus one lone subunit that forms a dimer by virtue of a 2-fold crystallographic symmetry axis. In all cases the protein could be traced from residue 2 or 3 to residue 158 or 160, out of the 172 residues in the sequence. The N-terminal His-tag was not seen in the electron density.

The two active sites of an *MtRpiB* dimer are located in clefts between the two subunits, with components drawn from both molecules; with the exception of Arg113, interactions with residue numbers less than 100 arise from the A molecule, and the others from the B molecule. A cartoon representation is shown in Fig. 3a. The electron density

in the active site was well defined and showed that the ligand was bound in all five molecules of the asymmetric unit in all three structures.

In the R5P complex (Fig. 3b), little or no furanose form of R5P was present; the electron density was most consistent with a mixture of the linear forms of both R5P and Ru5P. This observation is in agreement with the equilibrium constant value of ~ 0.33 reported for the isomerization of R5P.²⁰ At 1.65 Å resolution, it is not possible to investigate the relative proportions in detail, and both R5P and Ru5P were included in the refinement at occupancies of 0.5, which resulted in well-behaved maps, and temperature factors for the ligands that were similar to those of the surrounding protein (15-20 Å²). The manner in which R5P and Ru5P bind in the active site of *MtRpiB* is highly similar to the binding of the substrate analogue inhibitor 4-deoxy-4-phospho-D-erythronohydroxamic acid, 4PEH (Fig. 1).¹⁷ The two structures superimpose with an r.m.s. difference of 0.2 Å when all C^α atoms of the 5 protein molecules of the asymmetric unit are aligned. The active site residues are positioned in the same way, except in the immediate neighbourhood of the catalytic base Glu75, where the carboxylate moiety assumes slightly different angles in the two models (Fig. 3b).

The density seen in the active sites when *MtRpiB* was co-crystallized with 5PRH does not account for the whole ligand (Fig. 4a), and we concluded that the compound had been hydrolyzed to produce another good inhibitor, 5-deoxy-5-phospho-D-ribonate (5PRA, Fig. 1). 5PRA binds with the phosphate group in the same position as for the R5P, with interactions to residues Arg113 and His12 in the A molecule, and Arg137 and Arg141 in the B molecule. The proposed ring-opening catalyst, His102-B,¹⁷ interacts with O4, while O3 makes a hydrogen bond with Asp11-A; both interactions are seen in the other structures. Differences in binding occur at the top of the ligand, where O2 of 5PRA lies in the same position as C1 of the substrate and hydrogen bonds to Glu75-A, C1 of 5PRA points in the same direction as O2 of the substrate, and the carboxylate moiety interacts with Glu75 and backbone nitrogen atoms of the oxyanion hole (Fig. 4a).

The interactions of O2 and one of the carboxylate oxygens of 5PRA to the two carboxylate oxygens of Glu75-A involve short, tight hydrogen bonds of 2.7 and 2.4 Å, respectively. This implies that at least one of the partners in each case is protonated. These crystals were grown in the presence of 0.2 M Li₂SO₄, and two molecules of SO₄²⁻ are also found in the electron density. Neither ion appears to have an important role in the structure, although they may enhance dimer and crystallographic contacts.

In the *MtRpiB*/All6P complex, the density in the active site (Fig. 4b) reveals the α -pyranose (6-membered ring) form of the sugar phosphate, in contrast to the linear forms of the sugars discussed above. The ring oxygen (O5) of All6P assumes the position expected for the ring oxygen (O4) of the R5P. Asp11-A interacts with O4, which also has a hydrogen bond to the backbone nitrogen of Gly70-A. The group with most hydrogen bonding contacts is O3, which interacts with both oxygens of the Glu75-A side chain, and the backbone nitrogens of Gly70-A and Ser71-A. The side chains of Ser71-A and Asn103-B, as well as the backbone nitrogen of Gly74-A, have hydrogen bonds to O2. O1, observed as the α -anomer, interacts only with His102. Tyr46-A, a conserved residue in all *RpiBs*, lies with its ring almost parallel to the sugar ring, and ~4 Å away (Fig. 4b). Favourable stacking interactions between the aromatic side chain of the protein and the nonpolar surface of the sugar ring are expected.

The *EcRpiB*-H99N mutant

An *EcRpiB* His99 to Asn mutant constructed by site-directed mutagenesis exhibited a K_m for R5P of 0.8 mM, similar to the wild type enzyme, but k_{cat} was reduced to 2 s⁻¹, as compared to 52 s⁻¹ (Table 1).

EcRpiB-H99N was crystallized together with the substrate R5P, in a space group different from that observed for the original apo *EcRpiB* crystals.¹⁶ Six molecules were located in the asymmetric unit by molecular replacement, arranged as 1 dimer and 1 tetramer. The final model was refined to 2.1 Å. The electron density allowed the structure

to be traced from residue 1 (not including the 6-His tag) to 146 or 147 in all 6 molecules (out of 149 residues in total). Data collection and refinement statistics are summarized in [Table 2](#).

