Biochimica et Biophysica Acta 1822 (2012) 1516–1526

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Altered cofactor binding affects stability and activity of human UDP-galactose 4′-epimerase: Implications for type III galactosemia

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ARTICLE INFO

Article history:
Received 12 March 2012
Received in revised form 8 May 2012
Accepted 10 May 2012
Available online 18 May 2012

Keywords:
Type III galactosemia
Yeast model
GALE
Disease-associated mutation
UDP-galactose 4′-epimerase
Differential scanning fluorimetry

ABSTRACT

Deficiency of UDP-galactose 4′-epimerase is implicated in type III galactosemia. Two variants, p.K161N-hGALE and p.D175N-hGALE, have been previously found in combination with other alleles in patients with a mild form of the disease. Both variants were studied *in vivo* and *in vitro* and showed different levels of impairment. p.K161N-hGALE was severely impaired with substantially reduced enzymatic activity, increased thermal stability, reduced cofactor binding and no ability to rescue the galactose-sensitivity of *gal10*-null yeast. Interestingly p.K161N-hGALE showed less impairment of activity with UDP-N-acetylgalactosamine in comparison to UDP-galactose. Differential scanning fluorimetry revealed that p.K161N-hGALE was more stable than the wild-type protein and only changed stability in the presence of UDP-N-acetylglucosamine and NAD^+. p.D175N-hGALE essentially rescued the galactose-sensitivity of *gal10*-null yeast, was less stable than the wild-type protein but showed increased stability in the presence of substrates and cofactor. We postulate that p.K161N-hGALE causes its effects by abolishing an important interaction between the protein and the cofactor, whereas p.D175N-hGALE is predicted to remove a stabilizing salt bridge between the ends of two α-helices that contain residues that interact with NAD^+. These results suggest that the cofactor binding is dynamic and that its loss results in significant structural changes that may be important in disease causation.

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1. Introduction

Galactosemia is an autosomal recessive disease that results from impaired ability to metabolize the sugar galactose due to compromised expression or activity of the enzymes of the Leloir pathway [1–4]. Patients may develop a pathology that includes failure to thrive, vomiting, jaundice and sometimes bacterial infection in the most severe cases [4]. In the long term, disabilities in learning, speech, ovarian function, and movement can manifest [5–7]. The disease is classified into three types that vary in severity, pathology and occurrence and depend on which enzyme is affected. Type I is associated with galactose-1-phosphate uridylyltransferase deficiency (E.C. 2.7.7.12; OMIM#230400) and is the most commonly detected clinically severe form [8]. The occurrence of this type is geographically dependent and patients can show the most severe pathology of the three types. Over 230 different mutations have been detected by genetic screening, each associated with varying degrees of impairment. These mutations cause structural changes in the corresponding enzyme resulting in less efficient catalysis and/or reduced protein stability (for reviews see [9–11]). Galactokinase deficiency (EC 2.7.1.6; OMIM# 230200) is implicated in type II galactosemia [12] and this type is the least clinically severe of the three. Patients tend to develop galactose-dependent early onset cataracts and do not appear to suffer any other acute or long-term complications [5,12].

Human UDP-galactose 4′-epimerase (E.C. 5.1.3.2; hGALE) catalyses the inter-conversion of both UDP-galactose and UDP-N-acetylgalactosamine to their glucose counterparts and contains a bound NAD^+ as an essential cofactor [3,13–16]. Deficiency of this enzyme is implicated in type III galactosemia (OMIM #230350) [17,18] and this is the least understood form of galactosemia [2,19]. Only one nonsense and 21 missense mutations of hGALE are known [20]. Originally, epimerase deficiency was identified as a biochemical oddity that impacted only red and white blood cells in apparently healthy individuals ([21], and reviewed in [22]); this condition was termed "peripheral" epimerase deficiency because it impacted only cells in

