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Critical Review

Structural and Molecular Biology of Type I Galactosemia: Disease-associated Mutations

Thomas J. McCorvie and David J. Timson
School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, Belfast, Northern Ireland, UK

Summary

Type I galactosemia results from reduced galactose 1-phosphate uridylyltransferase (GALT) activity. Signs of disease include damage to the eyes, brain, liver, and ovaries. However, the exact nature and severity of the pathology depends on the mutation(s) in the patient's genes and his/her environment. Considerable enzymological and structural knowledge has been accumulated and this provides a basis to explain, at a biochemical level, impairment in the enzyme in the more than 230 disease-associated variants, which have been described. The most common variant, Q188R, occurs close to the active site and the dimer interface. The substitution probably disrupts both UDP-sugar binding and homodimer stability. Other alterations, for example K285N, occur close to the surface of the enzyme and most likely affect the folding and stability of the enzyme. There are a number of unanswered questions in the field, which require resolution. These include the possibility that the main enzymes of galactose metabolism form a supramolecular complex and the need for a high resolution crystal structure of human GALT.

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Keywords galactose metabolism; disease-associated mutation; GALT; galactose 1-phosphate uridylyltransferase; histidine triad transferase; UMP-histidine.

Abbreviations GALT, galactose 1-phosphate uridylyltransferase; GALE, activities of GALT and UDP-galactose 40-epimerase.

INTRODUCTION

Type I, or classical, galactosemia (OMIM #230400) results from mutations in the gene encoding the enzyme galactose 1-phosphate uridylyltransferase (GALT, EC 2.7.7.12) (*1*). The signs of disease include early-onset cataracts (which can be resolved by surgery) and damage to the liver, kidneys, brain, and ovaries (*2*). Thus, patients often develop developmental, cognitive, and reproductive defects in childhood (*3*). The exact pathology depends on the precise mutation(s) in the patient's genes and on his/her environment. The only treatment currently available is the removal of galactose and lactose (a disaccharide of glucose and galactose) from the diet. This treatment improves the prospects of patients and reduces the risk of death in childhood. However, this treatment is unable to reverse organ damage.

STRUCTURAL AND ENZYMOLOGICAL CONSEQUENCES OF DISEASE-ASSOCIATED MUTATIONS

GALT catalyses the transfer of an uridyl group from UDP-glucose to galactose 1-phosphate (Fig. 1a). The structure of GALT from *Escherichia coli* has been solved and revealed that the enzyme is a dimer with one active site, one zinc ion, and one iron ion per subunit (Fig. 1b) (*4*). Over 230 different mutations have been described in the gene encoding human GALT. A database of these mutations is maintained at www.arup.utah.edu/database/GALT/GALT_welcome.php and readers are encouraged to consult this database for up-to-date information on mutations in human GALT (*5*). Most of these mutations result in single amino acid changes within the enzyme. However, some result in premature termination of the polypeptide chain. Although some of the disease-associated mutations are close to the active site of the enzyme, many are not (Fig. 2). Therefore, it must be assumed that they exert their effects by conformational changes, which are transmitted through the enzyme's structure to the active site.

Considerable efforts have been made to understand the biochemical consequences of these mutations. The determination of enzymological parameters for variant forms of human GALT yields important, quantitative information about the level and nature of impairment of enzyme activity. This line of enquiry is especially useful when combined with other biochemical inves-

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Address correspondence to: David J. Timson, School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, Northern Ireland BT9 7BL, UK. Tel: +44-28-9097-5875. Fax: +44-28-9097-5877. E-mail: d.timson@qub.ac.uk