In each active site, density strongly suggests a mixture of α - and β -furanose forms of R5P, together with linear R5P or Ru5P. Interestingly, the two active sites in each dimer have different species bound. One active site (molecules A, D and F) has primarily the linear form and very little furanose ([Fig. 5a](#)). The other subunit of each dimer (molecules B, C and E) has mostly furanose sugar, but with a considerable fraction of the linear form ([Fig. 5b](#)). The distinctions were even clearer when density from the equivalent active sites was averaged. The differences can be traced back to crystal packing and involve different conformations of the His17 side chain located on the surface of the first helix. In the A, D and F molecules, the histidine points toward Phe13-A, possibly shielding it from water. In the B, C and E molecules, this histidine points straight out toward a symmetry related molecule's Arg133 ([Fig. 5c,d](#)). This arginine is involved in binding the phosphate group of the ligand; in the active sites that have the His17/Arg133 interaction, the predominant form of the substrate is the furanose. Other residues in the same helix, glutamates 18 and 25, could form salt links to Arg40 of the neighboring active site, although the density for this arginine is not well defined. Presumably it is interacting with the phosphate of the ligand some of the time, as well as with the neighboring glutamates.

In the β -furanose form of R5P, O1 interacts with Asn100-A and is close to one of the carbon atoms of Tyr43-B (3.3 Å; equivalent to *MtRpiB* Tyr46). The hydroxyl group is ~2.9 Å from a water molecule that is within hydrogen bonding distance of His134 and O4. O4 has an additional hydrogen bond to Asn99-A. O3 interacts with the backbone nitrogen of residue 67-B and the carboxylate of Asp9-B, while O2 is next to Cys66-B. An α -furanose has the same interactions apart from O1, which in this case is ~3.4 Å from Asn99.

Comparisons of available RpiB structures

Comparison of the mutant *EcRpiB* AB-dimer to the original wild type *EcRpiB* AD-dimer (PDB entry 1NN4) shows that the r.m.s. difference is only 0.3 Å for 287 C $^{\alpha}$ atoms using a cutoff of 0.8 Å. The small changes include several residues with alternative side chain conformations. The most striking difference is the conformation of Thr68, which now has its OH group oriented in the same direction as that of the equivalent serine residue in *MtRpiB*. We believe this to represent a minor error in the original structure.

The various *MtRpiB* models are also very similar, with r.m.s. differences between 0.15 and 0.36 Å when comparing the AB dimers. As mentioned previously the *MtRpiB* with R5P is very similar to PDB entry 2BES, the complex of the same enzyme with 4PEH.

Superpositioning of the *EcRpiB*-H99N model onto the different *MtRpiBs* gives r.m.s. differences of ~ 1 Å with 265 C $^{\alpha}$ atoms of the AB dimers matching within a cut-off of 2 Å (Fig. 6a). The only other RpiBs in the PDB represent the enzyme from *Thermotoga maritima* (*TmRpiB*; entry code 1O1X²¹) and *Streptococcus pneumoniae* (*SpRpiB*; entry code 2PPW, unpublished data). The *TmRpiB* crystal structure only has one molecule in the asymmetric unit, which forms a dimer through crystallographic symmetry. The subunit when aligned to *EcRpiB* has an r.m.s difference of 0.8 Å, with 137 C $^{\alpha}$ atoms matching within a cut-off of 2.0 Å. With *MtRpiB*, such an alignment resulted in an r.m.s. difference of 0.9 Å for 129 C $^{\alpha}$ s, with two gaps in the alignment. *SpRpiB* does not align as well to the two RpiBs studied here, and the sequences have low identity. However, the general topology is very similar, with the main differences in the loops connecting the secondary structural elements; the C-terminal end also has additional α -helices that extend the fold of the subunit.

Comparisons of RpiB active sites

When *Ec*RpiB and *Mt*RpiB are superimposed, the transformation brings the C $^{\alpha}$ atoms of the N-terminal end of the helix at the bottom of the active site cleft very close together. The backbone nitrogen atoms of the helix (residues 70-74 in *Mt*RpiB) are thought to be important for stabilizing the *cis*-enediolate intermediate of the reaction (Fig. 1). The active-site base, a glutamic acid in *Mt*RpiB and a cysteine in *Ec*RpiB, originates from different positions in the sequence; Glu75 of *Mt*RpiB lies at the N-terminal end of the helix, while Cys66 of *Ec*RpiB is located in the loop preceding it. However, as noted previously,¹⁵ the side chains terminate in the same position within the active site (Fig. 6b). The side-chain hydroxyl groups of Ser71 of *Mt*RpiB and Thr68 of *Ec*RpiB (the proposed proton shuttle between O1 and O2 of the substrate in the isomerization step) lie on top of each other, as do Asp11/9-A (naming for *Mt*RpiB/*Ec*RpiB, respectively), Tyr46/43-A and Arg141/137-B. Differences are seen in the arrangement of histidines 12/10-A, 102/99-B and 138/134-B, and Arg137/133-B; most residues of *Ec*RpiB are shifted by 1-2 Å relative to *Mt*RpiB, although the C $^{\alpha}$ atoms of His10/12 are closer. The shifts seen in the residues of the B molecule appear to originate from the substitution of Gln94 in *Mt*RpiB with Tyr91 in *Ec*RpiB. The bulky tyrosine side chain causes Leu98/Leu95 to take on different conformations. In *Ec*RpiB, the leucine is forced down toward Asn99 (the mutated residue), which in turn pushes His134 down (Fig. 6b). In all *Mt*RpiB structures presented here, Arg113-A interacts with the phosphate group; in the original apo structure (PDB entry 1USL), this residue had two different conformations, one pointing toward the phosphate and one pointing out into solution. In *Ec*RpiB, the equivalent Arg110 is not involved in phosphate binding (Fig. 6b). Although the phosphate group interacts with a similar set of positively charged residues, its density places this part of a bound ligand slightly further away from the catalytic groups in *Ec*RpiB when compared to the situation in all of the *Mt*RpiB complexes.

