the FEBS Journal

Functional analysis of disease-causing mutations in human UDP-galactose 4-epimerase

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Keywords
galactosemia; GALE; Leloir pathway; SDR family enzyme; UDP-glucose

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(Received 14 July 2005, revised 2 September 2005, accepted 17 October 2005)

doi:10.1111/j.1742-4658.2005.05017.x

UDP-galactose 4-epimerase (GALE, EC 5.1.3.2) catalyses the interconversion of UDP-glucose and UDP-galactose. Point mutations in this enzyme are associated with the genetic disease, type III galactosemia, which exists in two forms – a milder, or peripheral, form and a more severe, or generalized, form. Recombinant wild-type GALE, and nine disease-causing mutations, have all been expressed in, and purified from, *Escherichia coli* in soluble, active forms. Two of the mutations (N34S and G319E) display essentially wild-type kinetics. The remainder (G90E, V94M, D103G, L183P, K257R, L313M and R335H) are all impaired in turnover number (k_{cat}) and specificity constant (k_{cat}/K_m), with G90E and V94M (which is associated with the generalized form of galactosemia) being the most affected. None of the mutations results in a greater than threefold change in the Michaelis constant (K_m). Protein–protein crosslinking suggests that none of the mutants are impaired in homodimer formation. The L183P mutation suffers from severe proteolytic degradation during expression and purification. N34S, G90E and D103G all show increased susceptibility to digestion in limited proteolysis experiments. Therefore, it is suggested that reduced catalytic efficiency and increased proteolytic susceptibility of GALE are causative factors in type III galactosemia. Furthermore, there is an approximate correlation between the severity of these defects in the protein structure and function, and the symptoms observed in patients.

UDP-galactose 4-epimerase (GALE; EC 5.1.3.2) catalyses the interconversion of UDP-galactose and UDP-glucose as part of the Leloir pathway of galactose catabolism [1]. Mutations in the gene encoding the human enzyme can lead to a disease known as type III galactosemia (OMIM 230350), the symptoms of which can include early-onset cataracts, liver damage, deafness and mental retardation. Galactosemia can also be caused by defects in two other Leloir pathway enzymes, galactose-1-phosphate uridyltransferase (type I; OMIM 230400) and galactokinase (type II; OMIM 230200) [2–4]. Two forms of epimerase-deficiency galactosemia are recognized – the more severe, or generalized, form and the much milder, peripheral form. In the generalized form little, or no, epimerase activity can be detected in any tissues, and patients suffer from restricted growth and mental development, even when placed on lactose-free diets [5]. As dietary galactose is the patient's only source of this sugar for glycoconjugate biosynthesis, it cannot be withdrawn completely. In the peripheral form, epimerase activity is reduced in blood cells, but appears normal in other tissues. It is not clear why this is so. The symptoms are milder; indeed some patients may suffer no symptoms beyond raised levels of galactose-1-phosphate in the blood, and no therapy is required [6].

Abbreviations
BS^3, suberic acid *bis*(3-sulpho-*N*-hydroxysuccinimide ester); EDC, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide; GALE, UDP-galactose 4-epimerase.

GALE is a member of the short-chain dehydrogenases/reductases family [7,8], and crystal structures from bacteria, yeast, trypanosomes and humans have been solved [9–17]. These structures reveal a homodimeric enzyme, with each subunit containing one tightly bound NAD^+ molecule. Structural and kinetic studies suggest that this cofactor plays a key part in the catalytic mechanism. It is proposed that it transiently oxidizes the sugar moiety at carbon-4 and then re-reduces it in a nonstereospecific manner, permitting inversion of configuration [18–20]. This reaction is facilitated by a tyrosine residue (Tyr157 in the human enzyme) acting as an active-site base [16,21].

Nine disease-causing mutations have been identified so far in human GALE [5,22–26]. Of these, V94M, which is associated with the generalized form of the disease, has been studied in greatest detail [27,28]. The other mutations (N34S, G90E, D103G, L183P, K257R, L313M, G319E and R335H) are associated with peripheral forms of the disease. In this study, all nine disease-causing mutations have been expressed in, and purified from, *Escherichia coli*, and their steady-state kinetic parameters, ability to dimerize and susceptibility to proteolytic digestion have been compared.

