Eur. J. Biochem. **270**, 1767–1774 (2003) © FEBS 2003 doi:10.1046/j.1432-1033.2003.03538.x

Functional analysis of disease-causing mutations in human galactokinase

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Galactokinase (EC 2.7.1.6) catalyzes the first committed step in the catabolism of galactose. The sugar is phosphorylated at position 1 at the expense of ATP. Lack of fully functional galactokinase is one cause of the inherited disease galactosemia, the main clinical manifestation of which is early onset cataracts. Human galactokinase (GALK1) was expressed in and purified from *Escherichia coli*. The recombinant enzyme was both soluble and active. Product inhibition studies showed that the most likely kinetic mechanism of the enzyme was an ordered ternary complex one in which ATP is the first substrate to bind. The lack of a solvent kinetic isotope effect suggests that proton transfer is unlikely to be involved in the rate determining step of catalysis. Ten mutations that are known to cause galactosemia were constructed and expressed in *E. coli*. Of these, five (P28T, V32M, G36R, T288M and A384P) were insoluble following induction and could not be studied further. Four of the remainder (H44Y, R68C, G346S and G349S) were all less active than the wild-type enzyme. One mutant (A198V) had kinetic properties that were essentially wild-type. These results are discussed both in terms of galactokinase structure-function relationships and how these functional changes may relate to the causes of galactosemia.

Keywords: galactosemia; cataracts; GHMP family kinase; GALK1.

Galactose is metabolized by the enzymes of the Leloir pathway [1]. The sugar is first phosphorylated at position 1, then converted to UDP-galactose and glucose-1-phosphate (which can enter the glycolytic pathway) by reaction with UDP-glucose. Defects in the enzymes of the Leloir pathway can result in galactosemia in humans [2,3]. The main symptom of this disease is early onset cataracts although mental retardation is also seen in some patients. In the absence of a functional Leloir pathway, galactose accumulates in the lens of the eye where the enzyme aldose reductase catalyzes its conversion to galactitol [4]. High levels of this compound in lens fibre cells cause the uptake of water by osmosis, swelling of the cells, cells lysis and ultimately cataracts. The condition is treated by removal of galactose and lactose from the diet.

Galactokinase belongs to a family of small molecule kinases, the GHMP (galactokinase, homoserine kinase, mevalonate kinase, phosphomevalonate kinase) family as defined by sequence similarity [5]. Although there has been no three-dimensional structure of a galactokinase reported to date, structures of homoserine kinase [6,7], mevalonate kinase [8,9] and phosphomevalonate kinase [10] have been completed along with another family member mevalonate-5-diphosphate decarboxylase [11]. Five highly conserved motifs have been identified in galactokinases from different species [12]. The structures of GHMP kinases show a high degree of overall similarity. From this, functions can be inferred for some of the conserved motifs in galactokinase. Motif III is well conserved throughout the GHMP family and interacts with the phosphates of ATP. Motif V, which is also well conserved, is close to the substrate binding sites and makes several interactions with residues that themselves contact the substrates. Motif I is unique to galactokinases but occurs in approximately the same place in the sequence as the non-ATP ligand binding site in the other family members. Therefore it is likely that this motif forms part of the galactose-binding site.

A number of mutations in the first enzyme of the pathway, galactokinase (GALK1), which are associated with reduced blood galactokinase activity have been characterized [13–17]. A variety of different mutations have been observed including insertions, deletions, and single base changes. Many of the latter group result in a change to a stop codon and thus premature termination of the protein. However, 11 mutations that result in an altered amino acid sequence have been reported. Of these, four (P28T, V32M, G36R and H44Y) cluster in, or near, motif I (the galactokinase signature motif). One (T288M) occurs in motif IV and two (G346S and G349S) in motif V. Three others (R68C, A198V and A384P) are located outside the conserved motifs. One (M1I) abolishes the start codon of the gene (Fig. 1).

Disease causing mutations can be a valuable tool in helping to assign functional roles to motifs and regions of proteins. Furthermore, biochemical analysis of mutant proteins can help in understanding the causes and symptoms of

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Abbreviations: GHMP, galactokinase homoserine kinase mevalonate kinase phosphomevalonate kinase; $K_{m,gal}$, the Michaelis constant for galactose; $K_{m,ATP}$, the Michaelis constant for ATP; k_{cat}, the turnover number; k_{cat}/K_m, the specificity constant; K_{IC}, the competitive inhibition constant; K_{IU}, the uncompetitive inhibition constant.
Enzymes: galactokinase (EC 2.7.1.6).

