Alteration of substrate specificity by a naturally-occurring aldolase B mutation (Ala³³⁷ → Val) in fructose intolerance

Peter RELLOS^{*1}, Manir ALI^{*1,2}, Michel VIDAILHET[†], Jurgen SYGUSCH[‡] and Timothy M. COX^{*3}

^{*}Department of Medicine, University of Cambridge, Level 5, Addenbrooke's Hospital, Cambridge CB2 2QQ, U.K., [†]Centre Hospitalier Régional et Universitaire de Nancy, Vandoeuvre, France, and [‡]Université de Montréal, Faculté de médecine, Département de biochimie, C.P. 6128 Station Centre Ville, Montréal, Québec, Canada.

A molecular analysis of human aldolase B genes in two newborn infants and a 4-year-old child with hereditary fructose intolerance, the offspring of a consanguineous union, has identified the novel mutation Ala³³⁷ → Val in homozygous form. This mutation was also detected independently in two other affected individuals who were compound heterozygotes for the prevalent aldolase B allele, Ala¹⁴⁹ → Pro, indicating that the mutation causes aldolase B deficiency. To test for the effect of the mutation, catalytically active wild-type human aldolase B and the Val³³⁷ variant enzyme were expressed in *Escherichia coli*. The specific activities of the wild-type recombinant enzyme were 4.8 units/mg and 4.5 units/mg towards fructose 1,6-bisphosphate (FBP) and fructose 1-phosphate (F-1-P) as substrates with Michaelis constants of 4 μ M and 2.4 mM respectively. The specific activities of purified tetrameric Val³³⁷ aldolase B, which affects an invariant residue in the C-terminal region, were 4.2 units/mg and 2.6 units/mg towards FBP and F-1-P as substrates respectively; the corresponding Michaelis constants were 22 μ M and 24 mM. The FBP-to-F-1-P substrate activity ratios were 0.98 and 1.63 for

wild-type and Val³³⁷ variant enzymes respectively. The Val³³⁷ mutant aldolase had an increased susceptibility to proteolytic cleavage in *E. coli* and rapidly lost activity on storage. Comparative CD determinations showed that the Val³³⁷ protein had a distinct thermal denaturation profile with markedly decreased enthalpy, indicating that the mutant protein is partly unfolded. The undegraded mutant had preferentially decreased affinity and activity towards its specific F-1-P substrate and maintained appreciable activity towards FBP. In contrast, fluorescence studies of the mutant showed an increased binding affinity for products of the aldolase reaction, indicating a role for the C-terminus in mediating product release. These findings in a rare but widespread naturally occurring mutant implicate the C-terminus in the activity of human aldolase B towards its specific substrates and demonstrate its role in maintaining the overall stability of the enzyme tetramer.

Key words: catalytic defect, enzyme mutant, human gene.

INTRODUCTION

Inherited deficiency of aldolase B (fructose-1,6-bisphosphate aldolase, EC 4.1.2.13) in the liver, kidney and intestine is associated with the human metabolic disorder hereditary fructose intolerance (HFI) [1]. After consuming fructose and its congeners in the diet, affected individuals suffer abdominal symptoms associated with hypoglycaemia and metabolic acidosis [2]. Newborn infants, who might be given the noxious sugars indiscriminately, are most vulnerable to their effects. As a result, potentially fatal hepatic and renal injury can occur [3,4]. Provided that dietary exclusion of fructose and related sugars is instituted promptly, the severe manifestations of the disorder can be prevented and the infant can survive to healthy adulthood [5]. Metabolic studies of patients with HFI show that infusions of D-fructose rapidly inhibit hepatic glycogenolysis and gluconeogenesis, causing hypoglycaemia and lactic acidosis [1]. These disturbances are attributed to the accumulation of undegraded fructose 1-phosphate (F-1-P) in the affected tissues, which inhibits fructose-1,6-bisphosphate aldolase activity in the gluconeogenic pathway: in a milieu that is depleted of free inorganic phosphate, glycogen phosphorylase is also inhibited [1,6].

Three genetically distinct isoenzymes of fructaldolase (aldolases A, B and C) are found in vertebrates [7]. The enzymes show differential activities towards the two substrates F-1-P and

fructose 1,6-bisphosphate (FBP). Aldolase B is expressed selectively in tissues that metabolize exogenous fructose; unlike the ubiquitous glycolytic A isoform, which is principally active towards FBP, it catalyses the breakdown of both substrates about equally. Approx. 98 % of the aldolase protein in normal human liver is aldolase B; aldolase A accounts for the remainder. Determinations of fructaldolase activities in liver from patients with fructose intolerance show that FBP aldolase activity is usually decreased to 16–25 %, whereas the activity towards F-1-P is 0–10 % of control values [1,8].