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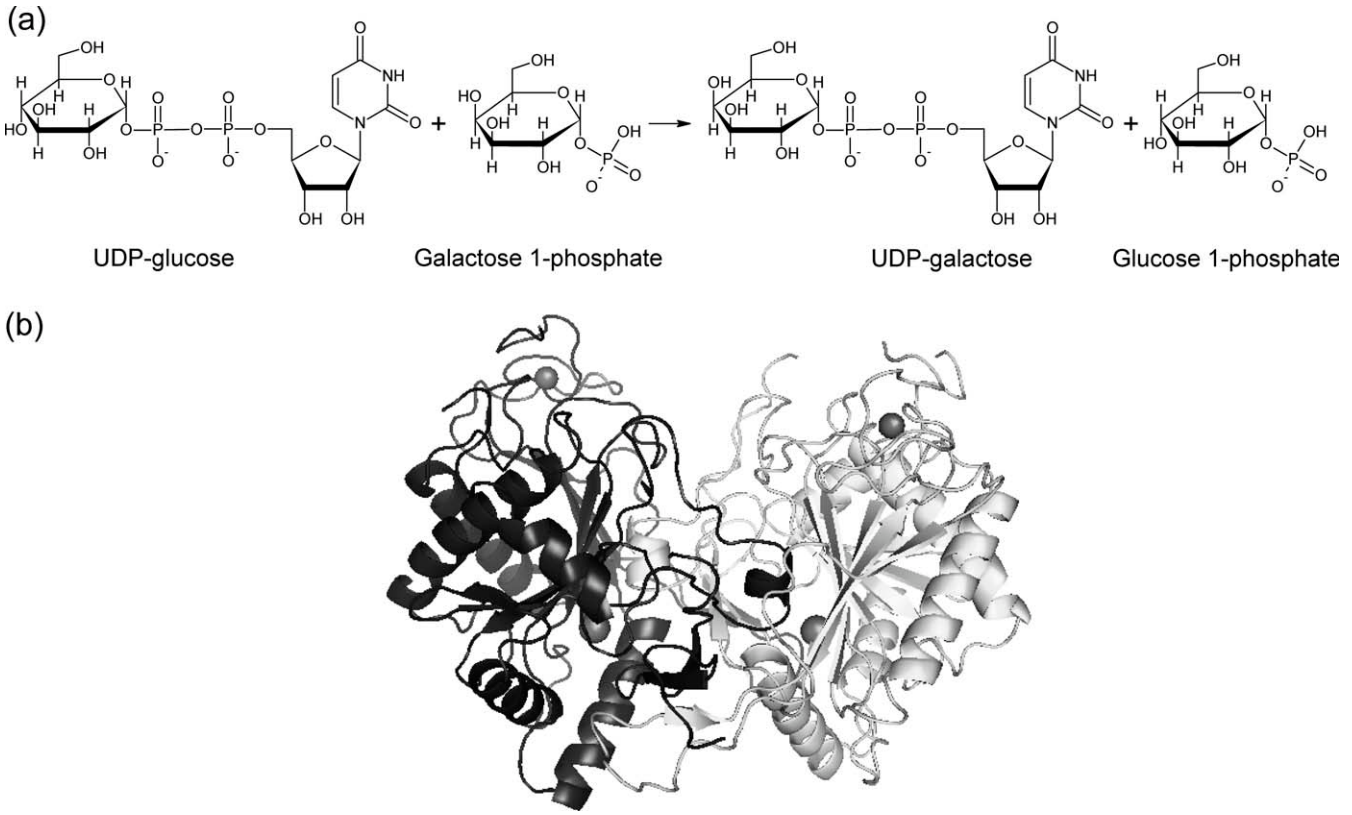

Figure 1. (a) The reaction catalyzed by GALT. (b) The structure of *E. coli* GALT shows that the enzyme is a dimer. Here the two sub-units are shown in light and dark grey. The metal ions are shown as spheres with the zinc ions towards the top of the sub-units as shown in the figure and the iron ions towards the lower middle. The figure was produced from PDB file 1HXQ (*4*) using PyMol (www.pymol.org).

