J. Inherit. Metab. Dis. 23 (2000) 713–729

Studies of the V94M-substituted human UDPgalactose-4-epimerase enzyme associated with generalized epimerase-deficiency galactosaemia

T. M. WOHLERS[1] and J. L. FRIDOVICH-KEIL[2]*
[1] *Graduate Program in Genetics and Molecular Biology, Emory University,*
[2] *Department of Genetics, Emory University School of Medicine, Atlanta, Georgia, USA*

* *Correspondence: Emory University School of Medicine, Department of Genetics, 1462 Clifton Rd, NE, Atlanta, GA 30322, USA. E-mail: jfridov@emory.edu*

MS received 17.04.00 Accepted 30.06.00

Summary: Impairment of the human enzyme UDPgalactose 4-epimerase (hGALE) results in epimerase-deficiency galactosaemia, an inborn error of metabolism with variable biochemical presentation and clinical outcomes reported to range from benign to severe. Molecular studies of the hGALE loci from patients with epimerase deficiency reveal significant allelic heterogeneity, raising the possibility that variable genotypes may constitute at least one factor contributing to the biochemical and clinical heterogeneity observed. Previously we have identified a single substitution mutation, V94M, present in the homozygous state in all patients genotyped with the severe, generalized form of epimerase-deficiency galactosaemia. We report here further studies of the V94M-hGALE enzyme, overexpressed and purified from a null-background yeast expression system. Our results demonstrate that the mutant protein is impaired relative to the wild-type enzyme predominantly at the level of V_{max} rather than of K_m. Studies using UDP-*N*-acetylgalactosamine as a competitor of UDPgalactose further demonstrate that the K_m values for these two substrates vary by less than a factor of 3 for both the wild-type and mutant proteins. Finally, we have explored the impact of the V94M substitution on susceptibility of yeast expressing human GALE to galactose toxicity, including changes in the levels of galactose 1-phosphate (gal-1-P) accumulated in these cells at different times following exposure to galactose. We have observed an inverse correlation between the level of GALE activity expressed in a given culture and the degree of galactose toxicity observed. We have further observed an inverse correlation between the level of GALE activity expressed in a culture and the concentration of gal-1-P accumulated in the cells. These data support the hypothesis that elevated levels of gal-1-P may underlie the observed toxicity. They further raise

the intriguing possibility that yeast may provide a valuable model not only for assessing the impact of given patient mutations on hGALE function, but also for exploring the metabolic imbalance resulting from impaired activity of GALE in living cells.

Epimerase-deficiency galactosaemia (McKusick 230350) is an inborn error of metabolism resulting from impaired function of the human enzyme UDPgalactose 4-epimerase (hGALE; EC 5.1.3.2). This bifunctional enzyme normally catalyses the third step of the Leloir pathway of galactose metabolism (Figure 1) (Holton et al 2000), as well as the interconversion of UDP-*N*-acetylgalactosamine (UDP-galNAc) and UDP-*N*-acetylglucosamine (UDP-glcNAc) (Kingsley et al 1986; Maley and Maley 1959; Piller et al 1983). Historically, epimerase-deficiency galactosaemia has been subdivided into two forms—peripheral and generalized. The peripheral form is usually considered benign (Gitzelmann 1972; Gitzelmann and Steimann, 1973; Gitzelmann et al 1976) and is associated with impaired or absent enzyme activity in the circulating red and white blood cells but apparently normal activity in other tissues (e.g. fibroblasts or transformed lymphoblasts) (Mitchell et al 1975). Peripheral epimerase deficiency can be quite common, at least in some ethnic groups (Alano et al 1997; Gitzelmann and Steimann 1973; Gitzelmann et al 1976).

In contrast, generalized epimerase-deficiency galactosaemia is both clinically severe and extremely rare. Patients demonstrate absent or impaired GALE activity in all tissues tested, and present with acute symptoms and long-term complications that are similar but not identical to those typically associated with classic transferase-deficiency galactosaemia (Henderson and Holton 1983; Holton et al 1981; Sardharwalla et al 1988; Walter et al 1999). Only five patients, representing two families, have been reported to date with this disorder (Walter et al 1999); all have shown poor growth and learning difficulties despite long-term dietary galactose restriction (Walter et al 1999). Unfortunately, the consanguineous nature

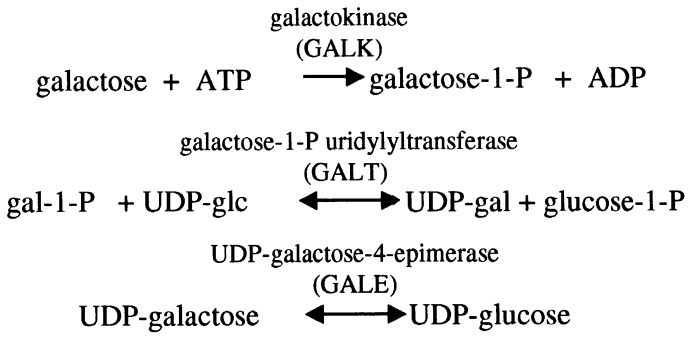


Figure 1 The Leloir pathway of galactose metabolism

of both families confounds clear assignment of specific traits to epimerase deficiency alone.

Finally, recent biochemical analyses and clinical observations by ourselves and others suggest that epimerase-deficiency galactosaemia may be a continuum rather than a truly binary disorder (Alano et al 1998; Quimby et al 1997; Shin et al 2000; Wohlers et al 1999). The identification of a small number of ostensibly peripheral epimerase deficiency patients with impaired enzymatic activity in nonperipheral cells raises the possibility that others may also exist. More alarming is the observation that at least one such child with intermediate levels of lymphoblast epimerase activity was clinically well as an infant but experienced clear development delay after his first year of life (Alano et al 1998; Quimby et al 1997). This child was maintained on a normal diet for all but a brief period in infancy. Since most patients with epimerase deficiency are lost to follow-up as infants, the prevalence of complications appearing later in life for these individuals remains unknown.

The human cDNA and gene encoding hGALE have been cloned and sequenced (Daude et al 1995; Maceratesi et al 1998), enabling the characterization of patient alleles. Previously, we have expressed and studied a number of these alleles in a null-background yeast model system. Our results demonstrate that some mutations cause marked catalytic impairment, while others do not (Quimby et al 1997; Wohlers et al 1999). One of the more severe substitutions identified is V94M, which in the homozygous state accounts for all five reported cases of generalized epimerase deficiency (Wohlers et al 1999).