(Received 19 December 2002, revised 29 January 2003, accepted 24 February 2003)

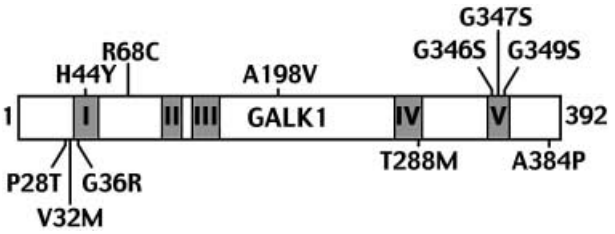

Fig. 1. Disease causing mutations in human galactokinase. The numbers I to V represent the conserved motifs in galactokinases [12]. Mutations that resulted in soluble protein on induction in *E. coli* are shown above the bar representing the sequence of the protein, while those that were insoluble are shown below.

the inherited disease. We have established a bacterial expression system for human galactokinase and have purified active enzyme from this source. As there are a number of different kinetic mechanisms reported for galactokinases from different sources [18–23], we first determined the kinetic mechanism. The kinetic consequences of the point mutations described above (with the exception of M1I) were determined. Half were insoluble and four exhibited altered kinetic constants with respect to the wild-type enzyme. One was essentially unchanged in its enzymological properties compared to wild-type.

Experimental procedures

Cloning, expression and purification of GALK1

cDNA coding for the *GALK1* gene was obtained from the I.M.A.G.E. consortium (Clone ID: 3501788) [24]. The sequence was amplified using PCR with primers designed to introduce an *Nco*I restriction enzyme site and a His_6-tag at the 5′ end and an *Eco*RI restriction enzyme site at the 3′ end. This PCR fragment was then cloned into the *Nco*I and *Eco*RI sites of pET21d (Novagen). The DNA sequence of the entire GALK1 coding sequence was determined (University of Manchester, Faculty of Medicine DNA Sequencing Facility).

The recombinant plasmid was transformed into *Escherichia coli* HMS174(DE3) cells (Novagen) for expression. One to two litres of these cells were grown shaking in LB media at 37 °C until the absorbance at 600 nm was approximately 0.6. The cultures were then induced with isopropyl thio-β-D-galactoside (2 mM, final concentration) and grown for a further 2 h. Cells were harvested by centrifugation (10 min at 5000 ***g***), resuspended in approximately 20 mL 50 mM Hepes/OH pH 7.5, 150 mM NaCl, 10% (v/v) glycerol and stored at −80 °C.

Cells were broken by sonication and cell debris removed by centrifugation (20 min at 20 000 ***g***). The supernatant was passed over a column of 1–2 mL ProBond nickel-agarose resin (Invitrogen) which had previously been equilibrated in Buffer A (50 mM Hepes/OH, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol). The column was washed in this buffer until the absorbance at 280 nm was negligible and then washed again in Buffer A supplemented with 30 mM imidazole. Protein was eluted in Buffer A supplemented with 250 mM imidazole. Fractions containing GALK1 (as judged by SDS/PAGE) were dialysed overnight at 4 °C against 50 mM Hepes/OH pH 7.5, 150 mM NaCl, 2 mM EDTA, 1.4 mM 2-mercaptoethanol, 10% (v/v) glycerol. Protein concentrations were measured by the method of Bradford [25]. The protein solution was frozen in small aliquots in liquid nitrogen and stored at −80 °C.

Generation of point mutations

Mutations were introduced in to the GALK1-pET21d construct using the Quik-Change method [26]. Briefly, the PCR was used to amplify the entire plasmid from two, complementary primers which both contained the desired mutation. Template plasmid was then digested using the restriction enzyme *Dpn*I. Following transformation into *E. coli* XL-1 Blue (Stratagene) and the isolation of single colonies, plasmids were purified and the GALK1 coding region sequenced in full to confirm the presence of the mutation and that no other mutations had been introduced during the PCR. All mutants were expressed and purified by the same method as the wild-type.