tigations, for example, into structural stability and dimerization ability. However, it is also important to understand how the enzymes function in vivo. Although some work has been done with cultured cells derived from patients with type I galactosemia, more has been done with genetically modified model organisms. In this approach, the organism's own GALT gene is replaced with either the wild-type human GALT or a disease-associated variant. The budding yeast, *Saccharomyces cerevisiae*, is a powerful system for these experiments. This organism can use either glucose or galactose as a carbon and energy source. Therefore, strains can be developed using glucose containing media without any risk of negative selective pressures resulting from defective galactose metabolism. However, if the yeast strain is then transferred to media containing galactose as the sole energy source, it rapidly induces the genes encoding Leloir pathway enzymes. Human GALT substitutes well for the yeast's own enzyme (Gal7p) (*8*). However, many of the disease-associated mutations result in impaired growth on this sugar (see below). A further advantage of using *S. cerevisiae* is that the concentration of galactose in the medium can be controlled experimentally thus permitting effects due to the build up of galactose 1-phosphate to be assessed. The major disadvantage of using a single-celled organism for these experiments is that it does not have differentiated cell types or organ systems. Therefore, the experiments have only limited predictive value when assessing molecular pathology. For this reason, mouse models have also been developed, although these do not display as severe pathology as humans with the same alleles (*9*). The recent development of a fly (*Drosophila melanogaster*) model for type I galactosemia is an exciting advance, which will provide a genetically tractable multicellular model system (*10*).

THE Q188R ALLELE

The most commonly detected mutation in Caucasians results in the Q188R allele. Homozygotes for Q188R show very little to no GALT activity in their erythrocytes and this variant of the enzyme shows no activity when expressed in GALT-null yeast (*8, 11, 12*). In transformed COS cells, however, the enzyme retained 10% of its activity, but it is thought that endogenous GALT in the cell line contributed to this discrepancy (*11, 13, 14*). The majority of heterozygotes are found to have no activity, while others have a low value (up to 20% of the wild type). This suggests that allelic heterogeneity plays a role in the range

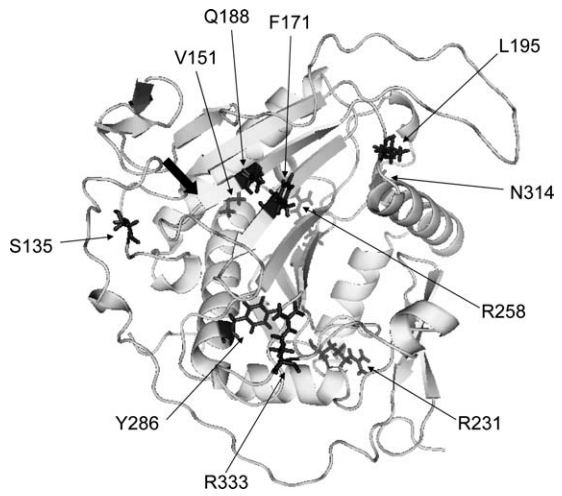

Figure 2. Location of amino acids that are altered by disease-associated mutations in human GALT. For clarity, only those variants discussed in this review are shown (in dark grey) in one subunit of the homodimer. The position of the active site histidine (His-186) is indicated by the black block arrow. The model was obtained using the online molecular modeling service, Phyre (*6*). The resulting model, based on PDB file 1HXQ (*4*), was then computationally solvated and minimized using YASARA (*7*).

of activities found in patients and may ultimately contribute to the progression of the disease (*15, 16*).

