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Use of Site-Directed Mutagenesis in the Diagnosis, Prognosis and Treatment of Galactosemia

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1. Introduction

Site-directed mutagenesis (**SDM**) is undoubtedly one of the most powerful techniques in molecular biology. In this chapter, we will describe the use of SDM in the study of the human inherited metabolic disorder, Galactosemia (Type I, II, and III) and the development of novel therapies for the disease. This powerful technique not only helped confirm suspected *GAL* gene mutations in Galactosemia, but also played a significant role in unraveling the catalytic mechanisms of the GAL enzymes in the conserved Leloir pathway of galactose metabolism. To date, more than thirty disease-causing mutations in the human *GAL* genes have been characterized in great detail; and these findings have paved the way for innovative, state-of-the-art therapies, such as chaperone therapy. Recently, in order to optimize small molecule GALK inhibitors for the treatment of Type I Galactosemia, we have employed SDM to identify amino acids of the GALK enzyme that interact with its selective inhibitors. These studies exemplified the expanding roles of SDM in innovative drug design and in kinase inhibitor selectivity.

2. Background

2.1 What is galactosemia?

Galactose is a hexose that differs from glucose only by the configuration of the hydroxyl group at the carbon-4 position. Often present as an anomeric mixture of α-D-galactose and β-D-galactose, this monosaccharide exists abundantly in milk, dairy products and many other food types such as fruits and vegetables (Acosta and Gross, 1995; Berry et al., 1993). However, galactose can also be produced endogenously in human cells, mainly as products of glycoprotein and glycolipid turnover.(Berry et al., 1995, 2004). Once freely present inside the cells, β-D-galactose is epimerized to α-D-galactose through the action of a mutarotase (Beebe and Frey, 1998; Thoden and Holden, 2002a). α-D-galactose is then metabolized by the Leloir pathway (Leloir 1951), an evolutionarily conserved biochemical pathway which begins with the phosphorylation of galactose by the enzyme galactokinase (GALK) to form galactose-1 phosphate (gal-1P) (Cardini and Leloir, 1953). Gal-1P is subsequently, together with the substrate UDP-glucose, converted by galactose-1-phosphate uridylyltransferase

(GALT) to form UDP-galactose and glucose-1 phosphate (glu-1P) (Kalckar et al., 1953). The Leloir pathway is completed by reversibly forming UDP-glucose from UDP-galactose *via* UDP-galactose-4-epimerase (GALE) (Leloir 1953; Darrow and Rodstorm, 1968). Inherited deficiencies of GALK, GALT, and GALE activities in humans have all been observed, studied, and reviewed extensively (Bosch et al., 2002; Elsas 1993; Fridovich-Keil et al., 1993a). The clinical manifestations of each enzyme deficiency, however, differ markedly (Berry et al., 1995; Berry and Elsas, 2011; Fridovich-Keil et al., 1993a; Lai et al., 2009;). For instance, patients with GALK deficiency (MIM 230200) (Type II Galactosemia) have the mildest clinical consequences, as they may present only with cataracts (Bosch et al., 2002). On the other hand, GALT-deficiency (MIM 230400) (Type I or Classic Galactosemia) is potentially lethal in infancy, if undiagnosed and untreated, and is also associated with long-term, organ-specific complications (Berry et al., 1995). GALE-deficiency (MIM 230350) (Type III Galactosemia) has been somewhat controversial with regards to clinical manifestations, as this disorder is rare; and information is mostly derived from case reports (Fridovich-Keil et al., 1993a). Until newborn screening for GALE deficiency is available, the natural history will likely remain unknown. The differences in clinical outcome between GALT and GALK deficiencies reflect the differences in tissue response to the characteristic changes in the levels of galactose metabolites as a result of the respective enzyme deficiencies.

2.2 How are the different types of galactosemia detected and diagnosed?

Newborn screening programs worldwide have greatly facilitated the early detection of Galactosemia (Kaye et al., 2006; Levy 2010). The screening tests often involve the detection of elevated level of blood galactose and/or specific GAL enzyme in the dried blood spots on filter paper. Elevated galactose will detect GALK deficiency and GALT deficiency, but it may not detect GALE deficiency. Other states screen for GALT activity, and may therefore diagnose Type I Galactosemia. However, this screen will miss GALK and GALE deficiency. The final diagnosis is secured once the specific enzyme deficiency is confirmed by enzymatic assays or by DNA genotyping; these tests are available commercially in the USA (http://www.ncbi.nlm.nih.gov/sites/GeneTests/, Tests #3437, #2229 and #53782).

2.3 What are the current treatments for galactosemia, and what is the outlook for patients?

The main aspect of management for all forms of Galactosemia is withdrawal of lactose/galactose from the diet as soon as the diagnosis is made, or even considered (Segal 1995). In infants, this means the replacement of breast/cow milk with soy-based formula. However, it has become clear that, despite early detection and (early) dietary intervention, there still is a significant burden of the disease, particularly for Classic Galactosemia where chronic problems persist through adulthood. The most common medical complications of Type I Galactosemia are speech dyspraxia, ataxia, premature ovarian insufficiency, and intellectual deficits, which are rarely seen in other forms of galactosemia (Waggoner et al., 1990; Waisbren et al., 2011). GALK deficiency (Type II Galactosemia) is managed also with lactose/galactose restriction, though the complications are mainly confined to the eye (cataracts) (Bosch et al., 2002). GALE deficiency is treated similarly, though complications of this deficiency may not be preventable with such restriction, as is GALT deficiency (Fridovich-Keil et al., 1993a).