Galactokinase kinetics

Galactokinase activity was measured by coupling the production of ADP to the reactions catalyzed by pyruvate kinase and lactate dehydrogenase [12,23]. The decrease in absorbance at 340 nm, which results from the oxidation of NADH, was measured in a Multiskan Ascent microtitre plate-reader. Reactions were carried out at 37 °C in a total volume of 150 μL and each contained 20 mM Hepes/OH pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM KCl, 10% (v/v) glycerol, 1.0 mM NADH, 1 mM dithiothreitol, 400 μM phospho*enol*pyruvate, 7.5 U pyruvate kinase (Sigma) and 10 U lactate dehydrogenase (Sigma). Reactions were initiated by the addition of enzyme (concentrations ranged from 32 to 67 nM with the wild-type enzyme and from 67 to 700 nM with the mutants).

All data were analyzed by nonlinear curve fitting [27] using the program GraphPad Prism (GraphPad Software Inc.). Rates of reaction were obtained by fitting the absorbance data to straight lines. These rates (v) were fitted to the equation $v = V_{max,app}[S]/(K_{m,app} + [S])$ where $V_{max,app}$ is the apparent maximum rate of reaction and $K_{m,app}$ is the apparent Michaelis constant for the substrate, S [28]. The turnover number (k_{cat}) was calculated from the equation $k_{cat} = V_{max}/[E]_0$ where $[E]_0$ is the total enzyme concentration. From this the specificity constant, k_{cat}/K_m could be determined.

Product inhibition studies

The nature and magnitude of the inhibition by the product galactose 1-phosphate was determined by observing the effect of increasing concentrations of the compound on the apparent turnover number and the apparent specificity constants for both substrates. One substrate was held constant at a saturating concentration (5 mM) and the kinetic constants determined over a range of inhibitor concentrations. This was then repeated while holding the other substrate at a constant concentration. Competitive inhibition is characterized by an unchanging apparent

turnover number and variation in the apparent specificity constant according the equation $(k_{cat,app}/K_{m,app}) = (k_{cat}/K_m) \times K_{IC}/([I] + K_{IC})$ where K_{IC} is the competitive inhibition constant and [I] is the concentration of the inhibitor. In contrast, in uncompetitive inhibition the specificity constant is invariant and the apparent turnover number varies according to the equation $k_{cat,app} = k_{cat} \times K_{IU}/([I] + K_{IU})$ where K_{IU} is the uncompetitive inhibition constant. In mixed inhibition the apparent turnover number and specificity constant vary and both K_{IU} and K_{IC} define the inhibition [28].

Solvent kinetic isotope effect

The solvent kinetic isotope effect was measured by determining the kinetic constants as described above in the presence of increasing mole fractions of D_2O (Aldrich).

Kinetic constants of the mutants

The equation for a two-substrate ternary complex reaction is: $v = (k_{cat}.[E]_0.[gal].[ATP])/(K_{I,ATP}.K_{m,gal} + K_{m,gal}.[ATP] + K_{m\ ATP}.[gal] + [ATP].[gal])$ where [gal] and [ATP] are the concentrations of galactose and ATP, respectively, $K_{I,ATP}$ is a constant relating to the dissociation of the enzyme-ATP complex and $K_{m,gal}$ and $K_{m,ATP}$ are the Michaelis constants for galactose and ATP, respectively. At any constant value of [gal] this simplifies to $v = k_{cat,app}.[E]_0.[ATP]/(K_{m,ATP,app} + [ATP])$ where $k_{cat,app} = k_{cat}.[gal]/(K_{m,gal} + [gal])$. A similar situation holds if [ATP] is held constant [28]. Values for $k_{cat,app}$ were obtained over a range of subsaturating constant concentrations of ATP and galactose using a 5 × 5 concentration grid. Nonlinear curve fitting was then used to derive values for the kinetic constants.

Results

Active human galactokinase can be expressed in *E. coli*

Human galactokinase was expressed as an N-terminal His_6 fusion protein and purified on nickel-agarose resin (Fig. 2). Typical yields were approximately 2 mg of GALK1 per litre of bacterial culture. The protein is a monomer as judged by analytical gel filtration (data not shown). The enzyme is active (Fig. 3) with a turnover number (k_{cat}) of 8.7 s^{-1}, $K_{m,gal}$ of 970 μM and $K_{m,ATP}$ of 34 μM. These values are of the same order of magnitude as previously reported for the yeast [23], rat [18,19] and human [29] enzymes. There is no evidence for the glycosylation of human galactokinase described during the purification or isolation of the enzyme from human tissues, nor is there any anomalous migration of bands on gels [29]. We therefore believe that post-translational modifications do not play a significant role in the functioning of the protein, and the activity that we observe for the bacterially produced protein reflects that of the native enzyme.

