Identification and characterisation of human aldose 1-epimerase☆

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Abstract Aldose 1-epimerase or mutarotase (EC 5.1.3.3) is a key enzyme of carbohydrate metabolism catalysing the interconversion of the α- and β-anomers of hexose sugars such as glucose and galactose. We identified an open reading frame in the human genome (BC014916) which has high sequence similarity to previously identified bacterial aldose 1-epimerases. This sequence was cloned into a bacterial expression vector, and expressed and purified from this source. Enzyme assays show that the protein has aldose 1-epimerase activity and exhibits a preference for galactose over glucose. Site-directed mutagenesis confirmed the involvement of three residues involved in catalysis and substrate binding.

Key words: Mutarotase; Galactose; Glucose; Leloir pathway; Carbohydrate metabolism; Galactosemia

1. Introduction

Common hexose sugars such as glucose and galactose exist in two predominant forms in aqueous solution, the α- and β-pyranose structures [1]. These differ in the configuration of the hydroxyl group at carbon-1 of the ring. The two forms are interconverted readily in aqueous solution until an equilibrium mixture is formed [2]. Many enzymes of carbohydrate metabolism exhibit specificity for one anomer or the other. For example, galactokinase, which phosphorylates galactose at position 1, uses only α-galactose producing α-galactose-1-phosphate [3,4]. In contrast, glucose and galactose dehydrogenases exhibit specificity for the β-anomer of their respective sugars [5,6].

Although the anomers will interconvert in water, the rate of interconversion in the cytoplasm does not seem to be sufficient to provide the needs of metabolic pathways [7,8]. Indeed, the conditions prevailing in the cytoplasm may be such that very little spontaneous interconversion occurs [9]. Aldose 1-epimerases (mutarotases) have been isolated from a number of sources and catalyse rapid anomeric interconversion [7,10–14]. The enzyme is required for normal lactose metabolism in *Escherichia coli*. Strains in which the gene has been deleted grow much more slowly on media containing either sugar [7]. Recently, the crystal structure of galactose mutarotase from *Lactococcus lactis* has been solved [15,16]. This, together with recent enzymological work [17], suggests that the enzyme-catalysed reaction proceeds via an acid–base mechanism.

Although the enzyme has been purified from human tissues [12,18], the gene has not been identified nor has the protein been sequenced. Here we report the identification of the human gene for aldose 1-epimerase, the expression of the coding sequence in *E. coli* and the characterisation of the purified, recombinant protein.

2. Materials and methods

2.1. Cloning, expression and purification of human aldose 1-epimerase

A cDNA clone containing the sequence of putative human aldose 1-epimerase was obtained from the I.M.A.G.E. consortium (I.M.A.G.E. clone number 3502667) [19]. The polymerase chain reaction (PCR) was used to amplify the coding sequence. The primers were designed to introduce the sequences encoding a hexahistidine tag and an *Nco*I restriction enzyme site at the 5′-end and an *Eco*RI site at the 3′-end of the coding sequence. The fragment was then cloned into these restriction sites in the expression vector pET-21d (Novagen). The DNA sequence of the insert was verified in order to ensure no PCR-derived mutations had occurred (University of Manchester, Faculty of Medicine DNA Sequencing Service).

The resulting plasmid (pET21-GALM) was transformed into *E. coli* HMS174(DE3) cells (Novagen) for expression. One to two litres of these cells were grown shaking in LB media at 37°C until $A_{600\,nm}$ was between 0.6 and 1.0. The cultures were then induced with IPTG (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 of buffer A (50 mM HEPES-NaOH (pH 8.0), 150 mM NaCl, 10% (v/v) glycerol) and frozen at −80°C until required.

The protein was purified essentially as previously described for human galactokinase [20]. Briefly, the cells were disrupted by sonication, the lysate cleared by centrifugation (20 000×*g* for 20 min) and the supernatant loaded onto a 2 ml ProBond nickel-agarose column (Invitrogen). The column was washed extensively in 50 mM HEPES-NaOH (pH 8.0), 500 mM NaCl, 10% (v/v) glycerol and then in the same buffer supplemented with 30 mM imidazole. Protein was eluted in buffer supplemented with 250 mM imidazole. Fractions containing protein were dialysed overnight against 50 mM HEPES-NaOH (pH 8.0), 150 mM NaCl, 10% (v/v) glycerol, 1.4 mM β-mercaptoethanol, 2 mM EDTA before being snap frozen in liquid nitrogen and stored at −80°C.

