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ARTICLE

Novel six-nucleotide deletion in the hepatic fructose-1,6-bisphosphate aldolase gene in a patient with hereditary fructose intolerance and enzyme structure-function implications

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Hereditary fructose intolerance (HFI) is an autosomal recessive human disease that results from the deficiency of the hepatic aldolase isoenzyme. Affected individuals will succumb to the disease unless it is readily diagnosed and fructose eliminated from the diet. Simple and non-invasive diagnosis is now possible by direct DNA analysis that scans for known and unknown mutations. Using a combination of several PCR-based methods (restriction enzyme digestion, allele specific oligonucleotide hybridisation, single strand conformation analysis and direct sequencing) we identified a novel six-nucleotide deletion in exon 6 of the aldolase B gene (Δ6ex6) that leads to the elimination of two amino acid residues (Leu182 and Val183) leaving the message inframe. The three-dimensional structural alterations induced in the enzyme by Δ6ex6 have been elucidated by molecular graphics analysis using the crystal structure of the rabbit muscle aldolase as reference model. These studies showed that the elimination of Leu182 and Val183 perturbs the correct orientation of adjacent catalytic residues such as Lys146 and Glu187.

Keywords: inborn errors of metabolism; hereditary fructose intolerance; human aldolase B; mutation-causing enzyme alterations; molecular graphics analysis

Introduction

Aldolase is a homotetrameric enzyme that catalyses the reversible aldol cleavage of fructose 1,6-bisphosphate (FBP) and fructose 1-phosphate (F1P). There are three aldolase isoenzymes in mammals: A (muscle), B (liver) and C (brain).[1] Hereditary fructose intolerance (HFI), inherited in an autosomal recessive fashion with an estimated frequency of 1 in 20000 live births, is an inborn error of carbohydrate metabolism caused by a deficiency of aldolase B. The clinical manifestations of HFI, following ingestion of fructose, sucrose or sorbitol, consist in abdominal pain, vomiting, and such metabolic disturbances as hypoglycaemia that may be fatal.

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Symptoms usually regress once fructose is totally excluded from the diet.[2]

Aldolase B is encoded by a single gene and more than 20 gene mutations have been described. The most frequent in Europe are three missense mutations, A149P and A174D in exon 5, and N334K in exon 9.[3–6]

Crystallographic studies of human aldolase B at high resolution (J A Littlechild, personal communication, 1998) are in progress, although only low-resolution (3.8Å) diffracting crystals have been published so far.[7] Differently, the 3-D structure of rabbit aldolase A has been extensively studied at high resolution (about 2Å)[8–12] and recently a study of the rabbit aldolase A crystal structure containing dihydroxyacetone phosphate casts light on the molecular structure in the bound and unbound states.[12]

We have found a novel mutation in an Italian HFI patient consisting of a deletion of six nucleotides in exon 6 of the aldolase B gene, which alters the structure of the hepatic aldolase, thus resulting in a barely active enzyme.

Materials and Methods

Patient and Aldolase B Activity

The subject is a 6-year-old child. Hereditary fructose intolerance was suspected at 5 months of age on the basis of typical clinical symptoms and elevated serum aspartate transaminase levels. The clinical diagnosis was confirmed by greatly reduced aldolase B activity in liver biopsy. The fructose-1-phosphate (F1P) aldolase activity was 0.056 U/g (normal value 1.82–3.40 U/g), the fructose-1,6-bisphosphate (FBP) aldolase activity was 0.41 U/g (normal value 2.07–3.79 U/g), and the substrate activity ratio FBP/F1P was 7.3 (normal value 1.0–1.2); lastly, the fructose-1,6-bisphosphatase (FBPase) activity was 1.29 U/g (normal value 2.73–4.98 U/g).