Results

Expression and purification of human GALE

Human GALE was expressed as an N-terminal hexahistidine fusion protein in *E. coli* and purified on nickel–agarose resin (Fig. 1). Typical yields were $\approx$10 mg per litre of bacterial culture. The protein is active and shows saturation kinetics when increasing amounts of substrate are added (Fig. 2). The kinetic parameters determined from these data (Table 1) are similar to those published for human GALE and for GALEs from other species [21,27,29,30]. There is no evidence that human GALE is glycosylated *in vivo*, and there is no anomalous migration of recombinant human GALE produced in yeast [27], and thus the observed activity probably reflects that of the native enzyme.

With the exception of L183P, all the mutant proteins could be expressed and purified using similar conditions and procedures. Yields and purity were similar to those achieved with the wild-type protein. In contrast, L183P was expressed at much lower levels, and repeated attempts to purify the protein resulted in material that contained many contaminants of lower molecular

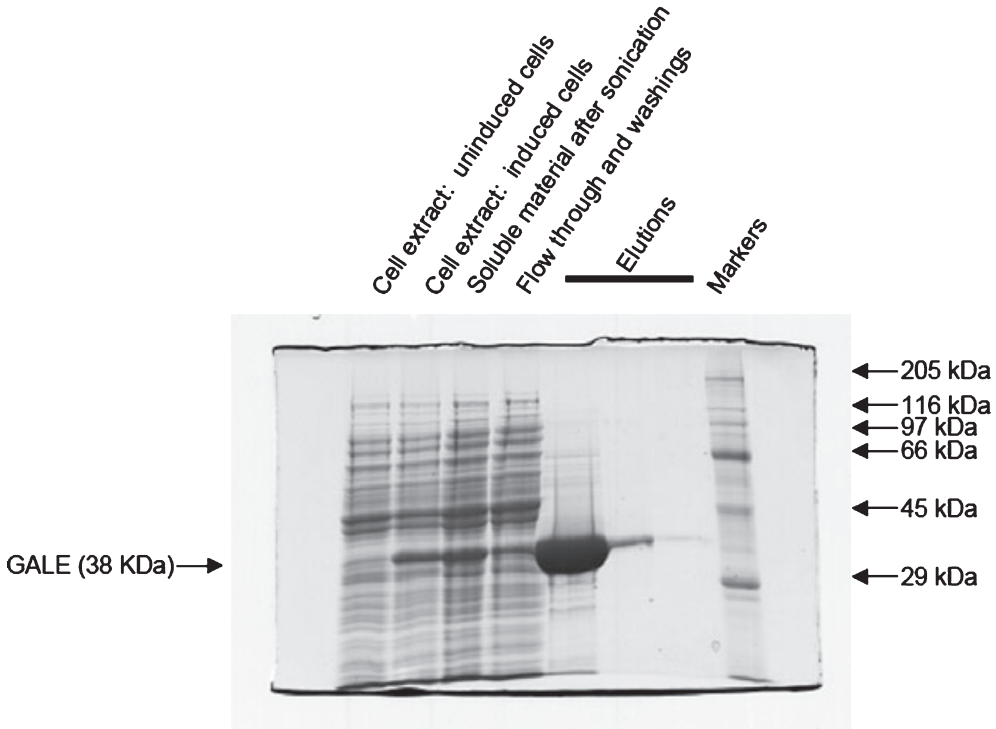

Fig. 1. Expression and purification of human UDP-galactose 4-epimerase (GALE). The hexahistidine-tagged protein was expressed in *Escherichia coli* HMS174(DE3) cells and purified on nickel agarose. Samples at various stages of the process were analysed by 10% SDS/PAGE and stained with Coomassie blue.

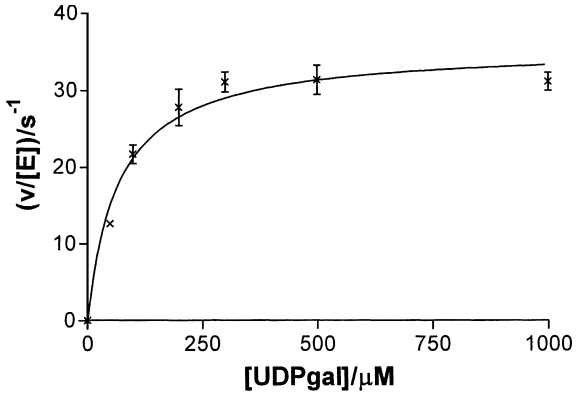

Fig. 2. Steady-state kinetics of recombinant human UDP-galactose 4-epimerase (GALE). Rates (v) were measured in the forward direction (i.e. the conversion of UDP-galactose to UDP-glucose). Each point represents the mean of three separate determinations. Error bars represent the standard deviation of these mean values.