2.2. Generation of point mutants

Point mutations in aldose 1-epimerase sequence were generated using the QuikChange method [21]. DNA sequencing was used to verify the mutations and that no other changes had occurred in the coding sequence. Mutant proteins were expressed and purified using the same protocol as used for the wild-type enzyme.

2.3. Analytical gel filtration

Analytical gel filtration was carried out using a Pharmacia S200 column equilibrated in 50 mM HEPES-NaOH (pH 8.0), 150 mM NaCl, 10% (v/v) glycerol, 1.4 mM β-mercaptoethanol. The column

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was calibrated using molecular mass standards (Pharmacia). Fractions (0.5 ml) were collected and subjected to acetone precipitation (as described previously [22]) prior to analysis by 10% SDS–PAGE.

2.4. Enzyme assays

The α-pyranose forms of glucose and galactose [23] were obtained from Sigma-Aldrich. The rate of sugar mutarotation was measured using an Optical Activity AA-100 polarimeter (Department of Chemistry, University of Manchester). All reactions were carried out in 25 mM HEPES-NaOH (pH 8.0), 50 mM NaCl at 20°C. Plots of optical rotation against time were fitted to single exponential curves, $\alpha = k_1 \cdot \exp(-k_2 \cdot t) + k_3$ (where α is the observed optical rotation at time t and k_1, k_2 and k_3 are constants) using non-linear curve fitting [24] as implemented in the program GraphPad Prism (GraphPad Software, San Diego, CA, USA). The initial rate is the gradient of this curve at $t = 0$ which is equal to $-k_1 \cdot k_2$. Measurement of the mutarotation rate in the absence of enzyme (v_0) allowed the first order rate constant (k) to be calculated from the relationship $v_0 = k[\text{sugar}]$. Knowing k permitted the calculation of v_0 for each sugar concentration used in the enzyme assay and this spontaneous rate was subtracted from the overall rate to give the initial, enzyme-catalysed rate (v_i). Initial rates were plotted against sugar concentration and fitted to the Michaelis–Menton equation $v_i/[E]_T = k_{cat} \cdot [\text{sugar}]/(K_m + [\text{sugar}])$ where k_{cat} is the turnover number, $[E]_T$ is the total enzyme concentration and K_m is the Michaelis constant [25].

The mutant enzymes were assayed by coupling the reaction to that of either glucose or galactose dehydrogenase (ICN and Sigma, respectively) following the methods of Beebe and Frey [17]. These enzymes are specific for the β-anomer of their respective sugars and the increase in absorbance at 340 nm was measured in a Multiskan Ascent plate reader. Reactions were carried out at 27°C in 10 mM HEPES-NaOH (pH 8.0), 3 mM NAD^+ in the presence of three units of the appropriate sugar dehydrogenase and were initiated by the addition of freshly dissolved sugar. The total reaction volume was 100 μl. Plots of $A_{340\,nm}$ were fitted to the equation $A_{340\,nm} = k_1 \cdot (1 - \exp(k_2 \cdot t)) + k_3$ and initial rates calculated from the product $k_1 \cdot k_2$. These rates were corrected by subtraction of the initial rate of mutarotation in the absence of enzyme.

3. Results

3.1. Identification, expression and purification of his-tagged human aldose 1-epimerase

In order to identify a putative aldose 1-epimerase in the human proteome, we used the protein sequence of the *L. lactis* galactose mutarotase (GalM) in a BLAST search [26] against all human proteins. The best match ($P = 7 \times 10^{-40}$) was unknown protein BC014916 (accession number NP_620156). Furthermore, the N-terminal sequence of this protein (ASVTRAVFGE...) agrees well with that of the hog kidney aldose 1-epimerase (VSVTRSVFGE...) which has been determined by mass spectrometry [27]. Overall, the hog kidney sequence (accession number BAB18973) shows 89% similarity to BC014916 at the amino acid level. A sequence alignment between the putative human aldose 1-epimerase and the *L. lactis* galactose mutarotase is shown in Fig. 1. The I.M.A.G.E. cDNA clone corresponding to this sequence was used to clone the protein coding sequence into the bacterial expression vector pET-21d such as to introduce a hexahistidine tag at its N-terminus. This tag enabled the rapid purification of the protein (Fig. 2A) from bacterial cell extracts. Typical yields were approximately 1 mg per litre of culture.

