Minireviews:

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Minireview

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Structure and Function of Enzymes of the Leloir Pathway for Galactose Metabolism*[S]

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In most organisms, the conversion of β-D-galactose to the more metabolically useful glucose 1-phosphate is accomplished by the action of four enzymes that constitute the Leloir pathway (Scheme 1). In the first step of this pathway, β-D-galactose is epimerized to α-D-galactose by galactose mutarotase. The next step involves the ATP-dependent phosphorylation of α-D-galactose by galactokinase to yield galactose 1-phosphate. As indicated in Scheme 1, the third enzyme in the pathway, galactose-1-phosphate uridylyltransferase, catalyzes the transfer of a UMP group from UDP-glucose to galactose 1-phosphate, thereby generating glucose 1-phosphate and UDP-galactose. To complete the pathway, UDP-galactose is converted to UDP-glucose by UDP-galactose 4-epimerase. In humans, defects in the genes encoding for galactokinase, uridylyltransferase, or epimerase can give rise to the diseased state referred to collectively as galactosemia (1, 2). Although galactosemia is rare, it is potentially lethal with clinical manifestations including intellectual retardation, liver dysfunction, and cataract formation, among others. Indeed, the enzymes of the Leloir pathway have attracted significant research attention for well over 30–40 years, in part because of their important metabolic role in normal galactose metabolism.

As of this year, the three-dimensional structures of all of the enzymes of the Leloir pathway have now been defined. It is thus timely to present in this minireview recent advances in our understanding of the structure and function of these enzymes. For a discussion of the literature prior to 1996, see Ref. 3.

Galactose Mutarotase

Galactose mutarotase activity was first reported in *Escherichia coli* in 1965 (4), and the gene encoding it was defined in 1994 (5). Since 1986, genes encoding for proteins with mutarotase activities have been identified in other organisms including *Lactococcus lactis* (6). With respect to the catalytic mechanism of galactose mutarotase, it was first suggested by Hucho and Wallenfels (7) that the reaction proceeds through the abstraction of the proton from the 1-hydroxyl group of the sugar by an active site base and donation of a proton to the C-5 ring oxygen by an active site acid, thereby leading to ring opening. Subsequent rotation of 180° about the C-1–C-2 bond followed by abstraction of the proton on the C-5 oxygen and donation of a proton back to the C-1 oxygen generates product. A kinetic analysis of the enzyme from *E. coli* was recently reported (8).

In 2002, the first structure of a galactose mutarotase (from *L. lactis*) was determined by Thoden *et al.* (9, 10). A ribbon representation of the dimeric enzyme is displayed in Fig. 1. Each subunit contains 339 amino acid residues and adopts a distinctive β-sandwich motif. Despite the lack of amino acid sequence homology, the overall topology of the β-sandwich is similar to that first observed in domain 5 of β-galactosidase from *E. coli* (11). This β-sheet architecture has since been seen in the central domain of copper amine oxidase (12), the C-terminal domain of chondroitinase (13), the C-terminal domain of hyaluronate lyase (14), and the N-terminal domain of maltose phosphorylase (15).

The active site of galactose mutarotase is positioned in a rather open cleft with the hydroxyl groups of galactose lying within hydrogen bonding distance to a number of side chains, including His-96, His-170, and Glu-304.[1] These three residues are strictly conserved in the galactose mutarotase sequences deposited to date in the Swiss Protein Database. To address the roles of Glu-304, His-96, and His-170 in catalysis, site-directed mutant proteins were constructed, their structures were solved and fully refined to high resolution, and their kinetic parameters were determined (16). Taken together these investigations have led to the proposed catalytic mechanism for galactose mutarotase whereby Glu-304 serves as the active site base to abstract the C-1 hydroxyl hydrogen and His-170 functions as the active site acid to protonate the C-5 ring oxygen.[2] A similar mechanism has been proposed for the enzyme from *E. coli* (17).