3. Use of SDM to confirm disease-causing mutations in human GALT, GALK, and GALE genes identified clinically

3.1 The issues

Advances in federal and state newborn screening programs worldwide have resulted in the inclusion of the potentially lethal disorder, Galactosemia, in the list of diseases for which newborns are screened. Very often, once an affected newborn is identified by the biochemical assays, it is helpful to know the genotype of *GAL* gene involved because there appears to be a genotype-phenotype correlation for a few selected *GAL* gene mutations. The confirmation of the *GAL* genotypes in the affected patients will provide better prognosis. Additionally, a few well-characterized GAL enzyme variants have been shown to retain significant residual enzyme activities. Consequently, patients with selected mutations might benefit from novel therapies, such as chaperone therapies.

Unfortunately many patients with Galactosemia identified to-date have novel (private) nucleotide changes in their *GAL* genes. For instance, the *GALT* gene database set up by the ARUP Laboratories (Salt Lake City, USA) has recorded over 200 nucleotide changes of the *GALT* gene identified in patients with Type I Galactosemia (*www.arup.utah.edu/database/GALT/GALT_welcome.php*). Without clinical correlation, it is impossible to tell if any of these novel changes actually results in impaired GALT enzyme activities seen in the patients. Moreover, many patients are compound heterozygous for the *GAL* gene mutations. In other words, a single patient may have a unique nucleotide change in each of the two *GAL* alleles; and it is difficult to conclude which one is responsible for the reduction in total enzyme activity. Thus there is a real need to perform *in vitro* expression studies of the identified "variant" *GAL* genes.

3.2 Research design

Our laboratory and others have largely used similar strategies in confirming the suspected human *GAL* gene mutations in patients with Galactosemia (Fridovich-Keil et al., 1995a; Lai et al., 1999; Reichardt et al., 1992;). In almost all cases, we sub-cloned the cDNA of the respective *GAL* gene into expression plasmid vectors, before we performed SDM of the sub-cloned fragments to obtain "mutant" cDNAs with the same sequence changes observed in patients. We then expressed the wild-type and mutated cDNAs in heterologous expression systems, such as *Escherichia coli, Saccharomyces cerevisiae* or even mammalian expression systems. Subsequently, we tested for differences in kinetic parameters of the GAL enzymes, such as K_M and V_{max}, and the expression efficiencies, such as protein and/or mRNA abundances, between mutant and control cDNAs.

3.3 The results

The primary goal for expression analysis of the suspected disease-causing mutations in the *GAL* genes is to show that the nucleotide changes observed are causing impaired GAL enzyme activities and could therefore be the causes for the diseases. In addition, in the course of the analysis, kinetic parameters of the variant enzymes are often determined, which are expected to help advance the structural knowledge of the GAL enzymes.

3.3.1 Type I (GALT-deficiency) galactosemia

As mentioned above, more than 200 nucleotide changes in the *GALT* gene have been identified so far, mostly single nucleotide substitutions. The most common human *GALT* mutation, *Q188R*, is detected in over 70% of galactosemic patients in Europe and North America. The *Q188R* mutation is associated with a poor clinical outcome, even with a galactose-restricted diet (Guerrero et al., 2000; Murphy et al., 1999; Webb et al., 2003). *K285N* is the second most common mutation found in patients in Europe, especially in the countries of central and Eastern Europe, where it can account for up to 34% of *GALT* alleles (Greber-Platzer et al., 1997; Kozak et al., 2000). In the African-American population, the *S135L* mutation is predominant. The corresponding enzyme leads to a relatively benign outcome, if the mutation is identified and the patient is treated with a galactose-restricted diet in the newborn period (Lai et al., 1996, 2001; Landt et al., 1997). A more common mutation, *N314D*, occurs in all populations mentioned above and can lead to two different phenotypes, depending on the presence or absence of a 4-bp deletion in the coding region for the carbohydrate response element. When *N314D* is associated with a four-nucleotide deletion in the promoter region (the Duarte type 2), homozygosity for N314D and this altered promoter region causes a 50% decrease of GALT activity, with a mild or even undetected phenotype (Elsas et al., 1994). In the absence of this deletion in the promoter, homozygosity for the N314D missense mutation (the Los Angeles variant) results in normal GALT in erythrocytes (Shin et al., 1998). A 5-kb deletion is found so far exclusively in Ashkenazi Jewish patients (Coffee et al., 2006).

Due to its frequency among GALT-deficiency galactosemic patients and its association with a poor clinical outcome, the *Q188R* mutation has been extensively studied. The initial study using the COS cell expression system surprisingly showed that this mutation had about 10% of normal enzymatic activity (Reichardt et al., 1991). This result was not consistent with the clinical finding that patients homozygous for *Q188R* have no detectable enzyme activity in their red blood cells. Another study, carried out in a yeast model that was completely devoid of GALT activity, used a PCR-mediated SDM technique and clarified that the *Q188R* mutation did cause loss of function of both human and yeast GALT (Fridovich-Keil et al.,1993b). Interestingly, this study also showed that the mutant yeast, with its loss of GALT activity, could not survive in galactose media if the *Q188R* missense mutation was introduced, while reconstitution of wild-type GALT resulted in normal growth (Fridovich-Keil and Jinks-Roberson, 1993b). The confounding result of the first study is likely to be explained by the presence of endogenous GALT activity in the COS cells, highlighting the importance of studying mutations in a null background system, such as the *gal7*-deleted yeast model used in the second study. Alternatively, one should use purified mutant proteins in the analysis of the enzymatic activities. Subsequent studies further confirmed that the *Q188R* mutation not only totally abolishes GALT enzyme activity, but also acts as a partial dominant-negative mutation, as the heterodimer of *Q188R*/wild type has only 15% of wild-type activity (Fridovich-Keil et al., 1995a; Elsevier and Fridovich-Keil,1996). Kinetic analysis showed this mutation mainly causes impaired specific activity of the heterodimer without altering the K_M for both substrates. In order to further understand how mutation at this site could affect the enzyme, Lai and coworkers mutated glutamine-188 (Gln^{188}) to arginine and asparagine, respectively, through SDM (Lai et al., 1999). More detailed kinetic measurement showed that mutating glutamine to arginine or asparagine did not affect the first step of the double-displacement action (UDP-Glu to glu-1p). In fact, Q188R-GALT even

