CMLS, Cell. Mol. Life Sci. 58 (2001) 1650–1665
1420-682X/01/111650-16 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Epimerases: structure, function and mechanism

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Received 20 March 2001; received after revision 11 May 2001; accepted 31 May 2001

Abstract. Carbohydrates are ideally suited for molecular recognition. By varying the stereochemistry of the hydroxyl substituents, the simple six-carbon, six-oxygen pyranose ring can exist as 10 different molecules. With the further addition of simple chemical changes, the potential for generating distinct molecular recognition surfaces far exceeds that of amino acids. This ability to control and change the stereochemistry of the hydroxyl substituents is very important in biology. Epimerases can be found in animals, plants and microorganisms where they participate in important metabolic pathways such as the Leloir pathway, which involves the conversion of galactose to glucose-1-phosphate. Bacterial epimerases are involved in the production of complex carbohydrate polymers that are used in their cell walls and envelopes and are recognised as potential therapeutic targets for the treatment of bacterial infection. Several distinct strategies have evolved to invert or epimerise the hydroxyl substituents on carbohydrates. In this review we group epimerisation by mechanism and discuss in detail the molecular basis for each group. These groups include enzymes which epimerise by a transient keto intermediate, those that rely on a permanent keto group, those that eliminate then add a nucleotide, those that break then reform carbon-carbon bonds and those that linearize and cyclize the pyranose ring. This approach highlights the quite different biochemical processes that underlie what is seemingly a simple reaction. What this review shows is that each position on the carbohydrate can be epimerised and that epimerisation is found in all organisms.

Key words. Epimerase; bacteria; carbohydrate; pathogen; epimerisation; stereocentre.

Introduction

Carbohydrates are essential to life in all its forms; their synthesis and modification are common to every organism. They fill many roles in biology, including such vital tasks as molecular recognition markers, structural elements and energy sources. Carbohydrates are also used as convenient precursors for the biosynthesis of important building blocks such as aromatic amino acids. In addition, they have many applications in industrial processes such as sweetening agents in the food industry and raw materials for fermentation [1]. This review will focus on the epimerisation of carbohydrates, and principally the epimerases whose structures have been elucidated (table 1).

Pathogenic bacteria have re-emerged as a serious health problem in the developed world. All bacteria make complex carbohydrate polymers which, when incorporated into their cell walls and envelopes, are often used to protect them from the host immune system. In Gram-negative bacteria such as *Shigella, Salmonella* and *Escherichia*, these are vital components of the lipopolysaccharide (LPS) and capsule; in Gram-positive bacteria they can be part of the capsule and cell wall, whereas in *My-*

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cobacterium tuberculosis there is a series of complex carbohydrate polymers at the cell surface [2]. Many of these sugars, such as L-rhamnose, have no counterpart in humans, and are also highly conserved amongst microorganisms [3]. Deletion of one of the four genes involved in the dTDP-L-rhamnose pathway of *Streptococcus mutans* prevents the bacteria from initiating or sustaining an infection [4]. In *Vibrio cholerae*, mutants affected in genes encoding enzymes of the dTDP-L-rhamnose pathway show a severe colonisation defect [5]. Heptose (L-glycero-D-mannoheptose) is one of several rare sugar components of the LPS core domain of Gram-negative bacteria [6]. *Escherichia Coli* K12 mutants defective in heptose synthesis are less pathogenic and more sensitive to classical antibiotics [7, 8]. These observations confirm the importance in bacterial pathogenicity of enzymes implicated in the biosynthesis of LPS and capsular sugar precursors. Attention has therefore turned to the pathways involved in the biosynthesis of sugars, as the enzymes required for their manufacture are often (but not always) absent from humans.

Other carbohydrate epimerisation reactions include the conversion of D-glucuronic acid to L-iduronic acid by glucuronyl C5-epimerase, which is a key enzyme in the biosynthesis of heparin and heparin sulfate. This is the only one of the biosynthetic polymer-modification reactions that cannot be achieved by chemical means alone [9]. The important polysaccharide alginate is formed commercially by the polymer level epimerisation of β-L-mannuronic acid to α-L-glucuronic acid. This reaction can be catalysed by the Ca^{2+} dependent mannuronic C5-epimerases from *Azotobacter vinelandii* [10, 11]. Although these enzymes have been characterised, no structures have yet been deposited in the Protein Data Bank.

In many cases the biosynthetic pathways involving epimerases are complex, involving multiple chemical steps. The most common processes are oxidation, epimerisation, acetylation, dehydration and reduction of the carbohydrate. This review will focus on the epimerisation of carbohydrates.

Epimerisation is the inversion of configuration of an asymmetrically substituted carbon in linear or cyclic sugars (fig. 1a). This can be thought of as a process of hydrogen removal from one face and its return to the opposite face of a central carbon. Although simple to draw, the chemistry behind such a transformation is more complex; carbohydrates are extremely stable, and epimerisation does not occur spontaneously. However, almost every stereocentre of a carbohydrate is epimerised by one enzyme, some enzymes epimerising two centres during turnover. Furthermore, all branches of life utilise carbohydrate epimerases. In humans, the only mutations found in the UDP-galactose/glucose (change at C4) epimerase gene attenuate activity. It is thought that the mutants that abolish this conversion would be lethal [12]. In plants, the enzyme D-ribulose-5-phosphate 3-epimerase is a key enzyme in the Calvin pathway as well as in the oxidative pentose phosphate pathway [13, 14]. Epimerisation, although best characterised in bacteria, appears to be both ubiquitous and versatile.

Several different strategies have emerged to invert the chirality. Hydride abstraction (oxidation) from one face of the stereocentre, and hydride addition (reduction) to the opposite face of the same stereocentre has been employed for inversion of the 4 and 6 positions of the pyranose ring. During turnover a transient keto sugar is generated. This requires a redox active cofactor [NAD(P)] to achieve the necessary first oxidative step, the cofactor being regenerated during the reduction step. The abstraction of a proton attached to carbon is facilitated by the creation of keto sugars analogous to the intermediate for the redox epimerisation. These 'permanent' keto sugars significantly acidify the hydrogen α to the keto group. Even with the enolate, the pK_a of the proton remains high (over 10), the role of the protein being to stabilise the negative charge (thus lowering the pK_a), abstract the α proton and replace it from the opposite face. In contrast to both these mechanisms that involve hydrogen repositioning, other routes include carbon-carbon bond cleavage and elimination and readdition of nucleotide. This review will discuss each of these mechanisms of epimerisation.

Table 1. Table listing types of epimerisation reactions catalysed with example enzymes discussed in the text.