GALK1 has an ordered ternary complex mechanism

Galactokinases from different sources show a variety of kinetic mechanisms. The enzyme from *E. coli* has been

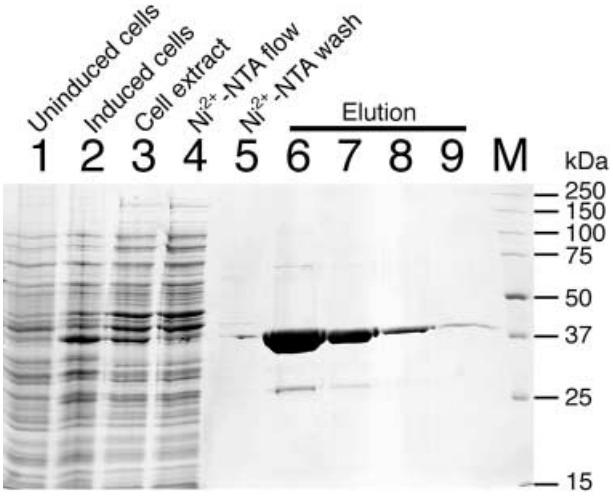

Fig. 2. Expression and purification of human galactokinase. The protein was expressed in *E. coli* HMS174(DE3) cells and purified on nickel agarose.

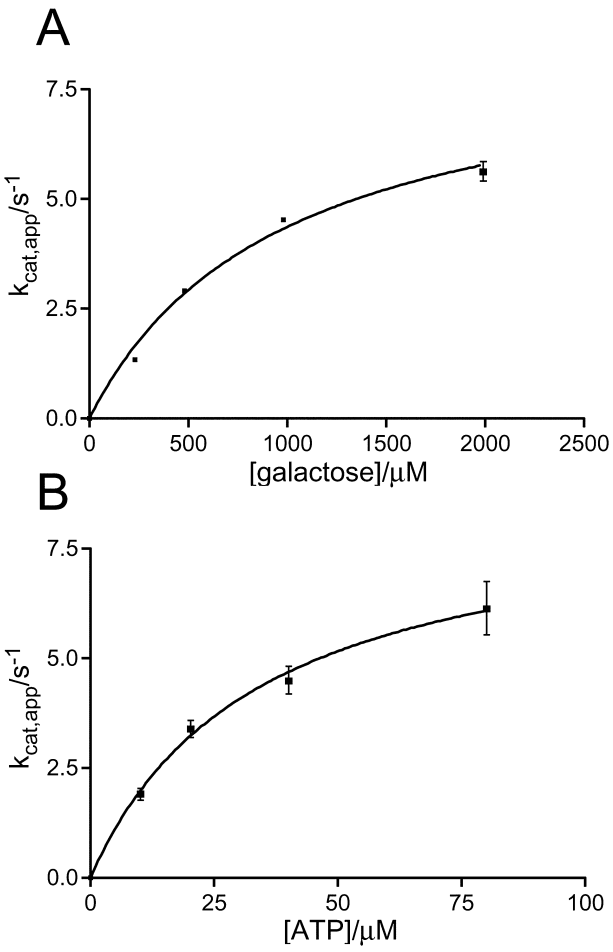

Fig. 3. Kinetics of human galactokinase. (A) Determination of $K_{m,gal}$. Apparent turnover numbers were determined at different galactose concentrations. The line shows the fit of these values to the equation $k_{cat,app} = k_{cat}.[gal]/(K_{m,gal} + [gal])$ as described in Experimental procedures. (B) The determination of $K_{m,ATP}$ by the same method.