had a better V_{max} as compared with the wild-type GALT. However, the *Q188R* mutation severely impaired the second step of the reaction. The crystal structures of *E. coli* GALT revealed that Gln^{168} (equivalent to Gln^{188} in human GALT) could stabilize the GALT-UMP intermediate through two hydrogen bonds formed between the amide side chain of Gln^{188} and the phosphoryl oxygen of the UMP moiety (Wedekind et al., 1996). Through molecular modeling studies (or "virtual SDM"), Lai and coworkers changed glutamine to arginine and asparagine, respectively, and found that the number of hydrogen bonds formed between new amino acid residues and UMP moiety decreased to one, which could have destabilized the GALT-UMP intermediate required for the second displacement reaction (Lai et al., 1999). This destabilization was well manifested in the increased V_{max} in the Q188R mutant in the first displacement reaction, as the destabilization speeded up the recycling of the enzyme for the first reaction (Lai et al., 1999). To complete the double-displacement reaction, a stable GALT-UMP intermediate was required to bind gal-1P, which was better accomplished by the two hydrogen bonds from glutamine than by the single hydrogen bond from arginine or asparagine.

The *S135L* mutation was identified initially as a polymorphism with near normal enzymatic activity in the COS cell expression system (Reichardt et al., 1992). However, subsequent SDM studies in the yeast-expression system, defined this as a missense mutation that significantly impaired enzyme activity; but, unlike the *Q188R* mutation, it still had minor residual activity (Fridovich-Keil et al., 1995a). Later on, more detailed SDM and expression studies in yeast and *E. coli* heterologous expression systems revealed this mutation decreased the abundance of mutant protein about 2-fold compared with the wild type, as well as caused 10-fold decrease of specific activity with less than 2-fold of differences of K_M values for both substrates (Lai and Elsas, 2001; Wells and Fridovich-Keil, 1997). There was no apparent difference in releasing glu-1P between the wild type and this mutant (Lai and Elsas, 2001). Mutating this serine to alanine, cysteine, histidine, threonine or tyrosine by SDM confirmed that a hydroxyl group is required on the side chain of amino acid 135, since only the threonine substitution resulted in active enzyme (Lai and Elsas, 2001).

The *K285N* mutation compromises the activity of the enzyme, as well as its abundance, in the yeast expression system (Riehman et al., 2001). As for the *N314D* mutation, it was regarded as the reason of reduced enzymatic activity in Duarte 2 patients; but detailed enzymatic studies facilitated by SDM revealed that the mutation itself only causes isoelectric point shifting, without affecting protein abundance, subunit dimerization or activity (Fridovich-Keil et al., 1995b). The decrease in GALT activity observed in the Duarte type 2 patients is likely caused by the 4-bp deletion at the promoter region associated with the *N314D* mutation, which abolishes the binding sites of two transcription factors to the GALT gene promoter (Carney, et al., 2009). The fact that the Los Angeles variant has normal activity in the erythrocytes supports this conclusion (Carney et al., 2009).

3.3.2 Type II (GALK-deficiency) galactosemia

More than 20 mutations associated with GALK deficiency have been reported to date. Through SDM studies, the majority of the mutations have been characterized. By expressing 10 variant GALK enzymes in GALK-less *E. coli*, Timson and Reece showed that five of mutant GALK enzymes (P28T, V32M, G36R, T288M and A384P), which are associated with more severe clinical phenotypes and near-zero blood galactokinase levels, are insoluble

(Timson and Reece, 2003). Further studies showed that these mutations disrupted the secondary structure of the enzymes, which could result in misfolding of the protein (Thoden et al., 2005). Four of the five soluble mutants (H44Y, R68C, G346S, and G349S, but not A198V) have impaired enzymatic properties, such as increased K_M for one or both substrates and decreased k_{cat}. All five are associated with low blood enzyme levels and milder symptoms. From the crystal structure of human GALK, it is clear that His^{44}, Gly^{346} and Gly^{349} are close to the active site. Additionally, these residues reside in the signature motif III of the GHMP kinase superfamily (Bork et al., 1993; Thoden et al., 2005). Therefore, it is not surprising that any changes in these resides would alter the kinetic parameters of the enzyme. As for A198V, its kinetic parameters are essentially indistinguishable from the wild-type enzyme. Compared to other mutations, from which patients will develop cataracts with high incidence within the first few years (without treatment), the A198V enzyme causes only a moderate incidence of cataracts in later life.

Similarly, Park and colleagues characterized another four missense mutations and one insertion (*G137R*, *R256W*, *R277Q*, *V281M* and *850_851insG*) by expressing the corresponding mutated genes in COS7 cells (Park et al., 2007). The steady-state expression level of R256W was lower than that of wild type. The stability of the mutant enzyme was significantly reduced, and it had no detectable activity. No protein was detected for the insertion variant. The other three mutations manifested enzymes with similar expression levels in the soluble fraction, as compared to the wild-type level. However, the *G137R* and *R277Q* enzymes had approximately 10%-15% of wild-type activity, and no activity was detected for the *V281M* enzyme.