Type of epimerisation	Example enzyme	Site of epimerisation
By transient keto intermediate	UDP-galactose 4-epimerase	C4
	ADP-L-glycero-D-mannoheptose 6-epimerase	C6
	CDP-tyvelose 2-epimerase	C2
Proton abstraction/addition	dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase	C3, C5
	GDP-4-keto-6-deoxy-D-mannose epimerase/reductase	C3, C5
	D-ribulose-5-phosphate 3-epimerase	C3
Nucleotide elimination and readdition	UDP-*N*-acetylglucosamine 2-epimerase	C2
	N-acyl-D-glucosamine 2-epimerase	C2
Carbon-carbon bond cleavage	L-ribulose-5-phosphate 4-epimerase	C4
Mutarotation (ring opening)	galactose mutarotase	C1

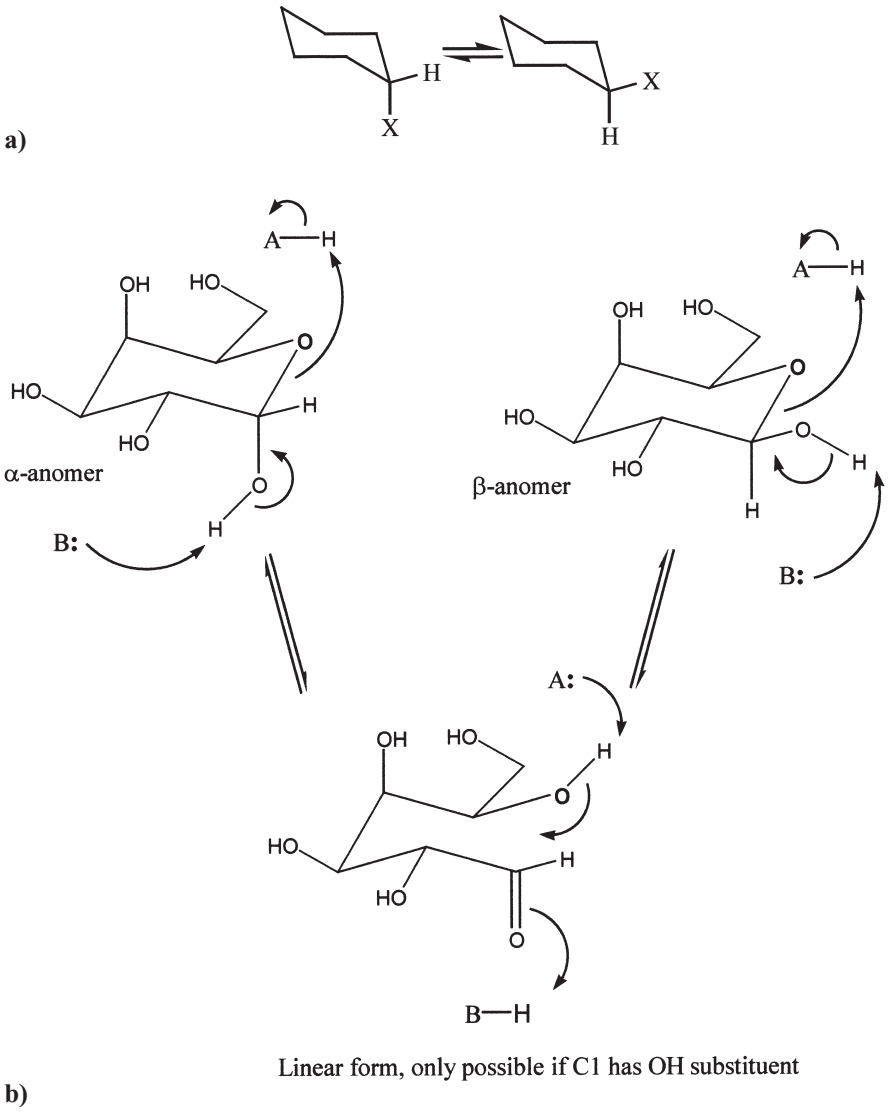


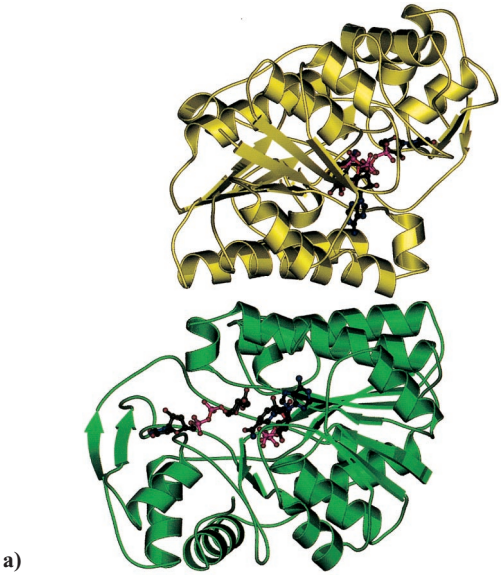
Figure 1. (*a*) Epimerisation; the inversion of configuration of an asymmetrically substituted carbon in a sugar. (*b*) Epimerisation by ring opening (mutarotation).

Epimerisation by a transient keto intermediate

UDP-galactose 4-epimerase

UDP-galactose 4-epimerase (GALE) is an archetypical example of this type of epimerisation. The enzyme catalyses the interconversion of UDP-glucose and UDP-galactose by inverting the stereochemistry at the C4 position (table 1, fig. 3). The enzyme obviates the need for the de novo synthesis of both sugars [15]. The biological interconversion of galactose and glucose is known as the Leloir pathway, and in addition requires the enzymes galactokinase and galactose-1-P uridylyltransferase [16, 17]. In humans, the role of GALE in galactose metabolism is critical. Two forms of GALE deficiency have been described: one is benign and involves only red and white blood cells and not other tissues (the 'peripheral' form). The other is very rare and presents with symptoms resembling transferase deficiency (the 'general' form) [12, 18, 19].

GALE belongs to the enzyme superfamily of short-chain dehydrogenases/reductases (SDRs). The SDR family is a diverse group of enzymes with a highly divergent sequence identity of typically 15–30%. The proteins bind the NAD(P)H cofactor and normally contain two characteristic signature sequences, a TyrXXXLys (where X represents any residue) couple and a GlyXGlyXXGly motif near the cofactor-binding pocket [20–23]. In SDR-catalysed reactions, hydrophobicity, size and rigidity of the possible substrates vary [21, 24, 25]. However, as the name suggests, these proteins are all involved in redox-based reactions.



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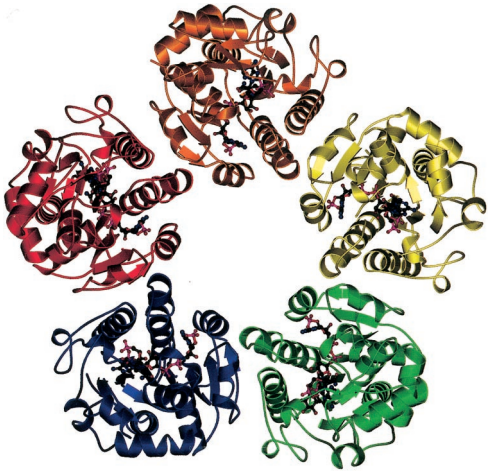

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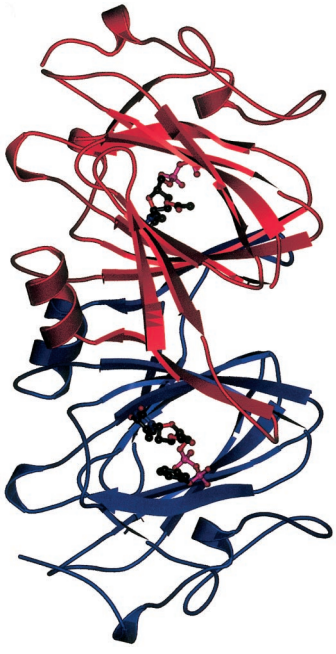