Several mutations in the human aldolase B gene have been identified in HFI [6,9,10]. Of these, the most predominant, Ala¹⁴⁹ → Pro, Ala¹⁷⁴ → Asp and Asn³³⁴ → Lys, have been shown to represent 65 %, 14 % and 6 % of European HFI alleles [11] and 32 %, 11 % and 2 % of North American alleles respectively [12]. Although it has been suggested that partial activity of mutant aldolase B might prevent the development of hypoglycaemia on fasting [13], we have shown that null alleles of aldolase B are associated with HFI that is compatible with normal adult life, even in homozygotes, when strict dietary treatment is instituted [14,15].

We report here a novel mis-sense mutation in the aldolase B molecule that has been identified in patients with HFI and that as a recombinant protein expressed in *Escherichia coli* is uniquely informative about aldolase function and disease pathogenesis.

Abbreviations used: F-1-P, fructose 1-phosphate; FBP, fructose 1,6-bisphosphate; HFI, hereditary fructose intolerance; IPTG, isopropyl β -D-thiogalactoside.

¹ These authors contributed equally to this study.

² Present address: Molecular Medicine Unit, Level 6 Clinical Sciences Building, St James's University Hospital, Leeds LS9 7TF, U.K.

³ To whom correspondence should be addressed (e-mail jbg20@medschl.cam.ac.uk).

The presence of a conservative substitution in the C-terminal α -helical region of the enzyme selectively inactivates its intrinsic catalytic activity towards the specific F-1-P rather than the generic FBP substrate.

MATERIALS AND METHODS

Subjects

The proposita was the firstborn infant of maternal first cousins originating from Terçan, a province in eastern Turkey. The infant was given undiluted fruit juice at 6 weeks of age and was admitted to hospital because of vomiting and liver enlargement. Blood examination revealed an elevated glutamic-pyruvic transaminase activity (1680 i.u./l; normal activity less than 40 i.u./l) and hypoglycaemia (blood glucose 1.8 mM, normal activity more than 3.5 mM). No deficiency of blood coagulation factors was identified and neither glucose nor amino acids were detected on urine analysis. An exclusion diet lacking galactose and fructose was instituted with accompanying clinical improvement (serum glutamic-pyruvic transaminase, 16 i.u./l). Intravenous infusion of fructose (0.5 g/kg bodyweight) under controlled conditions induced hypoglycaemia, hypophosphataemia and hyperuricaemia, indicating fructose intolerance. A liver biopsy specimen was obtained for enzymic analysis: F-1-P aldolase activity was 0.08 μ mol/min per g of tissue (normal 4–12); FBP aldolase activity was 1.96 μ mol/min per g (normal 4–12); the activity ratio of FBP to F-1-P was 24.5 (normal ratio less than 2.0). When last reviewed at age 6 years, while taking the fructose exclusion diet supplemented with ascorbic acid and folic acid, the child was in good health with normal clinical and laboratory tests of liver function. Both parents remain healthy and have had two further children (see the Results section), both of whom are identically affected by fructose intolerance as demonstrated by restriction endonuclease digestion of amplified exons 9 of aldolase B genes with *MscI*. These analyses were conducted with genomic DNA extracted from umbilical cord blood samples taken immediately after birth. All the offspring are receiving a strict diet free of fructose, sorbitol and sucrose.

Materials

DNA-modifying enzymes, which include restriction endonucleases, T4 DNA ligase and the Klenow fragment of DNA polymerase 1, were purchased from Boehringer Mannheim or New England Biolabs. *Taq* polymerase was obtained from Promega. *Pfu* DNA polymerase was obtained from Stratagene. Oligonucleotides were obtained from either Pharmacia or Genosys corporations. Gelase was purchased from Epicentre Technologies (Cambridge, U.K.). The reagents used for radio-nucleotide-based DNA sequencing were from USB Corporation. Reagents for dye terminator sequencing were from Amersham. Adenosine 5'-[α - 32 S]thiotriphosphate was purchased from DuPont-NEN. The *E. coli* strains CJ236 and TG1 as well as the bacteriophages M13mp19 were from Promega. The *E. coli* strain BL21(DE3) was purchased from Stratagene. The pET-21(+) expression vector was purchased from NBL Gene Sciences. The protease inhibitors PMSF, leupeptin and pepstatin A were obtained from Boehringer Mannheim. The aldolase substrates F-1-P and FBP were purchased from Boehringer Mannheim and Sigma. Ampicillin and isopropyl β -D-thiogalactoside (IPTG) as well as other chemicals and reagents were of the highest grade available from Sigma Chemical Company. Cibacron Blue thiazine affinity chromatographic gel (Blue Sepharose affinity dye CL-6B), DEAE-Sepharose, desalting Sephadex PD-10, Mono-S

and Superose 12 columns and resins were obtained from FP Pharmacia-LKB (Uppsala, Sweden).