The galactose mutarotase from *E. coli* is a monomer [17], whereas the crystal structure of the *L. lactis* enzyme suggests this may be a dimer [15]. Gel filtration was used to determine the native molecular mass of bacterially expressed human aldose 1-epimerase. The protein (11 μM) was applied to a Sephadex S200 column and eluted at 14.1 ml corresponding to a molecular mass of 32 kDa (Fig. 2B). The calculated molecular mass of protein BC014916 is 38 kDa and thus we conclude that human aldose 1-epimerase is a monomer in solution.

Table 1
Kinetic parameters of human aldose 1-epimerase

	Sugar	
	Galactose	Glucose
K_m/mM	37 ± 10	54 ± 11
k_{cat}/s^{-1}	12 000 ± 1 400	4 900 ± 370
(k_{cat}/K_m)/l mol^{-1} s^{-1}	340 000 ± 56 000	90 000 ± 12 000

Mutarotation rates were observed directly using polarimetry. Reactions were carried out at 20°C. Values were determined by non-linear curve fitting and are shown ± the standard error derived from this procedure.

3.2. Kinetic analysis and substrate specificity of human aldose 1-epimerase

Polarimetry was used to observe directly changes in the optical rotation of sugar solutions in the presence and absence of aldose 1-epimerase. Addition of the protein to a solution of galactose or glucose resulted in an increase in the mutarotation rate. These increases of rate showed a saturatable dependence on sugar concentration that could be fitted to the Michaelis–Menton equation (Fig. 3). The kinetic parameters derived from this experiment (Table 1) are of a similar order of magnitude to those reported for the *E. coli* enzyme [17].

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Ll:   4  TTKDFGLGSSLISLTNKNDVTIAFTNLGARIVDWQKDGKHFILGFDSAQEYLEKDAYPGATVGRTAGRIKDGLVDISGKTYHLN
         T + F L S L+ +   + ++   T     + D Q      +LGF   + YL+K  Y GA +GR A RI  G   + GK YHL
Hs:  18  TVEKFQLQSDLLRV---DIISWGCTITALEVKDRQGRASDVVLGFAELEGYLQKQPYFGAVIGRVANRIAKGTFKVDGKEYHLA

Ll:  88  QNEAPQTLHGGEDSIHTKLWTYEINDLGDEVQVKFSLVSNDGENGYPGKIEMSVTHSFDEENNWKIKYEAISDKDTVFNPTGHV
          N+ P +LHGG       LWT  +   G    V+FS +S DGE GYPG++++ VT++ D      + Y A + + T  N T H
Hs:  99  INKEPNSLHGGVRGFDKVLWTPRVLSNG----VQFSRISPDGEEGYPGELKVWVTYTLD-GGELIVNYRAQASQATPVNLTNHS

Ll: 172  YFNLNGDASKSIENHQLKLAASRFVPLKDQTEIVRGDIVDTKNTDLDFRQEKQLSKALESSMEQVQLVGGIDHPFLLDEQSLEK
         YFNL G AS +I +H++ + A  ++P+ D+T I  G++   + T  D R+  +L K L+         + G DH F L     +
Hs: 178  YFNLAGQASPNINDHEVTIEADTYLPV-DETLIPTGEVAPVQGTAFDLRKPVELGKHLQDFH-----LNGFDHNFCLKGSKEKH