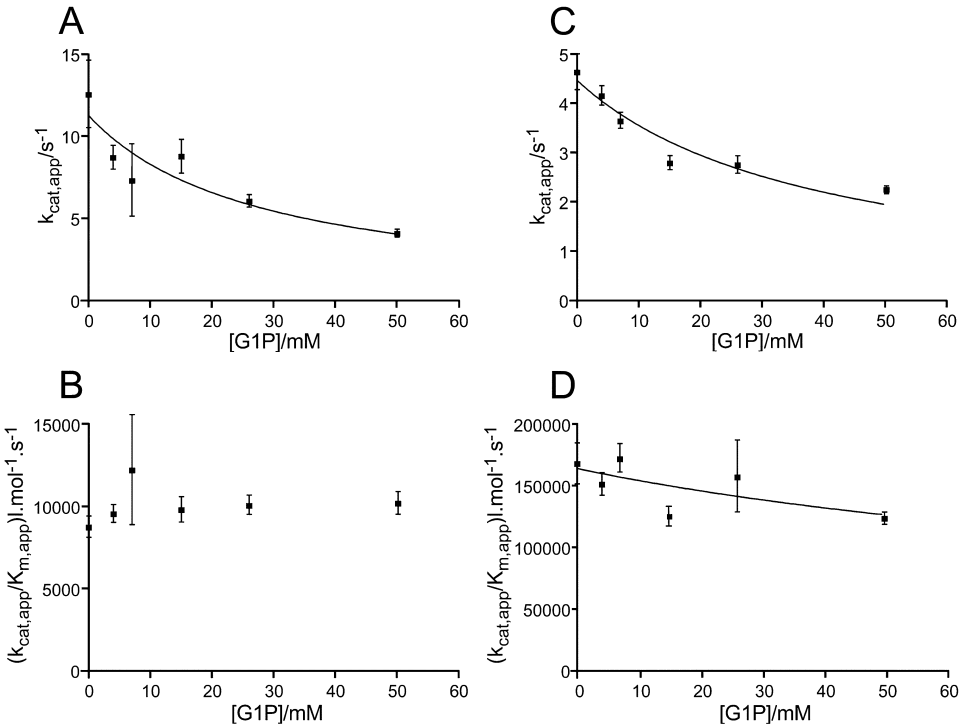

Fig. 4. Human galactokinase has an ordered, ternary complex mechanism. Galactose 1-phosphate (G1P) is an uncompetitive inhibitor with respect to galactose. (A) Galactose 1-phosphate causes a decrease in the apparent turnover number, $k_{cat,app}$. The concentration of ATP was 5 mM. (B) There is no change in the specificity constant, $k_{cat,app}/K_{m,app}$ under the same conditions. Galactose 1-phosphate is a mixed inhibitor with respect to ATP. (C) Galactose 1-phosphate causes a decrease in the apparent turnover number. The concentration of galactose was 5 mM. (D) Galactose 1-phosphate causes a decrease in the apparent specificity constant under the same conditions. This pattern of inhibition is consistent with an ordered ternary complex mechanism in which ATP binds first.

reported to have a random ternary complex mechanism [20] in which either ATP or galactose can be the first substrate to bind. In contrast, galactokinases from rat [18,19] and yeast [23] have an ordered, ternary complex mechanism in which ATP binding precedes galactose binding. Plant galactokinases also show an ordered mechanism, but one in which galactose is the first substrate to bind [21,22]. Product inhibition studies were undertaken with recombinant human galactokinase in order to see which mechanistic class it falls into (Fig. 4). α-D-Galactose 1-phosphate was found to be an uncompetitive inhibitor with respect to galactose ($K_{IU} = 28 \pm 11$ mM) and a mixed inhibitor with respect to ATP ($K_{IU} = 39 \pm 9$ mM; $K_{IC} = 130 \pm 90$ mM). If galactose and galactose-1-phosphate bound to the same form of the enzyme, competitive inhibition would be observed [28]. As this is not the case, these two molecules are unlikely to be the first substrate to bind and the last product to be released from the enzyme. Therefore, the most likely kinetic mechanism for GALK1 is an ordered ternary complex one, in which ATP binds first. Using ADP as an inhibitor was not possible using the enzyme-linked assay system described here. The inhibition pattern we observed was consistent only with an ordered ternary complex mechanism with ATP binding first (out of all the common mechanisms). If there were either a random mechanism or an ordered one with galactose binding first, galactose-1-phosphate would be a competitive inhibitor with respect to galactose.

Proton transfer is unlikely to play a significant role in the rate determining step of GALK1

Although the enzymes of the GHMP family share sequence and structural similarity, there are differences in the mechanism of catalysis. The structure of mevalonate kinase shows an aspartate residue at an appropriate place in the active site to act as catalytic base [9]. However, the active site of homoserine kinase has no residues capable of acting as a catalytic base [7] and catalysis is believed to be driven through the stabilization of a transition state. A recent study on the yeast enzyme, Gal1p, showed little variation of any kinetic constant with pH and no significant deuterium kinetic isotope effect [23]. This suggested that proton transfer was unlikely to be important in the mechanism of Gal1p and that this enzyme is likely to be similar mechanistically to homoserine kinase.