Molecular analysis of the human aldolase B gene

Genomic DNA, prepared from EDTA-anti-coagulated peripheral blood [16] was used in PCR to amplify the eight exons encoding the human aldolase B polypeptide. The amplified fragments also included the splice-site junctions [9]. The amplified DNA sequences were purified from agarose gels by using Gelase before direct sequencing by means of the chain-termination procedure with Sequenase [17]. To confirm the presence of the Val³³⁷ mutation identified in the amplified genomic products, freshly amplified exon 9 fragments were precipitated with ethanol and digested with the restriction endonuclease *MscI* in accordance with the manufacturer's instructions.

Construction of expression plasmid and oligonucleotide-directed mutagenesis

The truncated human aldolase B cDNA clone pHL413 [18], which lacks the first 35 amino acid residues of the polypeptide, was excised as a partial *PstI* fragment and subcloned into the *PstI* site of pEMBL 18 to give pHAB413. At the same time, amplified exon 2 of wild-type human aldolase B [9] was cloned into the *SmaI* site of pEMBL19 to give pEE.PCR200. This plasmid was digested with *NcoI*, which cleaves the ATG translation initiation site of exon 2, and blunt-ended with the Klenow fragment of DNA polymerase; the insert was excised as a 108 bp fragment with the endonuclease *HinfI*. The truncated aldolase B insert of pHAB413 was excised as an *XbaI*-partial *HinfI* fragment and the complete coding sequence was constructed by ligation of this fragment with the *HinfI*-*NcoI* (filled in) exon 2 sequence, into pEMBL19 that had been cleaved with *SmaI* and *XbaI*. The recombinant plasmid containing the desired sequence was termed pHABX1. The expression plasmid p Δ XB, containing the wild-type human aldolase B cDNA, was created by excising the insert with the complete coding sequence of aldolase B from the plasmid pHABX1, as a *HindIII*-partial *NcoI* fragment and subcloned into the vector pKK Δ 4, which had been cut with the same two enzymes. The vector pKK Δ 4 is a deletion derivative of pKK233.2 [19], which had been constructed after a double digestion with *PvuII* and *BamHI*, filling in with the Klenow fragment and self-ligation.

The mutant plasmid pA337V was created by the Kunkel method for mutagenesis [20]. The wild-type aldolase B cDNA was cloned into the phage M13mp19 and grown in the CJ236 *E. coli* strain. A synthetic DNA oligonucleotide SDA337V (5'-GCTAACTGCCAGGTGGCCAAAGG-3'), spanning the region to be mutated, was annealed to the uracil-rich single-stranded template then extended and ligated to form heteroduplex molecules that were then transfected into *E. coli* strain TG1. Plaques that formed were analysed by single-stranded M13 DNA sequencing. A mutagenized M13 clone was subcloned into p Δ XB by using the unique restriction endonuclease sites *ApaI* and *HindIII* to create the new aldolase B plasmid.

Expression of the wild-type and Val³³⁷ variant of aldolase B was increased by subcloning the genes into the T7 promoter expression vector pET-21(+). The wild-type and Val³³⁷ genes were amplified with *Pfu* DNA polymerase with the use of p Δ XB and pA337V as templates and with the primers ER1 (5'-CGGATAACGAATTCATAAGGAGGAAACAG-3') and HP (5'-GGAGCTAAGCTTGC GGGA-3'). The ER1 primer introduces an *EcoRI* site upstream of the gene and also incorporates a modification of the parent flanking ribosome-binding site so that it includes a consensus *E. coli* ribosome-binding site [21].

Inclusion of this primer also introduces an ATG initiation codon nine bases away from the ribosome-binding site and immediately upstream of the first Ala residue that is represented by the first codon of the human aldolase B polypeptide. The HP primer incorporates a unique *Hind*III site downstream of the human aldolase cDNA, thereby removing the poly(A) tail. The amplified products were cloned directionally into the expression vector pET-21(+), generating the plasmids designated pEBR+ and pEBR337V, corresponding to the wild-type and Val³³⁷ variant expression plasmids respectively. The structures of the plasmid inserts were confirmed by digestion with the restriction enzyme *Mse*I and by DNA sequencing. No adventitious mutations were identified.

Bacterial expression of human aldolase B

The plasmid to be expressed was transformed into the protease-deficient *E. coli* strain BL21(DE3). To obtain samples of enzyme exhibiting different physical conformations, recombinant aldolase B was expressed at either 37 or 22 °C. An overnight culture of the recombinant bacterial strain was used to inoculate 500 ml (for growth at 37 °C) or 250 ml (for growth at 22 °C) of ampicillin-supplemented 2YT medium and grown until D_{600} reached 0.6–0.7. The gratuitous inducer IPTG was added to a final concentration of 1 mM, and the culture was incubated for a further 2.5 h when grown at 37 °C or for a further 16 h when grown at 22 °C. Bacteria in the induced culture were harvested by centrifugation and cells were resuspended in 15 ml of ice-cold 50 mM Tris/HCl/2.5 mM EDTA (pH 7.6)/5 mM dithiothreitol/5% (v/v) glycerol/0.2 mM PMSF/20 µg/ml pepstatin A/20 µg/ml leupeptin. The cells were lysed by sonication and cell debris was removed by centrifugation at 30000 g for 45 min at 4 °C.