In *TmRpiB* the active site residues align reasonably well with those of the other two RpiBs. The active site cysteine, which aligns perfectly with Cys66 of *EcRpiB*, was found to be oxidized, and so was modeled as a cysteine sulfonic acid.²¹ This causes Thr67 to move slightly. Tyr91 of *EcRpiB* is replaced with Asp90, but Arg93 in *TmRpiB* causes a slight downward movement of Leu94 compared to this leucine in *MtRpiB*. Thus His98 and His133 lie between the equivalent histidines in *MtRpiB* and *EcRpiB*. Arg132 points out into solution, as there is no phosphate in the active site to hold it in position. Arg136 is slightly further away from the location where the phosphate would be expected to bind.

The residues lining the active site of *SpRpiB* are more variable. However, the C^α atoms at the N-terminal end of the helix in the active site (with the sequence CGTGVG), which also include the catalytic cysteine and threonine residues, align perfectly. Asn100 and Tyr43 are also conserved. Asp9 of the *E. coli* enzyme is replaced by a glutamate in *SpRpiB*, and His10 is missing, although a serine (Ser10) in this position could have interactions with the phosphate moiety of a ligand. A further candidate for a phosphate-binding residue is Arg147 of a symmetry related molecule. His99 and His134 are absent from the *S. pneumoniae* sequence but a glutamic acid (Glu151, again from the symmetry related molecule) is found in the equivalent position, approximately between the two histidines.

Sequence comparisons

BLAST²² searches of all completed genomes where protein sequences have been deduced (777 bacterial, 45 archaeal and 48 eukaryotic, at the time of writing), using the *E. coli* RpiA and RpiB sequences as probes, showed that many organisms, mainly bacteria, have both genes. Both types of RpiB sequence, as represented by *MtRpiB* and *EcRpiB*, are well represented. The most significant difference between the two sub-families is the nature of the catalytic base (glutamate or cysteine) and its position in the sequence. Other

residues implicated in catalysis are well conserved, except for Ser71 (*MtRpiB* numbering). In *EcRpiB* this residue is a threonine, but serines are also observed in *E. coli*-like Rpis. *MtRpiB*-like enzymes are mainly found in the actinobacterial phylum, while the *EcRpiB*-like protein appears scattered through all the other bacterial families. *TmRpiB* belongs to the *E. coli*-like family with a sequence identity of 46% to *EcRpiB*. No RpiA is observed in its genome. The same can be said for the genome of *Clostridium thermocellum* where the RpiB has a sequence identity of 57% to *EcRpiB*. In archaea, *Thermofilum pendens* is the only representative with a sequenced genome that has an *EcRpiB*-like enzyme. All others only have an RpiA. Unfortunately no Rpi sequences could be identified for the archaeal species *Aerobacter aerogenes* that can survive on allose;¹⁰ few of this organism's genes have been sequenced as yet. Most eukaryotes have only an RpiA, although there are some examples of fungal species that also have an *EcRpiB*-like enzyme. *Entamoeba histolytica*, *Trypanosoma* species and *Giardia lamblia* are noteworthy as eukaryotes that only have an *EcRpiB*-like protein. *Magnetospirillum magnetotacticum*, *Robiginitalea biformata*, *Blastopirellula marina* and some organisms within actinobacteria have both types of RpiB, but no RpiA. *Rhodococcus* sp. RHA1 and *Reinekea* sp. MED297 are the only two organisms known at present to have an *MtRpiB* together with an RpiA.

Apart from *E. coli*, only two other organisms (with completed genome sequences) appear to have the *als* operon: *Yersinia intermedia* and *Haemophilus somnus*. Of these, the operon of *Y. intermedia* is most similar to that of *E. coli*, with the same gene organization, and protein sequence identities ranging between 60 and 90%.

Discussion

Aggregation state

In the first *EcRpiB* apo structure,¹⁶ the enzyme was found as a tetramer, an observation that was also supported by dynamic light scattering experiments. However, the enzyme had previously been found to behave as a dimer during size exclusion chromatography.^{1,2} The tetramer seen in the crystal was thought to be partly due to the long N-terminal His-tag, which would promote dimer-dimer interactions. The high protein concentrations used in crystallization and light scattering experiments would also be expected to favor tetramer formation. In the present study, a construct with a shorter N-terminal His-tag was used; the protein still behaved as a dimer during size exclusion chromatography. However, the dimer-dimer interactions that build the tetramer are still observed in the new crystal form, resulting from both non-crystallographic and crystallographic symmetry. We conclude that both dimer and tetramer are likely to be relevant species for this enzyme in solution.