Given the diversity of kinetic mechanisms among galactokinases, we tested whether proton transfer is important in the reaction catalyzed by GALK1. Increasing the mole fraction of D_2O in the reaction mixture had essentially no effect on the turnover number or the specificity constants (Fig. 5). Other studies, in which there is a critical proton transfer event in the rate determining step of the mechanism, show a reduction in k_{cat} of between 25 and 50% at a deuterium mole fraction of 0.4 [30,31]. This level of reduction would certainly have been observable in our experimental system. Therefore in GALK1, like Gal1p and

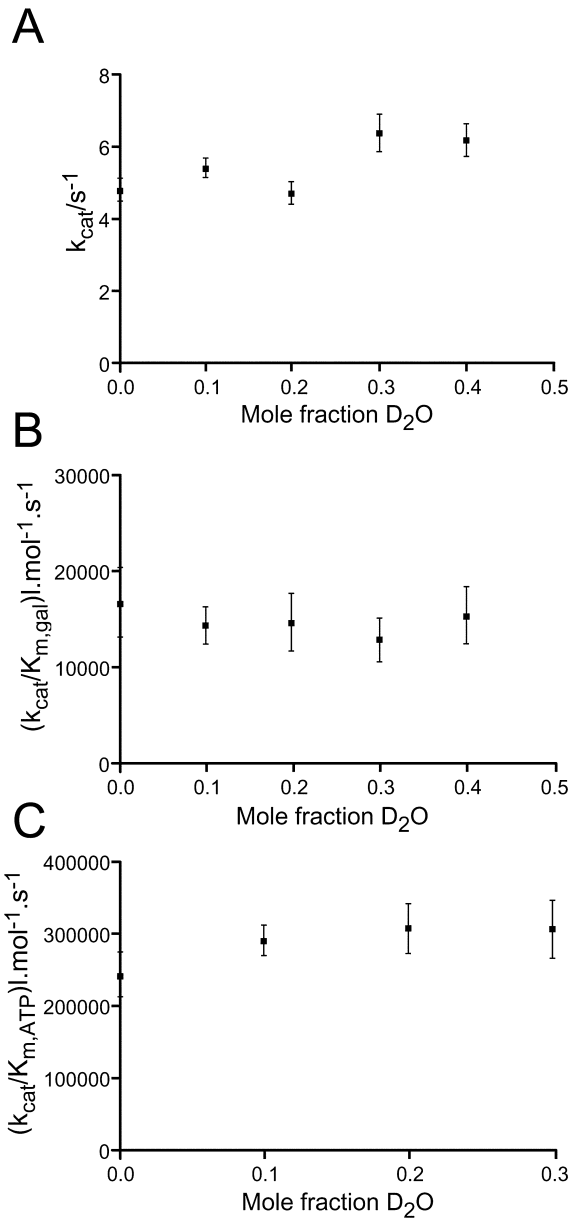

Fig. 5. There is no solvent kinetic isotope effect in human galactokinase. (A) The variation of k_{cat} with mole fraction of deuterium oxide. These values were obtained in an experiment in which the concentration of ATP was varied and galactose was maintained at a saturating level (5 mM). Similar results were obtained when ATP was the saturating ligand and galactose concentration was varied (not shown). (B) The variation in the specificity constant for galactose with mole fraction of deuterium oxide. (C) The variation in the specificity constant for ATP with mole fraction of deuterium oxide. Error bars show standard error.

homoserine kinase, proton transfer is unlikely to play a major role in the rate-determining step of catalysis.

Several of the disease-causing mutations are not soluble following induction in *E. coli*

Although all the mutant galactokinases constructed could be expressed in *E. coli* (as judged by the appearance of an additional band of the expected molecular mass on SDS/PAGE of cell extracts after induction), five (P28T, V32M, G36R, T288M and A384P) were not present in the soluble fraction after sonication and could not be purified (data not shown).

The soluble mutants show altered kinetic constants compared to the wild-type

The remaining mutants (H44Y, R68C, A198V, G346S and G349S) were soluble on induction in *E. coli* and could be purified in a similar manner to the wild-type enzyme. Yields were comparable to that obtained with the wild-type, except in the case of R68C where approximately fivefold less soluble enzyme per litre of starting culture was obtained. Each of these five mutants was an active galactokinase and the kinetic constants for each could be determined (Table 1). The kinetic consequences of a further mutation in the highly conserved part of motif V, G347S were also measured.