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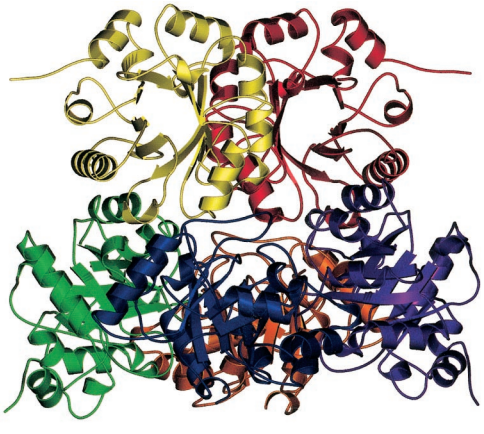
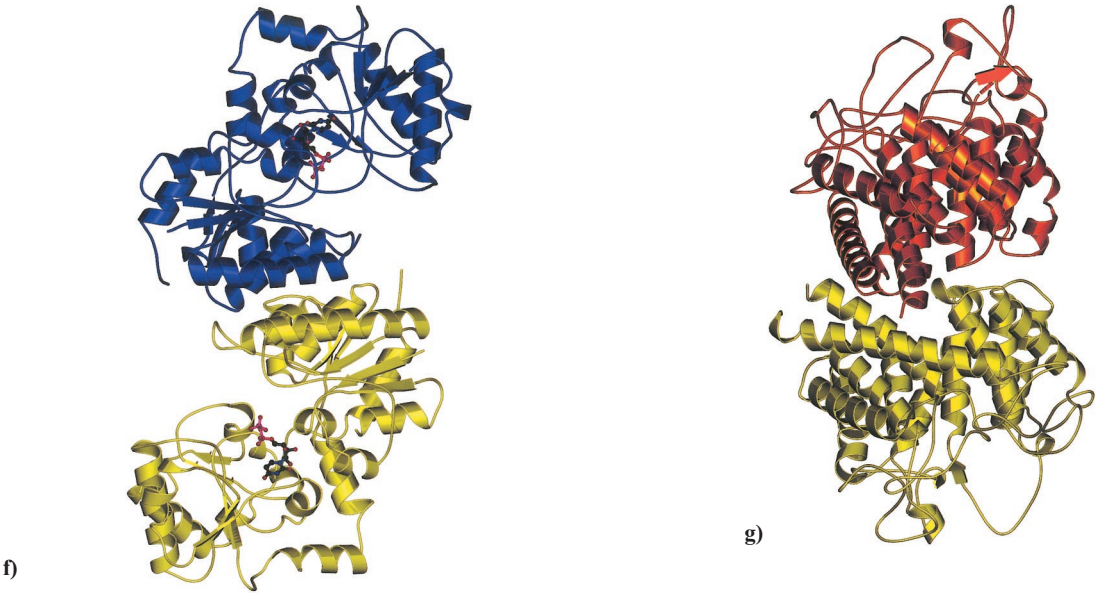


e)

Figure 2. The crystal structures of some of the enzymes discussed in the text; all structures are shown as ribbon representations with monomers illustrated in different colours. (*a*) The UDP-galactose 4-epimerase dimer from *E. coli* (EGALE) with the cofactor NAD^+ and substrate UDP-glucose shown bound in the active site. (*b*) AGME in complex with $NADP^+$ and ADP-glucose. The enzyme functions as a homopentamer with the five monomers arranged in a ring. The catalytic triad residues present in GALE are also present in AGME. They are in structurally similar positions and have analogous functions. (*c*) The RmlC dimer with 3′-*O*-acetylthymidine-(5′-diphosphate phenyl ester) bound in the active site. The catalytic residues are currently under investigation in our laboratory. (*d*) The GMER dimer with NADPH bound. The catalytic triad residues present in GALE are also present in GMER, where they perform the reduction function of the enzyme. The residues involved in the epimerisation process are still under investigation. (*e*) The hexameric RPEase enzyme; each monomer consists of a slightly modified TIM barrel.



f) g)

Figure 2 (continued). (*f*) The UDP-*N*-acetylglucosamine 2-epimerase dimer with UDP bound in the active site. (*g*) The *N*-acyl-D-glucosamine 2-epimerase dimer.

GALE has been extensively studied from both *Escherichia coli* (EGALE) [26–32] and more recently *Homo sapiens* (HGALE) [33, 34]. Structurally, HGALE is 55% identical to the homologous *E. coli* enzyme [33]. Both HGALE and EGALE are homodimers with molecular masses of 38.3 and 37.3 kDa for their respective monomers. Each monomer consists of 348 (HGALE) and 338 (EGALE) amino acids with one molecule of NAD^+ tightly bound in the syn-conformation with its si-face oriented towards the sugar substrate. The monomers fold into two distinct domains, an N-terminal or nucleotide binding domain dominated by a seven-stranded parallel β-pleated sheet flanked on either side by α helices, and a smaller C-terminal domain responsible for the proper positioning of the UDP-sugar substrates. The crevice created by the two domains contains the active site [27–29]. The structure of EGALE is shown in figure 2a. The structures of both EGALE and HGALE are virtually identical, as they can be superimposed with a root mean square deviation of 0.98 Å for 299 structurally equivalent α-carbon atoms [33]. A subtle difference is that the human enzyme has an active site volume ~15% larger than that observed for EGALE and the substitution of an active site amino acid Tyr299 (EGALE) for the less bulky Cys307 (HGALE) [34]. This is thought to be related to the secondary role of HGALE in catalysing the interconversion of UDP-*N*-acetylgalactosamine and UDP-*N*-acetylglucosamine.

GALE contains three amino acids that have been identified by site-directed mutagenesis as important in the catalytic process: Lys153, Tyr149 and Ser124 (numbers refer to EGALE enzyme) [28, 35]. The conservation of Lys and Tyr has already been highlighted across the SDR superfamily, with many of the other members also containing either a Ser or Thr residue as the third member of the catalytic triad.

The broad details of the GALE mechanism were worked out in the 1970s [36, 37]. The first step is an abstraction of the 4-hydroxyl proton by an enzymatic base and an abstraction of a hydride from the C4 position of the sugar to the C4 position on NAD^+ to form NADH. Studies have shown that Tyr149 is in the phenolic form and is stabilised in this form by Lys153 [38]. It therefore seemed obvious that Tyr149 acts as the main base deprotonating the 4-hydroxyl group. However, with the exception of the HGALE structure, Tyr149 is too far from the substrate to directly abstract this proton (4.3 Å) [28]. To solve this conundrum, a proton shuttle mechanism had been advanced, in which the hydroxyl side chain of the conserved serine (Ser124) abstracts the proton from the substrate and loses its own proton to Tyr149 [27]. At the active site a transient keto sugar is formed which of course has no chirality at the C4 position. The keto sugar is not released by the enzyme and remains bound [17]. The UDP-group serves a vital role in the mechanism by anchoring the pyranosyl groups of the galactosyl/glucosyl and 4-keto sugar intermediate in the active site. Binding studies have shown that in EGALE most of the binding free energy between the enzyme and these molecules is directed towards binding the nucleotide portion [39, 40]. In the final step of the process NADH transfers the hydride back to the C4 of the sugar, but this time to the opposite face, with inversion of configuration at C4 of the sugar. The proton extracted by Tyr149 (or Ser124) is transferred back to the sugar.

A key question then is how the hydride is abstracted from one face and transferred to the other. The NAD^+/NADH transformation in GALE is itself stereospecific, with hydrogen transfer exclusively to and from the *si*-face. This would seem to indicate that hydride transfer in GALE must follow a stereospecific and fairly well defined trajectory, even if the overall result is nonstereospecific. Cursory examination of the substrate complex confirms that it is not possible to abstract and add hydride to opposite faces without a change in the orientation of sugar substrate relative to the cofactor. Weak binding of the 4-keto sugar intermediate in the active site suggests that it may be conformationally mobile in the active site. This has led to the proposition that there is a rotation of the 4-keto sugar intermediate within the active site around the β-phosphate of UDP. The α-phosphate and uridine remain fixed in position during this rotation. This would place the 4-keto group in a position relative to the NADH that would allow it to accept hydride transfer from either face to form UDP-glucose or UDP-galactose. Indeed, space-filling models show that this rotation can allow virtually equivalent positioning of the hydroxyl groups at glycosyl C3 and C4 and the hydrogen at C4 in the substrate epimers [32]. This rotation of the carbohydrate portion of the substrate allows the NADH to transfer the hydride to the opposite face while conserving an optimum trajectory for hydride transfer. Such a revolving door mechanism is unusual in biology.