Ring opening

The present study with the *MtRpiB*-R5P complex demonstrates that the linear substrate or product does indeed bind in the same manner as the high-energy intermediate analogue studied earlier, 4PEH.¹⁷ It was considered likely that RpiB is involved in opening the R5P furanose ring, since only 0.1% of the sugar is in the linear form in solution.²³ We previously proposed that His102 of *MtRpiB* catalyzes this opening by donating a proton to O4 of the furanose. The *EcRpiB*-H99N mutant presented here is a conservative substitution of the equivalent histidine. Consistent with the proposed role, the mutant protein had a K_m similar to the wild type, but a 26-fold lower k_{cat} (Table 1). A comparable mutation in *Trypanosoma cruzi* RpiB, where His102 was changed to alanine, resulted in an enzyme with K_m s for both R5P and Ru5P similar to the wild type enzyme.²⁴ The k_{cat} was 10-fold lower in the forward reaction, whereas k_{cat} was almost the same for the reverse reaction. This implies that the mutant enzymes have no difficulty in performing the isomerization when they are given a linear substrate, but that they are slower if most

of the substrate is in the ring form, as they must wait for an open R5P molecule to diffuse into the active site.

These conclusions are supported by comparison of the structures of wild type *MtRpiB* and *EcRpiB*-H99N co-crystallized with R5P. In the wild type protein, only linear forms of the sugar were observed in the active site (Fig. 4a), while in the mutant protein the observed density corresponds to α - and/or β -ribofuranose 5-phosphate as well as linear molecules (Fig. 5a,b). The docking of either furanose in the active-site density of *EcRpiB*-H99N places O4 of the sugar in close proximity to Asn99. In *MtRpiB*, O4 of both linear sugars lies next to the equivalent His102 (Fig. 6b). The role of His99/His102 as a facilitator of ring opening thus seems clear. At the same time as the histidine donates a proton to O4 of the furanose, a second proton must be accepted from O1. Docking the β -furanose in the *EcRpiB*-H99N active site places O1 less than 3 Å from a water molecule that lies close to His134 and Arg137. This water molecule could accept the proton from O1. An equivalent water molecule is found near His138 and Arg141 in *MtRpiB*. The α -anomer would situate O1 within hydrogen-bonding distance of Asn100/Asn103, which cannot accept a proton. The only candidates for proton acceptors in this case are His99/102 itself and the water molecule. Thus for the α -anomer, it is possible that His99/102 protonates O4, and deprotonates O1, in a concerted mechanism. An alternate scenario is that the water could donate a proton to O4, and His99/102 accept that from O1.

Substrate specificity

The kinetic data show that *EcRpiB* is a much more effective catalyst than *MtRpiB* of the All6P-Allu6P interconversion (Table 1). Indeed, the 6-carbon sugar is an inhibitor of *MtRpiB* when R5P is substrate. Our numerous attempts to obtain the structure of a complex of *EcRpiB* with All6P resulted only in uninterpretable electron density in the active site. Presumably a mixture of sugar forms is present, similar to the results

described above for *MtRpiB* when crystallized together with R5P. By contrast, in the *MtRpiB*/All6P complex, we observe very clear density for the pyranose that is expected to be most common for All6P in solution. Although in D₂O solution the ratio of α : β -allopyranose is about 1:3,²³ the density in the active site of *MtRpiB*/All6P shows only the α -anomer (Fig. 4b). The preference is explained by the fact that the α -anomer has one additional hydrogen bond to protein (involving His102) compared with a docked β -anomer. The ring oxygen (O5) of All6P assumes the position expected for the ring oxygen (O4) of the substrate R5P. O5 also forms a hydrogen bond to a water molecule interacting with His138, although the distance to His102 is greater than in the *EcRpiB*-H99N structure. Thus, we conclude that His102 and the water molecule near His138 should be able to catalyze opening of the All6P pyranose ring. The equivalent groups will have the same roles in *EcRpiB*. In *MtRpiB*, the problems seem to arise at the next step. Once opened, the sugar phosphate is too long to fit into the *MtRpiB* active site with productive interactions to Glu75 and Ser71. In the All6P structure, Glu75 is already forced up, away from the ligand, incorrectly situated to catalyze the isomerization (Fig. 4b). We earlier proposed that the long, flexible arginine and histidine side chains that interact with the phosphate moiety of a substrate/inhibitor could swing further away from the catalytic residues, and so accommodate the longer 6-carbon substrate.¹⁸ We see no evidence from the combined structures that such is the case. However, differences that appear relevant are observed in the structures of *EcRpiB* and *MtRpiB*, which can be traced back to two residue substitutions. A Tyr91/Gln94 (*Ec*/*Mt*) substitution allows Leu95 of *EcRpiB* to take on a different conformation compared to the equivalent Leu98 in *MtRpiB*, bringing it down toward His99. This results in a shift of the two histidines (99 and 134) even further down toward the active site opening compared to their equivalents in *MtRpiB* (Fig. 6b). This shift places them in a good position for opening a furanose or pyranose ring placed further along the active site. The second change involves Arg40 in *EcRpiB*, which is replaced by Asp43 in *MtRpiB*. This residue is located in a loop at the