The patient had liver failure, characterised by hepatomegaly, steatosis and fibrosis of hepatic cells, ascertained after needle biopsy. In the first two years of life, the weight gain was within the 25 percentile and height increase within the 50 percentile with respect to controls of the same age. At present, his weight is within the 75 percentile and height within the 50 percentile, compared with age-matched controls.

DNA Analysis

Genomic DNA was isolated from nucleated peripheral blood cells according to a standard procedure.[13] The conditions used for DNA amplification (PCR) and for allele-specific oligonucleotide hybridisation (ASO) analysis for the most frequent mutations (A149P, A174D and N334K) were as previously described.[14,15] Amplified DNA products containing the coding regions of the aldolase B gene were examined by single strand conformation polymorphism (SSCP) analysis carried out at 4°C on a 10% non-denaturing polyacrylamide gel in the presence of 5% glycerol, as previously described.[15]

The amplified fragments were directly sequenced in both directions using the Sequenase PCR Product Sequencing Kit (USB, Cleveland, Ohio, USA), according to the manufacturer's instructions. PCR-amplified genomic fragments containing exon 6 were digested with Ava II and Rsa I endonucleases (Boehringer, Mannheim, Germany), according to the manufacturer's recommendations, and the products were analysed by electrophoresis on a 12% polyacrylamide gel and visualised after ethidium bromide staining.

Molecular Graphic Analysis

Modelling of human aldolase B was carried out using the structure of rabbit aldolase A at 1.9 Å resolution (Protein Data Bank, code 1ADO). The sequence identity between the two isozymes is 69%, with neither insertions nor deletions. In particular, the sequence identity is 85% in the encompassing region of the deletion site (aa 159–191). All the catalytic residues are conserved except from Asn41 which is replaced by a lysine in rabbit aldolase A.

Rabbit aldolase A structural motifs were analysed using the programme PROMOTIF.[16] Because the mutated fragment is far from the subunit interfaces in the tetramer, we confined our analysis to a single subunit that is known to possess catalytic activity, albeit at a level lower than the tetrameric structure.

The search for loops connecting the α-helix D (Ser159–Asn180) and the β-strand 5 (Val183–Leu191) was performed using a database of high resolution structures implemented in the programme O,[17] (Figure 3 was drawn using MOLSCRIPT[18] and RASTER-3D.[19])

Results and Discussion

DNA Mutation Analysis

Using PCR and ASO hybridisation techniques we found the patient was heterozygous for mutation N334K in exon 9. SSCP analysis of the entire coding region revealed a shift in the electrophoretic mobility only in exon 6 (Figure 1A). Direct sequencing analysis showed a six-nucleotide deletion (from 10072 to 10077), corresponding to codons 182 and 183, leaving the downstream coding region inframe (Figure 1B). This new mutation, assigned the trivial name Δ6ex6, and the systematic name g10072–10077del,[20] creates a new Ava II site (G/G(AT)CC) and abolishes an Rsa I site (GT/AC) (see Figure 2).

Restriction digestion analysis (Figure 2) revealed that the patient inherited the Δ6ex6 mutation from the father. The N334K mutation in exon 9, as revealed by ASO hybridisation (data not shown), was carried by the mother, heterozygous for this mutation. Direct sequencing of both parents DNA confirmed that the mutation followed Mendelian inheritance (data not shown). Restriction analysis of Δ6ex6 in the proband's 5-month-old brother showed normal patterns (see Figure 2).