The biochemistry of the Q188R mutation has been assessed by studying its enzyme kinetics and structural effects by modeling the human enzyme using the *E. coli* structure as a template (*17–19*). Importantly, the effect on GALT heterodimers has been studied by using a yeast recombinant expression system. Purified Q188R/WT heterodimers showed 15% activity in comparison to the wild-type homodimer. This is much smaller than the 50% activity predicted if no intersubunit interactions were present and suggests that Q188R acts as a partial dominant negative. It was also found that the K_m values for the substrates were not greatly affected and that the heterodimer was thermodynamically destabilized compared to the wild type (*20*). The Q188R variant does not form a random mixture of heterodimers and homodimers, but an excess of the latter that further confirms the partial dominant negative nature of this allele (*21*). In addition, two-dimensional electrophoresis and Western blot analysis showed two discrete spots from GALT purified from human red blood cells and with recombinant, wild-type GALT. This was attributed to the presence of uridylated and nonuridylated GALT and was confirmed by only finding one spot with the inactive variant, H186G. The Q188R variant was found to have a two-spot pattern, in agreement with other findings that this variant can still form the covalent intermediate (*17, 18, 22*). In kinetic studies, the corresponding *E. coli* GALT mutant, Q168R, showed a decrease in activity but not complete loss of activity as was found in human GALT. Decreased rates of uridylation and deuridylation were suggested to be the cause of the large overall decrease in activity and not any change in overall structure or stability of the intermediate (*17*). In contrast, initial modeling studies on the human GALT suggested that Gln-188 provides two hydrogen bonds to α and β phosphoryl oxygens of the UDP moiety and that these stabilize the covalent intermediate. Alteration of the residue to arginine decreases the number of hydrogen bonds to one thus decreasing the stability of the uridyl-enzyme intermediate (*18*). However, more recent modeling studies suggest that this change in hydrogen bonding patterns to the phosphates of UDP-glucose prevents the dispersion of electrons and slows the nucleophilic attack by galactose 1-phosphate. The addition of a positive charge and steric hindrance also decreases the number of hydrogen bonds between the subunits of the dimer (*19*). This would decrease the thermostability of the Q188R variant, in agreement with experimental findings (*20*).

COMMON ALLELES: A BIOCHEMICAL AND MOLECULAR PARADIGM FOR OTHER ALLELES

Apart from Q188R the most commonly detected mutations in European populations are K285N, S135L, and N314D. The K285N mutation is associated with 50% and 0% GALT activity in heterozygous and homozygous patients, respectively (*23, 24*). In agreement with this, GALT null yeast were not rescued by expression of human K285N GALT and showed no detectable activity. The amount of GALT was also found to be lower in comparison to wild-type levels, but the main cause of its decreased activity is unknown (*8, 25*). The change from lysine to asparagine may cause increased susceptibility to proteolysis, inability to fold correctly, and/or global changes to the structure. Molecular modeling has suggested that residue Lys-285 is on the protein's surface (*8*). Several other mutations associated with reduced protein abundance (V151A, R231H, and R259W) also occur in residues near the surface [Fig. 2; (*8*)].

S135L is almost only ever detected in African-Americans and negroid South Africans. They occur at 62% and 91% of all mutant alleles detected in these populations, respectively (*26, 27*). The allele has been associated with variable levels of activity: heterozygous subjects show a range from 0 to 17% of wild-type activity, whereas homozygous subjects show a much smaller range of 0 to 1.7% (*15*). These activities are derived from patient erythrocytes, but total body oxidation was found to be normal for homozygous patients (*26, 28*). COS cells expressing the S135L GALT showed normal GALT activity (*29*), but homozygous transformed lymphoblasts did not show any activity (*26*). Again, endogenously expressed GALT in the COS cells could explain the disparity (*12, 14*). The ability of homozygous subjects to metabolize galactose normally, indicated that S135L GALT may have a tissue or organ-specific effect, and it was suggested that the serine might be phosphorylated (*26*). The more likely possibility is that S135L GALT is more