Purification of aldolase B

Recombinant wild-type and Val³³⁷ human aldolase B in the bacterial extracts were fractionated on DEAE-Sepharose, which retains acidic *E. coli* proteins. The unbound fraction was then adsorbed on Cibacron Blue Sepharose CL-6B affinity matrix. After application to the Cibacron Blue thiazine affinity matrix and washing with 50 mM Tris/HCl/10 mM EDTA (pH 7.6) buffer, the recombinant protein was affinity eluted with a pulse of 2–5 mM FBP in 50 mM Tris/HCl/5 mM EDTA (pH 8.6) [22]. The eluted proteins were concentrated with either an Amicon concentrator fitted with a YM30,000 filter or a Millipore ultrafree filter device (30 kDa cut-off). The concentrated proteins were then fractionated by gel filtration in an FPLC Superose 12 column in 50 mM Tris/HCl/100 mM NaCl. Val³³⁷ protein expressed at 37 °C was then exchanged into 50 mM Mops, pH 7.0, with Sephadex PD-10 columns and bound to a Pharmacia Mono-S FPLC column; protein fractions were eluted with an NaCl gradient. Approx. 50% of the original wild-type and approx. 15% of the Val³³⁷ protein was recovered from cell lysates.

Electrophoresis and immunoblotting

Protein samples were subjected to electrophoresis through denaturing polyacrylamide gels containing SDS by the method of Laemmli [23]. The proteins were identified in the gel by staining with Coomassie Brilliant Blue G250. The differing proportions of intact mutant polypeptides fractionated in Coomassie Blue-stained SDS/polyacrylamide gels were determined by laser densitometry. For immunoblotting, the gel was electroblotted to a PVDF filter for binding to a polyclonal sheep anti-(human

aldolase B) antibody (Merck). Polypeptides reacting specifically with primary antibody were detected by sequential incubation with biotinylated mouse anti-sheep IgG followed by avidin–alkaline phosphatase conjugate (Sigma Immunochemicals). Human aldolase B was finally identified by incubating the washed filter in tetrazolium substrate, which confirmed the exact location of the primary reaction between sheep antibody and the recombinant aldolase B polypeptides.

Assay for aldolase activity

The catalytic activities of aldolase B towards the substrates F-1-P (45 mM) and FBP (1 mM) were determined by measuring NADH oxidation at 340 nm with the method of Nakamura et al. [34]. Enzymic assays were conducted at 22 °C in a 1 ml volume by the addition of aldolase protein containing substrate, 0.15 mM NADH, 50 mM Tris/acetate, 10 mM EDTA, pH 7.6, 100 µg/ml BSA and 10 µl of α -glycerophosphate dehydrogenase/triose phosphate isomerase (2 mg/ml). One unit of activity is equivalent to the cleavage of 1 µmol of hexose substrate/min. The protein concentration of pure aldolase was determined spectroscopically by determining A_{280} [25] and by using the bicinchoninic acid method (Pierce Chemical Co.). Kinetic estimates were determined in duplicate by least-squares analysis of at least three separate enzyme preparations by using the LEONORA program [26].

Fluorescence measurements

Fluorescence measurements were made with a Perkin–Elmer LS 50B spectrofluorimeter. The intrinsic fluorescence of aldolase B was measured in 50 mM Tris/acetate/10 mM EDTA (pH 7.6) at 25 °C with an excitation wavelength of 295 nm and emission wavelengths of 325–365 nm. Triose ligands were added sequentially and the quenching of fluorescence was estimated by the decrease in peak intensity. Optimal emission was controlled by varying the emission slit widths between 5 and 10 nm. A 0.5 ml cuvette with a pathlength of 1 cm along the excitation axis and 0.2 cm along the emission axis was used for the analysis. Binding constants were derived by using the GRAFIT equation-fitting program (Erithacus Software Ltd) and are expressed means $\pm$ S.E.M.

CD studies

CD measurements were made with a JASCO J-720 spectropolarimeter. CD spectra of samples were collected with a 0.05 cm pathlength quartz cuvette at a protein concentration of 0.25 mg/ml in 50 mM phosphate buffer, pH 7.4. Changes in the secondary structure of aldolase with time and temperature were measured by monitoring the CD signal at 222 nm. The effects of thermal unfolding on ellipticity were determined at a rate of 60 °C/h in 0.1 °C increments. The results were used to calculate the inflection point of the transition and the melting temperature, T_m , by using the formulation defined by Lawrence et al. [27] and plotted with GRAFIT (see above).