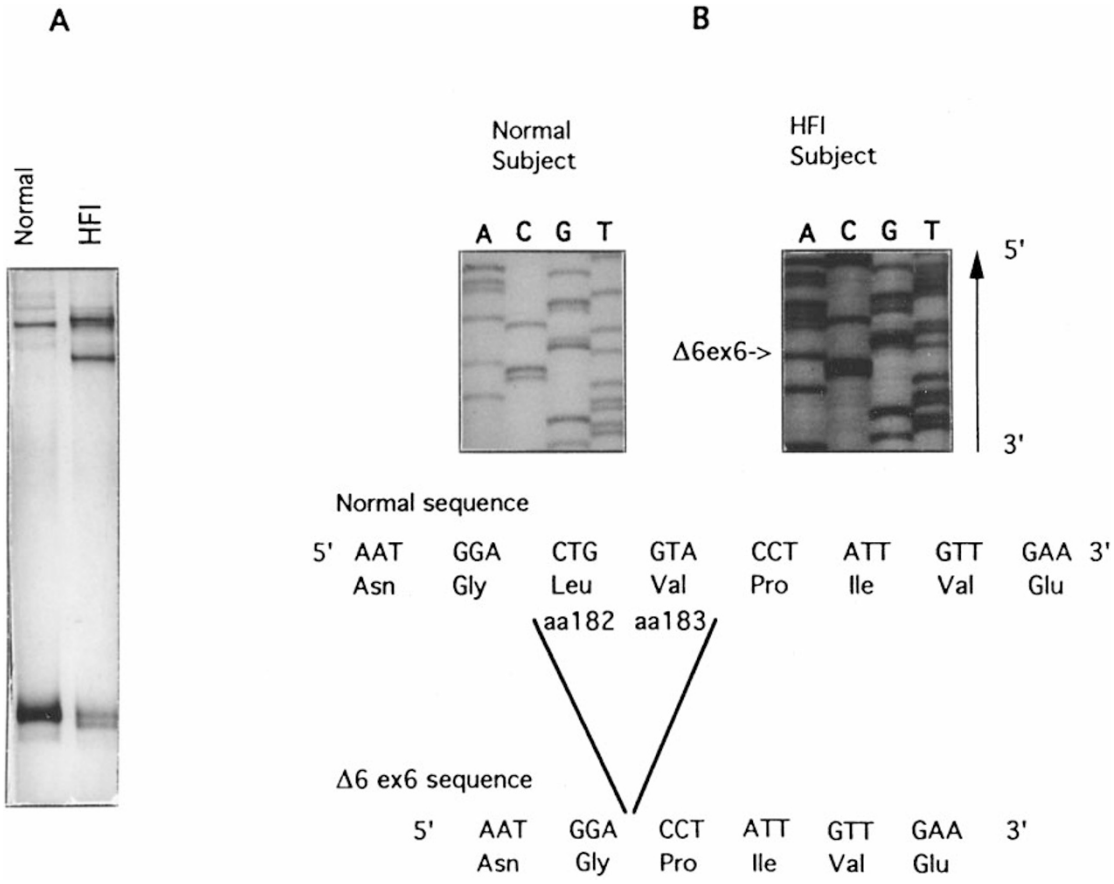

Figure 1 *Detection of the Δ6ex6 mutation in the human aldolase B gene.* ***A****: Single strand conformation polymorphism analysis of exon 6 amplified DNA in normal and HFI subjects.* ***B****: Direct sequencing of exon 6 amplified DNA from a normal subject and the proband. The deletion site is marked by an arrow; normal and mutated sequences and codon translation are indicated; the deleted region in the HFI subject includes the two amino acids, Leu 182 and Val 183*

The newly identified mutation (Δ6ex6) was not a polymorphism since it was absent from the normal population, as determined by restriction analysis of 60 chromosomes from 30 unrelated individuals.

The mutation described in this paper consists in the deletion of two amino acids which are highly conserved: Leu182, which is replaced in some vertebrates only by isoleucine, and Val183, which is present in all class I aldolases. The two amino acids are located near the catalytic site of the enzyme. Therefore, their elimination could cause inactivation of aldolase B. This is strongly supported by a negligible F1P aldolase B activity in the patient's liver. The other mutation N344K carried by our patient has previously been found in the homozygous state associated with HFI.[4] Consequently, it appears that the Δ6ex6 mutation is critical for aldolase B function. As verified in 60 normal chromosomes, mutation Δ6ex6 is not present in the normal population, except in the proband's father in the heterozygous state. Therefore, this mutation together with mutation N334K, is the cause of HFI in the proband.