Recently, the gene encoding for galactose mutarotase in humans was cloned and expressed, and the protein was purified to homogeneity (18). Unlike the bacterial enzyme, the human protein behaves as a monomer in solution. As observed for the *L. lactis* enzyme (10), however, the human galactose mutarotase demonstrates a preference for galactose over glucose as its substrate. On the basis of site-directed mutagenesis experiments and kinetic analyses, it is believed that the reaction catalyzed by human galactose mutarotase proceeds via Glu-307 and His-176 through a similar mechanism proposed for the enzyme from *L. lactis*. Thus far no diseases have been attributed to mutations in human galactose mutarotase.

Galactokinase

In the next step of the Leloir pathway, α-D-galactose is converted to galactose 1-phosphate via the action of galactokinase. Deficiencies in this enzyme can lead to galactosemia II in humans, which is characterized by the formation of cataracts at an early age. On the basis of amino acid sequence similarities, galactokinase has been placed into the GHMP superfamily with the other primary members being homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (19). The x-ray structures for the latter three proteins have been determined within the last 3 years, and as expected, they adopt similar molecular motifs (20–24).

Galactokinase was first isolated from mammalian liver (25, 26) and has since been studied from bacteria (27), yeast (28, 29), plants (30), and humans (31). The kinetic properties of the enzyme seem to differ according to the source of the protein. In the enzyme isolated from *E. coli*, it appears that the reaction mechanism is random with either ATP or galactose binding first (32). In the rat, yeast, and human enzymes, however, it is reported that the reaction mechanism is ordered with ATP binding first (25, 26, 29, 31). The reaction mechanism in plant galactokinases is ordered but with galactose rather than ATP binding in the first step (30). Recent investigations by Timson and Reece[3] have shown that for human galactokinase, 2-deoxy-D-galactose is also a substrate for the enzyme, whereas *N*-acetyl-D-galactosamine, L-arabinose, D-fucose, and D-glucose are not phosphorylated.

The three-dimensional architecture of galactokinase from *L. lactis,* complexed with α-D-galactose and inorganic phosphate, has recently been described (34). This bacterial enzyme demonstrates a

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[S] The on-line version of this article (available at *http://www.jbc.org*) contains supplemental Figs. 1–4 and Scheme 1.
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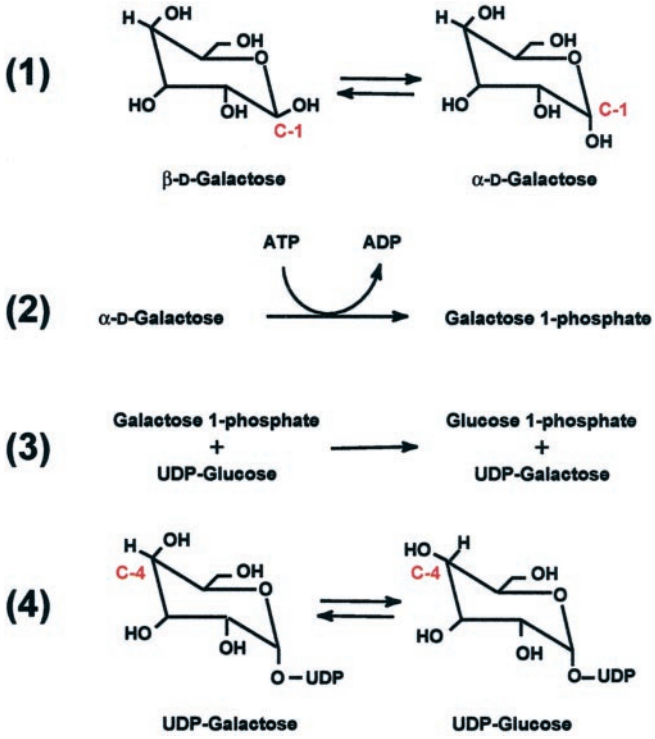
[1] A stereo view of the active site of galactose mutarotase from *L. lactis* is shown in Fig. 1 of the Supplemental Material.
[2] A schematic of the reaction mechanism for galactose mutarotase from *L. lactis* is presented in Scheme 1 of the Supplemental Material.
[3] D. J. Timson and R. J. Reece, submitted for publication.