3.3.3 Type III (GALE-deficiency) galactosemia

GALE deficiency exists in a continuum, from generalized to peripheral *via* intermediate (Openo et al., 2006). If GALE is deficient in all tissues, it is classified as generalized; and, if it is only deficient in red and white cells but normal in other tissues, it is known as peripheral deficiency. It is possible that the presence of bi-allelic amorphic mutations is incompatible with life (Sanders et al., 2010). Infants with generalized deficiency develop disease on a lactose-containing milk diet, while infants with peripheral disease remain well, at least in the newborn period. GALE deficiency has been extensively reviewed by Fridovich-Keil and coworkers (Fridovich-Keil et al., 1993a). Genomic *GALE* is about 5 kb in length, with multiple alternatively spliced transcripts. Some of the reported mutations are deposited in the HGMD database (http://www.hgmd.org/). Few case series have been reported, including a Korean study, reporting 37 patients with reduced GALE activity (Park et al., 2005), and two US-based studies, with one reporting 35 patients (Maceratesi et al., 1998) and the other, 10 patients (Openo et al.,2006). Others have reported a few cases (Alano et al., 1998; Wohlers et al., 1999). The *V94M* mutation has been reported in the homozygous state as being associated with generalized disease (Wohlers et al., 1999). In-depth studies of the *V94M* mutation through SDM in the yeast system showed that this mutation severely damages the specific activity of the enzyme predominantly at the level of V_M without affecting its abundance and thermal stability (Wohlers et al., 1999; Wohlers and Fridovich-Keil, 2000). In the same study, the *G90E* mutation was shown to have zero enzymatic activity, rendering the mutant enzyme to high temperature and protease (Wohlers et al., 1999). A more recent study further confirmed the impact of *V94M* and *G90E* on V_M (Timson 2005). Other missense mutations have not (yet) been

reported in patients, but they have been studied *in vitro* or in model systems. They are associated with severe enzyme deficiency; these include *G90E* and *L183P* (Quimby et al., 1997; Timson, 2005; Wohlers et al., 1999). Missense mutations associated with peripheral disease include *R169W*, *R239W* and *G302A* and have been described by Park and coworkers in individuals with peripheral GALE deficiency (Park et al., 2005). The *K257R* and *G319E* mutations have been described in African-Americans with peripheral deficiency (Alano et al., 1998). The *L183P* mutation encodes an enzyme that experiences severe proteolytic degradation during expression and purification. Also the authors showed that enzymes resulting from the *N34S*, *G90E* and *D103G* mutations exhibited increased susceptibility to digestion in limited proteolysis experiments (Timson 2005). An earlier study on *L183P* and *N34S* using SDM in a yeast model revealed that the L183P-hGALE mutant demonstrated 4% wild-type activity and 6% wild-type abundance, while N34S-hGALE demonstrated approximately 70% wild-type activity and normal abundance. However, yeast cells co-expressing both L183P-hGALE and N34S-hGALE exhibited only approximately 7% wild-type levels of activity, thereby confirming the functional impact of having both substitutions and raising the intriguing possibility that some form of dominant-negative interaction may exist between the mutant enzymes found in this patient (Quimby et al., 1997). Two other mutations, *D130G* and *L313M*, which are associated with intermediate epimerase deficiency, manifested enzymes with near normal GALE activity, but with compromised thermal stability and protease-sensitivity (Wohlers et al., 1999). Three other mutations associated with intermediate forms (*S81R*, *T150M* and *P293L*) were analyzed for their kinetic and structural properties *in vitro* and their effects on galactose-sensitivity of *S. cerevisiae* cells in the absence of Gal10p. All three mutations result in impairment of the kinetic parameters, principally the turnover number, k_{cat}, compared to the wild-type enzyme. However, the degree of impairment was mild compared with that seen with the mutation *V94M* (Chhay et al., 2008). Studies are limited by the fact the many patients are compound heterozygotes and by the observation that dominant-negative interactions may be involved in some of these cases.

4. Use of SDM in the understanding of catalytic mechanisms of the human GAL enzymes

4.1 The issue

Although the Leloir pathway is evolutionarily conserved and is indispensable for productive galactose metabolism, the catalytic mechanisms of the GAL enzymes are largely unknown.

4.2 Research design

Several groups have attempted to combine the techniques of SDM, analytical biochemistry and X-ray crystallography to advance the understanding of the catalytic mechanisms of the different GAL enzymes.

4.3 The results

4.3.1 GALK

GALK converts galactose to gal-1P by transferring γ–phosphate group of ATP to the O1 position of galactose. It belongs to a unique kinase superfamily – the GHMP kinase family,

which is named after four characteristic family members: galactokinase (GALK), homoserine kinase (HSK), mevalonate kinase (MVK) and phosphomevalonate kinase (PMVK) (Bork et al., 1993). This family of proteins was first identified by three highly conserved motifs among the four kinases mentioned above by sequence alignment and analysis. Motifs I and III are located at the N-terminal and C-terminal ends; and motif II, the most conserved, is located in the middle of the protein, with the consensus sequence of GLGSS(G/A/S) (Holden et al., 2004).

Interestingly, two different catalytic mechanisms have been proposed for this family. A common catalytic strategy to achieve nucleophilic attack is to use a negative charged residue, such as aspartate or glutamate, to act as a Brønsted base. This catalytic base can then abstract a proton from the hydroxyl group of the substrate converting the weakly nucleophilic hydroxyl group into the more strongly nucleophilic alkoxide ion, which then attacks the electron-deficient phosphorus atom in ATP (Fig. 1A). In such systems, it is common to find positively-charged lysine or arginine residues close to the catalytic site to help stabilize the negative charges on the enzyme and the substrates. Studies on MVK suggest this enzyme follows this mechanism. The crystal structure of MVK reveals an aspartate (residue 204 in the rat enzyme) positioned to act as an active site base. There is also a lysine (residue 13 in rat MVK), which is close to both the putative catalytic aspartate residue and the hydroxyl group of the substrate (Fu et al., 2002; Yang et al., 2002). Replacement of the lysine residue with a methionine by SDM resulted in a reduced, but non-zero, rate (V_{max} was reduced approximately 60-fold) (Potter et al., 1997). Similar results were observed when the equivalent lysine (residue 18) was changed to methionine in yeast mevalonate diphosphate decarboxylase (Krepkiy and Miziorko, 2004). These results are consistent with this positively-charged residue playing an assisting, but non-vital, role in catalysis. Crystal structures of GALK put it into this mechanism by revealing there are aspartate and arginine residues in the active center close to the galactose C1 hydroxyl group (Asp^{186} and Arg^{37} in the human structure, Asp^{183} and Arg^{36} in *Lactococcus lactis*) (Thoden and Holden, 2003; Thoden et al., 2005). Similarly, changing Arg^{37} of human GALT to alanine resulted in a nearly inactive enzyme; and lysine resulted in compromised k_{cat} and K_M for galactose (Tang, et al., 2010).