Enzyme Structure–Function Implications

The aldolase molecular architecture corresponds to an $(\alpha/\beta)_8$ barrel with the active site in a deep pocket at the C-terminal end. Consequently α-helices are identified by the letters from A to H and β-strands by numbers from 1 to 8 (Figure 3A). The pocket is lined primarily by charged amino acids that interact with the substrate. Among others, Lys146 and Glu187 are directly bound to DHAP, the product of the catalytic reaction.[12] These residues are supposed to interact with the C1-phosphate moiety and to act as proton donors/acceptors during the catalytic reaction which proceeds through a Schiff base intermediate involving proton transfers.[12]

In aldolases, Leu182 belongs to a loop connecting the α-helix D with the β-strand 5 (Figure 3B) and Val183 is

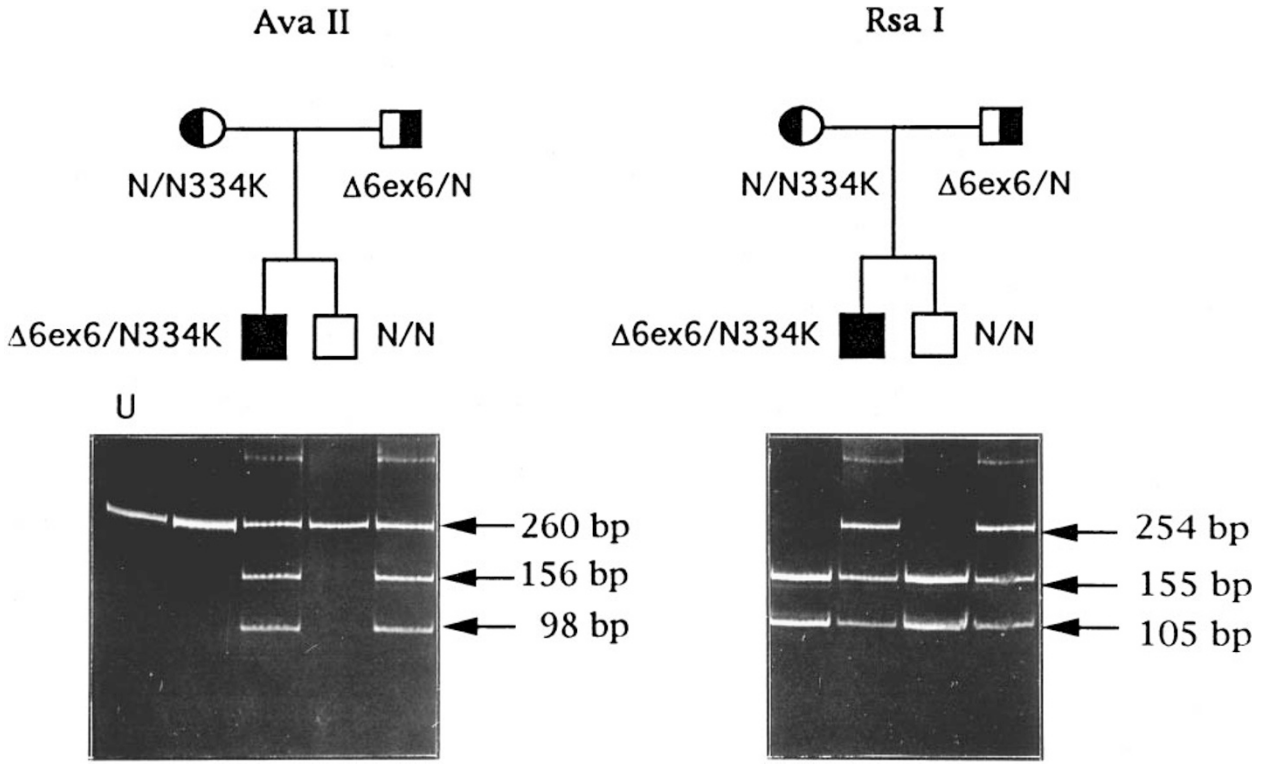

Figure 2 *Segregation of the Δ6ex6 mutation in the proband's family by restriction enzyme digestion analysis of exon 6 amplified DNA. The lanes of the electrophoregrams correspond vertically to the subjects indicated in the pedigrees of the family, which are reported in the upper part of the Figure together with the corresponding genotypes; U indicates the undigested 260-bp PCR product. Left: digestion with the restriction enzyme Ava II. The normal fragment of 260 bp is present in all subjects, and in the patient and in his father the fragments of 156 bp and 98 bp are present because of the mutation. Right: the pattern after digestion with the restriction enzyme Rsa I. The mother and the brother of the proband show the normal fragments of 155 bp and 105 bp; the patient and his father show both the normal fragments (155 bp and 105 bp), and the fragment of 254 bp (present because of the mutation) indicating a heterozygous state for this mutation*