Table 1. Kinetic constants of human UDP-galactose 4-epimerase (GALE) and the consequences of disease-causing mutations. Values were determined by nonlinear curve fitting, as described in the Experimental procedures, and are quoted plus/minus the standard error.

Protein	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($L \cdot mol^{-1} \cdot s^{-1}$)
Wild type	69 ± 12	36 ± 1.4	$5.2 \pm 0.72 \times 10^5$
N34S	82 ± 15	32 ± 1.3	$3.9 \pm 0.59 \times 10^5$
G90E	93 ± 24	0.046 ± 0.0028	$5.0 \pm 0.11 \times 10^2$
V94M	160 ± 38	1.1 ± 0.088	$6.9 \pm 1.2 \times 10^3$
D103G	140 ± 21	5.0 ± 0.23	$3.6 \pm 0.40 \times 10^4$
L183P	97 ± 40	11 ± 1.2	$1.1 \pm 0.35 \times 10^5$
K257R	66 ± 15	5.1 ± 0.29	$7.8 \pm 1.5 \times 10^4$
L313M	35 ± 11	5.8 ± 0.36	$1.7 \pm 0.46 \times 10^5$
G319E	78 ± 13	30 ± 1.3	$3.9 \pm 0.53 \times 10^5$
R335H	99 ± 12	15 ± 0.48	$1.5 \pm 0.14 \times 10^5$

mass. These problems could not be overcome by expressing the protein at lower temperatures (30 °C or 22 °C), by using an alternative expression host {BL21(DE3)[pLysS]} or by including protease inhibitors in the solutions used during purification.

Kinetic analysis of disease-causing mutations

The steady-state kinetic parameters of each of the nine disease-causing mutant proteins were determined (Table 1). In general, little change was seen in K_m (no change greater than threefold), whereas some mutants (especially G90E and V94M) showed large changes in k_{cat} and in k_{cat}/K_m. In contrast, two mutants (N34S and G319E) showed very little change in these parameters compared with the wild-type protein.

Dimerization of GALE

Human GALE is known to exist in solution as a homodimer [1]. One possible explanation for the *in vivo* effects of the disease-causing mutations is a failure to form dimers. However, cross-linking using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) showed that the wild-type protein and all the mutants were able to form dimers (Fig. 3). In all cases, but especially with the mutant proteins, some higher molecular mass species were also observed. Similar results were seen using suberic acid *bis*(3-sulpho-*N*-hydroxysuccinimide ester) (BS^3) (data not shown).

Limited proteolysis of GALE

As L183P appears to be highly susceptible to proteolysis during expression and purification, it is possible that the other disease-causing mutations may also have increased proteolytic sensitivity compared with the wild-type protein. Limited proteolysis with thermolysin (EC 3.4.24.27) showed that while the pattern of fragments produced is not changed by the disease-causing mutations, the sensitivity to proteolysis is altered, in some cases (Fig. 4). In particular, N34S, G90E and D103G are clearly more susceptible to proteolysis than the wild-type protein. In all cases, the presence of substrate at saturating levels (1 mM) partially protects the enzyme from proteolysis.

Discussion

Active recombinant human GALE can be produced in good yields in *E. coli*. This enabled the creation and analysis of all nine currently known disease-causing mutations in this protein. All but one of these mutant proteins (L183P) could also be produced in an active and soluble form. This contrasts with human galactokinase where approximately half the disease-causing mutations were insoluble on expression in *E. coli*, suggesting that a failure to fold and/or aggregation of the protein product could be a major factor in disease causation [31,32]. This is unlikely to be the case with most of the mutations in GALE.