A variety of different kinetic phenotypes were observed. G346S and G347S showed substantial reductions in turnover number. G347S also showed an increase in $K_{m,gal}$ as did H44Y. Less dramatic effects were observed on $K_{m,ATP}$ with no mutant showing more than a fivefold change. The most affected were H44Y and R68C. All three motif V mutants (G346S, G347S and G349S) along with H44Y have lower specificity constants for galactose and all the mutants with the exception of A198V have lowered specificity constants for ATP. Interestingly, A198V shows very similar kinetic parameters to the wild-type enzyme.

Discussion

Human galactokinase, GALK1, has been expressed in and purified from *E. coli*. The ability to produce good yields of active protein in this way makes it possible to study the biochemical consequences of mutations within the coding sequence of the *GALK1* gene.

The kinetic mechanism of GALK1 was shown to follow an ordered ternary complex pathway in which ATP binds first. GALK1 is therefore most similar to the rat and yeast enzymes in its kinetic mechanism. The most likely cause of this sort of mechanism is that ATP binding induces a conformational change in the enzyme, which creates a functional binding site for galactose. Identifying the nature of this change and the residues involved in transmitting information through the protein will be important challenges for the future. The absence of a deuterium kinetic isotope effect suggests that GALK1 belongs to that group of GHMP kinases in which proton transfer does not play a major role in the rate determining step of catalysis.

That five of the 10 disease-causing mutations resulted in insoluble protein in *E. coli* suggests that in these cases protein folding and/or stability of the folded state may be more important than enzymological defects. Generally these mutations are associated with more severe clinical phenotypes. Individuals who are homozygous for the P28T mutation (which is common in Roma and Bosnian populations [32,33]) develop cataracts in the first few months or years of life if galactose is not completely removed from the diet. Blood galactokinase activities are low or zero [14].

Table 1. Kinetic constants of disease-causing mutations in GALK1.

Enzyme	k_{cat} (s^{-1})	$K_{m,gal}$ (μM)	$K_{m,ATP}$ (μM)	$k_{cat}/K_{m,gal}$ ($L \cdot mol^{-1} \cdot s^{-1}$)	$k_{cat}/K_{m,ATP}$ ($10^5 \times L \cdot mol^{-1} \cdot s^{-1}$)
Wild-type	8.7 ± 0.5	970 ± 220	34 ± 4	8900 ± 2900	2.6 ± 0.4
H44Y	2.0 ± 0.1	7700 ± 4400	130 ± 9	270 ± 240	0.15 ± 0.02
R68C	3.9 ± 0.8	430 ± 150	110 ± 35	11000 ± 5600	0.35 ± 0.18
A198V	5.9 ± 0.1	660 ± 220	26 ± 1	8500 ± 4000	2.3 ± 0.2
G346S	0.4 ± 0.04	1100 ± 160	5 ± 2	400 ± 96	0.87 ± 0.37
G347S	1.1 ± 0.2	13000 ± 2000	89 ± 34	85 ± 21	0.12 ± 0.07
G349S	1.8 ± 0.1	1700 ± 480	39 ± 4	1100 ± 380	0.46 ± 0.07

A similar phenotype is seen in patients homozygous for V32M [13]. When DNA encoding GALK1 with this mutation was transfected into COS cells, no galactokinase activity above background could be detected [13]. The G36R mutation was detected in an individual who was heterozygous for this mutation and a frameshift [15]. Blood galactokinase activity was zero and transfection of this mutant into COS cells also gave no activity [15]. T288M was also observed in an individual who was heterozygous for this and a frameshift mutation [16]. The patient had low blood galactokinase activity and had been placed on a low galactose diet and so no other symptoms had been observed. A single individual was heterozygous for A384P and R68C [16]. Like the T288M patient, the patient had been placed on a low galactose diet before any symptoms could occur.

The M1I mutation [15] is assumed to cause loss of galactokinase activity because the protein lacks its start codon. If protein synthesis were to start at the next methionine in the sequence, this would be M55 and would result in deletion of the whole of motif I, the putative galactose binding site. It is therefore not surprising that transfection of this mutant sequence in to COS cells resulted in no galactokinase activity [15].

H44Y and G349S were detected in a patient who was heterozygous for these two mutations [15]. Although there was zero blood galactokinase activity, transfection of either mutant sequence in to COS cells gave low, but not zero, levels of galactokinase activity. G346S (which was detected in a patient who also had a seven base pair insertion in the gene) gave similar results [15]. In general therefore the soluble mutants tend to be those which occur in heterozygotes along with more drastic mutations. Furthermore where the activity of these mutants has been tested *in vivo* by transfection into COS cells [15] they tend to give much reduced, but not zero levels of activity in contrast to the insoluble mutants. This gives us added confidence that our conclusion that failure to produce soluble protein in *E. coli* means that the protein is insoluble or unstable in humans is correct.