very entrance to the active site (Fig. 6). In *MtRpiB*, the aspartic acid consistently points out into the surrounding solution, repelled by the overall negative charge of the phosphate. In the available *EcRpiB* structures, Arg40 frequently has poorly resolved density, although when it is ordered it is seen to bind the phosphate group. Minor differences between *EcRpiB* and *MtRpiB* include slight shifts in the phosphate-binding residues His10/His12 and Arg133/Arg137, which might make them better adapted to binding a phosphate molecule slightly farther away from the catalytic residues. However, these movements are less clearly linked to an amino acid substitution and may be simply due to natural flexibility in these residues.

Attempts to produce a structure of *EcRpiB* bound to 5PRH, a compound designed to mimic the enediolate intermediate of the All6P reaction, were unsuccessful. Similar experiments with *MtRpiB* produced clear electron density for a hydrolysis product, 5PRA. 5PRA lacks the terminal N-hydroxylamino group that mimics the C1H(=O) group of the All6P substrate (Fig. 1). The crystallization conditions and long time needed for the experiment are such that the hydrolysis could reasonably occur. Comparable breakdown of 5-deoxy-5-phospho-D-arabinohydroxamic acid to give 5-deoxy-5-phospho-D-arabinonate was reported to take place in co-crystallization experiments with phosphoglucose isomerase.²⁵ It would appear that *MtRpiB* selectively binds the shorter R5P-like molecule (Fig. 4a), in preference to the original All6P-like compound.

The isomerization of All6P and Allu6P in *EcRpiB* is thought to proceed in much the same manner as the isomerization of the 5-carbon sugar phosphate. The ring will presumably be opened by His99 and the water molecule. Once opened, the linear substrate will bind with its top part in the same orientation as observed for R5P in the *MtRpiB* structure, and consistent with the docking of the linear substrate in *EcRpiB*-H99N. Binding of the phosphate group lower down in the active site allows the 6-carbon molecule to be placed in this way. The proposed catalytic base, Cys66, will then accept a proton from C2, and Thr68 will catalyze the proton transfer between O1 and O2 of the

enediolate intermediate. The positive helix dipole near residues 67-71, together with Asn100, will help to bind and so stabilize the high-energy intermediate. Tyr43, which presumably has some favourable stacking interactions with the ring form of the substrate that will assist substrate binding (Fig. 4b shows this interaction in *MtRpiB* with Tyr46), could possibly have an additional role in fixing the position of Asn100 *via* a hydrogen bonding interaction. In the final step of catalysis, Cys66 returns a proton to C1.

The biological significance of the All6P-Allu6P isomerization remains a mystery. From exhaustive sequence searches, we conclude that all completed genomes (covering nearly 1000 organisms) have at least one type of Rpi. Where RpiA is found, it is assumed that the main role for the enzyme is to interconvert R5P and Ru5P. Where only an RpiB exists (regardless of the sub-family) similar conclusions can be drawn with regard to its main function. Slow-growing organisms are best suited to this latter scenario, because of the lower catalytic efficiency of RpiBs. In cases where an organism has two different Rpis, one of them could have another, secondary function. The combination of an *MtRpiB*-like enzyme and an RpiA is apparently very rare. Since *MtRpiB*-like enzymes are most efficient when processing the 5-carbon sugars, that combination offers no particular advantage; the RpiA is already quite adequate for the task. When two enzymes are present, either an RpiA/*EcRpiB* combination or *MtRpiB*/*EcRpiB* combination, the *EcRpiB*-like enzyme is likely to take on a different role in metabolism. The most relevant substrate is probably a 6-carbon sugar, although not necessarily All6P. At present, the best evidence that All6P is the true biological substrate of *EcRpiB* is found in the structure of the D-allose binding protein in the associated operon;⁵ typical of complexes between such transport receptors and their cognate ligand, the interactions observed are numerous, tightly networked and suggestive of high specificity. The only other organisms where a complete *als* operon could be identified were *Y. intermedia* and *H. somnus*. *Y. intermedia* is mainly associated with waste water although it is found in humans with gastrointestinal disorders. *H. somnus* can cause disease in humans and cattle; it inhabits

mucous membranes and is found in the blood stream. As *E. coli* resides in the gut, it seems possible that all three of these organisms are adapted to utilizing allose that accumulates through mammals' inability to metabolize the sugar. By virtue of a high-affinity transport system, the organism can accumulate the sugar, even when it is present at very low concentrations in the media. Links to pathogenicity provide for tempting speculation, but the bias of the currently available genome data toward pathogens suggests that this idea must be treated with caution.