Although all the mutant proteins were active, some of their kinetic properties differed from that of the wild-type protein and from each other. The main effect seen was in the turnover, k_{cat}, and in the specificity constant, k_{cat}/K_m. Again, this contrasts with disease-causing mutations in galactokinase where effects on all the kinetic parameters were observed [31]. The most severely affected protein was G90E, which showed an ≈800-fold decrease in k_{cat}. This mutation was

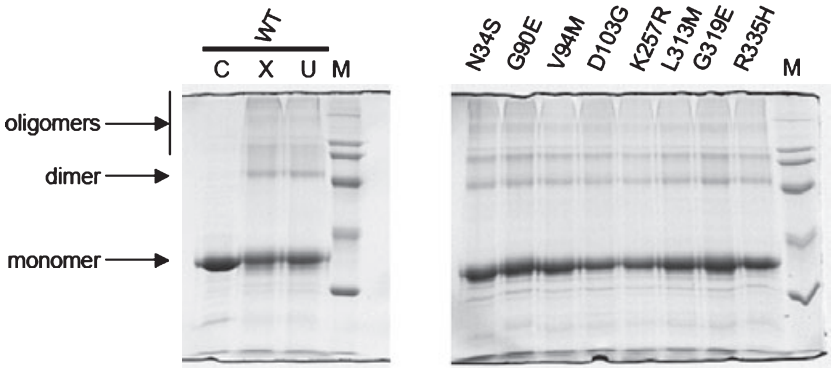

Fig. 3. Crosslinking of wild-type and disease-causing mutants of UDP-galactose 4-epimerase (GALE). Crosslinking was carried out with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), as described in the Experimental procedures. C, control lane containing 25 μM GALE not exposed to crosslinker; X, 25 μM GALE exposed to crosslinker; U, 25 μM GALE exposed to crosslinker in the presence of a saturating amount (1 mM) of UDP-galactose; M, molecular mass markers (29, 45, 66, 97, 116 and 205 kDa). All the mutant proteins were exposed to crosslinker. Bands were identified by reference to the uncrosslinked wild-type protein and by estimation of the molecular mass (QUANTITY ONE software; Bio-Rad, Hercules, CA, USA).

originally discovered in a patient who was compound heterozygous for this and another, uncharacterized, allele [24]. Previous *in vivo* studies on this mutant expressed in yeast showed that there was essentially no GALE activity present in extracts from cells carrying only this allele [25]. G90 lies close to the bound NAD^+ molecule (Fig. 5) and the increase in size of the side-chain resulting from mutation to glutamate may well disrupt the binding or conformation of the cofactor. The V94M mutation, which is associated with the more severe generalized form of the disease [5,25], also had an impaired k_{cat} value (≈ 30-fold reduction compared with the wild-type protein). This mutation also results in the biggest change in K_m (≈ 2.3-fold). These changes are consistent with those previously observed with proteins expressed in yeast [27]. The structure of this mutant has been solved, showing that a valine to methionine substitution at this position disrupts the packing close to the sugar-binding site, making it easier for the sugars to bind nonproductively [28]. This explanation would be entirely consistent with a reduction in k_{cat}.

Three mutations – D103G, K257R and L313M – are mildly impaired in k_{cat} (approx. six- to sevenfold decreased compared with the wild-type protein). All of these mutations are associated with the less severe, peripheral form of the disease. D103G was first observed in a patient homozygous for this mutation [24]. Cell extracts from yeast expressing only this form of GALE had ≈ 50% epimerase activity compared with wild-type protein, and growth on galactose was unaffected [25]. Similar results were found with L313M [25], which was first isolated from a heterozygous patient [24]. Two mutations, L183P and R335H, result in small changes in k_{cat} (approx. two- to threefold changes) and two, N34S and G319E, are essentially unchanged compared with the wild-type protein (i.e. no change greater than twofold in any kinetic parameter). Again, these mutations are associated with the peripheral form of the disease [22,24,26]. K257, L313 and G319 lie within 0.13 nm of each other on one face of the protein. G319 is on the surface and therefore the protein may be able to tolerate a larger side-chain at this position (in contrast to G90). R335 lies far from the other mutations and from the ligand-binding sites (Fig. 5).

The ability to dimerize does not appear to be impaired in the mutants. Crosslinking analyses showed that they are all capable of forming dimers. In all cases, some higher-order crosslinked products are seen. This seems to be slightly more pronounced in the case of the mutants, suggesting that there might be enhanced aggregation. However, this is not sufficient to affect the solubility of the proteins.