Previous studies of V94M-hGALE in yeast have revealed that this substitution impairs enzyme activity to only ~5% of wild-type levels with respect to UDPgalactose (UDP-gal), and to 25% wild-type levels with respect to UDP-galNAc (Wohlers et al 1999). At least two important questions remain, however. First, what is the kinetic defect caused by the V94M substitution, and why are the two substrates impacted differently? Second, what impact does this substitution have on the sensitivity of yeast to galactose, and on the *in vivo* galactose metabolite levels accumulated in these cells?

We report here further kinetic analyses of purified wild-type and V94M-hGALE enzymes, as well as initial studies of the *in vivo* impact of the V94M substitution on cell growth and galactose 1-phosphate (gal-1-P) accumulation in yeast. Our results demonstrate that the V94M substitution results in severe impairment of the enzyme's maximum velocity (V_{max}), but not substrate K_m. Furthermore, both wild-type and V94M-hGALE have K_m values approximately 2-fold lower for UDP-gal than for UDP-galNAc. Finally, we observed a strong relationship between the severity of the hGALE mutation expressed, the sensitivity of the culture to galactose, and the *in vivo* accumulation of gal-1-P. These data support the hypothesis that gal-1-P may underlie the galactose toxicity observed. They also raise the intriguing possibility that yeast may provide a valuable model not only for studies of specific human enzymes associated with galactosaemia, but also for studies of the metabolic imbalance that can result from impaired activity of these enzymes *in vivo*.

METHODS

Yeast strains and plasmids: The yeast strain yBBQ1 (MATa *gal80 gal10-120 ura3-52 his3-200 ade2-101 lys2-801 tyr1-501*) (Quimby et al 1997), which expresses no endogenous GALE, was used in all experiments. All recombinant DNA manipulations were performed according to standard techniques (Sambrook et al 1989) using the *Escherichia coli* strain XL1-Blue (Stratagene). All mutant hGALE alleles expressed in yeast were recreated from the wild-type sequence by site-directed mutagenesis and have been described previously (Wohlers et al 1999). The hexahistidine epitope tag added to the C-terminus of the wild-type hGALE open reading frame has been described previously (Quimby et al 1997). This same tag was added to the C-terminus of V94M-hGALE by subcloning of the appropriate cassette. Both hexahistidine-tagged proteins subjected to purification were expressed from a strong, constitutive yeast promoter on the high-copy-number plasmid MM195, a 2μ derivative of YEPlac195 (Gietz and Sugino 1988) and YEPGAP.BX (a generous gift of Dr Warren Kruger). Strains used in the galactose toxicity experiments all expressed the indicated alleles of hGALE from the low-copy-number plasmid BQy1 (Quimby et al 1997; Wohlers et al 1999).

Enzyme purification: Yeast were grown at 30°C to midlogarithmic phase in 1 litre of synthetic dextrose medium under selection for uracil, and harvested by centrifugation at 4°C. The cells were lysed by agitation with glass beads at 4°C in 0.1 mol/L glycylglycine pH 7.5, with protease inhibitors: 1 μg/ml pepstatin, 170 μg/ml phenylmethylsulphonyl fluoride, 0.1 μg/ml chymostatin, 0.6 μg/ml phosphoramidon, 0.5 μg/ml leupeptin. Nickel-affinity purification of hexahistidine-tagged proteins was performed using Hi-Trap chelating columns (Pharmacia) according to the manufacturer's instructions. The binding buffer contained 25 mmol/L imidazole, 20 mmol/L sodium phosphate and 0.5 mol/L sodium chloride at pH 7.4. The columns were washed with five volumes each of 50 mmol/L, 75 mmol/L, and 100 mmol/L imidazole in 20 mmol/L sodium phosphate, and 0.5 mol/L sodium chloride at pH 7.4, and the purified enzyme was eluted with 200 mmol/L imidazole in this same buffer. The imidazole was removed from the eluted protein using a Hi-Trap desalting column (Pharmacia) and eluting with PBS (75 mmol/L sodium phosphate, 68 mmol/L sodium chloride, pH 7.5). The near-homogeneity of each sample was demonstrated by SDS-PAGE followed by staining with colloidal Coomassie blue, as described previously (Neuhoff et al 1988). Concentrations of the purified proteins were determined using the Bio-Rad protein-assay reagent as recommended by the manufacturer, with BSA as the standard. Purified enzyme was diluted to the desired concentration in 0.1 mol/L glycylglycine pH 7.5, 1 mmol/L NAD, and 0.1% BSA. The purified enzyme, in 50% glycerol and 1 mmol/L NAD, was stored in liquid nitrogen for at least one month with no detectable loss of activity.

Enzyme assays and kinetic analyses: Enzymatic activities of the purified hGALE proteins were determined using a spectrophotometric coupled assay essentially

as described previously (Ng et al 1967). Briefly, each reaction contained 0.1 mol/L glycylglycine pH 8.7, 4 mmol/L NAD, 0.011 units UDPglucose dehydrogenase, 10–30 ng of wild-type enzyme or 75–150 ng of V94M substituted enzyme, and varying concentrations of UDP-gal in a total volume of 500 μl. The reactions were carried out at 37°C in a quartz cuvette for 5 min. The first 2 min were allowed for equilibration followed by continuous monitoring of absorbance at 340 nm for 3 min. The initial velocities at various substrate concentrations were thereby determined and the kinetic constants were calculated using a nonlinear least-squares regression fit to the Michaelis–Menten equation, using SigmaPlot. To approximate the K_m for UDP-galNAc, 0.25 mmol/L and 0.50 mmol/L UDP-galNAc were added to act as a competing substrate in the UDP-gal assay. Again, initial velocities at various concentrations of UDP-gal were determined and the apparent kinetic constants were calculated as described above. From these data an apparent K_i, which approximates the K_m for UDP-galNAc, was determined.