At a more subtle level, it is known that the uridine nucleotide also induces a conformational change in GALE. The conformational change activates the NAD^+ to reduction [17, 40–42]. The structure of the enzyme in its active conformation reveals that the 6-ammonium group of Lys153 is hydrogen-bonded to both the 2′- and 3′-hydroxyl groups of the nicotinamide riboside in NAD^+ [28]. The 6-ammonium group is seen to be much closer to the nicotinamide-N1 (5.3 Å) than to the nicotinamide C-4 (>7 Å). The positive electrostatic field between Lys153 and nicotinamide-N-1 in the active conformation should polarise the π-electrons in the nicotinamide ring, decreasing the positive charge on N-1 and increasing it on C4, thus activating it for hydride abstraction. The effect is brought about through electrostatic repulsion between the nicotinamide ring and a residue of the enzyme, Lys153, and not through enzymatic binding of the transition state [17].

Recently the structure of HGALE has been solved, which has led to some revision of the epimerase mechanism [33]. Upon binding of the UDP-glucose, there is a structural movement of one of the HGALE subunits relative to the other, causing the C-terminal domain of one monomer to clamp down more tightly over the active site. The major significance of this is that the active site tyrosine (Tyr157) is now within hydrogen-bonding distance not only of the nicotinamide ribose 2′-hydroxyl group and the 3′-hydroxy group of the glucose moiety, but also the 4′-hydroxyl group of the UDP-sugar. The net result of this would be to enable the tyrosine to act directly as the active site base, without the need for the serine (Ser132) to act as a proton shuttle. This subunit movement has not as yet been observed for EGALE, and the possibility that EGALE and HGALE have slightly different mechanisms cannot be ruled out. The function of Ser132 in HGALE could be to form a low-barrier hydrogen bond between the O^γ of Ser132 and the 4′-hydroxyl group of the UDP-glucose substrate. This would lead to both the pK_a values of the alcohol groups being similar, resulting in a proton being shared between two oxygen atoms. This close interaction would facilitate the removal of the 4-hydroxyl hydrogen of the sugar by the phenolic side chain of Tyr157 and the transfer of the hydride from C4 of the sugar to C4 of the dinucleotide cofactor. As pointed out by Thoden et al., these new data on the HGALE enzyme imply that the knowledge of one, or even a few members of an enzyme superfamily may be not sufficient to fully characterize either the structures or functions of all the members [33]. Further studies are probably required to fully appreciate the exact role of Ser124.

ADP-L-glycero-D-mannoheptose 6-epimerase

ADP-L-glycero-D-mannoheptose 6-epimerase is a 34-kDa enzyme required in Gram-negative bacteria for ADP-L-glycero-D-mannoheptose (heptose) synthesis, a component of the core domain of the LPS portion of the bacterial cell wall [6]. The substrate for this enzyme (ADP-D-glycero-D-mannoheptose) is a sugar with seven carbons, and the epimerisation occurs at the C6′ position (table 1, fig. 4). The structure of ADP-L-glycero-D-mannoheptose 6-epimerase (AGME) in complex with $NADP^+$ and ADP-glucose, a catalytic inhibitor of the enzyme, has been solved at a resolution of 2 Å [43]. The protein has two domains, the N-terminal domain consists of a modified 7-stranded Rossmann fold, which contains the $NADP^+$ binding site. The C-terminal domain is an α/β fold and provides residues that create the specificity for the substrate. The two domains interact to form a crevice where the substrate is brought close to the $NADP^+$ cofactor. AGME is very similar to GALE, and with other members of the SDR family. The three key catalytic residues identified in GALE are also conserved in AGME, and found to be located in structurally similar positions in both enzymes. This and other evidence confirms the epimerisation proceeds through a transient keto intermediate. However, the quaternary structure of AGME diverges from the other proteins of the SDR family since AGME forms homopentamers as opposed to the dimers or tetramers of most SDR proteins (fig. 2b) [44]. The five monomers are arranged in a ring, with the crevices containing the catalytic site oriented towards the

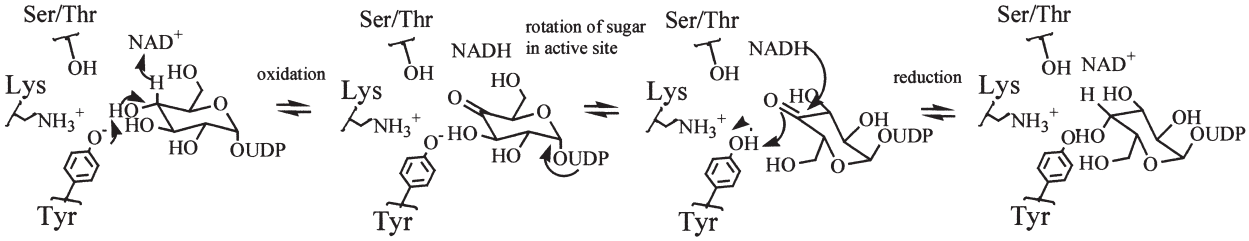


Figure 3. Epimerisation by a transient keto intermediate as carried out by UDP-galactose 4-epimerase with the interconversion of UDP-glucose and UDP-galactose.

centre. This position of the active site crevice varies between monomers and reflects the two binding modes of ADP-glucose. Although this may be a result of crystal packing, there are similarities to the domain movements in HGALE and in the different UDP-galactose/UDP-glucose binding modes discussed above. Depending on the C-terminal conformation AGME ('closed' or 'open'), the torsion angle, defined by the oxygen linking the two α and β phosphates, the β-phosphate, the glycosyl oxygen and the C1 atom of ADP-glucose, changes by 125°. Such a 'rotation' of substrate would permit epimerisation to take place and provides indirect evidence that such a rotation occurs in GALE also.

CDP-D-tyvelose 2-epimerase (table 1) is a bacterial epimerase that interconverts CDP-tyvelose and CDP-paratose. Tyvelose is a 3,6-dideoxyhexose present at the nonreducing end of the O-antigen of the LPS [45, 46]. The structure of this enzyme is unknown and the mechanism is under investigation. The presence of NAD^+/NADH in purified CDP-D-tyvelose 2-epimerase indicates that the enzyme may follow a redox mechanism similar to that of GALE with the formation of a keto sugar intermediate [1, 47].

Epimerisation by proton abstraction/addition

The keto-enol tautomerism is well known to organic chemists; in essence any ketone exists in equilibrium with the enol. Under normal conditions the equilibrium greatly favours the ketone, and the enol can be ignored in a structural description of the molecule. However, it does have a profound influence on the chemistry of the molecule. It acidifies the α-proton(s) by stabilising the formal carbocation generated by proton abstraction through an enolate. This type of stabilisation is routine in both biology and chemistry, occurring in many different classes of compound. In carbohydrates it is less well known principally because keto sugars are considerably less common than normal ketones. Examination of the L-rhamnose and L-fucose biosynthetic pathways shows that both create a D-keto sugar, which is epimerised. The keto group is then reduced in the final step to give the L-sugar product. In contrast to GALE, the keto sugars are isolable enzyme products, and epimerisation occurs at the stereocentres adjacent (or α) to the keto group not at the keto group directly.

dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase

The rhamnose biosynthetic pathway requires four distinct enzymes: RmlA, RmlB, RmlC and RmlD [48–51] and is highly conserved among Gram-positive and Gram-negative bacteria. L-rhamnose is a component of the O-antigen part of lipopolysaccharides of Gram-negative bacteria such as *Salmonella typhimurium*, *Shigella flexneri*, *E. coli* VW187, and *V. cholerae*. Its importance is illustrated by the fact that *V. cholerae* mutants devoid of RmlB or RmlD show a severe colonisation defect [5]. In Gram-positive bacteria, rhamnose is essential to the integrity of the cell wall, since it connects the inner peptidoglycan layer to arabinogalactan polysaccharides [2, 52]. In *Streptococcus mutans*, the deletion of one of the four *Rml* genes inhibits cell wall synthesis, and bacteria are unable to sustain an infection [4]. RmlB (dTDP-D-glucose 4,6-dehydratase), which is homologous and structurally similar to GALE, creates a keto sugar at C4 of the glucose ring and dehydrates the C6 to give dTDP-6-deoxy-D-xylo-4 hexulose [51]. This keto sugar is the substrate of RmlC (dTDP-6-deoxy-D-xylo-4 hexulose 3,5-epimerase, EC 5.1.3.13). RmlC catalyses a double epimerisation at positions C3 and C5 to give the product TDP-4-keto-L-rhamnose (table 1, fig. 5).