SCHEME 1

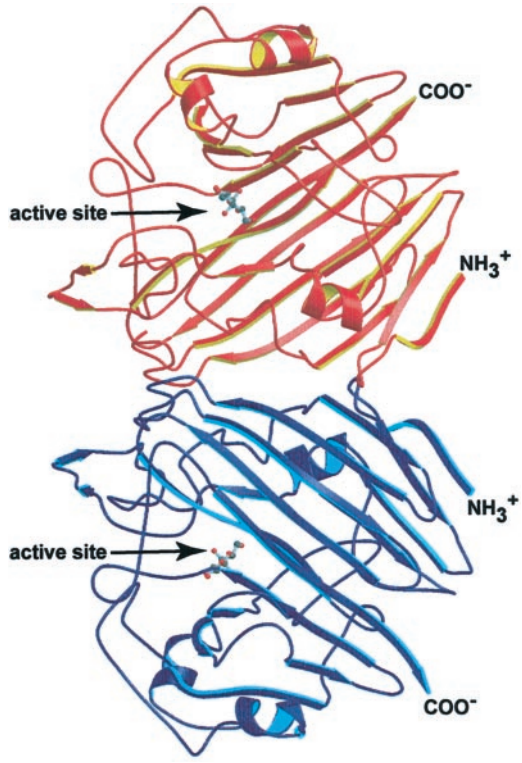


FIG. 1. **Ribbon representation of galactose mutarotase from *L. lactis*.** The two subunits of the dimeric protein are displayed in *red* and *blue*. Bound galactose molecules are depicted in *ball-and-stick* representations. X-ray coordinates were from Protein Data Bank number 1L7K. All figures were prepared with MOLSCRIPT (33).

34% identity and a 47% similarity with human galactokinase. A ribbon representation of the monomeric *L. lactis* enzyme (399 amino acid residues) is presented in Fig. 2, and as can be seen the polypeptide chain folds into two domains of roughly equal size. The N-terminal domain is dominated by a five-stranded mixed β-sheet surrounded by five α-helices whereas the C-terminal motif contains

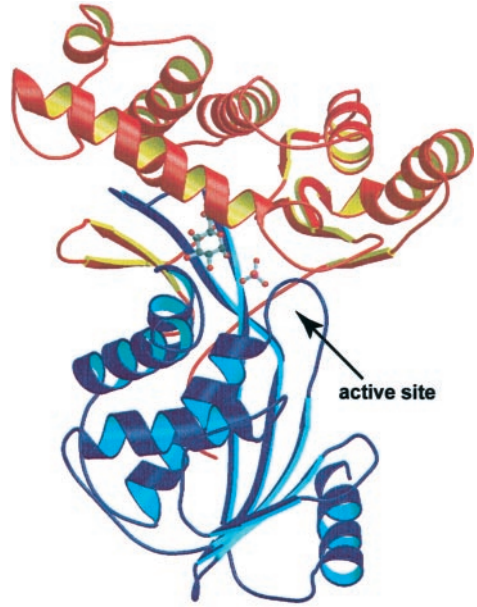


FIG. 2. **Ribbon representation of galactokinase from *L. lactis*.** The N- and C-terminal domains of the monomeric enzyme are displayed in *blue* and *red*, respectively. The bound galactose and inorganic phosphate moieties are depicted in *ball-and-stick* representations. X-ray coordinates were from Protein Data Bank number 1PIE.

two layers of β-sheet and six α-helices. As expected, the overall topology of galactokinase is similar to that first observed in homoserine kinase.

The active site for galactokinase is wedged between the N- and C-terminal domains.[4] The location of the bound inorganic phosphate in galactokinase is similar to the position observed for the γ-phosphorus of AMPPNP in homoserine kinase. Both the carboxylate side chain of Asp-183 and the guanidinium group of Arg-36 lie within hydrogen bonding distance of the substrate 1-hydroxyl group. These two residues appear to be absolutely conserved in galactokinase amino acid sequences examined thus far.