In contrast, phosphoryl transfer in HSK has been suggested to occur by direct nucleophilic attack on the γ-phosphate group of ATP by the δ-hydroxyl of homoserine (Fig. 1B) (Krishna et al., 2001). In this mechanism, the latter is stabilized by the formation of a hydrogen bond to a neighboring asparagine residue (Asn^{141}), which is not conserved in the superfamily. Catalysis is proposed to be assisted through activation of the γ-phosphate of ATP by the magnesium ion, which is coordinated by a conserved glutamate residue (Glu^{130}) with the deprotonation of the δ-hydroxyl possibly involving the γ-phosphate (Krishna et al., 2001).

4.3.2 GALT

GALT catalyzes the transfer of the uridine monophosphate group (UMP) from uridine diphosphate-glucose (UDP-Glu) to gal-1p to form uridine diphosphate-galactose (UDP-Gal) and glucose-1-phosphate (glu-1P) (Kalckar et al., 1953). The reaction follows the double displacement mechanism as shown in Fig. 2 (Arabshahi et al., 1986). The most characteristic

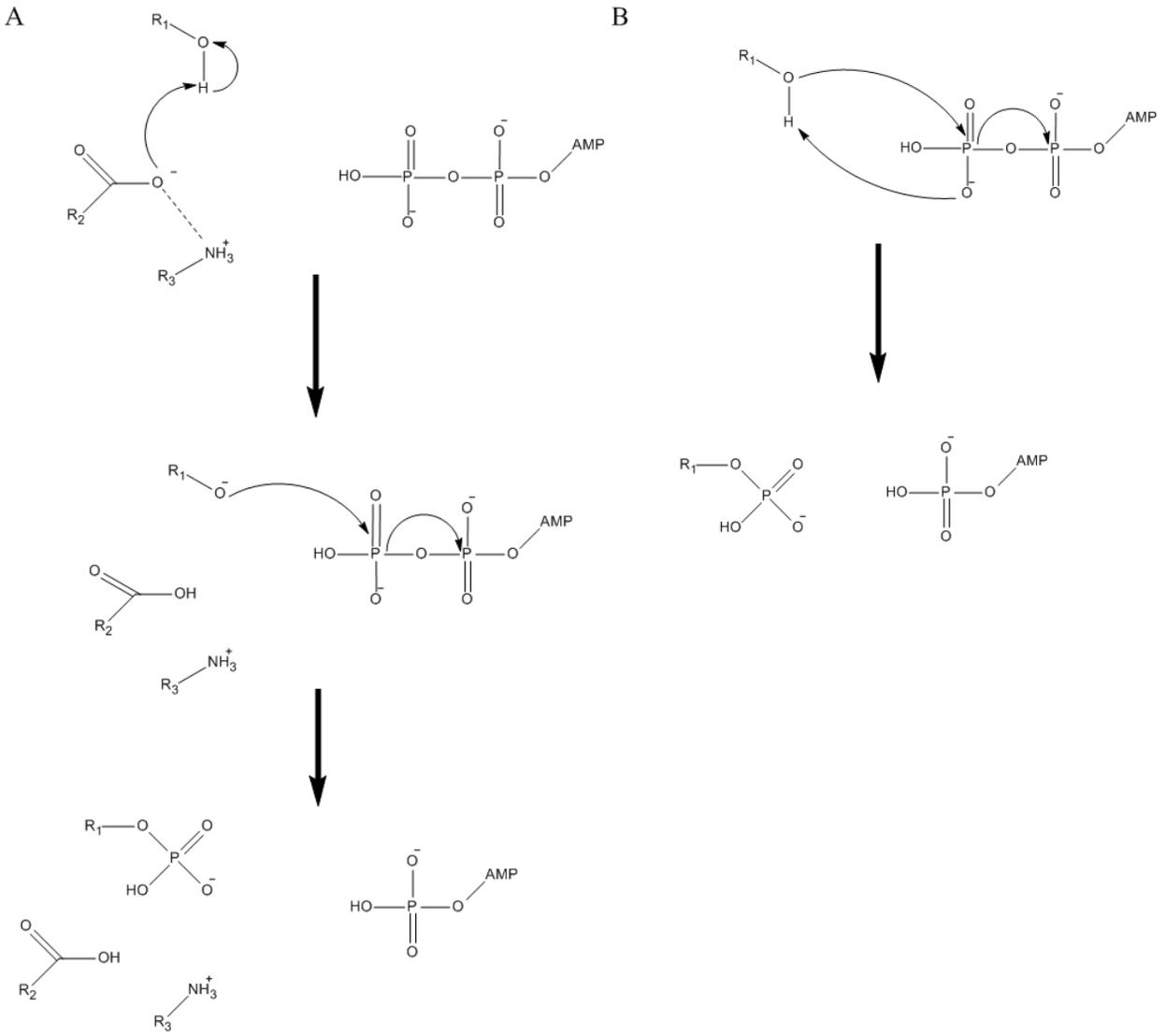

Fig. 1. Catalytic mechanisms proposed for GHMP kinase. **A**. The enzyme catalyzes the reaction through an active base residue R_1, which attracts a proton from the substrate R_3, converting the weakly nucleophilic hydroxyl to an alkoxide ion, which attacks the γ-phosphate of ATP. A positively charged residue R_2, sits close to the catalytic residue and stabilizes the alkoxide ion. **B**. There is no active base residue in the active center, the substrate directly attacks the γ–phosphate of ATP.

feature of the reaction is forming a covalent UMP-enzyme intermediate (Arabshahi et al., 1986). The intermediate was isolated by gel permeation chromatography in reaction mixtures containing the enzyme and radiolabeled UDP-Glu, and the radiolabeled intermediate could react with gal-1P or glu-1P to form the corresponding radiolabeled UDP sugar (Wong, et al., 1977a). This intermediate is very fragile in slightly acidic solutions but quite stable in strong basic solutions (Wong et al., 1977a; Yang and Frey, 1979), which indicates the intermediate is phosphoramides. Further degradation study of this intermediate confirmed that the nucleophile in GALT, to which the uridylyl group is bonded in the uridylyl-enzyme intermediate, is imidazole N3 of a histidine residue (Yang and Frey, 1979).