Decreased protein stability can be a factor in disease-causation [33]. To test for this, the proteins were exposed to low concentrations of the protease thermolysin, and the digestion products were observed. The presence of the mutations did not alter the pattern of digestion, but did affect the amount of digestion. These effects were reproducible. The most severe case was that of L183, which was difficult to obtain in a purified form. It appeared that this mutation causes the protein to be degraded in the bacteria following expression and also during the purification procedure. This suggests that reduced stability may be an important factor in disease causation in the case of N34S, G90E, D103G and, particularly, L183P. This is most likely to manifest itself in increased susceptibility to intracellular proteases and thus a decreased concentration of active

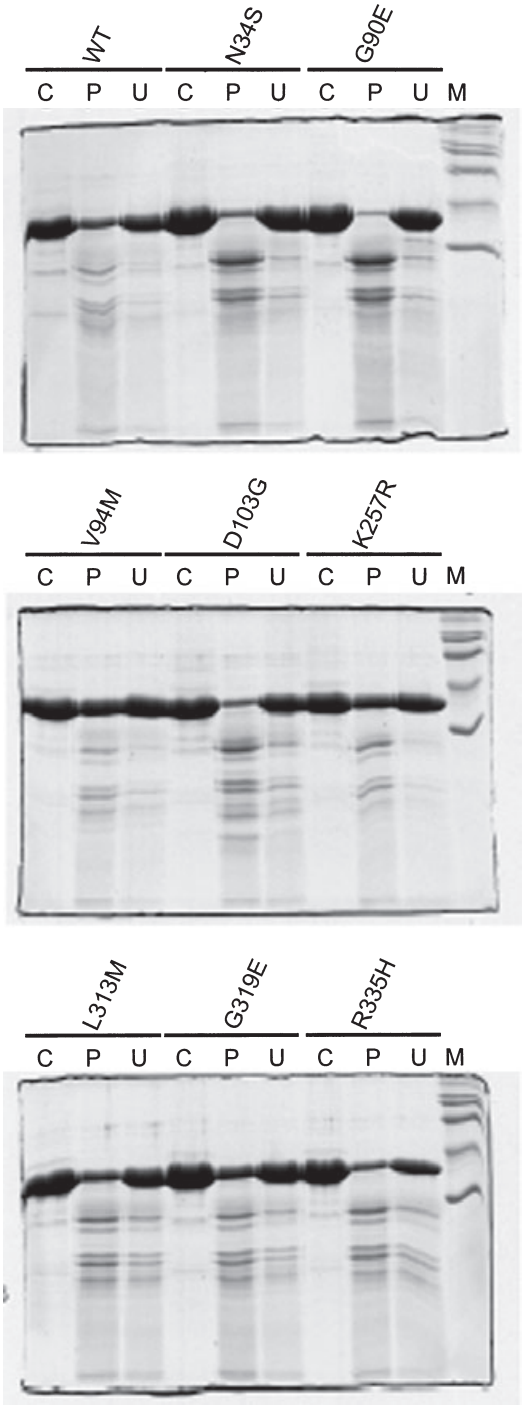

Fig. 4. Limited proteolysis of wild-type and disease-causing mutants of UDP-galactose 4-epimerase (GALE). Proteolysis was carried out as described in the Experimental procedures, and then the products were separated by 15% SDS/PAGE and stained with Coomassie blue. C, control lanes containing 50 μM GALE; P, GALE digested with 90 nM thermolysin for 30 min at 37 °C; U, GALE digested with 90 nM thermolysin for 30 min at 37 °C in the presence of 1 mM UDP-galactose; M, molecular mass markers (29, 45, 66, 97, 116 and 205 kDa).