susceptible to degradation and erythrocytes cannot synthesize any new GALT unlike other cells in organs such as the liver (*26, 28*). This hypothesis is supported by the detection of 10% activity in the liver (*30*) and intestine (*30*) of S135L patients, which correlates with the 10% activity found in cultured leukocytes (*26*). Purified, recombinant enzyme derived from a yeast expression system showed a twofold decrease in abundance of S135L GALT in comparison to the wild type. The K_m values were also twofold higher, but the greatest effect was a 10-fold decrease in the specific activity. No changes in the thermostability were detected, and it was concluded that the serine to leucine substitution causes some perturbations to the active site through steric clashes or alterations in charge (*31*). When this allele was expressed in GALT null yeast, it rescued growth; however, this was delayed by several days in comparison to wild-type GALT and other, milder, mutations (*8*). Similar experiments using an *E. coli* expression system with site saturation mutagenesis at Ser-124 (the equivalent of Ser-135 in the human enzyme) showed that all mutations, except S135T, decreased the overall activity to less than 5%. However, S135T showed 35.4% activity, suggesting the removal of the hydroxyl group is the main cause of the detrimental effect. The crystal structure of *E. coli* GALT shows that Ser-124 is close to His-115 in the active site. This residue binds to the β-phosphate of UDP-hexose (*32*). Unlike Q188R, the S135L allele showed the expected 50% activity as a heterodimer with the wild type, but interestingly the ratio of heterodimers to homodimers was skewed toward an excess of the latter; however, the effects this has on S135L patients are unknown (*33*).

The N314D mutation is detected at much higher rates than classic galactosemia in general (*34*) and this varies with ethnicity (*35, 36*). The N314D mutation, by itself, appears to contribute no effect and is most likely a polymorphism (*8*). Interestingly, it is associated with two variants, Duarte-1 (or Los Angeles) and Duarte-2 (*37*). Duarte-1 is a variant where subjects have a much higher GALT activity and protein abundance in their lymphoblasts and erythocytes than is normally detected (*37, 38*). The increased abundance is thought to be associated with a polymorphism in exon 7 that causes a base change of C to T at position 1721. This is a neutral mutation in the codon encoding Leu-218. No changes in mRNA levels and thermostability have been detected, and it has been proposed that codon bias might play a part, increasing the protein levels of this GALT variant (*38*). Duarte-2, however, has been associated with subjects who have lower than normal GALT activity but show a mild phenotype. It also shows a large variation due to allelic effects where homozygous (D/D) patients, heterozygous patients (D/N), and heterozygous patients with another galactosemic allele (D/G) show 50, 75, and 25% activity, respectively (*39–41*). Even with this low activity, D/G patients appear to have good clinical and developmental outcomes. This does not depend on whether or not galactose is restricted in the diet and D/G patients appear to have no abnormal liver function. They also appear to have a normal ability to oxidize galactose (*40, 42*).

D/G subjects have a decrease of ~89% in GALT abundance in their lymphoblasts (*38*), and lymphoblasts transformed so that they are homozygous for the Duarte-2 variant showed a 50% decrease (*41*) correlating well with the 50% activity found in subjects homozygous for Duarte-2. Three intronic, in cis variants linked to N314D have been found in Duarte-2 subjects: IV54nt-27g → c, IV55NT+62g → a, and IV55nt-24g → a. It was suggested that these alter mRNA slicing (*37, 43, 44*). However, the detection of a GTCA deletion in the 5′ upstream promoter region in cis with Duarte-2 suggested that the promoter sequence may be important (*33*). This deletion is not found in Duarte-1 patients (*45*). HepG2 cells transformed with a luciferase gene under the control of the promoter of human GALT with this GTCA deletion decreased the production of luciferase by 55%. Therefore, this deletion must be the main contributor to Duarte-2's 50% reduction in cellular GALT activity (*46*). Further conformation of the role of this GTCA deletion was provided by the detection of decreased mRNA levels in the blood cells of Duarte-2 subjects (*45*).