Determination of galactose toxicity and gal-1-P levels in yeast: Yeast expressing either wild-type, V94M-, G90E-, D103G-, or L313M-hGALE, or the BQy1 plasmid backbone alone, were grown with gentle shaking at 30°C as 100 ml cultures in synthetic histidine drop-out medium with 3% glycerol as the carbon source. All cultures were grown to midlogarithmic phase. At this time, galactose was added to a final concentration of 0.05%. At the times indicated after addition of galactose, culture densities were monitored by reading OD_{600}, and 20 ml aliquots were removed from the cultures. These cells were centrifuged and washed with water. Extracts were made by agitation with glass beads in 0.1 mol/L glycylglycine pH 8.7 at 4°C. The lysates were centrifuged for 10 min at high speed in a microfuge at 4°C. Supernatants were then transferred to clean tubes and the protein concentrations were determined using the Bio-Rad protein assay reagent, with BSA as the standard. These samples were then boiled for 10 min and centrifuged for 10 min at high speed in a microfuge at 4°C to remove most of the proteins. The remaining supernatants were then assayed for the presence of gal-1-P using a spectrophotometric assay with the enzyme galactose-1-phosphate uridylyltransferase (Elsevier et al 1996; Quimby et al 1996). In brief, reactions were performed at 37°C and contained 1.0 mol/L glycylglycine pH 8.7, 5 mmol/L dithiothreitol, 5 μmol/L glucose 1,6-diphosphate, 5 μmol/L $MgCl_2$, 0.8 mmol/L NADP, 0.6 mmol/L UDPG, 0.06 μg glucose-6-phosphate dehydrogenase (Boehringer Mannheim), 0.1 μg phosphoglucomutase (Boehringer Mannheim), and 50–150 μl of the appropriate yeast supernatant in a total volume of 400 μl. The concentration of gal-1-P in each supernatant was determined by comparison to a standard curve established using known concentrations of gal-1-P. The resulting values were normalized to the protein concentration of the original yeast lysate prior to boiling.

RESULTS

V94M-hGALE is impaired at the level of V_{max}: Previously, we have shown that V94M-hGALE exhibits 5% wild-type levels of activity with regard to UDP-gal when

assayed in yeast crude lysates (Wohlers et al 1999). To investigate further the nature of this catalytic impairment, we purified the protein for kinetic analysis. To facilitate these studies, a C-terminal hexahistidine tag was added to both the wild-type and mutant proteins, and both were expressed in yeast and affinity-purified to near homogeneity (Figure 2, Methods). We have demonstrated previously that the hexahistidine tag does not detectably alter expression or activity of the wild-type human epimerase enzyme (Quimby et al 1997). Kinetic studies of both the purified wild-type and V94M-substituted enzymes were performed as described in Methods. Kinetic constants were determined by a nonlinear least-squares regression fit to the Michaelis–Menten equation (Figure 3). The K_m calculated for wild-type hGALE-His6 was 0.15±0.02 mmol/L UDP-gal, a number that correlates well with published values for both the *E. coli* and human enzymes (Quimby et al 1997; Wilson and Hogness 1964). The K_m calculated for V94M-hGALE-His6 was 0.27±0.01 mmol/L UDP-gal, a value less than 2-fold different from that of the wild-type enzyme. A more striking difference observed between the wild-type and mutant enzymes was the maximum velocity; V_{max} V94M-hGALE-His6 was less than 3% of that measured for the wild-type enzyme (0.036 vs 1.22 mmol

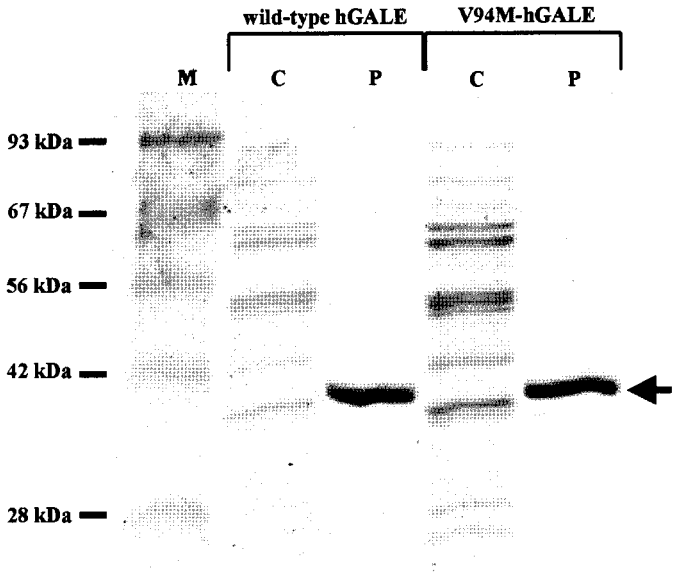


Figure 2 Purification of hexahistidine-tagged wild-type and V94M-hGALE enzymes expressed in yeast. Crude and purified samples were size-fractionated by SDS-PAGE and visualized by staining with colloidal Coomassie blue. Lanes, from left to right: molecular weight marker (M); 2 μg crude (C) and 300 ng purified (P) wild-type hGALE protein; 2 μg crude (C) and 300 ng purified (P) V94M-hGALE protein. The arrow indicates the position of the human epimerase protein

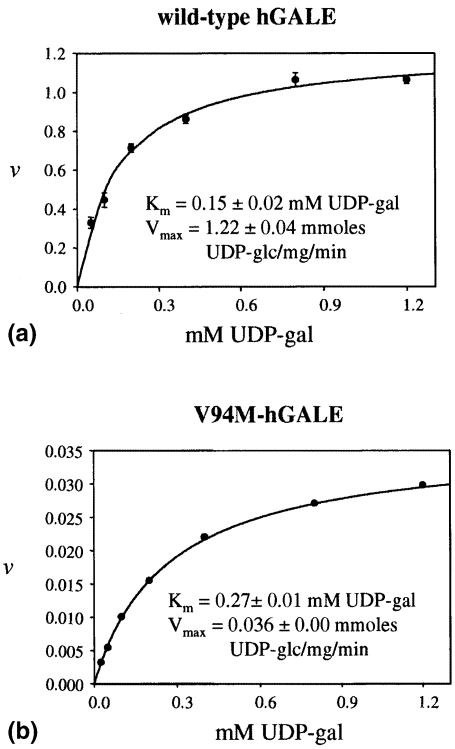


Figure 3 Kinetics of the conversion of UDP-gal to UDP-glc catalysed by wild-type or V94M-hGALE enzymes. Purified enzymes were assayed at six different substrate concentrations, each with $n \geqslant 3$. The resultant data were subjected to a nonlinear least-squares regression fit to the Michaelis–Menten equation (SigmaPlot) from which the values for K_m and V_{max} were derived. Although error bars were included in plots of both data sets, these values were so small in the V94M-hGALE set as to be invisible in the graph

UDP-glc/mg per min, respectively). This decrease correlates well with previous observations of enzyme impairment made in yeast crude lysates (Wohlers et al 1999).