Experimental Procedures

Protein cloning, production and purification

EcRpiB was cloned into the pBAD/TOPO™ vector (Invitrogen) using the pCRT7/TOPO™ construct previously described¹⁶ as the DNA template. An N-terminal 6-His tag was added to the sequence (GenBank accession number X82203, residues 1703 to 2147) using the primer 5'-ATGGCCCATCATCATCATCATCTTCTGGT-3'. The gene for thioredoxin, which is present in the original pBAD vector, was removed by restriction enzyme digestion with NcoI. The QuickChange® Site-Directed Mutagenesis Kit from Stratagene was used to construct the H99N mutant using the primers 5'-GCGCAACTTTGCCGGCAGAATAACGACACCAACGTGC-3' and the inverse complement 5'-GCACGTTGGTGTCTGTTATTCTGCCGCGAAAGTTGCGC-3' with Pfu Turbo® polymerase. The mutation was verified by DNA sequencing at the Uppsala Genome Center. Plasmids were transformed into *E. coli* Top10 cells (Invitrogen) by heat shock. Recombinant protein was produced at 37 °C in a 1 L culture of Luria broth supplemented with 50 µg/mL ampicillin, which was induced with L-arabinose at 0.2% (w/v) once the A₅₅₀ had reached 0.8. Cells were harvested after 4 h and stored at -20 °C.

EcRpiB was purified in much the same manner as *MtRpiB*, as previously described.¹⁵ Differences from the original protocol include cell lysis at 2 kBar using a cell

disruptor from Cell Disruption Systems, and a final purification step by size exclusion chromatography, with a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 10 mM β -mercaptoethanol, 0.1 mM EDTA. For crystallisation the protein was concentrated to 8 mg/mL and the buffer changed to 20 mM Tris-HCl (pH 8.3), 35 mM NaCl, 10% glycerol, 5 mM MESNA, 0.1 mM EDTA.

The *MtRpiB* protein was cloned, over-expressed and purified as described previously¹⁵ with the addition of a final purification step by size exclusion chromatography, in a buffer containing 20 mM Tris-HCl (pH 8.3), 150 mM NaCl, 10% glycerol, 10 mM β -mercaptoethanol, 0.1 mM EDTA. The protein was concentrated to 7.5 - 12 mg/mL during which the NaCl concentration in the buffer was decreased to 35 mM.

Kinetic assays

A spectrophotometric assay described previously,²⁰ in which the production of Ru5P is monitored as a change in absorbance at 290 nm, was used to characterize the *EcRpiB*-H99N mutant and both wild type *Rpis*. Kinetic constants were estimated from linear regression using Hanes-Woolf plots.

A spectrophotometric assay involving TBA was developed to test the conversion of All6P to Allu6P. The method^{26,27} was developed recently in one of our laboratories for the detection and quantification of D-fructose 6-phosphate.¹⁹ We found that TBA reacts similarly with Allu6P²⁸ which, following dehydration and dephosphorylation in the presence of hydrogen chloride, gives rise to 5-hydroxymethylfurfural, which in turn reacts with TBA to yield the characteristic yellow adduct. The adduct absorbs strongly at 438 nm ($\epsilon = 27800 \pm 100 \text{ M}^{-1} \text{ cm}^{-1}$). Under the same conditions, All6P²⁹ produced a TBA adduct with an extinction coefficient of 690, ~40 times smaller than that for Allu6P. Therefore, a blank without enzyme was included to correct for the absorbance due to All6P. Typically, the assay mixture contained 50 mM Tris-HCl (pH 7.5), 0.8 to 8 mM All6P and 5 mM MESNA, in a final volume of 1 mL. Following pre-incubation at 37 °C

for 5 min, the reaction was initiated by the addition of 52 nM *Ec*RpiB (5 μ L of a 0.165 mg/mL solution in buffer) or 316 nM *Mt*RpiB (5 μ L of a 1.1 mg/mL solution). Precisely 2 min later (established to represent initial rate conditions), the reaction was stopped by the addition of 1 mL of concentrated HCl and 1 mL of a 20 mM TBA solution (prepared fresh daily by dissolving 144.15 mg of TBA in 50 mL concentrated HCl), yielding a total volume of 3 mL. After heating to 80 °C for 6 min, the reaction mixture was cooled to room temperature in an ice-water bath. After 3 h, the amount of Allu6P formed was estimated from the absorbance at 438 nm. Kinetic constants were estimated from linear regression using Hanes-Woolf plots. Each point reported is the average obtained from 2-8 measurements. IC₅₀ values of All6P and Allu6P against *Mt*RpiB were measured using a constant R5P concentration of 2 mM.