Abbreviations: ANS, 1-anilinonaphthalene-8-sulphonic acid; BS^3, suberic acid bis(3-sulfo-N-hydroxysuccinimide ester); Gal-1P, galactose 1-phosphate; Glc-1P, glucose 1-phopshate; GALE, UDP-galactose 4′-epimerase; GuHCl, guanidine hydrochloride; hGALE, human GALE; FRET, Förster resonance energy transfer; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NAD(H), nicotinamide adenine dinucleotide (both oxidized and reduced); UDP-Gal, uridine diphosphate galactose; UDP-Glc, uridine diphosphate glucose; UDP-GalNAc, uridine diphosphate-N-acetylgalactosamine; UDP-GlcNAc, uridinediphosphate-N-acetylglucosamine

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doi:10.1016/j.bbadis.2012.05.007

peripheral circulation. Other cell types tested, including fibroblasts, liver, and even stimulated or EBV-transformed lymphoblasts, were not affected (reviewed in [22]). Subsequently, rare patients were identified who were severely symptomatic and demonstrated epimerase deficiency in all cell types tested; these patients were said to have "generalized" epimerase deficiency galactosemia [7]. Further studies of infants identified by newborn screening clearly established that there is a continuum of biochemical phenotypes [23].

The GALE enzymes of *Aeromonas hydrophila* and *Trypanosoma* sp. [24–26] are important in the viability of these pathogens, and GALE is also essential for survival of the "model" multicellular eukaryote *Drosophila melanogaster* [27]. The crystal structures of GALE from a variety of different species interacting with various substrates and substrate analogues are also known providing insight into how the enzyme functions [15,16,28–31]. The human GALE structure has been an invaluable tool in understanding how mutations can affect the enzyme and the structure of the variant associated with the most common severe phenotype, p.V94M, has been solved [32]. Coupled with a yeast model and biochemical work on the recombinant p.V94M protein, it was revealed that this variant's lower activity was due to improper binding of both UDP- and UDP-N-acetyl- sugars and this caused elevated galactose 1-phosphate levels *in vivo* [32,33]. Both the yeast model and recombinant proteins have been used to dissect how each mutation causes its effects. From these studies it has been shown that there is a correlation between enzyme activity and the ability to rescue growth in *gal10*-null yeast cells, which lack endogenous GALE [34–36]. Enzyme activity also correlates inversely with cellular Gal-1P accumulation [34,35], and Gal-1P and UDP-Gal accumulation correlate inversely with growth of *gal10*-null yeast [37]. The enzyme's turnover number (k_{cat}) is reduced in variant enzymes associated with type III galactosemia [20,38,39]. Biochemically, the stability of the enzyme is also important: decreased stability and protein aggregation *in vivo* have been reported for some of the disease-associated variants [20,34,38,40,41].

Here, both *in vivo* and *in vitro* approaches have been used to investigate two previously uncharacterized variants of hGALE, p.K161N and p.D175N. Each corresponding allele was originally detected in the heterozygous state in patients who had been diagnosed biochemically with a clinically benign form of epimerase deficiency [23]. Of note, the patient who is heterozygous for *hGALE.K161N* is also heterozygous for two intronic variants of unknown significance [23], and the patient who is heterozygous for *hGALE.D175N* is also heterozygous for two silent substitutions of unknown significance [23]. Parental DNAs were unavailable, leaving linkage of the different variants in each patient unclear. The relationship between functional significance of the K161N and D175N substitutions and the biochemical and clinical outcomes of the patients who carry them is considered in Conclusions below.

Both the K161N and the D175N variants of hGALE were investigated for their ability to rescue *gal10*-null yeast, for the levels of internal metabolites that accumulated in these yeast strains, and for enzyme structure, stability, activity, and ability to bind substrates and the NAD^+ cofactor. p.K161N-hGALE was found to be severely impaired while p.D175N-hGALE was found to be only mildly affected. That the patient carrying the K161N variant was nonetheless clinically mild may imply that the other allele present in that patient remained functional, as is discussed below. Results of stability studies and investigation of each residue's location in the overall enzyme structure suggest altered conformations affect substrate and cofactor binding as well as stability.