RESULTS

Identification of the aldolase B mutation

Genomic DNA obtained from leucocytes in peripheral blood drawn from the 4-year-old proposita was analysed for the presence of mutations in the human aldolase B gene. Molecular analysis excluded the presence of the common mis-sense alleles Ala¹⁴⁹ $\rightarrow$ Pro and Ala¹⁷⁴ $\rightarrow$ Asp [10]; however, allele-specific oligonucleotide hybridization for the mutation Asn³³⁴ $\rightarrow$ Lys [28] failed to show selective hybridization with either the wild-type or

Figure 1 Identification of the mutation Val³³⁷ in the human aldolase B gene in a pedigree affected by HFI

(A) Sequencing autoradiogram depicting the sense strands of exons 9 PCR amplified from genomic DNA template. The fragments were sequenced by the Sanger procedure [17] as described in the Materials and methods section with the E9+/403 oligonucleotide primer [10]. The figure shows a sequence obtained from a healthy individual and the probanda with fructose intolerance. (B) Confirmatory genotype analysis for the Val³³⁷ mutation in members of the affected pedigree. The figure shows DNA fragments of amplified exons 9 of the human aldolase B gene that have been digested with the restriction endonuclease *MscI* and separated by electrophoresis in a 2% (w/v) agarose gel. Lane 1, DNA obtained from the father; lane 2, mother (a first cousin); lane 3, infant with HFI; lane 4, an unrelated patient, homozygous for the N334K mutation [28] in aldolase B. In the presence of the mutation Val³³⁷, the endonuclease cleaves the 205 bp amplified products of exon 9 into two fragments of 111 and 94 bp.

the mutant oligonucleotides after discriminatory washing at 57 °C (results not shown). The Asn³³⁴ oligonucleotide primers span a 16 bp sequence of DNA encompassing the Asn³³⁴ → Lys, G → C transversion. The absence of hybridization at the discriminatory temperature suggested the presence of a mutation within the amplified region of exon 9 in the vicinity of the Asn³³⁴ → Lys site but not Asn³³⁴ → Lys itself. The nature of this putative mutation was investigated by a sequence analysis of exon 9 (Figure 1A). Sequencing of exon 9 of aldolase B from the probanda showed a C → T transition for the first base of codon 337, which would result in the replacement of an alanine residue by a valine, hence the designation Val³³⁷. This mis-sense mutation generates a new recognition sequence for the restriction endonuclease *MscI* (TGG↓CCA), thus facilitating confirmation of the presence of the allele in the probanda and her immediate family members. Complete digestion of the amplified 205 bp fragment containing exon 9 with the enzyme *MscI* resulted in two fragments of 111 and 94 bp by using DNA obtained from the probanda, indicating that she was homozygous for the mutation (Figure 1B). As expected, a comparable analysis of her parents showed partial cleavage of the 205 bp fragment after *MscI* digestion, indicating that they were heterozygous for the Val³³⁷ mutation. Similar analyses of two subsequent live-born siblings of the probanda have confirmed homozygosity for the Val³³⁷ mutant allele. All three siblings remain well after the institution of a strict fructose exclusion diet.

Similar analyses of a Finnish patient with HFI and a Swiss patient with this disorder has revealed the presence of the Val³³⁷ mutant allele in compound heterozygous form with the Ala¹⁴⁹ → Pro allele [6].

Analysis of recombinant human aldolase B

Mendelian segregation of Val³³⁷ in association with HFI and its independent association with the disease phenotype in individuals harbouring an additional aldolase B mutation indicated that the conservative replacement of an alanine residue by valine at position 337 leads to the pathological inactivation of the enzyme

molecule. To investigate the precise effects of this mutation on catalytic function, these human aldolases were expressed in *E. coli*. Bacterial expression of the recombinant proteins was tightly regulated and is driven by the highly processive T7 DNA polymerase enzyme on induction.

The expression plasmid encoding wild-type human aldolase B conferred abundant F-1-P and FBP aldolase-splitting activity, comparable to that possessed by purified aldolase B obtained from human liver [25,29] and human liver supernatant extracts (Figure 2 and Table 1), indicating that the addition of an N-terminal methionine residue to the recombinant enzyme did not detectably affect the activity of the enzyme. SDS/PAGE analysis of bacterial lysates and purified aldolase preparations under denaturing conditions showed the expected 38 kDa species corresponding to the aldolase B subunit polypeptide. Densitometric scanning estimates and substrate cleavage estimates indicated that the recombinant enzyme accounted for approx. 30% of the soluble protein fraction in the bacterial extracts bearing the wild-type aldolase B expression plasmid. It is noteworthy that the 38 kDa polypeptide was absent from bacterial lysates prepared with the non-recombinant vector (results not shown). A Western blot analysis with polyclonal anti-(human aldolase B) antibody was undertaken to determine whether the additional 38 kDa species in the extracts of *E. coli* transformed with the recombinant plasmids corresponded to polypeptides with aldolase B immunoreactivity. As expected, the 38 kDa species that was derived from the recombinant expression plasmids encoding the human aldolases reacted specifically with the antibody. No protein species bound antibody in the vector only and untransformed bacterial control extracts (results not shown).