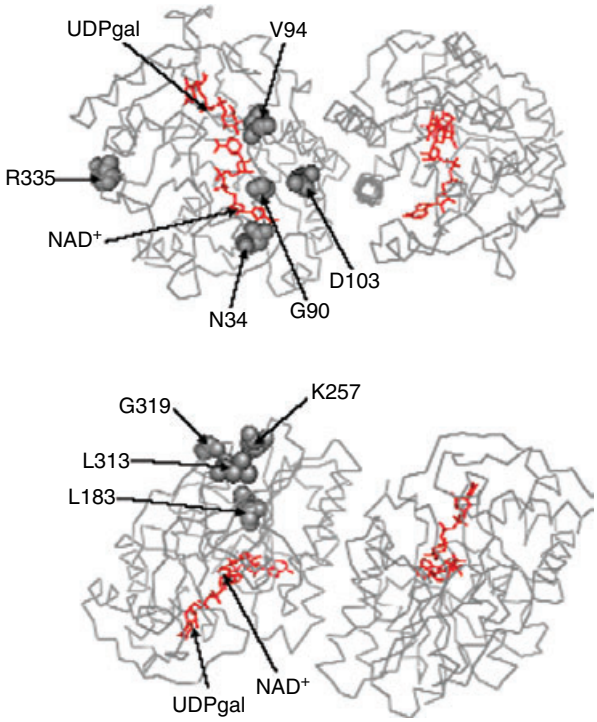

Fig. 5. Structure of human UDP-galactose 4-epimerase (GALE) showing the positions of the residues altered in the disease-causing mutations. The image was created from PDB entry 1EK6 [16] using the program PyMol (DeLano Scientific LLC, San Carlos, CA, USA; http://www.pymol.org). The lower image is rotated approximately 90° on a horizontal axis compared with the upper image. For clarity the residues are highlighted on only one of the two molecules in the homodimer.

enzyme present in the cell. Indeed, when L183P was expressed in yeast, its activity was reduced to 4% of that observed for the wild-type protein and its abundance to 6% [22]. Residue 183 forms part of a β-sheet in the interior of the protein (Fig. 5), and mutation from leucine to proline is highly likely to disrupt this secondary structure, potentially leading to wider changes in the overall fold of the enzyme, resulting in decreased stability and enhanced susceptibility to proteolysis. Enhanced protease sensitivity and reduced thermal stability has previously been observed for G90E and D103G [25]. In both cases, the mutation results in substantial changes to the volume of the side-chain that are likely to disrupt the local fold. These two residues, together with N34, are located within 0.15 nm of each other and all are at, or close to, the surface of the enzyme. Therefore, it is not surprising that alterations to these residues affect the sensitivity to proteolysis.

In conclusion, there is an approximate correlation between the severity of symptoms observed in patients

with the level of biochemical impairment of the purified protein. The mutation, V94M, associated with the generalized form of the disease, has a much reduced value for k_{cat}. A more complete genotype–phenotype correlation is difficult because of the relatively small number of patients afflicted with type III galactosemia. For example, there have been no reports of patients homozygous for G90E (which has an even lower k_{cat} than V94M) or for L183P (which is highly susceptible to proteolysis). It is reasonable to predict that if such patients were found, their symptoms would be closer to the generalized form than the peripheral form of disease. Some of the mutants have only small changes in the kinetic parameters. In the case of N34S and D103G, disease-causation may be explained by a combination of kinetic impairment and increased sensitivity to proteolysis. In other cases, additional factors may be important. For example, in the case of K257R and G319E, it has been suggested that these mutations are tightly genetically linked to other changes that give rise to the symptoms (perhaps by affecting the level of expression of GALE) [34]. That there are two clusters of disease-causing mutations (N34S/G90E/D103G and K257R/L313M/G319E) suggests that these two parts of the protein are sensitive to changes and that further mutations may be found in these regions in the future.

Experimental procedures

Expression and purification of human GALE

Human GALE was expressed in and purified from *E. coli*, essentially as described previously for galactose mutarotase and galactokinase [31,35]. The coding sequence was amplified by PCR using an IMAGE clone (ID number 3459004) as a template [36]. The primers were designed in order to add restriction sites (*Nco*I at the 5′ end and *Eco*RI at the 3′ end) and to encode a hexa-histidine tag at the N terminus of the expressed protein. PCR products were cut with these enzymes and inserted into the corresponding sites in the expression vector pET-21d (Merck, Nottingham, UK). The entire GALE coding sequence was verified by DNA sequencing.

The recombinant plasmid was transformed into *E. coli* HMS174(DE3) (Merck) for expression. An overnight culture (volume 5 mL) was diluted into 1 L of Luria–Bertani (Miller) media supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin. This 1 L culture was incubated, with shaking at 37 °C, for 3–4 h before induction with isopropyl thio-β-D-galactoside (final concentration, 2 mM). The culture was grown for a further 2 h and then harvested by centrifugation (4200 ***g*** for 10 min). Cells were resuspended in ≈20 mL of 50 mM Hepes-OH, pH 7.5, containing 150 mM NaCl and 10% (v/v) glycerol, and stored at −80 °C.