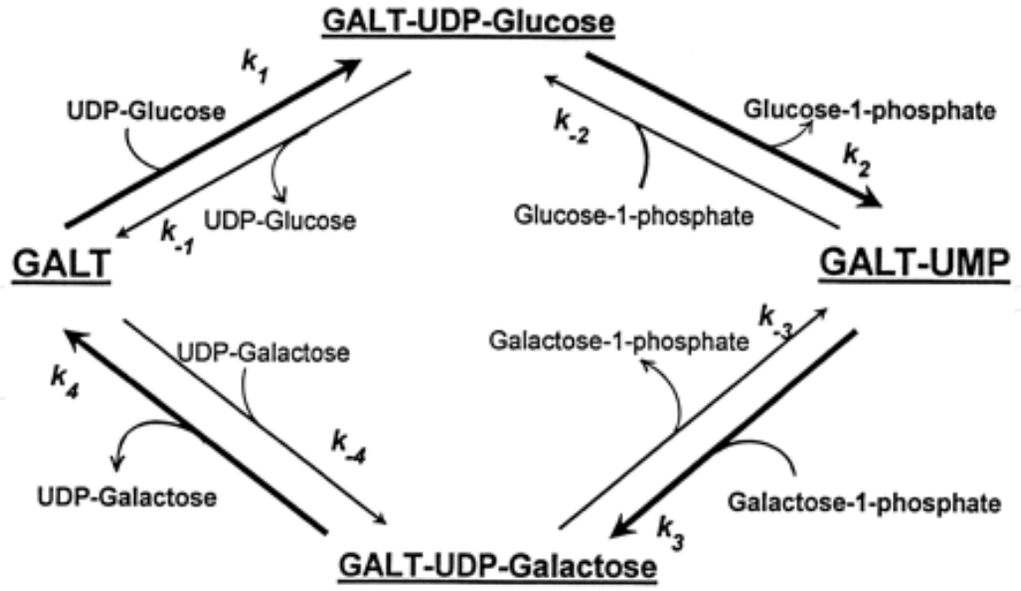

Fig. 2. Double displacement reactions of GALT. GALT binds to UDP-Glu to form a GALT-UDP-Glu intermediate. Glu-1-P is subsequently released, whereas the enzyme remains bound to UMP. Gal-1-P then reacts with the enzyme-UMP intermediate to form UDP-Gal, freeing the GALT enzyme for continued catalysis. k_n and k_{-n} denote rate constants of the forward and reverse reactions.

Substituting each of the 15 histidine residues in *E. coli* GALT with asparagines by SDM, proved that His^{164} and His^{166} were the only essential histidine residues in the enzyme (Field et al., 1989). In order to identify which of these two residues is the catalytic residue, two more specific mutations were introduced by SDM, *H164G* and *H166G*, which resulted in loss of function of the enzyme because of the missing imidazole ring of histidine, which might be filled and salvaged by adding exogenous imidazole ring. The experimental results showed that the activity of the H166G mutant could be recovered by adding exogenous imidazole ring, while mutant H164G could not. Therefore, His^{166} provides the catalytic nucleophilic imidazole ring in the reaction (Kim et al., 1990).

Also, as mentioned earlier, by mutating Gln^{188} of human GALT (equivalent to Gln^{168} in *E. coli* GALT), the most common mutation found in Type I Galactosemia, to arginine and asparagine, respectively, we were able to determine that glutamine at position 188 stabilizes the UMP-GALT intermediate through hydrogen bonding and enables the double displacement of both glucose-1-phosphate (glu-1P) and UDP-galactose. The substitution of arginine or asparagine at position 188 reduces hydrogen bonding and destabilizes UMP-GALT. The unstable UMP-GALT allows single displacement of glu-1P with release of free GALT but impairs the subsequent binding of gal-1P and displacement of UDP-Gal (Lai, et al., 1999).

4.3.3 GALE

GALE catalyzes the inter-conversion of UDP-Glu and UDP-Gal to finish the Leloir pathway of galactose metabolism. There are four key steps for the reaction of GALE as shown in Fig. 3: (1) abstraction of the 4'-hydroxyl hydrogen of the sugar by an enzymatic base, (2) transfer of a hydride from C4 of the sugar to the C4 of NAD^+ leading to a 4'-ketopyranose intermediate and NADH, (3) rotation of the resulting 4'-ketopyranose intermediate in the