The purified aldolase preparations were assayed for fructose aldolase activities to obtain values for the specific activities, K_m values and substrate activity ratios ($V_{\text{FBP}}^{\text{max}}/V_{\text{F-1-P}}^{\text{max}}$). Purified wild-type recombinant human aldolase B gave specific activities, K_m values and substrate activity ratios similar to those of the purified enzyme obtained from human liver [25,29] (Table 1).

The purification strategy initially adopted for the Val³³⁷ aldolase B variant utilized expression at 37 °C, lysis, anion-

Figure 2 Purification of the wild type and the Val³³⁷ variant of human aldolase B expressed as a recombinant protein in bacteria

After transformation of the protease-deficient BL21(DE3) *E. coli* strain, with the modified expression plasmids pEBR+ and pEBR337V respectively, and induction by the addition of 1 mM IPTG, the cells were harvested and lysed as described in the Materials and methods section. Aldolase B was purified from bacterial supernatants by using thiazine dye affinity chromatography and substrate elution as described [22]. **(A)** Recombinant proteins stained with Coomassie Blue G250 after separation by SDS/PAGE under denaturing conditions as described by Laemmli [23] with a 9% (w/v) resolving gel. Lane 1, aldolase B purified from normal human liver [29]; lane MW, standard protein markers with the indicated molecular masses [phosphorylase b (97.2 kDa), BSA (66.4 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa)]; lane 2, *E. coli* strain BL21(DE3) supernatant with non-recombinant vector (pET-21a); lane 3, BL21(DE3) with pEBR+ (expressing wild-type human aldolase B); lane 4, recombinant wild-type human aldolase B purified from bacterial lysates; lane 5, supernatant of BL 21(DE3) with mutant expression vector pEBR337V; lane 6, recombinant human aldolase B variant Val³³⁷ purified from bacterial lysates. Note the partly cleaved Val³³⁷ polypeptides in lane 6; these species react with bovine anti-(human aldolase B) antiserum in immunoblotting experiments (results not shown). **(B)** Recombinant aldolase B variant Val³³⁷ stained with Coomassie Blue G250 after separation by SDS/PAGE under denaturing conditions with a 10% (w/v) resolving gel. The positions of molecular mass markers are indicated (in kDa) at the left: BSA (66.4 kDa), glutamic dehydrogenase (55.6 kDa), maltose-binding protein (42.7 kDa) and lactate dehydrogenase M (36.5 kDa). Lane 1, recombinant Val³³⁷ expressed at 37 °C and separated on mono-S cation-exchange chromatography; lane 2, recombinant Val³³⁷ expressed at 22 °C and purified by affinity elution and gel filtration; lane 3, recombinant wild-type aldolase B purified by affinity elution and gel filtration.

exchange affinity chromatography followed by gel filtration. This procedure generated mixed populations of Val³³⁷ enzyme with a decreased content of the 38 kDa polypeptide species and included two smaller polypeptides at apparent molecular masses of 36 and 33 kDa (results not shown). These smaller species were taken to represent truncated variants of the Val³³⁷ peptide because West-

Table 1 Properties of wild-type and mutant human aldolase B expressed in *E. coli*

Activities are expressed as $\mu\text{mol}/\text{min}$ per mg of protein at 22 °C. Abbreviation: n.d., not determined.

Enzyme	Specific activity ($\mu\text{mol}/\text{min}$ per mg)		Activity ratio (FBP to F-1-P)	K_m^{FBP} (μM)	$K_m^{\text{F-1-P}}$ (mM)
	FBP	F-1-P			
Wild type	4.787	4.507	0.98	4.0 ± 0.6	2.4 ± 0.24
Val ³³⁷	4.174	2.557	1.63	22 ± 0.03	24 ± 5
Val ^{337*}	0.30	0.06	5.00	80 ± 10	n.d.

* Purified sample after 1 cycle of freeze-thawing.

Table 2 Affinity of wild-type and mutant human aldolase B for triose ligands

Abbreviation: DHAP, dihydroxyacetone phosphate.

Enzyme	K_d^{DHAP} (nM)	$K_d^{\alpha\text{-glyceraldehyde}}$ (nM)
Wild type	2397 ± 243	57.5 ± 8.1
Val ³³⁷	151 ± 11	2.0 ± 0.2

ern blot analysis with polyclonal anti-(human aldolase B) antibody confirmed that the 38 kDa species and the two additional polypeptides of 36 and 33 kDa reacted strongly. The aberrant species comprised 22% and 43% respectively of the recombinant products. These findings suggested that the Val³³⁷ variant polypeptide, unlike wild-type aldolase B, is highly susceptible to limited proteolytic cleavage in *E. coli*.