Melo and Glaser [53] showed that in the epimerisation reaction, hydrogen atoms at C3 and C5 were derived from the solvent. Substitution of the C3 hydrogen by deuterium decreased the rate of the reaction by 3 to 4 fold, whereas substitution of the C5 hydrogen decreased the reaction rate by twofold. This isotope effect indicates that after deuterium substitution, either C3 or C5 epimerisation can become rate limiting. Incubation of the enzyme in the presence of 4-keto-6-deoxyglucose and D_2O, followed by reduction and analysis of the different compounds by mass spectroscopy, led to the identification of molecules having deuterium at positions C3, C3 and C5, but only a small fraction of molecules with deuterium at C5. This suggests an obligate order with epimerisation first at C3

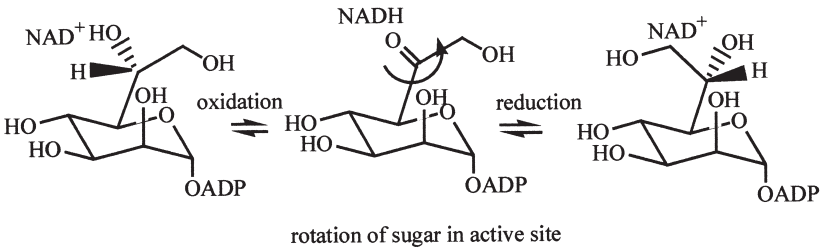


Figure 4. Epimerisation mechanism of ADP-L-glycero-D-mannoheptose 6-epimerase (AGME); the epimerisation occurs at the C6′ position.

followed by epimerisation at C5. Other investigations, using highly purified RmlC, have demonstrated that only 3% of RmlC substrate is converted into dTDP-4-keto-L-rhamnose [54]. A similar product/educt ratio was observed in the *E. coli* strain Y10 defective in 4-keto-6-deoxy-L-rhamnose reductase, RmlD, and for *Mycobacterium tuberculosis* RmlC [55, 56]. Despite this low product/substrate ratio, the quasi-total reaction catalysed by RmlA (which catalyses the transfer of a thymidylmonophosphate nucleotide to glucose-1-phosphate) [50], together with the thermodynamically favourable reaction catalysed by RmlD, leads to a very efficient rhamnose pathway.

The structure of RmlC from *S. typhimurium* was reported last year [49] closely followed by the enzyme from *Methanobacterium thermoautotrophicum* [57]. RmlC is a mainly β-class protein, with a β barrel constituted by 13 β strands. RmlC exists as a dimer (fig. 2c), the dimer interface created by the antiparallel interaction of the β3 strand of one monomer with the β5 strand of the other monomer. The RmlC active site is formed by both subunits and is located at the entrance to the β barrel. There is a very strong sequence identity in this region, in particular two conserved His-Asp diads are found in this cleft. An absolutely conserved lysine residue (Lys73) sits between the two His residues. The structure of a dTDP-phenol/RmlC complex has also been determined, confirming that the substrate binding site was located in the cluster of conserved residues. The thymidine ring of the ligand is stacked between the aromatic rings of Tyr139 from one monomer and Phe27 from the other monomer of the dimer. The phenyl ring of dTDP-phenol sits on the exterior of the protein and is twisted out of the active site. More recently, data have been obtained [C.-J. Dong and J. H. Naismith, unpublished results] on a product complex of RmlC. This confirms the location of the sugar nucleotide binding site and shows the conserved active site residues interacting with the product. As had been predicted [48], the tight cluster of conserved hydrophobic residues Val75, Phe131 and Phe122 acts as a hydrophobic pocket and recognises the C5 position. Val75 and Phe131 are not absolutely conserved but are only replaced with nonpolar residues. The C3 position (hydroxyl) points to a more open pocket in which water molecules and polar side chains predominate.

The epimerisation of the C3 and C5 positions requires that the hydrogens attached to the C3 and C5 carbons are removed and replaced at the opposite face. The removal of one proton from C3 substantially raises the pK_a of the remaining proton on C5 (or vice versa). As the pK_a of the remaining proton is well beyond any base found in biology, the abstracted proton must be replaced (at the opposite face) before the second epimerisation takes place. It follows that the enzyme mechanism is a series of at least four sequential steps rather than a concerted mechanism. The key question in reaching a mechanistic understanding is what are the acids and what are the bases involved in proton abstraction/addition. The recently sequenced *Streptococcus suis RmlC* gene shows that only one His-Asp diad is conserved, the *Streptococcus* sequence being in contrast to all other organisms in which both His-Asp diads are conserved. The absolutely conserved His-Asp diad is set up to act as one base; the basicity of a histidine bound to an aspartic acid is substantially higher than that of a free histidine. In the product complex, the His-Asp diad is close to the both the C3 and C5 position, suggesting this might indeed function as a base. Based on the structure of the *S. typhimurium* enzyme [49], only the absolutely conserved Tyr133 appears able to act as an acid; no other appropriate conserved residue is close by. The WbcA gene product is 34% identical to RmlC, and encodes an epimerase (CDP-4-keto-6-deoxyglucose epimerase) involved in the biosynthesis of dCDP-6-deoxy-D-gulose from dCDP-4-keto-6-deoxyglucose, the precursor of D-gulose, a deoxysugar present in the O-antigen part of *Yersinia enterocolitica* [58]. This epimerase is particularly relevant since it only catalyses a single epimerisation at the C3 atom of the sugar ring [58]. Comparison of the RmlC and the WbcA gene product shows that Tyr133 is missing from WbcA. Since WbcA does not epimerise the C5 position, it represents strong evidence that Tyr133 from RmlC is involved in the C5 position epimerisation. Currently, only the identification of one acid and one base has been possible. Further experiments will be required to understand the mechanism fully.

In a subtle twist, the RmlC structure shows Lys73 sitting at the bottom of an anion hole and perfectly positioned to stabilise the formally negatively charged enolate ion, probably by proton donation to form the neutral enol. It remains an intriguing possibility that the Lys may in fact form a Schiff base with the keto sugar. Such a Schiff base would further acidify the hydrogens at C3 and C5, significantly lowering the transition state energy. Preliminary experiments [M. Asuncion and J. H. Naismith, unpublished results] suggest that water is eliminated from and added back to the sugar nucleotide during the epimerisation reaction. This provides evidence for the formation of a transitory immine during turnover, since such an immine would form by elimination of water and be decomposed by addition of water. If indeed the mechanism turns out to involve immine formation, this would be highly unusual and an entirely new chemical mechanism.