THE BIOCHEMISTRY OF LESS COMMON ALLELES

Other less common mutations have also been studied using yeast as a model organism and patterns have been observed between the site of the mutation and severity of its effects. One such mutation is F171S, which is mainly detected in the African-American population and is associated with low activity (*29*). Yeast studies correlate well with this (*8, 47*). Modeling studies suggested that Phe-171 is near the active site and the subunit interface (Fig. 2). Importantly, Phe-171 is near Gln-188 and modeling suggests that replacement of the bulky phenylalanine side chain with the smaller leucine creates a void, repositioning Gln-188 (*47*). Heterodimers of F171S with wild type have much lower activity suggesting that the dimer interface is altered by the mutation (*21, 47*). This was also found with another mutation at the same site, F171W (*21*) and both suggest that mutations at, or close to, the dimer interface can affect the activity of both subunits. This hints that there may be allosteric communication between the active sites, via this interface.

R333W is associated with low GALT activity (*13*) and modeling suggests that this residue is also part of dimer interface [(*8*); Fig. 2]. However, no effects on the formation of the heterodimer with the wild-type protein have been detected, and very little change in the K_m values for the substrates were seen (*20*). The homodimer, however, cannot rescue GALT-null yeast and showed similar activity to S135L, L195P, F171S, and Q188R in this system. These residues are also involved in the dimer interface as well as the active site leading to the conclusion that mutations at this location will cause severe reductions in GALT activity (*8*). This may be due to decreased stability, altered ability to dimerize or inability to stabilize the covalent intermediate.

FUTURE PERSPECTIVES

Data from the most commonly detected alleles suggest that sequence alterations at the protein level may not be the only contributing factor in disease causation and to obtain a deeper understanding a combination of the yeast and fruit fly models (*10, 11*) along with biochemical and structural studies will be required. Currently, structural interpretations of the disease-associated variants rely on homology modeling based on the *E. coli* structures. Resulting from this, a large database has been created (http://bioinformatica.isa.cnr.it/GALT/) that includes the results of the effects of over 100 single-point mutations in relation to the changes in interactions between residues, stability, secondary structure, and ligand binding (*48*). Such databases already provide valuable insights; however, these would be enhanced by the solution of the structure of human GALT, which would enable more accurate modeling of the variants.

One important issue when considering the activity of the enzyme in vivo, is the possibility that the Leloir pathway enzymes may form a multienzyme complex or metabolon. Such an association has been suggested by studies on *S. cerevisiae* GALT (Gal7p). GFP-tagged Gal7p localized to discrete spots in the cytoplasm. This discrete localization was abolished in yeast strains in which the galactokinase or UDP-galactose 4′-epimerase genes had been deleted. This suggested that the localization of Gal7p was determined by the other enzymes, consistent with the formation of discrete, multienzyme complexes (*49*). Interestingly, when the human GALT gene was substituted for the yeast one, similar localization was observed (*49*). To date, no localization studies have been carried out on the Leloir pathway enzymes in human cells. However, if the enzymes do form a metabolon, this will have profound effects on the in vivo kinetics of the overall pathway and it will be necessary to consider the effect of disease-associated mutations on the metabolon as a whole and not just the individual enzymes. This may be the reason for the discrepancy observed with R67C between its intermediate activity in vitro and inability to rescue GALT-null yeast in vivo. Arg-67 is predicted to be on the protein's surface and, unlike other alterations on the surface, R67C did not cause any decrease in GALT abundance (*8*).

In this context, it is interesting to note that the activities of GALT and UDP-galactose 4′-epimerase (GALE) from human cells are positively correlated (*50*). This suggests that reduction of the activity of one of these enzymes (for example, by a disease-associated mutation) would affect the activity of both enzymes in vivo. This could arise via protein–protein interactions in a Leloir pathway metabolon, although other explanations cannot be ruled out, for example, coordinated regulation of the genes encoding the proteins. It would be interesting to know if galactokinase activity is also correlated with GALT and GALE activity.

CONCLUSIONS

The knowledge of the enzymology and structure of GALT has greatly assisted our understanding of type I galactosemia. It has enabled us to make rational predictions about the likely molecular consequences of disease-associated mutations. The integration of structural, enzymological, and animal model data will continue to be vital in advancing our understanding of the biochemical basis of this disease.

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