Kinetic properties of wild-type and V94M-hGALE-His6 with respect to UDP-galNAc: The kinetics of the reaction UDP-galNAc→UDP-glcNAc are of interest for two reasons. First, although several groups have isolated active GALE and studied the UDP-gal→UDP-glc reaction (Bergren et al 1973; Darrow and Rodstrom 1968; Frey 1996; Langer and Glaser 1974; Maley and Maley 1959; Mitchell et al 1975; Piller et al 1983; Quimby et al 1997; Salo et al 1968; Thoden et al 1996; Tsai et al 1970; Wilson and Hogness 1964), to our knowledge only one group has previously explored the kinetics of the UDP-galNAc→UDP-glcNAc reaction, and that involved porcine GALE (Piller et al 1983). No kinetic data for purified human GALE have been reported previously with respect to UDP-galNAc. Second, we observed previously that V94M-hGALE was impaired

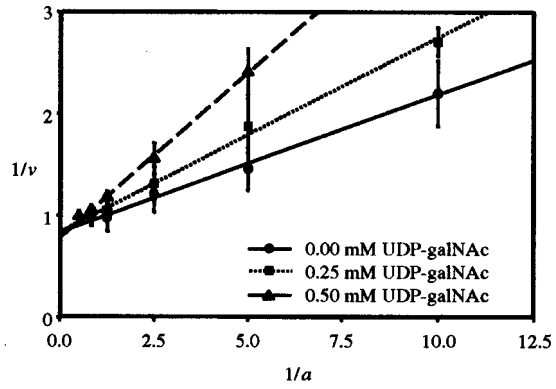
to a much lesser extent with regard to UDP-galNAc than with regard to UDP-gal (Wohlers et al 1999). To explore the cause of this differential effect, studies to define the K_m of each enzyme with regard to UDP-galNAc were performed. Owing to technical constraints, we were unable to assay epimerization of UDP-galNAc directly, and so used this compound as a competitive inhibitor in assays with UDP-gal. In the presence of a competitive inhibitor (I), the apparent K_m for UDP-gal should be increased by the value $(1+[I]/K_i)$, but the V_{max} should remain unchanged. As illustrated in Figure 4, the apparent K_i calculated for UDP-galNAc using wild-type hGALE was 0.286±0.05 mmol/L UDP-galNAc. For the V94M-substituted enzyme, the corresponding value was less than 2-fold greater, at 0.445±0.01 mmol/L UDP-galNAc. Since the apparent K_i for a given competitive substrate determined in this way gives a close approximation of its apparent K_m (Piller et al 1983; Teipel et al 1968), we infer that for both wild-type and mutant enzymes the K_m for UDP-galNAc is approximately 2 times greater than the corresponding K_m for UDP-gal.

Galactose toxicity in yeast expressing impaired human GALE: Yeast lacking *GAL7* (transferase) or *GAL10* (epimerase), but expressing *GAL1* (kinase), exhibit galactose toxicity, and are unable to grow in the presence of galactose, even if an alternative carbon source such as glycerol or ethanol is present (Douglas and Hawthorne 1964). We have exploited this toxicity as a measure of hGALE function in yeast.

Previously we have demonstrated that epimerase-deficient yeast expressing V94M-hGALE are able to grow, albeit slowly, on media containing galactose as the sole carbon source (Wohlers et al 1999). To assess further the *in vivo* impact of this mutation on galactose metabolism in yeast, we examined the possibility that these cells may experience galactose toxicity in the presence of another carbon source. In brief, yeast cells null for endogenous epimerase (yBBQ1) but expressing either wild-type hGALE, V94M-hGALE, or plasmid backbone alone, were grown in duplicate cultures in liquid synthetic medium deficient in histidine, with 3% glycerol provided as the carbon source. After 22 h of growth, galactose was added to one culture of each set, for a final concentration of 0.05%. All cultures were incubated and monitored at OD_{600} in parallel for the indicated times following addition of galactose (Figure 5). The results were striking. Yeast expressing

Figure 4 Inhibition of the UDP-gal to UDP-glc reaction by competition with UDP-galNAc. Purified enzymes were assayed for the ability to convert UDP-gal to UDP-glc in the presence of 0.00, 0.25 and 0.50 mmol/L UDP-galNAc, each with $n \geqslant 3$. The resultant data were subjected to a nonlinear least-squares regression fit to the Michaelis–Menten equation (SigmaPlot) from which the values for K_m, V_{max} and apparent K_i for the competing substrate were determined (values listed below each graph). Individual data sets also were graphed using a Lineweaver–Burk double-reciprocal plot (Robyt and White 1990) strictly to demonstrate visually the competitive nature of the inhibition by UDP-galNAc. Values listed for K_m and V_{max} at zero inhibitor concentration for both the wild-type and mutant enzymes differ slightly from those presented in Figure 3 because independent data sets were used to derive these values

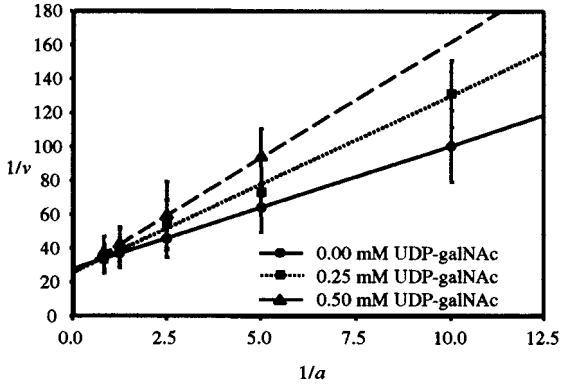
wild-type hGALE



mM UDP-galNAc	0.00	0.25	0.50
apparent K_m (mM UDP-gal)	0.15 ± 0.02	0.25 ± 0.02	0.40 ± 0.01
apparent V_{max} (mmoles UDP-glc/mg/min)	1.16 ± 0.05	1.23 ± 0.03	1.27 ± 0.01

apparent K_i for UDP-galNAc = 0.286 ± 0.05 mM

V94M-hGALE



mM UDP-galNAc	0.00	0.25	0.50
apparent K_m (mM UDP-gal)	0.26 ± 0.01	0.40 ± 0.23	0.55 ± 0.40
apparent V_{max} (mmoles UDP-glc/mg/min)	0.036 ± 0.004	0.036 ± 0.008	0.041 ± 0.013