GDP-4-keto-6-deoxy-D-mannose epimerase/reductase

Fucose is a deoxysugar found in many organisms and plays a role in various events like cell stem development in plants [59], nodulation in *Azorhizobium* [60], and adhesion in animals [61]. Humans deficient in the biosynthesis of GDP-fucose suffer from the immune disorder leukocyte adhesion deficiency type II [62]. Fucose is also a component of bacterial cell walls [63]. Its precursor, GDP-fucose, is synthesised from GDP-mannose in a three-step reaction catalysed by two enzymes [64]. The first enzyme, GDP-mannose 4-6 dehydratase (GMD) converts GDP-D-mannose to GDP-4-keto 6-deoxy-D-mannose. The second enzyme, GDP-4-keto-6-deoxy-D-mannose epimerase/reductase [GMER, also known as GDP-L-fucose synthetase (GFS)], catalyses a two-step reaction: the epimerisation at the C3 and C5 of the sugar ring and a NADPH-dependent reduction at C4 (table 1, fig. 6). GMER combines both RmlC and RmlD activity in a single enzyme. It was initially believed that GDP-4-keto-6-deoxy-D-mannose was transformed to GDP-L-fucose by two different enzymes, as is the case in the dTDP-L-rhamnose pathway. However, an enzyme able to transform GDP-4-keto-6-deoxy-D-mannose to GDP-L-fucose was isolated from porcine thyroid, showing that this enzyme contains both epimerisation and reduction activities [65]. This was also demonstrated with the homologous human enzyme, the protein FX [66, 67].

Epimerisation at C3 and C5 most likely proceeds via an enediol-enolate intermediate [68]. Incubations of ^{14}C-labeled GDP-4-keto-6-deoxymannose with either GMER alone or with GMER in the presence of $NADP^+$, followed by reduction of the products with $NaBH_4$ and acid cleavage of the nucleotide, result in the formation of 6-deoxyglucose and fucose [69]. Both of these compounds can only be generated if a double epimerisation occurs at positions C3 and C5. Thus, GMER does not require a nicotinamide cofactor to epimerise its substrate. The three-dimensional structure of GMER was determined by two independent groups in 1998 (see fig. 2 d) and confirmed the expectation that the enzyme is a member of the SDR family, with the classical catalytic Ser-Tyr-Lys triad [44, 70]. However, this structural similarity relates to the second reaction catalysed by GMER, the reduction of the 4-keto function. The two epimerised stereocentres are in α position to a keto group, directly analogous to the substrate of RmlC discussed above. There is no structural homology between GMER and RmlC. Analysis of the products formed after incubation of GDP-4-keto-6-deoxymannose with GMER confirms the epimerisation is enzymatic [69]. In the GDP-4-keto-6-deoxymannose binding model proposed by Somers et al. [70], the His179 side chain of GMER is in a suitable position to fulfil the

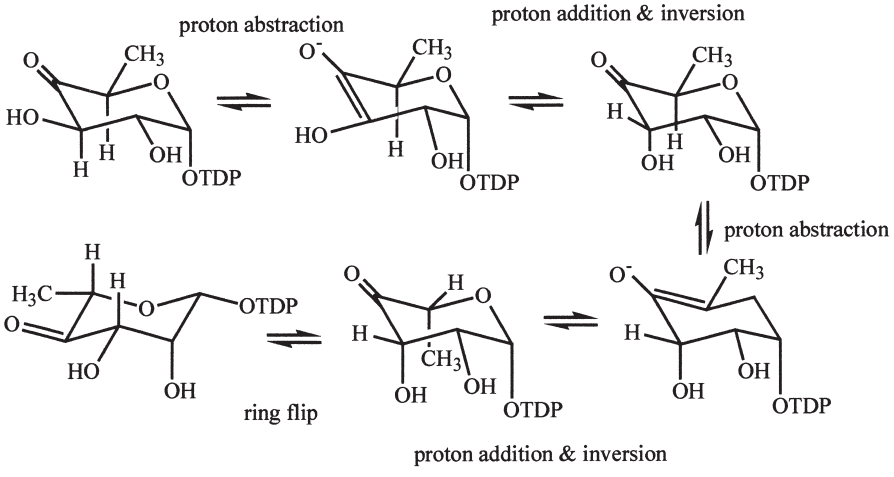


Figure 5. Epimerisation by proton abstraction/addition as illustrated by the mechanism of dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase (RmlC). The enzyme catalyses a double epimerisation at positions C3 and C5.

role of a general acid or base during catalysis. In their model, Tyr136 of GMER could transiently protonate the C4 oxygen, and stabilise the enediol/enolate intermediate. His179 could then act as a base and abstract a proton from the C3 position. This would be followed by a reprotonation from the opposite face of the sugar ring, either by a water molecule, or by a GMER residue that has not yet been identified. Finally, the C3 epimerisation would be completed by the deprotonation of the C4 hydroxyl group. The same residues could then proceed to epimerise at the C5 position following a slight movement of the sugar inside the catalytic site to put the C5 carbon in an ideal position. However, how the His base loses its proton, necessary for it to abstract the second protein, is unclear. This proposed mechanism is in agreement with the experiments from Chang et al. [65], where it was demonstrated that a tritium at the C3 position was lost during catalysis. It is significant that in both GMER and RmlC only one base has been identified.

D-ribulose-5-phosphate 3-epimerase

D-ribulose-5-phosphate 3-epimerase (RPEase) catalyses the conversion of D-ribulose 5-phosphate to D-xylulose 5-phosphate (table 1, fig. 7). In contrast to RmlC and GMER, both substrate and product exist in a linear not cyclic form. The keto group is situated on the C2 carbon, and the stereocentre at C3 is inverted. The enzyme is found in many organisms due to its obligatory step in the oxidative pentose phosphate pathway [13, 14]. RPEase incorporates one atom of tritium from T_2O during the reaction [71], and the proton abstracted from the C3 atom during catalysis is released to the solvent [72]. These observations, and the presence of the epimerised stereocenter in α position of a keto group, have led to the postulated catalytic mechanism in which deprotonation and reprotonation take place via an ene-diolate intermediate. The structure of the potato chloroplast RPEase has been elucidated by Kopp et al. [73]. The enzyme is a hexamer, and each monomer consists of a slightly modified $(\beta\alpha)_8$ barrel (TIM barrel), with an extra small α helix after the last strand of the classical TIM-barrel (fig. 2e). RPEase is topologically unrelated to RmlC. Alignments of 13 RPEase sequences from various organisms by Kopp et al. [73] indicate that two carboxylic residues, Asp43, Asp185 (all numbers refer to the potato chloroplast enzyme); three histidines, His41, His74, His98; and three methionines, Met45, Met76, Met147 are strictly conserved. These residues are located towards the centre of the TIM barrel, where they maintain a strong hydrogen network around a stable water molecule. In this structure a sulphate ion is located in the same position as the phosphate group of TIM-barrel enzymes that bind phosphorylated substrates [74]. Using this fact, Kopp et al. [73] modelled D-ribulose 5-phosphate into the enzyme. This model positioned the C3 atom between Asp185 and Asp43, two conserved residues that could play the role of general acid/base catalysts. This model is substantiated by the observation that an Asp185Asn mutant is completely inactive [75]. This symmetrical protonation and deprotonation mechanism is common to racemases, where two acid/base catalysts transfer protons to and from the α-carbon of their substrate and product enantiomers via an enolic intermediate [76, 77]. A potential RPEase mechanism taking into account the hydrogen network observed in the centre of the TIM barrel has been proposed. Residues His41, Asp72 , Glu183, His74, His98 and Glu100 are hydrogen-bonded in such a way that they can polarise the central water molecule that is ideally set up to stabilise a negative charge at residue Asp43. Asp43 deprotonates the C3 atom, and a cis-ene diolate intermediate is formed. According to Kopp et al. [73], the resulting oxyanion would be stabilised by the presence of the sulphur atoms of the three conserved methionines. The charge of the oxyanion would induce dipoles in the larger and softer shells of the sulphur atoms, stabilising the oxyanion. The advantage of having sulphur as opposed to hydroxyl is that stabilisation of the charged oxygen by a hydrogen bond could then favour an isomerisation rather than an epimerisation. The epimerisation is completed with the C3 abstracting a proton from Asp185. Water molecules from the bulk solvent then regenerate the initial state of the two aspartates for the mechanism to be in agreement with the observations of Davis et al. [72].