Two quite different catalytic mechanisms have been proposed for members of the GHMP superfamily. In mammalian mevalonate kinase, for example, it has been suggested that an aspartate residue (in the same position as Asp-183 in galactokinase) serves to abstract a hydroxyl hydrogen from the substrate whereas a lysine residue (in the same position as Arg-36 in galactokinase) functions to lower the pK_a of the hydroxyl group (23). In homoserine kinase, however, these two residues have been replaced with an asparagine and a threonine, respectively, and there is an apparent absence of a catalytic base in the region near the substrate hydroxyl group that is ultimately phosphorylated during the reaction (20, 21). In light of this, a catalytic mechanism for homoserine kinase has been proposed whereby the binding of homoserine and ATP positions the two ligands close enough for direct attack of the δ-OH group of homoserine onto the γ-phosphorus of ATP. It is believed that deprotonation of the δ-hydroxyl group of homoserine occurs through its interaction with the γ-phosphate of ATP rather than through the action of a general base. From the structure of galactokinase from *L. lactis*, it might be speculated that the conserved Asp-183 and Arg-36 are playing similar roles as those suggested for mevalonate kinase and that the reaction mechanism proceeds through proton abstraction by the side chain carboxylate of Asp-183. On the other hand, a recent study on human galactokinase by Timson and Reece (31) demonstrated the absence of a deuterium kinetic isotope effect, thus suggesting that proton transfer is not involved in the rate-determining step.

Galactose-1-phosphate Uridylyltransferase

The reversible transfer of a UMP moiety from UDP-glucose to galactose 1-phosphate is catalyzed by the third enzyme of the

[4] A stereo view of the active site of galactokinase from *L. lactis* is shown in Fig. 2 of the Supplemental Material.

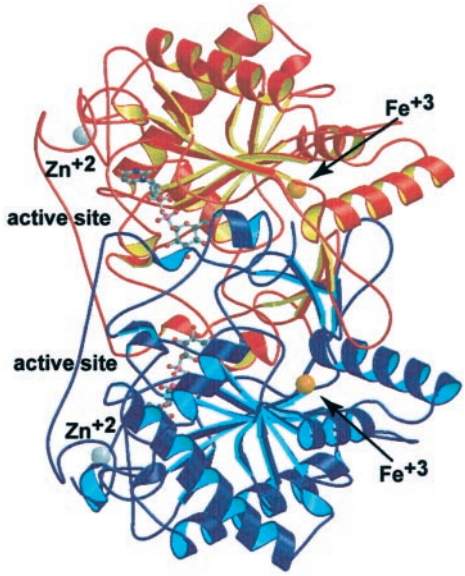


FIG. 3. **Ribbon representation of galactose-1-phosphate uridylyltransferase from *E. coli*.** The two subunits of the dimeric enzyme are displayed in *red* and *blue* with the positions of the metals indicated by the *round spheres*. The active site in this protein model contains bound UDP-glucose. X-ray coordinates were from Protein Data Bank number 1GUQ.

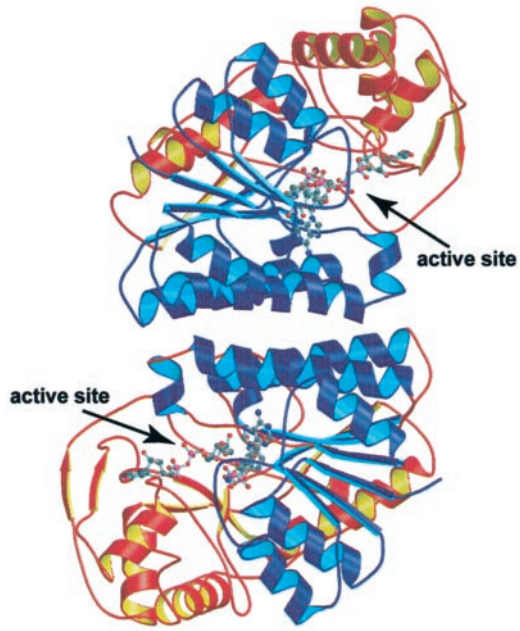


FIG. 4. **Ribbon representation of human UDP-galactose 4-epimerase.** Each subunit of the dimeric enzyme folds into two domains. The N- and C-terminal domains are color-coded in *blue* and *red*, respectively. Bound NADH and UDP-glucose molecules are drawn in *ball-and-stick* representations. X-ray coordinates were from Protein Data Bank number 1EK6.