apparent K_i for UDP-galNAc = 0.445 ± 0.01 mM

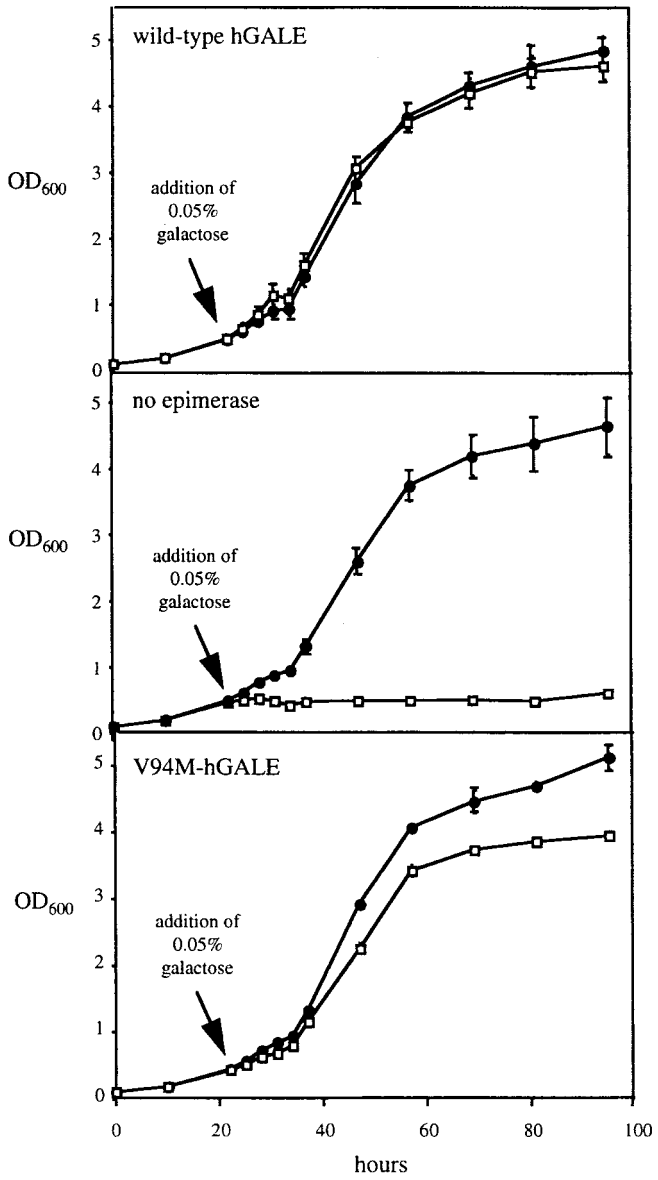


Figure 5 Effects of galactose on the growth of a *gal10*-deficient yeast strain expressing wild-type hGALE, V94M-hGALE or plasmid backbone alone, as indicated. Initially, all cultures were grown in liquid synthetic media with 3% glycerol as the sole carbon source. After 22 h of growth, galactose was added to half of the cultures representing each yeast genotype, to a final concentration of 0.05%. Growth was monitored by measuring absorbance at 600 nm. Cultures to which galactose was added are indicated by the open symbols; cultures that did not receive galactose are indicated by solid symbols. All values plotted represent averages ±SD for $n = 3$ cultures

wild-type hGALE grew slightly faster than their glycerol-grown counterparts immediately following the addition of galactose (Figure 5, top panel), although this minimal growth advantage disappeared as the cultures aged and presumably the galactose was consumed. In contrast, yeast expressing the plasmid backbone but no hGALE grew well in glycerol but completely ceased growing after the addition of galactose (Figure 5, middle panel). Finally, yeast expressing V94M-hGALE grew well in glycerol but experienced growth retardation to intermediate levels after the addition of galactose to the medium (Figure 5, bottom panel).

Galactose 1-phosphate accumulation in yeast expressing impaired human GALE: Elevated haemolysate gal-1-P levels are often cited as one of the initial diagnostic criteria for galactosaemia, and gal-1-P accumulation in a variety of tissues is hypothesized to be one important factor underlying at least some of the complications associated with the disorder in humans (Gitzelmann 1995). Recent studies in yeast also suggest that the accumulation of gal-1-P may be an important factor mediating galactose toxicity in these cells (Kabir et al 2000; Mehta et al 1999).

To explore the possibility that yeast expressing V94M-hGALE may accumulate unusually high levels of gal-1-P, and that this accumulation may correlate with the growth effects observed, lysates derived from these yeast were assayed for the presence of gal-1-P. At the zero time point (before addition of galactose to the media), there was no gal-1-P detectable in any of the strains (Figure 6). After the addition of 0.05% galactose to the medium, however, gal-1-P accumulated

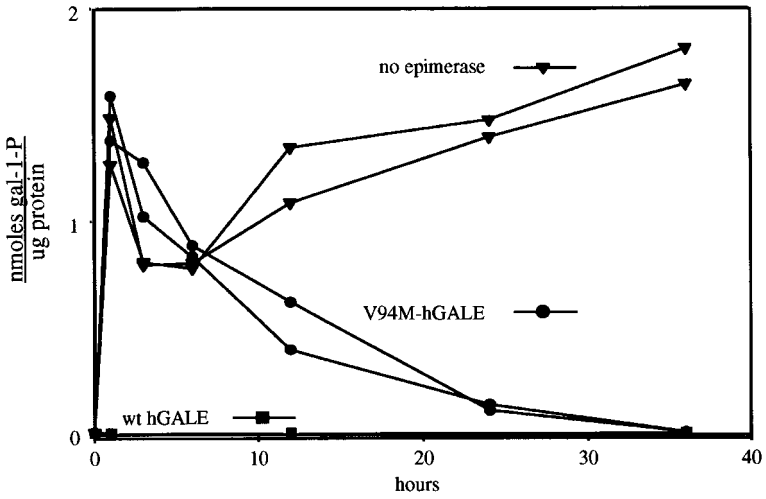


Figure 6 Gal-1-P accumulation in *gal10*-deficient yeast expressing wild-type hGALE (squares), V94M-hGALE (circles) or no hGALE (triangles). As illustrated, extracts prepared from samples of duplicate cultures were monitored (see Methods) at the times shown following the addition of galactose to the growth medium

