Archives of Biochemistry and Biophysics 585 (2015) 39–51

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

The structural basis of substrate promiscuity in UDP-hexose 4-epimerase from the hyperthermophilic Eubacterium *Thermotoga maritima*

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ARTICLE INFO

Article history:
Received 2 July 2015
Received in revised form
18 August 2015
Accepted 31 August 2015
Available online 3 September 2015

Keywords:
Crystal structure
UDP-galactose 4-epimerase
Substrate specificity
Hyperthermophiles
Evolution

ABSTRACT

UDP-galactose 4-epimerase (GalE) catalyzes the interconversion of UDP-glucose (UDP-Glc) and UDP-galactose (UDP-Gal), which is a pivotal step in the Leloir pathway for D-galactose metabolism. Although GalE is widely distributed in prokaryotes and eukaryotes, little information is available regarding hyperthermophilic GalE. We overexpressed the TM0509 gene, encoding a putative GalE from *Thermotoga maritima* (TMGalE), in *Escherichia coli* and characterized the encoded protein. To further investigate the molecular basis of this enzyme's catalytic function, we determined the crystal structures of TMGalE and TMGalE bound to UDP-Glc at resolutions of 1.9 Å and 2.0 Å, respectively. The enzyme was determined to be a homodimer with a molecular mass of 70 kDa. The enzyme could reversibly catalyze the epimerization of UDP-GalNAc/UDP-GlcNAc as well as UDP-Gal/UDP-Glc at elevated temperatures, with an apparent optimal temperature and pH of 80 °C and 7.0, respectively. Our data showed that TM0509 is a UDP-galactosugar 4-epimerase involved in D-galactose metabolism; consequently, this study provides the first detailed characterization of a hyperthermophilic GalE. Moreover, the promiscuous substrate specificity of TMGalE, which is more similar to human GalE than *E. coli* GalE, supports the notion that TMGalE might exhibit the earliest form of sugar-epimerizing enzymes in the evolution of galactose metabolism.

1. Introduction

Carbohydrates are essential for all forms of life. Many types of carbohydrate structures play vital roles in biological systems as source of energy, structural elements, molecular recognition markers, and precursors for the biosynthesis of other molecules. In nature, many sugar-decorating and -modifying enzymes (e.g., epimerases, isomerases, glycohydrolases, glycosyltransferases, oxidoreductases, and dehydrogenase) are involved in carbohydrate metabolism. Epimerases, which are widespread among animals, plants, and microorganisms, can catalyze the inversion of the configuration of asymmetrically substituted carbon in linear or cyclic molecules such as carbohydrates. These enzymes are responsible for removing hydrogen from one face of a central carbon and returning it to the opposite face. Although simple to draw, the chemistry behind such a transformation is complex because carbohydrates are extremely stable, and epimerization does not occur spontaneously except mutarotation. During the past decades, a variety of carbohydrate epimerases, such as UDP-α-D-glucose 4-epimerase (GalE) [1], D-ribulose-5-phosphate 3-epimerase [2], UDP-*N*-acetylglucosamine 2-epimerase [3], L-ribulose 5-phosphate 4-epimerase [4], and D-galactose mutarotase [5], have been intensively studied to unravel their epimerization reactions.

Abbreviations: GalE, UDP-α-D-galactose 4-epimerase; NAD(H), nicotinamide adenine dinucleotide; UDP, uridine diphosphate; Glc, D-glucose; Gal, D-galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; UDP-GlcA, UDP-glucuronate; UDP-GlcDH, UDP-Glc dehydrogenase.

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http://dx.doi.org/10.1016/j.abb.2015.08.025
0003-9861/

Epimerization reactions are categorized according to the reaction mechanism [6]: transient keto intermediate, proton abstraction/addition, nucleotide elimination/readdition, carbon–carbon bond cleavage, and mutarotation. These studies suggested that a variety of cofactors and/or coenzymes are involved in the evolution of epimerases.

In living organisms (animals, plants, and bacteria), galactosyl groups can be utilized for the biosynthesis of complex carbohydrates such as glycoproteins and lipopolysaccharides [7,8], as well as for catabolism of D-galactose (Gal) through the Leloir pathway [9]. UDP-α-D-galactose 4-epimerase (EC 5.1.3.2, GalE), which catalyzes the interconversion of UDP-α-D-glucose (UDP-Glc) and UDP-α-D-galactose (UDP-Gal), is essential for *de novo* biosynthesis of UDP-Gal in the Leloir pathway, by which β-D-galactose is converted to α-D-glucose 1-phosphate (Glc-1-P) to allow catabolism of D-galactose [9–11]. First, α-D-galactose is produced from β-D-galactose by galactose mutarotase [12]. The α-D-galactose is then phosphorylated to α-D-galactose 1-phosphate (Gal-1-P) by galactokinase (GalK). The third enzyme, Gal-1-P uridyltransferase (GalT), catalyzes the transfer of an uridyl group from UDP-Glc to Gal-1-P to produce UDP-Gal and Glc-1-P, which enters into the glycolytic pathway. The last enzyme in the pathway, GalE, regenerates UDP-Glc from UDP-Gal, thereby maintaining production of Glc-1-P. Accordingly, these enzymes are required for the interconversion of D-galactose moiety to D-glucose moiety in cells. In the absence of GalT or GalE, the accumulation of intermediary metabolites such as Gal-1-P and UDP-Gal [13], or the depletion of pyrimidine nucleotides [14] could cause the dys-regulation of robust metabolism if D-galactose added in the cell growth medium [15]. In humans, impaired galactose metabolism, ascribed to missense mutations in either GalT or GalE, results in galactosemia, a serious metabolic disease [10,16]. Moreover, GalE deficiency exacerbates D-galactose toxicity in plants [17] and yeast [18]. In light of this, GalE is of great importance, not only for understanding carbohydrate metabolism in various organisms, but also for its physiological relevance to diseases and cellular phenotypes.