Ll: 256  EQARL--SLDDLSVSVYTDQPSIVIFTANFGDLGTVYHGKNQV---HHGGITFECQVSPGSQQIPELGDISLKAGDEYQATT
           AR+  +     + VYT QP +  +T NF D GT+  GKN       H G   E Q  P +   P    + L+ G+EY  TT
Hs: 256  FCARVHHAASGRVLEVYTTQPGVQFYTGNFLD-GTL-KGKNGAVYPKHSGFCLETQNWPDAVNQPRFPPVLLRPGEEYDHTT
```

Fig. 1. Sequence alignment of human protein BC014916 (Hs) with galactose mutarotase from *L. lactis*, accession number CAB44215 [33] (Ll). Three key, conserved, residues are highlighted. The two histidines make hydrogen bonds to the ring oxygen of the sugar in the structure of the *L. lactis* enzyme and are potential proton donors in the catalytic mechanism. The glutamate makes a hydrogen bond with the hydroxyl group attached to carbon-1 and is believed to be the catalytic base [15].

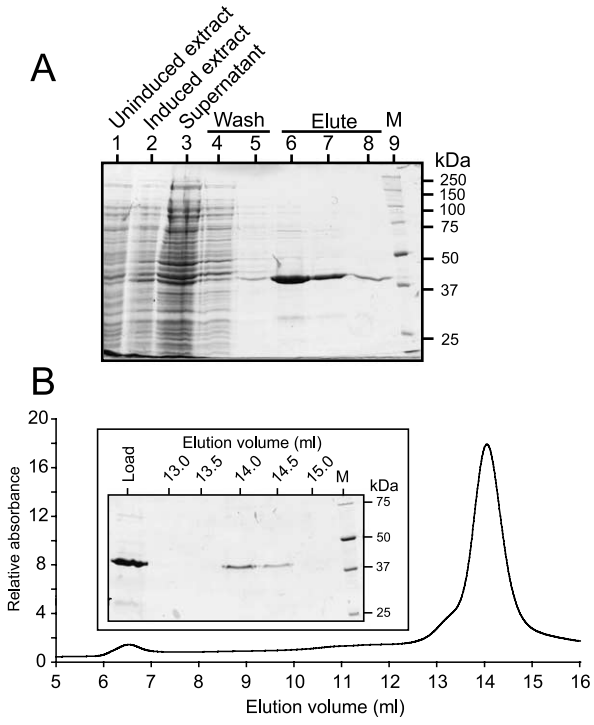

Fig. 2. Expression, purification and characterisation of the putative human aldose 1-epimerase. A: The protein was expressed in *E. coli* HMS174(DE3) cells as a hexahistidine fusion. This could be readily purified on nickel-agarose resin as shown on this 10% SDS–PAGE gel. B: Gel filtration analysis shows that the protein is a monomer. The inset shows a 10% SDS–PAGE gel of fractions across the main elution peak.

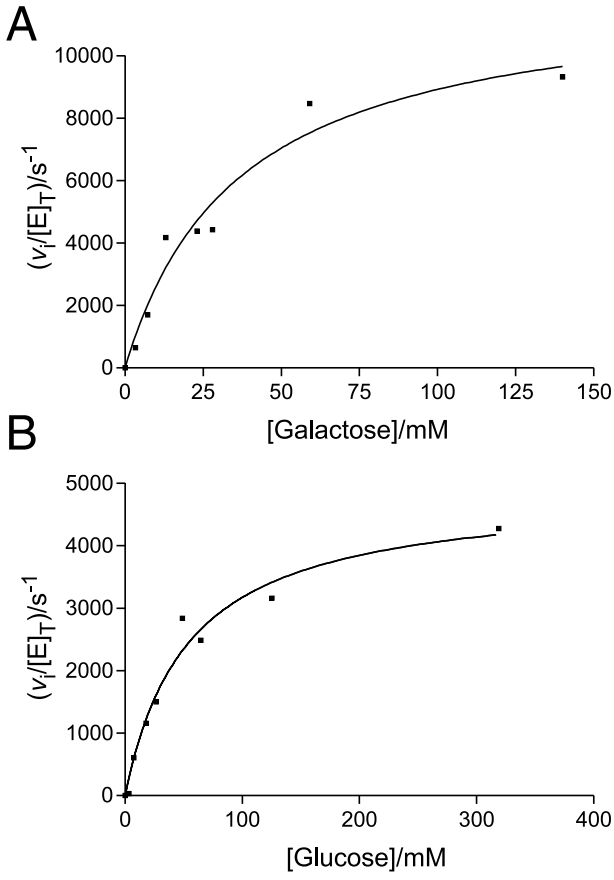

Fig. 3. Kinetic analysis of human aldose 1-epimerase with (A) galactose and (B) glucose as substrates. Rates of sugar mutarotation were measured directly using a polarimeter and initial rates determined as described in Section 2. The data were fit to the Michaelis–Menton equation using the non-linear curve fitting program GraphPad Prism.

The specificity constant, k_{cat}/K_m, is higher for galactose suggesting that the enzyme prefers this sugar as a substrate.

3.3. Identification of key catalytic residues in human aldose 1-epimerase

The crystal structure of *L. lactis* galactose mutarotase reveals a number of residues that contact the sugar [15,16]. These residues are all conserved in human aldose 1-epimerase. Of these three (His-96, His-170 and Glu-304) have been implicated in catalysis. The equivalent residues (His-107, His-176 and Glu-307, highlighted in Fig. 1) in human aldose 1-epimerase were all mutated to alanine. Although all could be expressed in *E. coli*, the amount of H176A following induction was less than for the wild-type or other mutants (data not shown). Furthermore the yield following purification of all these mutants was reduced 5–10-fold compared to the wild-type. The low yields and the expected low activity of these mutants made the polarimetry assay impractical. An alternative assay procedure was developed which involved coupling the aldose 1-epimerase reaction to the reaction catalysed by either galactose dehydrogenase or glucose dehydrogenase (both of which are specific for the β-anomer). The rate of reaction could be observed by measuring the increase in absorbance at 340 nm. This approach was complicated by the high rates of non-enzymatic mutarotation at sugar concentrations approaching the K_m of the enzyme. Therefore, we measured the rates at a very low level of sugar (approximately 1/100 of the K_m). At such low levels of substrate, the Michaelis–Menton equation simplifies to $v_i = (k_{cat}/K_m)\cdot[E]_T\cdot[S]$ [25]. Therefore, the specificity constant for each enzyme could be estimated (Table 2). That the specificity constants estimated this way and by direct polarimetric measurements are of the same order of magnitude gives us added confidence that this method is valid. H107A shows reduced activity (five- to eight-fold) with both substrates. In contrast H176A and E307A show no, or virtually no, aldose 1-epimerase activity. Since we were able to detect H176A activity at a level 300-fold reduced compared to the wild-type with galactose as a substrate, the activity of the E307A mutant must be considerably less than this.

Table 2
Specificity constants of the wild-type and mutant aldose 1-epimerases