The recombinant Val³³⁷ variant aldolase B purified after expression and growth at 37 °C had lower specific activities than the wild type. There was also a marked decrease in activity towards the F-1-P substrate and an appreciable retention of catalytic activity towards the general fructaldolase substrate FBP so that the substrate activity ratio was 5. Unlike wild-type aldolase B, the recombinant Val³³⁷ protein was also unstable when stored on ice or when subjected to a single episode of freeze-thawing, indicating a tendency to denature (Table 1).

Native Val³³⁷ protein was purified from bacterial cultures that were grown for expression of recombinant enzyme at 22 °C. The mutant protein was isolated rapidly in a pure undegraded form by anion-exchange affinity elution from thiazine dye resins; the final resolution from impurities was achieved by gel filtration. Native Val³³⁷ protein was isolated with catalytic activities as shown in Table 1. Val³³⁷ was highly active with respect to FBP with a specific activity very similar to that of wild-type enzyme preparations. The maximal activity of Val³³⁷ towards F-1-P was 58% of wild type but the intact protein had K_m values that were 10-fold those of wild type, indicating that the affinity for the specific aldolase B substrate was markedly impaired. The catalytic properties of the intact and truncated variants of the Val³³⁷ protein differed only slightly. This indicates that the predisposition of Val³³⁷ homozygotes towards fructose intolerance is attributable to the aberrant catalytic properties of the mutant enzyme and not solely to its tendency to denature.

The affinity of the Val³³⁷ protein for its triose substrates/products was examined by quenching of intrinsic fluorescence (Table 2). In the absence of triose, the maximum emission

Figure 3 CD measurements of recombinant human aldolases B

Ellipticity determinations at 222 nm of the wild-type (WT) and Val³³⁷ (A337V) proteins in relation to temperature were made at intervals of 0.1 min, as described in the Materials and methods section. The curves depict the best-fit analyses of each denaturation profile (see Table 3) [27].

Table 3 CD measurements of recombinant human aldolase B

Enzyme	Melting temperature (°C)	Enthalpy (J/mol)
Wild type	52	4355 ± 124
Val ³³⁷	59	1486 ± 40

wavelength, $\lambda_{\max}$, of the mutant protein was 340 nm compared with the wild-type aldolase B $\lambda_{\max}$ of 330 nm (results not shown). Triose binding studies showed a greater than 10-fold increase in apparent affinity for both triose substrates, including the aldolase B-specific substrate, D-glyceraldehyde, derived from the cleavage of F-1-P.

The effects of the Val³³⁷ substitution on aldolase B conformation were examined by determining the influence of temperature on CD measurement at 222 nm on mutant and wild-type proteins (Figure 3). The Val³³⁷ protein exhibited a distinct, flattened ellipticity profile during thermal denaturation; the markedly smaller slope reflects an abnormal α -helical structure and decreased enthalpy (Table 3) [27].

DISCUSSION

Here we report studies of a hitherto uncharacterized natural mutant of human aldolase B associated with HFI. The novel mutant allele occurred in three independent geographical isolates: it was originally identified in a homozygous form in the offspring of a consanguineous marriage in Turkey and has since been identified in patients with HFI from Switzerland and from Finland. This shows that, although rare, the mis-sense mutation is widespread in the human population.

The recombinant Val³³⁷ aldolase B has distinct structural and kinetic characteristics. When expressed in *E. coli* at 37 °C, the variant formed tetramers consisting of varying proportions of individual truncated subunits. Importantly, the native Val³³⁷ homotetramer exhibits an increased K_m for its specific F-1-P substrate while retaining an affinity for FBP resembling that of the wild type. This demonstrates that the invariant alanine residue at position 337 is required for the isoenzyme-specific activity of the enzyme towards its alternative fructose phosphoester substrates.

The class 1 aldolases possess a three-dimensional polypeptide backbone fold that corresponds to an α/β -barrel structure [30]. The alanine residue at position 337, which is located at one pole of the α -helical stretch of the C-terminal sequence, is invariant in all mammalian class 1 aldolases, irrespective of isoenzyme type. An alanine residue at this position is also found in plant aldolases, including those of spinach, rice, legumes and *Arabidopsis* as well as in the glycosomal aldolase of the protozoan *Trypanosoma brucei* ([31–35], and EMBL database accession number X89829). The Val³³⁷ substitution is located in an α -helix (residues 320–339) that precedes the conformationally flexible C-terminus ([30], and J. Sygusch, unpublished work). The presence of even an additional methyl group on the valine side chain is likely to disrupt the tight attachment between terminal helices and by causing misalignment induced partial denaturation of the human protein (J. Sygusch, unpublished work).