Leloir pathway, galactose-1-phosphate uridylyltransferase. This enzyme has been shown to belong to the histidine triad (HIT) superfamily (35, 36). Members of this family function as either nucleotide hydrolases or transferases that act upon the α-phosphorus of nucleotides.

Defects in galactose-1-phosphate uridylyltransferase result in galactosemia I or classic galactosemia with clinical manifestations including intellectual retardation, liver dysfunction, and cataract formation. Of the four enzymes in the Leloir pathway, only the reaction catalyzed by galactose-1-phosphate uridylyltransferase proceeds through a covalently bound intermediate (37, 38). According to the proposed mechanism, UDP-glucose binds to the enzyme, a uridylylated enzyme intermediate is generated, and glucose 1-phosphate is released. Subsequently, galactose 1-phosphate binds to the active site and the UMP moiety is transferred to generate UDP-galactose. In the enzyme from *E. coli*, His-166 has been shown to be the residue transiently modified and is thus the active site base that attacks the α-phosphorus of the incoming sugar-UDP substrate (39). Nucleophilic attack on the α-phosphate of the uridylyl enzyme intermediate by either galactose 1-phosphate or glucose 1-phosphate results in the transfer of the UMP moiety back to recreate the UDP-sugar.

The three-dimensional structure of the enzyme from *E. coli* was elucidated by Wedekind *et al.* in 1995 (40). A ribbon representation of the homodimer is presented in Fig. 3. Each subunit contains 348 amino acid residues and additionally one zinc and one iron. The overall fold of the subunit has been referred to as a "half-barrel" with nine strands of anti-parallel β-sheet flanked on either side by α-helices. The iron serves in a structural capacity by bridging two β-strands and an α-helix near the subunit:subunit interface of the dimer. The zinc ion is located within ~8 Å of the active site and is tetrahedrally ligated by Cys-52, Cys-55, His-115, and His-164. On the basis of amino acid sequence alignments, it appears that in higher organisms Cys-52 and His-115 are not conserved, thus suggesting that some uridylyltransferases do not bind a second metal (40).

To trap the uridylyl-enzyme intermediate, single crystals of the active enzyme were transferred to solutions containing UDP-glucose and moved to successively higher pH values up to 7.1 (41). Under these conditions, the enzyme was active, but the rate of acid-catalyzed hydrolysis of the intermediate was reduced. This study revealed a covalent bond between $N^{\epsilon 2}$ of His-166 and the α-phosphorus of UMP. Additionally it was shown that the side chain of Gln-168 provided important hydrogen bonds to both O2′ and O5′ of the nucleotide.[5] It should be noted that in the human enzyme, the mutation of this glutamine to an arginine is the predominant cause of galactosemia among the Caucasian population (42). This change to an arginine residue may result in overstabilization of the enzyme intermediate, thereby compromising its subsequent reaction with galactose 1-phosphate.

To address the manner in which UDP-glucose or UDP-galactose is accommodated in the active site of the uridylyltransferase, a site-directed mutant protein, H166G was constructed (43). This investigation revealed that the active site for the uridylyltransferase is formed by amino acid residues contributed by both subunits of the homodimer. Accommodation of the glucose *versus* galactose moieties is accomplished by simple movements of two side chains and by a change in the backbone dihedral angles of Val-314.

UDP-galactose 4-Epimerase

UDP-galactose 4-epimerase catalyzes the final step in normal galactose metabolism by regenerating UDP-glucose as indicated in Scheme 1. According to all presently available biochemical and kinetic data, the reaction mechanism of epimerase is presumed to occur via the following steps: 1) abstraction of the 4′-hydroxyl hydrogen by an enzymatic base and hydride transfer from C-4 of the sugar to the *si*-face the nicotinamide ring of NAD^+; 2) rotation of the resulting 4′-ketopyranose intermediate in the active site to present the opposite face of the sugar to the reduced dinucleotide; and 3) transfer of the hydride from the nicotinamide ring of NADH back to C-4 of the sugar and reprotonation of the C-4 oxygen.