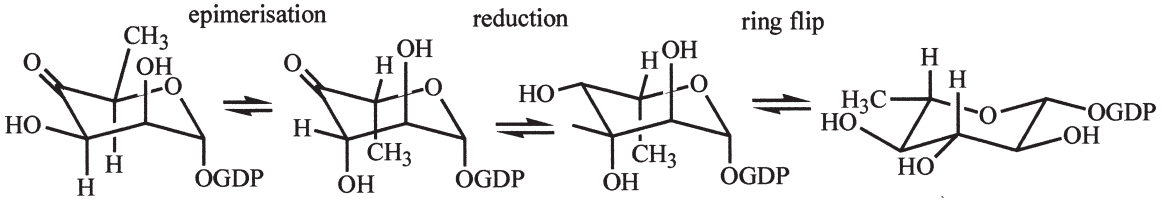


Figure 6. The epimerisation mechanism of GDP-4-keto-6-deoxy-D-mannose epimerase/reductase, which like RmlC, carries out a double epimerisation at the C3 and C5 of the glucose ring.

Epimerisation by nucleotide elimination and readdition

UDP-*N*-acetylglucosamine 2-epimerase

In bacteria, UDP-*N*-acetylglucosamine 2-epimerase catalyses the reversible interconversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylmannosamine (UDP-ManNAc). *N*-acetylglucosamine (ManNAc) is an amino sugar present in the antiphagocytic capsular polysaccharide of pathogenic strains of bacteria such as *Streptococcus pneumoniae* types 19F and 19A [78, 79]. It is also an essential component of the integrity of the cell wall of Gram-positive bacteria [80]. The mechanism involves the elimination and readdition of UDP [81, 82]. An initial anti elimination of UDP from UDP-GlcNAc generates the intermediate 2-acetamidoglucal (table 1, fig. 8). A subsequent *syn* addition of UDP gives the product UDP-ManNAc. The mechanism is supported by numerous experimental observations in which the proton is removed from C2, including the observation that a solvent-derived deuterium is incorporated at C2 during catalysis and that the epimerisation of UDP-[2-^{2}H]GlcNAc is slowed by a primary kinetic isotope effect [81, 82]. The scrambling of the ^{18}O label from the anomeric oxygen of UDP-*N*-acetyl-glucosamine to the nonbridging phosphate positions has also clearly demonstrated that the anomeric bond is cleaved during catalysis. Direct detection of the reaction intermediates UDP and 2-acetamidoglucal have confirmed this. All these observations have led to the postulated mechanism. The initial anti elimination of UDP could be triggered by a cationic elimination, leading to a transient oxocarbenium species. A catalytic base abstracts the C2 hydrogen, generating a 2-acetamidoglucal intermediate. Readdition of UDP with protonation of the C2 atom at the opposite face, effectively a *syn* addition, completes the cycle.

The structure of UDP-*N*-acetylglucosamine 2-epimerase from *E. coli* has been solved to 2.5 Å resolution [83]. The enzyme is a homodimer with each monomer composed of two $\alpha/\beta/\alpha$ domains that form a deep cleft at the domain interface (fig. 2f). The N-terminal domain consists of a seven-stranded parallel β sheet that is sandwiched between a total of seven α helices with a topology that is similar to the Rossmann dinucleotide binding domain [20]. The C-terminal domain contains a six-stranded β sheet that also has the topology of a Rossmann fold and is surrounded by a total of seven α helices. Each monomer has a single UDP-GlcNAc binding site. On binding substrate, one of the monomers may induce a conformational change across the dimer interface that converts the dimer partner to the catalytically active form. This topology is not related to any other epimerase but shows structural similarity with two phosphoglycosyl transferases, glycogen phosphorylase and T4 phage β-glucosyltransferase. This is consistent with the fact that these three enzymes proceed via the cleavage or the formation of a phosphoglycosyl bond.

The structure of UDP-*N*-acetylglucosamine 2-epimerase also shows that the two monomers differ in their global conformation [83], one subunit being found in a 'closed' conformation and the other in an 'open' conformation. UDP could be clearly modelled into the crevice of the closed form, defining the catalytic site. Six strictly conserved residues are located in the UDP binding region. However, since the glycosyl part of the substrate was not present in the crystallised complex, Campbell et al. [83] could not conclude the possible catalytic roles of these conserved residues. Nevertheless, the authors propose that His213, whose orientation is different in open and closed forms, could be involved in the elimination of UDP. In the closed subunit, His213 is hydrogen-bonded with the β-phosphate of UDP and could act as a general acid catalyst to activate UDP elimination from the sub-

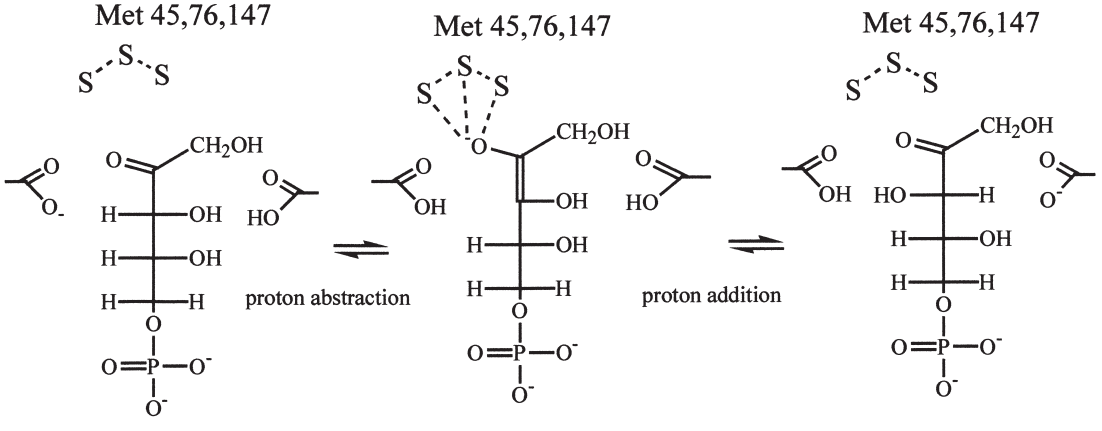


Figure 7. The mechanism of epimerisation of D-ribulose-5-phosphate 3-epimerase with the substrate and product existing in a linear, not cyclic form.

strate. Indeed, there is the possibility of the active site histidine (His213) being transiently phosphorylated, a process that takes place in other enzymes such as phosphoglycerate mutase [84]. Other conserved residues such as Glu117, Glu131 and Asp195 could also participate in the catalysis, either by stabilising the oxocarbenium intermediate or by acting as base or acid catalysts to promote C2 epimerisation.