the first residue of strand 5. Val183 links Asp143 and Ala145, which are located in the near β-strand 4, through two hydrogen bonds. Therefore, the N-terminal ends of strands 4 and 5, which are two adjacent staves of the β-barrel, are maintained close to each other by these hydrogen bonds. To explore the structural alterations resulting from the deletion, we carried out a systematic search for closure α-helix D with the β-strand 5, after omitting residues Leu182 and Val183. Various possible closures were found with satisfactory stereochemistry, indicating that the mutated enzyme should be able to fold. However, to rebuild this connection, a variation of the backbone conformation of at least three residues (amino acids 179–181) is required. The mutation produces, therefore, the unwinding of the C-cap amino acids 179–180 of the α-helix and the weakening of the contacts between N-ends of strands 4 and 5. This might severely alter the helix/strand connection. Furthermore, it is noteworthy that the catalytic residues Lys146 and Glu187 are located in β-strand 4 and 5 respectively and are not far from the deletion site. This proximity may induce small local changes to side chains of Lys146 and Glu187 and these variations may explain the remarkable reduction in enzyme activity towards both substrates (0.056 U/g for F1P and 0.41 for FBP). Indeed, the substrate activity ratio FBP/F1P of 7.3, which was observed in our patient's liver biopsy, is markedly higher than normal values. The more pronounced activity reduction towards F1P is consistent with a local rearrangement of Lys146 and Glu187 in the mutated enzyme. These residues are located in the active site region that recognises and interacts with the C-1 phosphate moiety;[11] even small movements of their side chains of Lys146 and Glu187 can weaken these favourable interactions.

In conclusion, the severe clinical symptoms of HFI observed in our proband support, albeit indirectly, the