Interestingly one mutant, A198V, has kinetic properties that are very similar to the wild-type enzyme. This mutation is also associated with the least severe clinical phenotype [17]. Homozygotes show reduced blood galactokinase activity (typically 10% of normal) and have a tendency to develop cataracts later in life [17]. Studies on crude blood extracts from homozygotes showed that $K_{m,gal}$ and $K_{m,ATP}$ were indistinguishable from the wild-type but that V_{max} was reduced by approximately 80%. The amount of protein that could be detected immunologically was also reduced by approximately the same amount [17]. This suggests that the reduced blood galactokinase activity results not from catalytic inefficiency of the enzyme but from reduced amounts of the protein. This mutation may cause the enzyme to be turned over more rapidly in human cells.

The five soluble mutations cause a variety of kinetic consequences. The turnover number, k_{cat}, reports on steps in the reaction that occur after the formation of the enzyme-ATP-galactose ternary complex including catalysis. All the mutants have reduced turnover numbers, with the most impaired being G346S and G347S. These residues are in motif V which is believed (on the basis of comparison to the structures of other GHMP family enzymes) to be adjacent to the residues that form the active site. It is unlikely that glycine can contribute much directly to stabilizing the transition state. However the change of glycine to serine is likely to make the peptide backbone much less flexible. This in turn may make interactions between the active site and the transition state less favourable, thereby reducing catalytic efficiency.

Although K_m values are often used as measures of enzyme-substrate affinity, this is not strictly correct. More accurately, it is an apparent dissociation constant referring to all enzyme bound species of the substrate [34]. For example, in the case of GALK1, $K_{m,ATP}$ does not just report on the initial interaction between the enzyme and ATP, but also on the dissociation of ATP from the ternary enzyme-ATP-galactose complex and from any conformational states that may occur prior to phosphate transfer. Two mutants have large changes in $K_{m,gal}$ – H44Y and G347S. H44 forms part of motif I, which is believed to interact with galactose [12]. In the case of G347S, it seems that the disruption of the peptide backbone that affects catalysis also affects the binding of galactose at some point in the reaction. Modest changes in $K_{m,ATP}$ are seen in H44Y and R68C. That H44 influences the binding of both substrates suggests that the binding sites are probably close in space. R68 is not part of any conserved galactokinase motif, nor is the residue well conserved between species. It is possible that its kinetic changes result from structural alterations that are propagated to the active site.

Specificity constants (k_{cat}/K_m) report on the interaction between the enzyme and a particular substrate. Thus in the case of GALK1, $k_{cat}/K_{m,ATP}$ reports on the enzyme–ATP interaction and $k_{cat}/K_{m,gal}$ on the interaction between the enzyme-ATP complex and galactose. The three mutations in motif V (G346S, G347S and G349S) all have much reduced specificity constants for galactose as does H44Y. Failure to form a proper galactose-binding site is the most

likely cause of this in all these cases. All the mutants except A198V have impaired specificity constants for ATP. Interestingly, G346S has only a modest reduction in $k_{cat}/K_{m,ATP}$ despite having a k_{cat} that is approximately 20-fold reduced compared to the wild-type. In this mutant $K_{m,ATP}$ is also reduced (approximately sevenfold) and this compensates partially. This must mean that although the free enzyme has a slightly reduced affinity for ATP, a later stage in the reaction pathway (perhaps the ternary complex) has an enhanced affinity.

The enzymological consequences of disease-causing mutations in human galactokinase have been investigated *in vitro*. In general proteins produced from mutations which give rise to the most severe clinical phenotypes are insoluble when purified from *E. coli*, which may suggest that gross structural changes have occurred in these proteins. The results from the soluble mutants support the hypothesis that motif I interacts with galactose and that motif V plays a role in maintaining the structural integrity of the substrate binding sites. The data represents the first step in the analysis of the metabolic control of flux through the Leloir pathway. Analysis of the galactokinase, its mutants, and the other enzymes of the metabolic pathway using the principles of a quantitative framework, such as metabolic control analysis [35], may yield significant insights into the syndrome of galactosemia.

Acknowledgements

We are grateful for members of the Reece Laboratory for helpful comments and suggestions. This work was funded by the Biotechnology and Biological Sciences Research Council, UK and The Leverhulme Trust, UK.

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