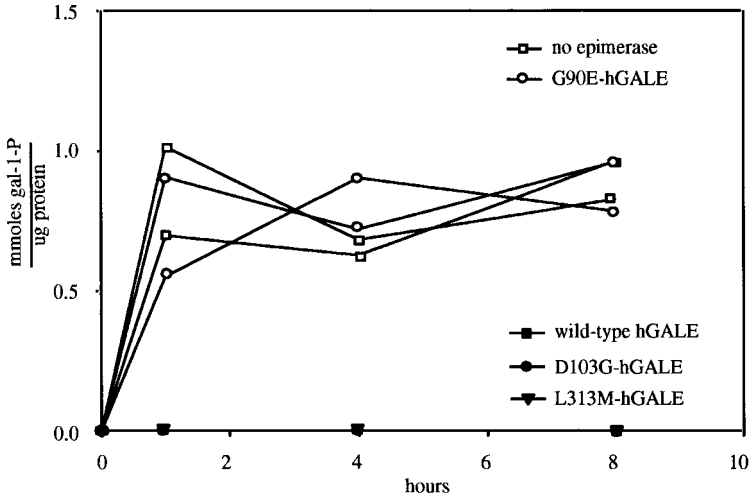


Figure 7 Characterization of additional patient-derived alleles of hGALE with regard to impact on gal-1-P accumulation in yeast. *Gal10*-deficient yeast cells expressing no hGALE, wild-type hGALE or G90E-, D103G- or L313M-hGALE were assayed in duplicate for accumulation of gal-1-P at the indicated times following addition of galactose to the culture medium

rapidly in cells either null for epimerase or expressing V94M-hGALE. Gal-1-P levels remained elevated in cells null for epimerase throughout the course of the experiment. In contrast, yeast expressing V94M-hGALE demonstrated only a transient elevation of gal-1-P. In these cells, gal-1-P levels rose rapidly and then gradually declined until gal-1-P was no longer detectable. In yeast expressing wild-type hGALE, no gal-1-P was detectable at any time during the course of the experiment (Figure 6).

To determine whether gal-1-P accumulation in yeast could be used as a criterion for discriminating degrees of impairment for patient-derived alleles of hGALE, yeast cells expressing G90E-, D103G- and L313M-hGALE were also monitored for the accumulation of gal-1-P, as described above. Previous studies of these alleles have shown that the G90E substituted enzyme has no detectable activity *in vitro*, whereas both the D103G and L313M substituted enzymes have near wild-type levels of activity (Wohlers et al 1999). As illustrated in Figure 7, high levels of gal-1-P accumulated in duplicate cultures of cells null for epimerase, or expressing the G90E variant. Yeast expressing the wild-type enzyme, or one of the mildly impaired alleles, D103G- or L313M-hGALE, revealed no detectable gal-1-P. These data support the hypothesis that gal-1-P levels can serve as an indicator of function for different alleles of human GALE expressed in yeast.

DISCUSSION

The results reported here both confirm and extend our prior work characterizing the impact of V94M and other substitutions in patients on human GALE function. In particular, we have explored kinetic properties of both the wild-type and V94M substituted hGALE enzymes with regard to both natural substrates, UDP-gal and UDP-galNAc. We have further begun to explore the impact of expression of these and other patient-derived hGALE alleles on galactose toxicity and accumulation of gal-1-P in yeast.

Enzyme kinetics: With regard to the nature of the functional impairment of V94M-hGALE, our data demonstrate that both the wild-type and V94M substituted enzymes have similar affinities for UDP-gal; the K_m values for this substrate for the two enzymes differ by less than 2-fold. In contrast, V94M-hGALE has a V_{max} for the UDP-gal→UDP-glc reaction that is more than 33-fold reduced relative to that of the wild-type enzyme. Whatever enzymatic step or steps are impaired by the V94M substitution in hGALE must therefore impact predominantly on functions reflected in V_{max} but not in K_m; current data are insufficient to extrapolate accurately beyond this point. Further insight into the underlying cause of this impairment should be revealed when the crystal structure of the V94M substituted protein is determined and compared with that of the wild-type enzyme (Thoden et al 2000); this project is currently underway.

Although numerous reports have explored aspects of human epimerase function with regard to the interconversion of UDP-gal and UDP-glc, much less is known about the interconversion of UDP-galNAc and UDP-glcNAc. Furthermore, little is known about the impact of patient mutations on this activity. In previous work we have found that V94M-hGALE activity is impaired to a 5-fold lesser extent with regard to UDP-galNAc than with regard to UDP-gal (Wohlers et al 1999). The explanation for this differential effect remains unclear. The data presented here demonstrate that UDP-galNAc does compete with UDP-gal, implying that both substrates share the same active site, or at least have overlapping active sites on the enzyme. Indeed, for both wild-type and V94M-hGALE, the K_m for UDP-galNAc was approximately 2-fold higher than the K_m for UDP-gal. These data demonstrate that the differential impairment of V94M-hGALE with regard to the two substrates is not a function of altered affinities for these compounds. The simplest conclusion, therefore, is that a differential impairment of V_{max} must be responsible, although direct measures of the maximal reaction velocities for both substrates will be required to confirm this possibility.

As we have described previously (Quimby et al 1997), K_m values for UDP-gal reported by other researchers working with GALE from a variety of mammalian and microbial sources range from 0.02 mmol/L to 0.16 mmol/L (Chacko et al 1972; Maxwell 1957; Piller et al 1983; Tsai et al 1970; Wilson and Hogness 1964). The apparent K_m value for UDP-gal reported here for wild-type human GALE is 0.15 mmol/L. Variations in sample origin, purity, or assay method may account for some of these differences. It is interesting to note that although the apparent

K_m for UDP-galNAc reported here for wild-type human GALE varies from that reported previously by Piller and colleagues from their studies of purified porcine GALE (Piller et al 1983), the ratio of apparent K_m values for UDP-galNAc and UDP-gal reported for both enzymes is strikingly similar—1.8 (Piller et al 1983) and 1.9 (this report, Figure 4). This similarity suggests that whatever factors account for the differences in apparent K_m values are likely to be extrinsic, rather than intrinsic, to the enzymes themselves.

Galactose toxicity: The physiological significance of UDPgalactose 4-epimerase in mammals extends beyond its role in the Leloir pathway. Epimerase maintains a balance between UDP-gal and UDP-glc, as well as between UDP-galNAc and UDP-glcNAc. All of these moieties are important sugar donors for the assembly of complex polysaccharides, and are necessary for the modification of glycoproteins and glycolipids. Moreover, in the absence of dietary galactose, epimerase activity is essential for the generation of endogenous galactose. As a result, impairment of this enzyme can have widespread consequences in many tissues, as demonstrated by the clinical outcomes of the 5 patients reported with generalized epimerase-deficiency galactosaemia (Walter et al 1999).