active site, and (4) return of the hydride from NADH to the opposite face of the sugar (Maitra and Ankel, 1971). When purified, this enzyme contains tightly bound NAD+, which functions as an essential coenzyme to catalyze the reaction (Darrow and Rodstorm, 1968). The binding of the UDP group is strong, while binding with the galactosyl, glucosyl and 4-ketohexopyranosyl moieties is weak (Kang et al., 1975; Wong and Frey, 1977b). Early study on the catalytic mechanism of GALE focused on Lys^{153}, since it is close to the NAD^+, and the positively-charged ammonium group of Lys^{153} may perturb the electron distribution in the nicotinamide ring of NAD+ through charge repulsion upon substrate binding (Swanson and Frey, 1993). Replacing this residue with alanine or methionine renders the inability of the mutant proteins to be reduced by the sugar in the presence or absence of UMP. As a result, the catalytic activities of the mutants decreased by a factor over 1000. Also the purified mutant contained much less NADH as compared with wild type (Swanson and Frey, 1993). These results indicate that Lys^{153} plays an important role in the UMP-dependent reduction of GALE-NAD^+. Further studies identified two more important residues, Tyr^{149} and Ser^{124}, which are involved in glucose moiety binding (Thoden et al., 1996). SDM studies on the latter two residues revealed that that Tyr^{149} provides the driving force for general acid-base catalysis, while Ser^{124} plays an important role in mediating proton transfer (Liu et al., 1997). The crystal structure of human GALE confirmed that Tyr^{149} (Tyr^{157} for human GALE) sits at the proper position to interact directly with the 4'-hydroxyl group of the sugar and attracts the proton from the hydoxy group and transfers it to NAD^+ (Thoden et al., 2000).

Unlike what was observed for the *E. coli* enzyme, the human enzyme can also convert UDP-*N*-acetylglucosamine (UDP-GlcNAc) to UDP-*N*-acetylgalactosamine (UDP-GalNAc) (Kingsley et al., 1986; Piller et al., 1983). Through structure analysis and alignment, investigators found that, when the human enzyme equivalent of Tyr^{299} in the *E. coli* protein is replaced with a cysteine residue (Cys^{307}), the active site volume for the human protein is calculated to be approximately 15% larger than that observed for the bacterial epimerase (Thoden 2001). Substituting Tyr^{299} of *E. coli* GALE with a cysteine residue by SDM confers UDP-GalNAc/UDP-GlcNAc converting activity to the bacterial enzyme with minimal changes in its three-dimensional structure. Specifically, although the Y299C mutation in the bacterial enzyme resulted in a loss of epimerase activity with regard to UDP-Gal by almost 5-fold, it resulted in a gain of activity against UDP-GalNAc by more than 230-fold (Thoden et al., 2002b).

5. Use of SDM in the development of novel treatment of Type I (classic or GALT-deficiency) galactosemia

5.1 The issues

Unlike Type II or the peripheral Type III Galactosemia, patients with Type I (GALT-deficiency) Galactosemia, also the most common type of Galactosemia, suffer a range of debilitating long-term complications, which include premature ovarian insufficiency, learning deficits, ataxia and speech dyspraxia (Lai et al., 2009; Berry and Elsas, 2011). The current galactose-restricted diet fails to prevent these complications, and the medical/ patient communities are yearning for a more effective therapy. The causes of these organ-specific complications remain unknown, but there is a strong association with the intracellular accumulation of gal-1P. But what is the source of gal-1P in these patients with Classic Galactosemia if they limit their galactose intake? Recent studies have shown that the

patients on a galactose-restricted diet are never really "galactose-free. A significant amount of galactose is found in non-dairy foodstuffs, such as vegetables and fruits (Berry et al., 1993; Acosta and Gross, 1995). More importantly, galactose is produced endogenously from the natural turnover of glycolipids and glycoproteins (Berry et al., 1995). Using isotopic labeling, Berry and coworkers demonstrated that a 50kg adult male could produce up to 2 grams of galactose per day (Berry et al., 1995, 2004). Once galactose is formed intracellularly, it is converted to gal-1P by GALK and in GALT-deficient patient cells. As a result, gal-1P is concentrated more than one order of magnitude above normal, even with strict adherence to a galactose-restricted diet. Accumulation of gal-1P is regarded as a major, if not sole, factor for the chronic complications seen in patients with Classic Galactosemia, as suggested by both clinical observation and experimental results from yeast models. Patients with inherited deficiency of GALK, who do not accumulate gal-1P, do not experience the brain and ovary complications seen in GALT-deficient patients (Gitzelmann et al., 1974; Gitzelmann 1975; Stambolian et al., 1986). While *gal7* (*i.e*, GALT-deficient) mutant yeast stops growing upon galactose challenge, a *ga17 ga11* double mutant strain (*i.e*, GALT- and GALK-deficient) is no longer sensitive to galactose (Douglas and Hawthorne, 1964, 1966). Based on these observations, in conjunction with dietary therapy, inhibiting GALK activity with a safe small-molecule inhibitor might prevent the squeals of chronic gal-1P exposure in patients with Classic Galactosemia.