	Specificity constant, (k_{cat}/K_m)/l mol^{-1} s^{-1}	
	Galactose	Glucose
Wild-type	310 000 ± 31 000	56 000 ± 3 000
H107A	63 000 ± 1 500	7 200 ± 330
H176A	1 000 ± 290	ND
E307A	ND	ND

Mutarotation rates were observed at 27°C by coupling the reaction to the appropriate sugar dehydrogenase. Galactose was present at a concentration of 360 μM and glucose at 670 μM. ND – no detectable activity. Values were determined by non-linear curve fitting and are shown ± the standard error derived from this procedure.

4. Discussion

Human protein BC014916 has been shown to be an active aldose 1-epimerase. The enzyme shows a small (approximately four-fold) preference for galactose over glucose. A key function of this enzyme is likely to be the maintenance of the equilibrium between the α- and β-anomers of galactose, thus ensuring that galactokinase is kept supplied with sufficient quantities of the α-anomer. In light of this we propose that the gene should be renamed *GALM* (galactose mutarotase) in order to be consistent with the other genes encoding enzymes of the Leloir pathway (*GALK1*, *GALT* and *GALE*).

The crystal structure of *L. lactis* galactose mutarotase [15,16] and enzymological studies of the *E. coli* enzyme [17] suggest that the reaction proceeds via an acid–base mechanism. The crystal structure implicates two histidine residues and one glutamate. The proposed mechanism involves the simultaneous protonation of the ring oxygen of the sugar by one of the histidines and the abstraction of a proton from the hydroxyl attached to carbon-1 leading to ring opening. Reversal of these protonation events results in ring closure and reversal of the configuration about carbon-1. Mutation of the equivalent residues in human aldose 1-epimerase severely reduced the activity of the enzyme. Furthermore, the yields of all three mutants (especially H176A) from over-producing bacteria were reduced suggesting that they are structurally less stable than the wild-type. The E307A mutant has virtually no activity, consistent with the role of this residue as a catalytic base. The results from the histidine mutants are more ambiguous. Our results are similar to those obtained with the *E. coli* enzyme. In this system mutation of the equivalent residue to H107 resulted in reduced, but not zero, activity whereas mutation of the equivalent residue to H176 resulted in no measurable activity [17]. Based on this, it has been postulated that this second histidine residue is the main proton donor [15]. Our results suggest that the mechanism of human aldose 1-epimerase is similar to that of bacterial mutarotases.

The other enzymes of the human Leloir pathway – galactokinase (GALK1), galactose-1-phosphate uridyl transferase (GALT) and UDP-galactose 4-epimerase (GALE) – are all known [28–30]. Furthermore, mutations in all these genes can give rise to the hereditary disease galactosemia [31,32]. The main symptom of this disease is early onset cataracts although mental retardation, kidney and liver damage are also known. Now that the gene for the fourth enzyme of the pathway, GALM, has been identified, it will be interesting to see if any disease-causing mutations map to this locus.

Identification of the gene encoding human aldose 1-epimerase will permit other studies on the structure and function of this enzyme. Although the maintenance of the anomeric equilibrium of galactose is likely to be a major function, the enzyme may also play a role in the metabolism of glucose and other sugars. Early work on this enzyme hypothesised that it may play a role in the transport of glucose into cells [13]. Knowledge of the gene and protein sequence will permit the tissue expression pattern and sub-cellular location of the enzyme to be determined. Such information will help us start to elucidate the range of metabolic functions performed by human aldose 1-epimerase.

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