The pathophysiological bases for the complications associated with both classic transferase-deficiency galactosaemia and severe epimerase-deficiency galactosaemia are not well understood. In both disorders a galactose-restricted diet alleviates the acute symptoms, although long-term complications remain (Holton et al 2000). Abnormal accumulation or depletion of specific Leloir pathway metabolites, or their derivatives, has been suggested to underlie the complicates in galactosaemia. For example, accumulation of galactitol, derived from galactose by the action of aldose reductase, has been correlated with cataract formation in the lens (Holton et al 2000). Similarly, several hypotheses have been proposed for the mechanism gal-1-P toxicity in different tissues. These include gal-1-P interference with the function of other enzymes (Gitzelmann 1995), or sequestration of phosphorus in gal-1-P, coupled with the depletion of cellular energy due to repetitive phosphorylation and dephosphorylation of galactose (Donnell et al 1963, 1967; Kozak and Wells 1969; Mayes and Miller 1973). Altered levels or ratios of UDP-gal and UDP-glc, or UDP-galNAc and UDP-glcNAc, are also likely to contribute to galactose toxicity, at least in some tissues (Holton et al 2000).

The question remains whether gal-1-P is a toxic metabolite and, if so, why. The answer is likely to be complex, and may be species-specific or even tissue-specific, as demonstrated by the observation that knockout mice deficient for GALT accumulate gal-1-P to significant levels yet remain phenotypically well (Leslie et al 1996). Clearly, there must be other factors that work either in sequence with gal-1-P or in parallel, to cause galactose toxicity in transferase-deficient or epimerase-deficient humans. Studies in a genetically and biochemically amenable model system, such as yeast, may facilitate the identification and characterization of at least some of those factors.

Galactose sensitivity of yeast deficient in enzymes of galactose metabolism was first reported over 30 years ago by Douglas and Hawthorne (Douglas and

Hawthorne 1964), who observed that yeast deficient in *gal7* (GALT) or *gal10* (GALE) could not grow in media containing galactose, even if another carbon source, such as ethanol, was present. Of particular interest, when these authors screened for *gal10*-deficient colonies that had become resistant to galactose toxicity, the predominant genotype recovered was *gal1*-deficient (GALK) as well as *gal10*-deficient. In short, loss of galactokinase, which converts galactose to gal-1-P, relieved the galactose toxicity observed. A recent study by Mehta and colleagues (Mehta et al 1999), and one by Kabir and colleagues (Kabir et al 2000), have confirmed and extended many relevant aspects of this earlier work. The genetic data from all of these reports consistently support the idea that gal-1-P accumulation contributes to galactose toxicity in yeast.

In this report, we have used phenotypic evidence of galactose toxicity, as well as biochemical evidence of gal-1-P accumulation, as additional tools for monitoring function *in vivo* of wild-type and patient-derived alleles of human GALE in yeast. We have observed that yeast devoid of endogenous GALE but expressing the wild-type human enzyme demonstrate no apparent galactose toxicity and accumulate no detectable gal-1-P upon exposure to galactose. In contrast, yeast completely devoid of epimerase protein cease to grow in glycerol-containing medium upon the addition of galactose. These cells also accumulate high levels of gal-1-P following exposure to galactose. Finally, yeast expressing V94M-hGALE, which exhibits about 5% wild-type levels of activity *in vitro*, demonstrate partial galactose toxicity, and only transiently accumulate high levels of gal-1-P. These data further implicate gal-1-P as at least correlated with, if not causal of, the observed galactose toxicity in yeast.

Clearly, many questions remain regarding the mechanisms and mediators of galactose toxicity in humans and other species impaired for epimerase activity. Most of these same questions also apply to issues of transferase deficiency—indeed from a population perspective these latter questions may be the more clinically relevant. Insights gained from studies in yeast and other model systems should help to provide a framework of possible answers within which observations from the human system may be more easily interpreted.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr Dale Edmondson for his insightful comments about the kinetic aspects of this work and for critical reading of the manuscript, Dr Ravi Nandigama for his invaluable help with nonlinear least-squares regression analysis of some of the data, and Jonathan Lochamy for contributions in the early stages of the galactose toxicity work. We also thank Dr Anita Corbett, Brian Lang and Nicole Christacos for their helpful comments. This work was supported by NIH grant 46403 (to J.L.F.K). T.M.W. was supported in part by funds from the NIH Predoctoral Training Program in Genetics (GMO8490-06).

REFERENCES

Alano A, Almashanu S, Maceratesi P, Reichardt J, Panny S, Cowan TM (1997) UDP-galactose-4-epimerase deficiency among African-Americans: evidence for multiple alleles. *J Invest Med* **45**: 191A.

Alano A, Almashanu S, Chinsky JM, et al (1998) Molecular characterization of a unique patient with epimerase-deficiency galactosaemia. *J Inherit Metab Dis* **21**: 341–350.

Bergren WR, Ng WG, Donnell GN (1973) Uridine diphosphate galactose 4-epimerase in human and other mammalian hemolysates. *Biochim Biophys Acta* **315**: 464–472.

Chacko CM, McCrone L, Nadler HL (1972) A study of galactokinase and glucose 4-epimerase from normal and galactosemic skin fibroblasts. *Biochim Biophys Acta* **284**: 552–555.

Darrow RA, Rodstrom R (1968) Purification and properties of uridine diphosphate galactose 4-epimerase from yeast. *Biochemistry* **7**: 1645–1654.

Daude N, Gallaher TK, Zeschinigk M, et al (1995) Molecular cloning, characterization, and mapping of a full-length cDNA encoding human UDP-galactose 4′-epimerase. *Biochem Mol Med* **56**: 1–7.

Donnell GN, Bergen WR, Perry G, Koch R (1963) *Pediatrics* **31**: 802–810.

Donnell GN, Bergen WR, Ng WG (1967) *Biochem Med* **1**: 29–53.

Douglas HC, Hawthorne DC (1964) Enzymatic expression and genetic linkage of genes controlling galactose utilization in *Saccharomyces*. *Genetics* **49**: 837–844.

Elsevier JP, Wells L, Quimby BB, Fridovich-Keil JL (1996) Heterodimer formation and activity in the human enzyme galactose-1-phosphate uridylyltransferase. *Proc Natl Acad Sci USA* **93**: 7166–7171.

Frey PA (1996) The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. *FASEB J* **10**: 461–470.

Gietz RD, Sugino A (1988) New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**: 527–534.

Gitzelmann R (1972) Deficiency of uridine diphosphate galactose 4-epimerase in blood cells of an apparently healthy infant. *Helv Paediatr Acta* **27**: 125–130.