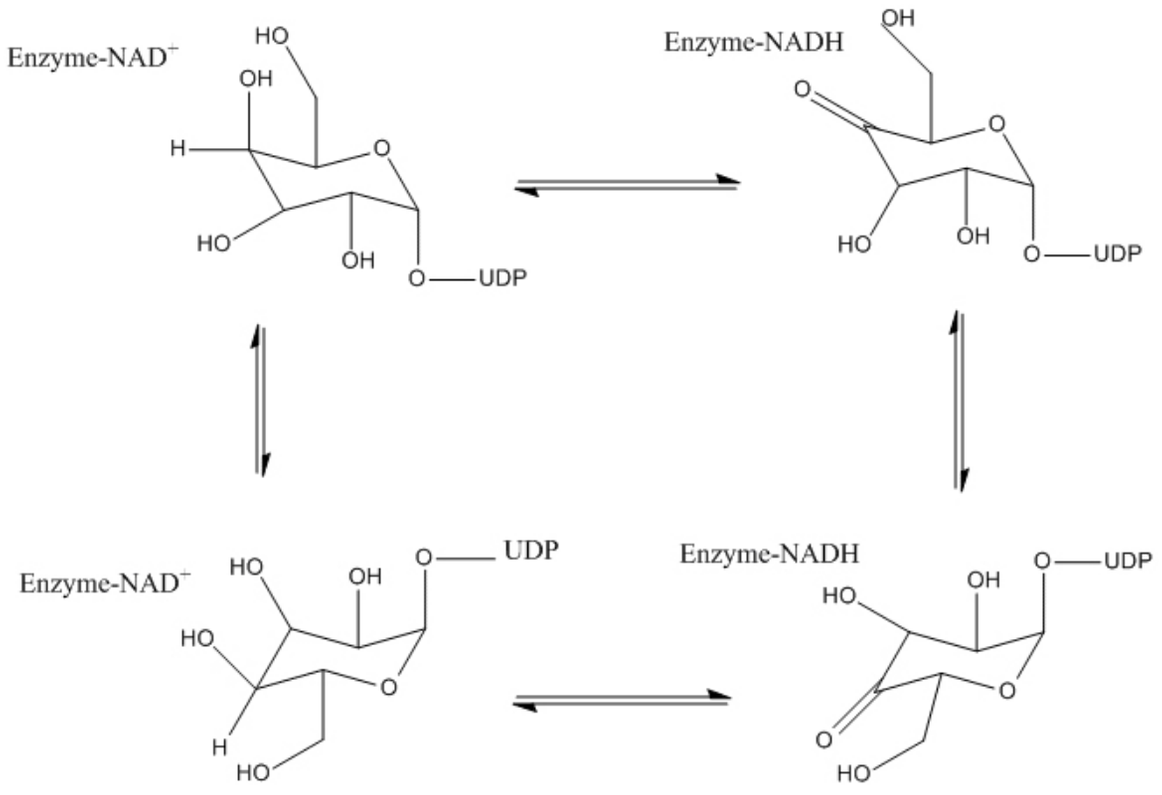

Fig. 3. Catalytic mechanism of GALE.

5.2 Research design

For the past few years, our group has conducted high-throughput screening (HTS) of small molecule compounds, which could inhibit human GALK enzyme *in vitro* (Tang et al., 2010;

Wierenga et al., 2008). To date, we have screened over 300,000 compounds of diverse chemical structures and identified a few promising hit compounds for further characterization. One of the characterization steps involved the use of SDM to change the respective amino acids of the GALK active site in order to confirm the predicted molecular interactions between the selected inhibitors and it target, GALK, through high-precision docking programs such as GLIDE (*Schrödinger*). Another characterization step that is noteworthy to mention is the assay for the kinase selectivity of the selected GALK inhibitors. As alluded above, GALK belongs to a unique small molecule kinase family, the GHMP kinase family (Bork et al., 1993). While the substrates of the GHMP kinases differ widely, the ATP-binding sites of the enzymes share a significant degree of structural homology (Tang et al., 2010). It is, therefore, important to ensure our selected GALK inhibitors did not cross-inhibit other GHMP kinases or other kinases in general.

5.3 The results

Selectivity is always one of the most important properties for developing therapeutic kinase inhibitors because of potential side-effects from unwanted inhibition of other kinases. During the characterization phase of our hit compounds, we found six compounds that selectively inhibit GALK but not any of the other GHMP kinases. These included MVK, which shares a high degree of structural similarity with GALK (Tang et al., 2010). In order to understand what structural elements conferred the specificity of these compounds, we aligned the crystal structure of human GALK and human MVK and focused on the ATP-binding site. Eight amino acid residues and the L1 loop were found to be different in these two kinases. SDM was employed to mutate each residue individually or the L1 loop, and the effects of the changes on the inhibitory capabilities of the compounds were tested. Two compounds were found to be affected by the mutation *S140G* (Table 1) (Tang et al., 2010). Ser^{140} of GALK resides in the signature motif of the GHMP kinase family, Motif II; but this amino acid is not conserved among the GHMP kinases. GALK is the only member that has a serine at this site. This could explain the selectivity of these two compounds. Furthermore, computational molecular docking confirmed that these two compounds interacted with Ser^{140} through hydrogen bonds; substituting serine with glycine abolished the hydrogen bonds and totally compromised the binding of the compounds to the enzymes.

Our use of SDM in the characterization of promising GALK inhibitors not only helped identify and confirm the amino acids of GALK with which these small molecules interact, but also exemplified a more rapid and cost-effective way to study the structural interactions between small molecule modifiers and their targets. This novel approach is particularly useful when large-scale co-crystallization projects are not feasible. These studies paved the way for more in-depth investigations to identify the structural determinants required for the inhibitor selectivity of GALK and GHMP kinases.

6. Concluding remarks

Using the disease Galactosemia as an example, we showed that site-directed mutagenesis (**SDM**) plays a vital role in biomedical research. As in the case of Galactosemia, in which the diagnosis begins at the bedside of the affected newborns, **SDM** can be employed in every step of basic and translational research in an attempt to improve the prognosis and

treatment of the patients. Further, not only did we show that **SDM** can be applied in traditional applications, such as expression analysis, we have also expanded its use in innovative drug design and the basic understanding of kinase inhibitor selectivity.

Mutations	k_{cat} (S^{-1})	K_M of ATP (μM)	K_M of Galactose (μM)	Effects on IC_{50} of compound 1	Effects on IC_{50} of compound 4	Effects on IC_{50} of compound 24
T77L	4.1	218.4	1305.2	None	None	None
S79N	4.8	303.4	1227.3	None	None	None
L145Y	11.6	259.7	222.7	None	None	None
L145A	6.4	379.9	356.8	None	None	None
W106A	No protein expression	-	-	-	-	-
W106T	No protein expression	-	-	-	-	-
Y109L	43.2	70.2	963.2	None	None	None
Y109A	8.7	579.3	268.7	None	None	None
GALK Loop to MVK Loop	0.1	695.4	1857.3	None	None	None
S140G	2.1	8.2	141.9	None	Increased 10-fold	Increased 20-fold
L135P	13.3	51.1	544.9	None	None	None
R37K	0.4	6.4	623.8	None	None	None
R37A	No activity	-	-	-	-	-
WT	17.5	20.9	319	-	-	-

Table 1. Effect of amino acid changes in human GALK on their enzymatic properties and the IC_{50} of selected inhibitors

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