N-acyl-D-glucosamine 2-epimerase

The structure of *N*-acyl-D-glucosamine 2-epimerase (AGE) from porcine kidney has been determined (table 1, fig. 2g) [85]. AGE is involved in the biosynthesis of *N*-acetylneuraminic acid, an important component of the carbohydrate chain of glycoproteins and glycolipids [86]. AGE epimerisation does not depend on an NAD^+ cofactor, but ATP modulates activity. Although epimerisation takes place on a nonactivated hydrogen at the C2 position, and on the same glycosyl substrate moiety as that of UDP-*N*-acetylglucosamine 2 epimerase, AGE and UDP-*N*-acetylglucosamine 2 epimerase have a completely distinct fold. AGE is a dimer, and its tertiary structure with an α_6/α_6 fold is related to sugar-metabolising enzymes such as glucoamylase [87], endoglucanase CelA [88], cellulase [89] and alginate lyase [90]. AGE has been crystallised in the presence of N-acetyl-D-glucosamine, and this amino sugar could be detected in the cleft created by the inner α barrel near conserved residues (His248, His382, Glu251 and Arg60). These residues may play an important role in catalysis; however, proposal of a mechanism for AGE requires further structural investigations and mutagenesis analyses.

Epimerisation by carbon-carbon bond cleavage

L-ribulose-5-phosphate 4-epimerase and L-fuculose-1-phosphate aldolase

The bacterial enzyme L-ribulose-5-phosphate 4-epimerase interconverts L-ribulose 5-phosphate and D-xylulose 5-phosphate by inverting the configuration at the C4 stereocentre (table 1, fig. 9). This enzyme enables bacteria to use arabinose as an energy source by connecting the arabinose pathway to the pentose phosphate pathway [91]. The enzyme is a homotetramer and requires one divalent cation per subunit for activity [92]. L-ribulose 5-phosphate 4-epimerase catalyses an epimerisation on a stereocentre that does not have an acidic hydrogen and is NAD(P) independent [92, 93]. The reaction is known to proceed without the incorporation of a solvent-derived oxygen or hydrogen [72, 94]. Furthermore, measurements of ^{13}C and deuterium isotope effects have provided evidence that an aldol cleavage occurs during catalysis [95]. This is in agreement with sequence similarities between L-ribulose-5-phosphate 4-epimerase and the class II L-fuculose-1-phosphate aldolase (FucA), a reversible enzyme that synthesises L-fucose-1-phosphate from L-lactaldehyde and dihydroxyacetone phosphate. The structure of FucA has been solved [96–98], and a putative mechanism has been proposed for this metal-assisted enzyme. The structure reveals that four residues, a glutamate and three histidines, are involved in metal binding. Dihydroxyacetone, upon binding, would disrupt the interactions between the glutamate residue and Zn^{2+}, and bind to the metal atom via its carbonyl and hydroxyl groups. The displaced glutamate would then protonate the bound substrate and generate an enediolate intermediate that would be stabilised by its interaction with the metal. The intermediate would then attack the carbonyl of lactaldehyde, creating a C–C bond. A tyrosine residue controls the stereospecificity of the attack, by hydrogen-bonding to the aldehyde. Alignments between FucA and L-ribulose-5-phosphate 4-epimerase show that the FucA residues involved in metal binding are also conserved in L-ribulose-5-phosphate 4-epimerase. In L-ribulose-5-phosphate 4-epimerase, these residues correspond to Asp76, His95, His97 and His171. Mutants His95Asn, His97Asn and His171Asn exhibit a decrease in k_{cat} and a reduced affinity for Zn^{2+} [99]. The His97Asn mutant showed that the mutated enzyme was capable of condensing dihydroxyacetone and glycolaldehyde, thus establishing that the mutated enzyme has an aldolase activity [99], suggesting that the epimerase proceeds through a C–C bond cleavage. From all these observations a potential mechanism, analogous to the FucA mechanism, can be proposed for L-ribulose-5-phosphate 4-epimerase, the only difference between the two mechanisms being

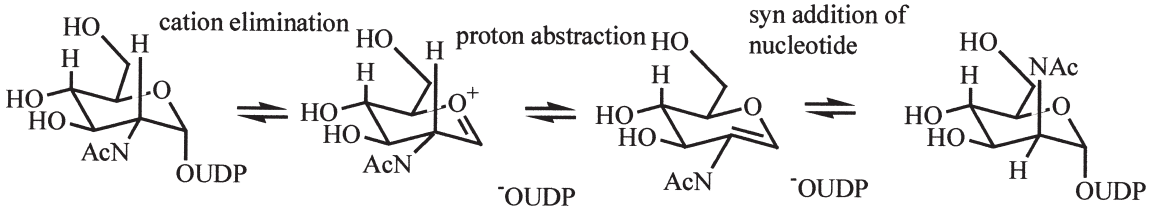


Figure 8. Epimerisation by nucleotide elimination and re-addition as carried out by UDP-*N*-acetylglucosamine 2-epimerase. The mechanism involves the elimination and readdition of UDP.

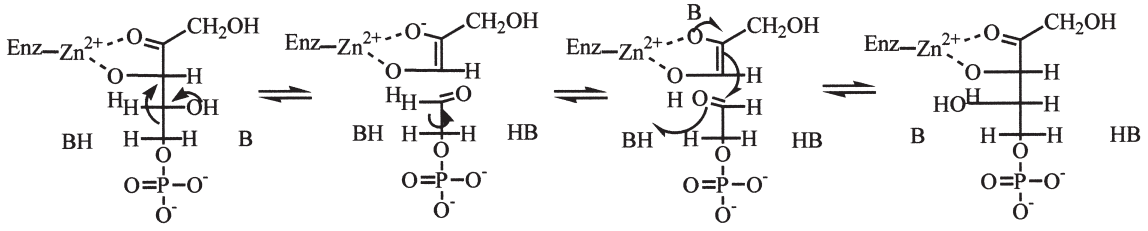


Figure 9. Epimerisation by carbon-carbon bond cleavage as carried out by L-ribulose-5-phosphate 4-epimerase. B represents base, and HB protonated base.

that the attack on the aldehyde is not stereospecific. This mechanism would start by abstraction of the proton from the C4 hydroxyl group, followed by the C3–C4 bond cleavage. Dihydroxyacetone endiolate and glycolaldehyde would be generated. The enediolate would be stabilised by interactions with the metal in a proton-free environment. The C–C glycolaldehyde bond has to be rotated by 180° to allow the inversion of stereochemistry at C4 after the regeneration of the C3–C4 bond. This mechanism seems to be confirmed as the first crystallographic studies obtained on this epimerase [N. Strynadka, unpublished results] show His95, His97 and H171 form an interaction with the metal. The publication of this structure will probably provide a clear mechanism for this enzyme.

Epimerisation by ring opening (mutarotation)

Sugars can exist either as α or β anomers, depending on the chirality of the alcohol substituent at the C1 position. In solution for reducing sugars (OH at C1), the interconversion between these forms is quite rapid. Interconversion can be measured by observing the change in the rotation of polarised light and was a classical experiment in undergraduate chemistry. The process occurs during the conversion of the cyclic sugar to its linear form. The linear form has a keto group at C1; ring closure creates a statistical mixture of α and β forms. Non reducing cyclic sugars cannot open and are thus locked in the α or β form. The enzyme galactose mutarotase is a ubiquitous enzyme and catalyses the conversion from the β anomer to the α anomer (inversion of chirality at C1) of galactose (table 1, fig. 1b) [100]. It has recently been identified as a member of the *gal* operon of *E. coli* and has been shown to be involved in the Leloir pathway. [101]. The catalysis is thought to occur by acid-base mechanism, with a histidine residue identified as a catalytic base [102].

Summary

The inversion of chirality of the hydroxyl substituents of sugars, although straightforward to describe, appears to have led biology to devise a diverse collection of enzymatic strategies. The process is clearly important as all organisms have the ability to perform the epimerisation of carbohydrates. The strategies adopted range from redox chemistry to carbon bond cleavage. The detailed molecular mechanism of many of these processes remains unclear, and ongoing research in this area will continue to provide fascinating insights into carbohydrate epimerisation.

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