2. Materials and methods

2.1. Expression and purification of recombinant proteins in Escherichia coli

Recombinant wild-type GALE proteins were expressed in *E. coli* and purified as previously described [36,38]. The "QuikChange" protocol [42] was used to change the appropriate codons in the expression vector. The following primers were used: hGALE.K161N for (5′-CCTTACGGCAAGTCCAATTTCTTCATCGAGGAA-3′) with hGALE.K161N rev (5′-TTCCTCGATGAAGAAATTGGACTTGCCGTAAGG-3′) and hGALE.D175N for (5′-GACCTGTGCCAGGCAAACAAGACTTGGAACG TA-3′) with hGALE.D175N rev (5′-TACGTTCCAAGTCTTGTTTGCCTGGCACAGGTC-3′). The sequences were verified (MWG-Biotech, Ebersburg, Germany) and the mutated plasmids were used to express p.K161N-hGALE and p.D175N-hGALE using essentially the same protocol as used with the wild-type protein.

Recombinant human UDP-glucose dehydrogenase was expressed and purified as previously described [36]. All protein concentrations were determined using the Bradford assay [43] with bovine serum albumin as a standard.

2.2. Measurement of the steady state kinetic parameters for UDP-galactose 4′-epimerase

The activity of each purified variant protein was determined as previously described [36]. Here the conversion of UDP-galactose to UDP-glucose was measured by the UDP-glucose dehydrogenase catalyzed oxidation of UDP-glucose by NAD^+ [44]. Since the oxidation of one molecule of UDP-glucose consumes two molecules of NAD^+, the rate of production of NADH, measured by absorbance at 340 nm, corresponds to twice the rate of production of UDP-glucose.

Initial rates of NADH production were determined from the linear segment of each progress curve. Each rate was halved and then plotted against the corresponding substrate concentration. The data were fitted to Eq. (1) using non-linear curve fitting using GraphPad Prism (GraphPad Software, CA, USA) with all points weighted equally.

$$v = \frac{V_{max}[\mathrm{UDP-Gal}]}{K_m + [\mathrm{UDP-Gal}]} \quad (1)$$

where V_{max} is the maximum, limiting rate and K_m is the Michaelis constant.

2.3. Biophysical characterisation of variant proteins

Chemical cross-linking with BS^3 was carried out as previously described [38,45]. Limited proteolysis was carried out as described [36] with a few modifications. In addition to incubating with UDP-galactose, protein samples were also incubated with 1 mM and 10 mM NAD^+. The amount of protection from limited proteolysis was analysed by 15% SDS-PAGE. The ANS unfolding assay was carried out as previously described [36].

2.4. Fluoresence spectra

Each hGALE variant (200 μl of 20 μM), dissolved in 10 mM HEPES, pH 8.8 was aliquoted in triplicate into a black 96 well plate. Samples were excited at 280 nm or 350 nm and emission was measured from 300 to 510 nm or 400 to 500 nm respectively. All measurements were done at room temperature using a Spectra Max Gemini X U.V. plate reader fluorimeter.

2.5. Plasmids and yeast strains

The p.K165N and p.D171N *hGALE* alleles were each re-created by site-directed mutagenesis (Quikchange, Stratagene, Inc) of a wild-type *hGALE* coding sequence within a low-copy-number yeast expression plasmid, pMM33, as described previously [34]. The corresponding positive (wild-type hGALE) and negative (plasmid backbone only) controls have been reported previously [34].

All yeast strains used in this study were derived by transformation of JFy3835, a *gal10*-null haploid yeast strain that lacks endogenous GALE, and that has been reported previously [35]. For enzyme assays, yeast were cultured at 28 °C in synthetic medium containing 2%

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