Crystallization, data collection, data processing and refinement

*Mt*RpiB and *Ec*RpiB-H99N were generally co-crystallized with the different compounds by vapour diffusion in hanging drops of 1 μ L protein and 1 μ L reservoir solution at 20 °C. Compound and protein solution were mixed together 5-10 min prior to setting up the crystallization drops. *Mt*RpiB (0.35 mM) together with 25 mM 5PRH crystallized in 15% PEG 8000, 0.1 M MES pH 6, 5% PEG 1000, 0.2 M Li₂SO₄, and with 10 mM All6P in 1 M tri-Na citrate, 0.1M Na cacodylate pH 6.5. *Mt*RpiB (0.22 mM) co-crystallized with R5P (2 mM) appeared in sitting drops in the Wizard 96-well crystallization screen (Emerald Biosystems) condition F6, 20% PEG 3K, 0.1 M Tris pH 7, 0.2 M Ca acetate. The *Ec*RpiB mutant (0.24 mM) with 10 mM R5P was crystallized in 20% PEG 3350, 0.1 M MES pH 6. For *Mt*RpiB needle-like crystals of dimensions 0.3 mm x 0.03 mm x 0.01 mm appeared after about one week, with slight improvements in size seen when the drops were streak seeded. *Ec*RpiB-H99N crystals grew in 2-3 days as chunky hexagonal plates of dimensions 0.2 mm x 0.2 mm x 0.03 mm. After transferring crystals to a drop of a cryo-protectant (10% ethylene glycol, 10% glycerol, ~80% well solution and 10 mM

ligand) they were flash cooled in liquid nitrogen. X-ray data were collected at 100 K (Oxford Cryosystems) at the European Synchrotron Radiation Facility, ESRF, Grenoble, France. All data were indexed with MOSFLM³⁰ and processed in SCALA³¹ via the CCP4 interface.³² Information related to data collection is presented in [Table 2](#).

EcRpiB-H99N was solved by molecular replacement with AMoRe³³ using the functional dimer of the original *EcRpiB* structure (PDB code 1NN4 with water molecules and histidine tag removed) as a search model. *MtRpiB* space group dimensions were similar enough for the five molecules in the original structure (PDB code 1USL with the water and phosphate ligands removed) to be placed by rigid body refinement in Refmac5.³⁴ The models were then subjected to restrained refinement, and the resulting maps inspected for bound ligand density. Water molecules were placed after cycling with ARP/wARP.³⁵ R5P and 5PRA ligands of *MtRpiB* were placed with the ARP/wARP ligand tool³⁶ while others were placed manually in O.³⁷ Rounds of rebuilding and refinement resulted in the final models described in [Table 2](#).

Structural analysis, sequence and structural comparisons

Structures were compared using the lsq options in the graphics program O.³⁷ Similar sequences were identified using BLAST²² and obtained from GenBank.³⁹ Figures were prepared with O and OPLOT,⁴⁰ Molray,⁴¹ MacPymol,⁴² and ChemDraw (CambridgeSoft Corp).

Protein Data Bank accession

Atomic coordinates and structure factor data have been deposited in the Protein Data Bank³⁸ with entry codes 2VVP, 2VVQ, 2VVO, and 2VVR for *MtRpiB*/R5P-Ru5P, *MtRpiB*/5PRA, *MtRpiB*/All6P and *EcRpiB*-H99N/R5P, respectively.

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Figure legends

Fig. 1. Isomerization reactions catalyzed by *MtRpiB* and *EcRpiB*. Residues involved in the different steps of the R5P to Ru5P catalysis are indicated, based on the previous structural results. *EcRpiB* residues are italicized. Structures of the inhibitors 4PEH, 5PRH and 5PRA are shown at bottom.

Fig. 2. Kinetic data. Hanes-Woolf graphical representations of the activity assays of: (a) *EcRpiB* (93 nM) with R5P, (b) *EcRpiB* (52 nM) with All6P, (c) *MtRpiB* (95 nM) with R5P, and (d) *MtRpiB* (316 nM) with All6P. Panels (e) and (f) show graphical determination of the IC₅₀ values for the inhibition of the R5P to Ru5P isomerization reaction catalyzed by *MtRpiB* (95 nM) in presence of (e) All6P and (f) Allu6P.

Fig. 3. Overall structure of *MtRpiB* and active site. (a) A cartoon representation of *MtRpiB* with a molecule of R5P in the active site, drawn with yellow carbon atoms. The A molecule is coloured pink and the B molecule is grey; these colours will be utilized throughout. The N- and C-termini of the A molecule have been marked. (b) Electron density, in blue mesh, from the final $2|F_o|-|F_c|$ map contoured at $1\sigma = 0.33\text{ e}^-/\text{\AA}^3$. The density corresponding to a mixture of R5P (yellow) and Ru5P (black) is shown together with the superimposed *MtRpiB* structure in complex with 4PEH (dark turquoise, PDB entry 2BES).

Fig. 4. Binding of *MtRpiB* to inhibitors. (a) The density of 5PRA (green carbon atoms) and mode of binding compared with R5P (yellow). (b) *MtRpiB* with All6P (purple) superimposed onto *MtRpiB* with R5P (yellow). The density for α -All6P is shown as blue mesh. Tyr46 is visible in the background.

Fig. 5. *EcRpiB*-H99N mutant. The dimer of the *EcRpiB* asymmetric unit is presented, with the A molecule colored dark red, and the B molecule dark grey. Density is shown for both the A-type (a) and B-type (b) sites. (c, d) Analysis of the crystal packing of *EcRpiB*-H99N reveals that the two active sites of a dimer have different environments. Symmetry related molecules have darker coloring.