During the last decade, genome sequences of hyperthermophiles have revealed that multiple carbohydrate metabolic pathways are also available to hyperthermophilic bacteria and archaea [19]. These genomic data shed light on metabolism under extreme environments, indicating that sugar catabolism in hyperthermophiles is different relative to those of other domains of life. In this regard, functional annotation and characterization of gene products derived from hyperthermophiles are essential for investigation and understanding of early forms of life and the molecular evolution of metabolic enzymes [20]. The order *Thermotogales*, consisting mainly of anaerobically fermenting extremophilic bacteria, can grow on a range of both simple and complex carbohydrates, including glucose, starch, cellobiose, xylan, and pectin [21–23]. The genome sequence of *Thermotoga maritima*, which has an optimal growth temperature of 80 °C [24], revealed that many genes in this organism (10–15%) are involved in carbohydrate metabolism [25–27]. In addition, about half of the protein coding genes of *T. maritima* are highly conserved relative to bacterial genes, whereas a quarter of genes are more similar to archaeal genes, providing evidence for lateral gene transfer [28]. Moreover, sugar-nucleotide C4 epimerase is of special interest because it is a key enzyme in broad-spectrum microbial carbohydrate utilization. Sugar-nucleotide C4 epimerases can be classified into three groups based on their substrate specificity [29,30]: group 1 (e.g., *Trypanosoma brucei* GalE) is highly specific for non-acetylated sugars; group 2 (e.g., human GalE) can epimerize acetylated and non-acetylated sugars equally well; and group 3 (e.g., WbpP from *Pseudomonas aeruginosa*) exhibits a strong preference for acetylated sugars. Intriguingly, sequence alignments of the TM0509 gene encoding a putative sugar-nucleotide C4 epimerase from *T. maritima* with *galE* genes derived from a wide range of prokaryotes and eukaryotes demonstrated that although the sequence of the TM0509 gene is more similar to those of prokaryotic homologues, its substrate specificity is more similar to those of its eukaryotic homologues [31]. Although GalEs are widely distributed across bacteria and eukaryotes, little information is available regarding hyperthermophilic GalEs.

In this study, we overexpressed the TM0509 gene encoding the *T. maritima* GalE (TMGalE) in *Escherichia coli*, characterized the enzyme, and determined its crystal structure. In addition, we discuss the molecular evolution of GalE in the context of D-galactose metabolism under extreme environments, as well as the functional annotation of the TM0509 gene with respect to substrate specificity.

2. Materials and methods

2.1. Materials

Restriction enzymes, PrimeSTAR DNA polymerase, deoxynucleotide triphosphates, and chemicals for PCR were obtained from Takara Biomedicals (Takara Co., Shiga, Japan). The pTOP Blunt V2 vector for cloning was obtained from Enzynomics (Daejeon, Korea). The pET-15b and pET-22b expression vectors and the His-Bind Resin kit were obtained from Novagen (Madison, WI, USA). The pBAD-hisA expression vector was obtained from Invitrogen (Carlsbad, CA, USA). Genomic-tip, gel extraction, PCR purification, and plasmid miniprep kit were obtained from Qiagen (Hilden, Germany). Electrophoresis reagents were obtained from Bio-rad (Hercules, CA, USA). All chemicals used for enzyme assays and characterization (e.g., UDP-glucose, UDP-galactose, UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, and nicotinamide adenine dinucleotide) were purchased from Sigma (St.Louis, MO, USA).

2.2. Bacterial strains and plasmids

E. coli strains, plasmids, and primers used in this study are listed in Table S1. Cultures were grown in Luria-Bertani (LB) and M9 media at 37 °C with appropriate concentrations of antibiotics (kanamycin at 50 μg/ml and ampicillin at 100 μg/ml, respectively). *E. coli* strains DH5α and BL21 (DE3) were used as bacterial hosts for recombinant plasmids. For complementation experiments, *E. coli* BW25113 Δ*galE* Keio strain was used as a host and grown in minimal M9 medium, supplemented with 0.5% D-galactose as the sole carbon source and 0.2% L-arabinose as an inducer. Growth was monitored by determining the absorbance at 600 nm with an Ultra8000 spectrophotometer (GE healthcare). The pTOP Blunt V2 vector was used for cloning and sequencing, and the pET-15b(+), pET-22b(+), and pBAD-hisA were used for expression.

2.3. Cloning and expression of the TM0509 gene

A search of the microbial genome sequences in GenBank revealed a *galE* homologue (TM0509) in *T. maritima* encoding a putative UDP-glucose 4-epimerase (GalE). The *galE* gene was amplified by PCR by using genomic DNA as the template. The PCR mixture (total volume, 50 μl) contained 20 ng of genomic DNA, 10 pmol of primer TM G4E-*Nde*I-F, 10 pmol of primer TM G4E-*Xho*I-R (Table S1), 1× PCR buffer, 0.2 mM dNTP mix, and 2.5 U of PrimeSTAR polymerase (Takara). The PCR product was cloned into pTOP Blunt V2, and the resultant construct was transformed into *E. coli* DH5α competent cells. Transformants containing pTOP Blunt V2 harboring the gene encoding *T. maritima* GalE (TM0509) were