Of the four enzymes in the Leloir pathway, the epimerase is by far the best structurally characterized. Most of the original x-ray crystallographic studies were conducted on the enzyme from *E. coli* (Ref. 44, and references therein). From this original work the overall molecular architecture of the dimeric enzyme was defined and the manner in which UDP-glucose or UDP-galactose is accommodated in the active site was elucidated. It was also shown that UDP-galactose 4-epimerase belongs to a set of proteins referred to as the short chain dehydrogenase/reductase superfamily. These enzymes are widely distributed in nature and are involved in a number of physiological processes including normal and metastatic growth, fertility, and hypertension (45). All of these enzymes contain a conserved Tyr-*X*-*X*-*X*-Lys motif that is involved in catalysis.

[5] A schematic of the hydrogen bonding pattern around the uridylyl-enzyme intermediate of galactose-1-phosphate uridylyltransferase is shown in Fig. 3 of the Supplemental Material.

Since 2000, most attention has focused on the human form of the enzyme. Impairment of this enzyme results in galactosemia III that can lead to symptoms ranging from benign to severe (1, 2). Shown in Fig. 4 is a ribbon representation of human epimerase complexed with NADH and UDP-glucose (46). Each subunit folds into two distinct motifs. The N-terminal domain contains seven strands of a parallel β-sheet with an overall topology similar to that of a Rossmann fold. The NAD(H) lies across the C-terminal end of this β-sheet. The C-terminal motif contains six strands of β-sheet and provides the binding site for the UDP portion of the UDP-sugar. In the ternary complex, C-4 of UDP-glucose (which transfers its hydride to NAD^+ during catalysis) is positioned within 3.5 Å of C-4 of the nicotinamide ring. Additionally, O^{η} of Tyr-157 lies at 3.1 Å from the 4′-hydroxyl oxygen.[6] This tyrosine is part of the conserved Tyr-*X*-*X*-*X*-Lys motif and given its location in the crystalline structure and the results of site-directed mutagenesis experiments most likely functions as the catalytic base in UDP-galactose 4-epimerase (47, 48).

Of particular interest is the ability of the human form of epimerase to interconvert UDP-GlcNAc and UDP-GalNAc. The *E. coli* enzyme does not display such activity. To address the manner in which UDP-GlcNAc is bound to the human protein, the structure of the enzyme complexed with this ligand was solved and refined to 1.5-Å resolution (49). This x-ray crystallographic analysis demonstrated that to accommodate the additional *N*-acetyl group at the C-2 position of the sugar there is a simple movement of an asparagine residue toward the interior of the protein. It was also noted in this study that the structural equivalent of Tyr-299 in the *E. coli* protein is replaced with Cys-307 in the human epimerase, thereby resulting in an active site that is ~15% larger. To address whether or not the increased active site volume is the underlying factor for the additional catalytic activity of the human epimerase against UDP-GlcNAc/UDP-GalNAc substrates, a site-directed mutant protein of the *E. coli* enzyme was constructed, namely Y299C (50). Indeed, although this Y299C mutation resulted in a loss of epimerase activity with regard to UDP-galactose by almost 5-fold, it resulted in a gain of activity against UDP-GalNAc by more than 230-fold.

In summary, the importance of normal galactose metabolism was recognized well over 30 years ago when researchers began their pioneering efforts on the four enzymes of the Leloir pathway (4, 25, 51, 52). Since that time an enormous amount of biochemical, kinetic, and structural data has been generated on these fascinating enzymes. Interestingly, in the past it has been speculated that enzymes within a given metabolic pathway evolved from one another because of the need to accommodate similar substrates (53). Clearly this is not the case for enzymes of the Leloir pathway. Indeed, questions remain regarding the evolutionary history of this important metabolic cycle.

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[6] A stereo view of the active site of human UDP-galactose 4-epimerase is shown in Fig. 4 of the Supplemental Material.