Gitzelmann R (1995) Galactose-1-phosphate in the pathophysiology of galactosemia. *Eur J Pediatrics* **154**(supplement 2): S45–49.

Gitzelmann R, Steimann B (1973) Uridine diphosphate galactose 4-epimerase deficiency. *Helv Paediatr Acta* **28**: 497–510.

Gitzelmann R, Steinmann B, Mitchell B, Haigis E (1976) Uridine diphosphate galactose 4′-epimerase deficiency. *Helv Paediatr Acta* **31**: 441–452.

Henderson MJ, Holton JB (1983) Further observations in a case of uridine diphosphate galactose-4-epimerase deficiency with a severe clinical presentation. *J Inherit Metab Dis* **6**: 17–20.

Holton JB, Gillett MG, MacFaul R, Young R (1981) Galactosemia: a new severe variant due to uridine diphosphate galactose-4-epimerase deficiency. *Arch Dis Child* **56**: 885–887.

Holton JB, Walter JH, Tyfield LA (2000) Galactosaemia. In Scriver CR, Beaudet AL, Sly SW, Valle D, eds., Childs B, Kinzler KW, Vogelstein B, assoc. eds. *The Metabolic and Molecular Bases of Inherited Disease*, 8th edn McGraw Hill (in press).

Kabir MA, Khanday FA, Mehta DV, Bhat PJ (2000) Multiple copies of MRG19 suppress transcription of the GAL1 promoter in a GAL80-dependent manner in *Saccharomyces cerevisiae*. *Mol Gen Genet* **262**: 1113–1122.

Kingsley D, Kozarsky KF, Hobbie L, Krieger M (1986) Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. *Cell* **44**: 749–759.

Kozak LP, Wells WW (1969) *Arch Biochem Biophys* **135**: 371–377.

Langer R, Glaser L (1974) Interaction of nucleotides with liver uridine diphosphate-glucose-4′-epimerase. *J Biol Chem* **249**: 1126–1132.

Leslie ND, Yager KL, McNamara PD, Segal S (1996) A mouse model of galactose-1-phosphate uridyl transferase deficiency. *Biochem Mol Med* **59**: 7–12.

Maceratesi P, Daude N, Dallapiccola B, et al (1998) Human UDP-galactose 4′-epimerase (GALE) gene and identification of five missense mutations in patients with epimerase-deficiency galactosemia. *Mol Genet Metab* **63**: 26–30.

Maley F, Maley GF (1959) The enzymatic conversion of glucosamine to galactosamine. *Biochim Biophys Acta* **31**: 577–578.

Maxwell ES (1957) The enzymic interconversion of uridine diphosphogalactose and uridine diphosphoglucose. *J Biol Chem* **229**: 139–151.

Mayes JS, Miller LR (1973) *Biochim Biophys Acta* **313**: 9–16.

Mehta DV, Kabir A, Bhat PJ (1999) Expression of human inositol monophosphatase suppresses galactose toxicity in *Saccharomyces cerevisiae*: possible implications for galactosemia. *Biochim Biophys Acta* **1454**: 217–226.

Mitchell B, Haigis E, Steinmann B, Gitzelmann R (1975) Reversal of UDP-galactose 4-epimerase deficiency of human leukocytes in culture. *Proc Natl Acad Sci USA* **72**: 5026–5030.

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**: 255–262.

Ng WG, Donnell GN, Hodgman JE, Bergren WR (1967) Differences in uridine diphosphate galactose-4-epimerase between haemolysates of newborns and of adults. *Nature* **214**: 283–284.

Piller F, Hanlon MH, Hill RL (1983) Co-purification and characterization of UDP-glucose 4-epimerase and UDP-*N*-acetylglucosamine 4-epimerase from porcine submaxillary glands. *J Biol Chem* **258**: 10774–10778.

Quimby BB, Wells L, Wilkinson KD, Fridovich-Keil JL (1996) Functional requirements of the active site position 185 in the human enzyme galactose-1-phosphate uridylyltransferase. *J Biol Chem* **271**: 26835–26842.

Quimby BB, Alano A, Almashanu S, DeSandro AM, Cowan TM, Fridovich-Keil JL (1997) Characterization of two mutations associated with epimerase-deficiency galactosemia using a yeast expression system for human UDP-galactose-4-epimerase. *Am J Hum Genet* **61**: 590–598.

Robyt JF, White BJ (1990) *Biochemical Techniques: Theory and Practice*. Prospect Heights: Waveland Press.

Salo WL, Nordin JH, Peterson DR, Bevill RD, Kirkwood S (1968) The specificity of UDP-glucose 4-epimerase from yeast *Saccharomyces fragilis*. *Biochim Biophys Acta* **151**: 484–492.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Sardharwalla IB, Wraith JE, Bridge C, Fowler B, Roberts SA (1988) A patient with severe type of epimerase-deficiency galactosaemia. *J Inherit Metab Dis* **11**: 249–251.

Shin YS, Korenke GC, Huddke P, Knerr I, Podskarbi T (2000) UDPgalactose epimerase in lens and fibroblasts: activity expression in patients with cataracts and mental retardation. *J Inherit Metab Dis* **23**: 383–386.

Teipel JW, Hass GM, Hill RL (1968) The substrate specificity of fumarase. *J Biol Chem* **243**: 5684–5694.

Thoden JB, Frey PA, Holden HM (1996) Crystal structures of the oxidized and reduced forms of UDP-galactose 4-epimerase isolated from *Escherichia coli*. *Biochemistry* **35**: 2557–2566.

Thoden JB, Wohlers TM, Fridovich-Keil JL, Holden HM (2000) Crystallographic evidence for tyr157 functioning as the active site base in human UDP-galactose 4-epimerase. *Biochemistry* **39**: 5691–5701.

Tsai CM, Holmberg N, Ebner KE (1970) Purification, stabilization, and properties of bovine mammary UDP-galactose 4-epimerase. *Arch Biochem Biophys* **136**: 233–244.

Walter JH, Roberts REP, Besley GTN, et al (1999) Generalised uridine diphosphate galactose-4-epimerase deficiency. *Arch Dis Child* **80**: 374–376.

Wilson DB, Hogness DS (1964) The enzymes of the galactose operon in *Escherichia coli*. *J Biol Chem* **239**: 2469–2481.

Wohlers TM, Christacos NC, Harreman MT, Fridovich-Keil JL (1999) Identification and characterization of a mutation, in the human UDP galactose-4-epimerase gene, associated with generalized epimerase-deficiency galactosemia. *Am J Hum Genet* **64**: 462–470.