This proposition is confirmed by the abnormal intrinsic fluorescence and CD measurements of the Val³³⁷ aldolase B: the protein has a greatly altered thermal denaturation profile with reduced enthalpy that indicates a significant disruption of its secondary structure. The marked shift of the fluorescence towards longer wavelength maxima suggests that at least one of the three tryptophan residues (at positions 147, 294 and 312) in the mutant protein is more exposed to solvent than in the wild type.

The catalytic properties of the Val³³⁷ protein are consistent with the suspected involvement of the C-terminus in modulating the substrate specificity of the human aldolase B isoenzyme. The sequence of the C-terminal 30 residues of type I aldolases is highly variable, whereas the active-site residues are invariant [30,36,37]. Thus the C-terminus might confer the specific substrate activities that correspond to the functional differentiation of the fructaldolases [30]. A recognition of the influence of the C-terminus on substrate specificity has emerged from studies of carboxypeptidase treatment and artificial mutagenesis [36,38,39].

The established role of the C-terminus involves the protonation of the enzyme–dihydroxyacetone phosphate complex, which is the limiting catalytic step for the A isoform of aldolase [40]. Kinetic studies of both carboxypeptidase-treated enzymes show that the release of dihydroxyacetone phosphate is affected [40,41]. Mutagenesis and kinetic studies of the nine penultimate, C-terminal residues of the maize aldolase indicates that the primary effect of each point mutation is purely kinetic, affecting the attachment or release of substrate or product from the enzyme [39]. Some mutants of maize aldolase that extend the C-terminus arm seem to be defective in the release of D-glyceraldehyde 3-phosphate. Hence residues present at positions 346 and 349 perturb the interaction of the C-6 phosphate-binding locus of the enzyme with substrate and/or product, modulating F-1-P/FBP discrimination. Thus the role of the C-terminus is not limited to proton exchange. The C-terminus is tightly associated with the active site in conformations that are probably unfavourable for efficient catalysis so that the limiting step in aldolase B catalysis is product release [39,42]. This is clearly illustrated with the Val³³⁷ human B isoenzyme: studies of fluorescence quenching induced by triose quenching indicate a greatly increased binding

affinity for triose products, as predicted for a C-terminal mutant. Structural analysis of the rabbit liver aldolase shows that D-glyceraldehyde 3-phosphate is released by a concerted conformational transition of the entire subunit including the C-terminus. Once expelled, the C-terminus is free to interact with the enzyme-dihydroxyacetone phosphate complex in protonation/proton abstraction. Val³³⁷ is unable to clear the triose products efficiently; this includes the specific D-glyceraldehyde product of F-1-P cleavage.

Val³³⁷ is more prone to proteolysis in *E. coli* when expressed at 37 °C, a finding compatible with partial denaturation of the C-terminus. Proteolytic degradation *in vivo* might contribute to the catalytic deficiency of aldolase B in the liver but it is unlikely to account for all the characteristics of the Val³³⁷ protein. Treatment of liver aldolase with carboxypeptidase decreases the activity towards both hexose substrates by approx. 50% [40,42]; starvation-induced proteolysis of liver aldolase in mice, which also cleaves C-terminal residues, shows no selective effects on F-1-P activity [43]. Although the partly denatured Val³³⁷ protein might be degraded more rapidly *in vivo* compared with the wild-type enzyme, this would not explain the loss of function specific to aldolase B.

In HFI, kinetic studies have consistently shown a decreased affinity for the specific substrate F-1-P, even in tissues where there was appreciable residual aldolase activity [8,29,44]. Here the catalytic properties of the purified recombinant Val³³⁷ variant aldolase, with its substrate activity ratio approaching 2:1 for FBP to F-1-P (5:1 for stored enzyme), resemble the enzymic activities determined *in situ* in the *proposita*, who was homozygous for the mutation. Although the presence of constitutive aldolase A might account partly for the substrate activity ratios observed in HFI liver samples, the presence of abundant residual FBP aldolase-splitting activity of the Val³³⁷ enzyme does not prevent symptoms. Impaired cleavage of F-1-P in the specialized fructose pathway is therefore pathogenic. We have previously reported that patients with HFI who are homozygous for null alleles of aldolase B [14] remain asymptomatic while they abstain from fructose. The selective inability to cleave the F-1-P substrate derived from dietary fructose and cognate sugars is therefore necessary and sufficient for the development of hypoglycaemia and other manifestations of this disease.

We thank Dr. Nicholas Cross for his contributions to the construction of the expression vector in this study, Dr. Tim Dafforn for his assistance with the fluorescence and CD spectroscopy, and Michael Frost and Mrs. Joan Grantham for kindly assisting in the preparation of the manuscript. This work was supported by a grant from the Wellcome Trust.

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Received 26 August 1998/2 February 1999; accepted 4 March 1999