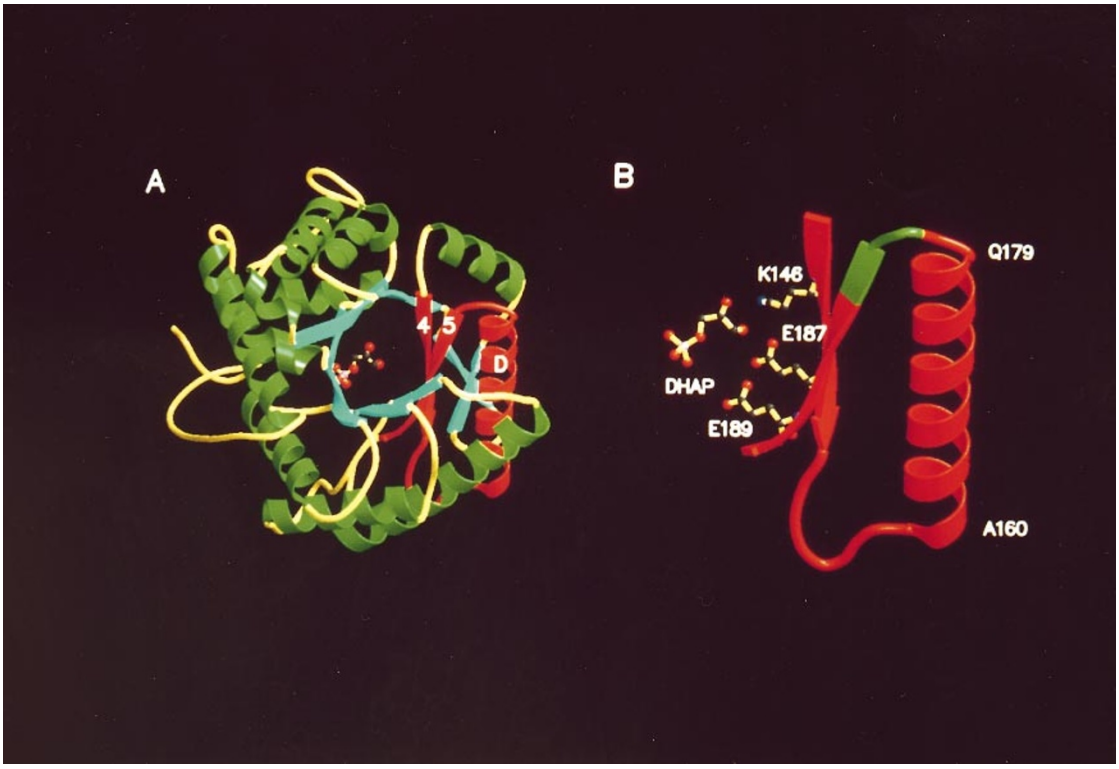

Figure 3 *Three-dimensional structure of rabbit aldolase A with α-helices and β-strands drawn as a coiled ribbon and flattened arrows, respectively.* ***A****: Overall* $(\alpha/\beta)_8$ *barrel of a subunit of rabbit aldolase A: α-helices and β-strands are shown in green and blue, respectively, other connecting regions are in yellow. The N-terminal α-helix A has been omitted, for clarity. The region corresponding to the α-helix D and the β-strands 4 and 5, where the latter includes the deleted residues 182–183, is shown in red. DHAP bound to the enzyme is shown in the centre.* ***B****: Detail of panel* ***A****, showing the region of the deletion site. Residues 182–183 are shown in green. Side chains of Lys146 (strand 4), Glu187 and Glu189 (strand 5), that belong to the active site, are also shown. Note the DHAP molecule at the active site*

role ascribed to Lys146 and Glu187 in agreement with findings emerging from the crystal structures.[8,12] Therefore, the analysis of altered protein conformation by molecular graphic modelling contributes to the understanding of the proper functioning at the enzyme active site.

Disease Mutation Frequency and Genetic Screening

In the 28 Italian patients studied by us the most frequent HFI mutations A149P and A174D account for 66% of mutant alleles, whereas the other HFI mutations account for the remaining 34% (see Table 1). Interestingly, the heterogeneity of the mutation increases going from northern to southern European countries, as is the case of other hereditary diseases, eg cystic fibrosis.[21] This higher heterogeneity of southern versus northern Europe accurately reflects the migrations typical of the Mediterranean basin.

Table 1 Allele frequency distribution of HFI mutations in Italy and North European countries

Mutation	*Italy*[a] *(%)*	*North European countries*[b] *(%)*
A149P	30.0	67.0
A174D	36.0	12.5
N334K	3.6	8.0
Other mutations	30.4	12.5

[a]Data from our case-mix on 56 alleles; [b]data from Cox[5]

The experimental approach to the diagnosis of HFI, based on DNA analysis, can replace the intravenous fructose tolerance challenge test and invasive liver biopsy. Therefore this approach is useful for the prevention and therapy of this hereditary disease.

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