Fig. 6. Comparison of *MtRpiB* and *EcRpiB*. (a) *MtRpiB* is superimposed on *EcRpiB*-H99N. The A molecule is colored in pink (*Mt*) or dark red (*Ec*), and the B molecule is light grey (*Mt*) or black (*Ec*). (b) Stereo view of the active site, showing *MtRpiB* with linear R5P (yellow), and *EcRpiB*-H99N (blue) with the β -furanose form of the sugar.

Table 1. Kinetic parameters of the ribose-5-phosphate isomerase (Rpi) and allose-6-phosphate isomerase (Allpi) activities determined for *Ec*RpiB and *Mt*RpiB, and the *Ec*RpiB-H99N mutant with R5P as substrate.

	<i>Ec</i> RpiB		<i>Mt</i> RpiB		<i>Ec</i> RpiB-H99N
	R5P	All6P	R5P	All6P	R5P
K_m (mM)	1.1 ± 0.2	0.5 ± 0.2	1.0 ± 0.4	16 ± 2	0.8
k_{cat} (s ⁻¹)	52 ± 2	6.1 ± 0.3	47 ± 2	~ 0.22	2
k_{cat}/K_m (mM ⁻¹ s ⁻¹)	47 ± 9	12 ± 5	50 ± 20	~ 0.014	2.5

Table 2. Data collection and refinement statistics. Information in parentheses refers to the highest resolution shell.

Data collection statistics	<i>MtRpiB</i>/R5P-Ru5P	<i>MtRpiB</i>/5PRA	<i>MtRpiB</i>/All6P	<i>EcRpiB</i>-H99N/R5P
Data collection beamline/detector	ID14EH3/Marmosaic CCD 165	BM14/Marmosiac CCD 225	ID14EH4/ADSC Q315R 315	ID23EH1/ADSC Q315r
Wavelength λ	0.931	0.974	0.940	0.940
Space group	C2	C2	C2	C2
Molecules in A.U	5	5	5	6
Cell axial lengths (Å)	136.8, 103.1, 69.7	136.4, 102.1, 69.6	152.7, 92.5, 61.0	89.70, 51.86, 207.21
Cell angles (°)	90.0, 95.5, 90.0	90.0, 96.2, 90.0	90.0, 98.9, 90.0	90.0, 115.55, 90.0
Resolution range (Å)	30-1.65 (1.69-1.65)	81.65-2.0 (2.11-2.0)	60.19-1.85 (1.95-1.85)	40.49-2.1 (2.21-2.1)
Number of reflections measured	431,788	163,030	273,406	181535 (26641)
Number of unique reflections	122,228	62,962	65,360	50635 (7305)
Average multiplicity	3.5 (1.8)	2.6 (2.6)	4.2 (4.1)	3.6 (3.6)
Completeness (%)	98.1 (86.0)	98.5 (97.7)	91.7 (61.8)	99.9 (99.9)
R _{meas} ^a	0.087 (0.39)	0.12 (0.48)	0.08 (0.59)	0.13 (0.20)

$\langle I \rangle / \sigma(\langle I \rangle)$	12.6 (1.9)	9.7 (3.0)	12.8 (2.6)	14.1 (5.8)
Wilson B ($\text{\AA}^2$)	19.8	17.0	23.4	19.1
Solvent content (%)	57 ($V_M^a=2.9$)	56 ($V_M=2.8$)	50 ($V_M=2.5$)	42 ($M=2.1$)
Refinement statistics				
Resolution range ($\text{\AA}$)	30-1.65	40-2.0	40-1.85	40-2.1
Number of reflections used in working set	109,555	59,765	62,048	48,057
No. of reflections for Rfree calculation	5804	3193	3307	2574
R-value, Rfree (%)	18.0, 19.9	17.2, 20.4	17.0, 20.4	20.3, 23.0
No. of non- hydrogen atoms	7053	6555	6398	6967
No. of solvent waters	984	571	421	346
Mean B-factor, protein atoms ($\text{\AA}^2$)	17.0	13.7	21.4	16.5
Mean B-factor, solvent atoms ($\text{\AA}^2$)	28.9	20.7	31.8	20.9

Mean B-factor, ligand atoms ($\text{\AA}^2$)	15.9 (R5P), 13.9 (Ru5P)	12.2, 50.7 (SO_4)	23.0	not included in refinement
Ramachandran plot outliers (%) ^b	0.14	0.14	0.14	0.65
r.m.s. deviation from ideal bond length ($\text{\AA}$) ^c	0.007	0.008	0.008	0.009
r.m.s. deviation from ideal bond angle ($^\circ$)	1.14	1.21	1.13	1.04
PDB entry code	2VVP	2VVQ	2VVO	2VVR

^a V_M is defined as the density of the protein in the crystal in $\text{\AA}^3$ per Da.⁴³

^b Calculated using a strict-boundary Ramachandran plot.⁴⁴

^c Using the parameters of Engh & Huber.⁴⁵