Cells were disrupted by sonication on ice (three times: 30 s at 100 W, with 30 s intervals between for cooling) and cell debris was removed by centrifugation (27 000 ***g*** for 20 min at 4 °C). The supernatant was applied to a 1 mL nickel–agarose (His-select; Sigma, Poole, UK) column and allowed to pass through by gravity flow. The column was washed with 20 mL of cold buffer A [50 mM Hepes-OH, pH 7.5, containing 500 mM NaCl and 10% (v/v) glycerol] and the protein was eluted in three, 2 mL washes of cold buffer B (buffer A supplemented with 250 mM imidazole). Fractions containing GALE, as judged by SDS/PAGE, were dialysed overnight at 4 °C against 50 mM Hepes-OH, pH 8.0, containing 150 mM NaCl, 1 mM dithiothreitol and 10% (v/v) glycerol. Protein concentrations were estimated by the method of Bradford [37] using BSA as a standard. The protein was stored frozen in small aliquots at −80 °C.

Generation of point mutations

Point mutations in the GALE sequence were generated using the QuikChange method [38]. DNA sequencing was used to verify each mutation and to ensure that no other changes had been introduced into the coding sequence. All mutant proteins were expressed and purified using the same techniques as for the wild-type protein.

GALE assay

GALE activity was assayed according to the method of Ng *et al.* [39], as modified by Wohlers & Fridovich-Keil [27]. The conversion of UDP-galactose to UDP-glucose was coupled to the oxidation of UDP-glucose using the enzyme UDP-glucose dehydrogenase (Sigma; EC 1.1.1.22). This results in the reduction of two molecules of NAD^+ per molecule of UDP-glucose, which can be followed spectrophotometrically at 340 nm. All assays were carried out at 37 °C in a volume of 1 mL, and each contained 50 mM Tris/HCl, pH 8.8, 4 mM NAD^+ and a variable amount of UDP-galactose (Merck). Reaction mixes were preincubated at 37 °C for at least 5 min. Then, 0.02 units of UDP-glucose dehydrogenase (manufacturer's unit definition) were added and the absorbance at 340 nm was monitored for 2–3 min. This is necessary as commercial UDP-galactose contains a small amount of UDP-glucose [29]. Reactions were initiated by the addition of GALE (to a final concentration of 2.6 nM for the wild-type protein and ranging from 2 nM to 330 nM for the mutants). The absorbance at 340 nm was measured for a further 7–8 min. Rates (v) were calculated by fitting the linear part of the A_{340} vs. time plot to a straight line and corrected by subtraction of the rate in the absence of GALE. The kinetic constants K_m and k_{cat} were derived by nonlinear curve fitting [40] to the Michaelis–Menten equation:

$$v/[\text{GALE}] = \{k_{cat} \cdot [\text{UDP-galactose}]/(K_m + [\text{UDP-galactose}])\},$$

using the program GRAPHPAD PRISM 3.0 (GraphPad Software, San Diego CA, USA). Specificity constants (k_{cat}/K_m) were determined directly by fitting to a modified form of the Michaelis–Menten equation:

$$v/[\text{GALE}] = \{k_{cat}/K_m \cdot [\text{UDP-galactose}]/(1 + [\text{UDP-galactose}]/K_m)\}.$$

All points were weighted equally and values are quoted plus/minus the standard error determined by the program.

Crosslinking of GALE

Wild-type or mutant GALE (25 μM) was incubated for ≈5 min at 37 °C. Cross-linker – either EDC, to a final concentration of 70 mM, or BS^3, to a final concentration of 100 μM – was added and the reaction was allowed to proceed for 30 min. Reactions were stopped by the addition of an equal volume of SDS/PAGE loading buffer [125 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 1% (w/v) dithiothreitol, 0.002% (w/v) bromophenol blue] and heating at 95 °C for 5 min. Products were analysed by 10% SDS/PAGE.

Limited proteolysis of GALE

Wild-type or mutant GALE (50 μM) was incubated for ≈5 min at 37 °C. Thermolysin (Sigma) was added to a final concentration of 90 nM and digestion allowed to proceed for 30 min. Reactions were stopped by the addition of an equal volume of SDS/PAGE loading buffer and heating at 95 °C for 5 min. Products were analysed by 15% SDS/PAGE.

Acknowledgements

The original GALE-expressing clone was constructed while I was working in the laboratory of Richard J. Reece (University of Manchester, UK) to whom I am grateful for continuing support and advice. This work was funded by The Royal Society (London, UK).

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