Human aldolase A natural mutants: relationship between flexibility of the C-terminal region and enzyme function

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We have identified a new mutation in the FBPA (fructose 1,6-bisphosphate) aldolase A gene in a child with suspected haemolytic anaemia associated with myopathic symptoms at birth and with a subsequent diagnosis of arthrogryposis multiplex congenita and pituitary ectopia. Sequence analysis of the whole gene, also performed on the patient’s full-length cDNA, revealed only a Gly346→Ser substitution in the heterogeneous state. We expressed in a bacterial system the new aldolase A Gly346→Ser mutant, and the Glu306→Lys mutant identified by others, in a patient with an aldolase A defect. Analysis of their functional profiles showed that the Gly346→Ser mutant had the same kcat as the wild-type enzyme, but a 4-fold lower Km, the Glu306→Lys mutant had a kcat approx. 2-fold higher than that of both the Gly346→Ser mutant and the wild-type enzyme, and a kcat value 40% less than the wild-type. The Gly346→Ser and wild-type enzymes had the same Tm (melting temperature), which was approx. 6–7 °C higher than that of the Glu306→Lys enzyme. An extensive molecular graphic analysis of the mutated enzymes, using human and rabbit aldolase A crystallographic structures, suggests that the Glu306→Lys mutation destabilizes the aldolase A tetramer at the subunit interface, and highlights the fact that the glycine-to-serine substitution at position 346 limits the flexibility of the C-terminal region. These results also provide the first evidence that Gly346 is crucial for the correct conformation and function of aldolase A, because it governs the entry/release of the substrates into/from the enzyme cleft, and/or allows important C-terminal residues to approach the active site.

Key words: aldolase A, aldolase A mutant expression, aldolase A gene mutation, fructose 1,6-bisphosphate, molecular modelling.

INTRODUCTION

Vertebrate fructose 1,6-bisphosphate (FBP) aldolase (EC 4.1.2.13), which exists in three isoforms, namely aldolase A, B and C, catalyses the reversible conversion of FBP and fructose 1-phosphate into two triose molecules in the glycolytic, gluconeogenic and fructose metabolic pathways [1]. Aldolase B is mainly expressed in the liver, kidney and small intestine, where it serves a role in exogenous fructose utilization. Aldolase A is a ubiquitous and constitutively expressed housekeeping enzyme that drives the glycolytic metabolic pathway in most mammalian cells. Aldolase C is expressed predominantly in the brain [1]. Aldolase B deficiency has been widely described in humans, because it causes HFI [hereditary fructose intolerance; OMIM (Online Mendelian Inheritance in Man) #229600], which is an autosomal recessive disease that may induce severe liver damage, leading, in extreme cases, to death if fructose is not eliminated from the diet [2]. To date, nearly 25 HFI-related aldolase B mutants have been isolated [3–7]. In contrast, cases of aldolase A deficiency, which has been associated with non-pherocytic haemolytic anaemia (OMIM #103850), are much rarer [8–12].

Aldolase A deficiency is an autosomal recessive trait, with alterations in the aldolase A gene leading to amino acid substitutions (Asn128→Gly and Glu306→Lys) in two of the few cases already described [10–12]. It is conceivable that, given the essential function of this enzyme in almost all human tissues, human embryos affected by severe aldolase A mutations do not survive. Biochemical and thermodynamic data are available for the Asn128→Gly mutant [10], whereas the Glu306→Lys mutant has never been characterized. Here, we report a novel defect in the aldolase A gene, i.e. a natural mutation at amino acid residue 346 (Gly→Ser), which appears to be a bending site that is important for the functioning of the enzyme. We also report the expression and characterization of the Gly346→Ser and Glu306→Lys natural mutant enzymes.

EXPERIMENTAL

Materials

Restriction endonucleases were from New England Biolabs. Taq polymerase, T4 DNA ligase, IPTG (isopropyl β-D-thiogalactoside), ampicillin, PMSF and GDH/TIM (α-glycerol phosphate dehydrogenase/triose phosphate isomerase) were from Roche Molecular Biochemicals. Imidazole, FBP and NADH were from Molecular Biochemicals. Imidazole, FBP and NADH were from Sigma–Aldrich. The pET-16b vector and the Escherichia coli strain BL21(DE3) were purchased from Novagen.

The patient

The proband is a 7-year-old Italian boy examined at the age of 3 months [13], in whom aldolase A deficiency was initially suspected because of muscle weakness and presumed haemolytic anaemia. Both parents are healthy and non-consanguineous.
Table 1: Primers used for ARMS analysis

<table>
<thead>
<tr>
<th>Primer type</th>
<th>Sequence</th>
<th>Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARMS 346 Norm</td>
<td>5′-AGGAAAGTACCTCGTGCC-3′</td>
<td>0.5</td>
</tr>
<tr>
<td>ARMS 346 Mut</td>
<td>5′-AGTAAATACCTCGTGCA-3′</td>
<td>0.5</td>
</tr>
<tr>
<td>Common</td>
<td>5′-GACTGCCAGAACCTTGT-3′</td>
<td>0.15</td>
</tr>
<tr>
<td>Forward control</td>
<td>5′-CCTGGACACAGAGG-3′</td>
<td>0.1</td>
</tr>
<tr>
<td>Reverse control</td>
<td>5′-AACATCGTCTGTTCCCT-3′</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Genetic analysis

Genomic DNA was purified from peripheral blood leucocytes by the NaCl extraction method [14]. The whole aldolase A-coding region, the splice junctions and the promoter regions were amplified by PCR using appropriate primer pairs (GenBank® #NT_010604). The amplified DNA fragments were automatically sequenced using fluorescence-based methods (Big Dye™ Terminator, ABI Prism 377 DNA Sequencer; PE Applied Biosystems, Foster City, CA, U.S.A.) with the ABI-Prism 377 Applied Biosystems Genetic analyser (PE Applied Biosystems). The sequence data were analysed by a visual check for heterozygous mutations. We used the ARMS (amplification refractory mutation system) analysis [15] to examine Mendelian inheritance of the Gly346 → Ser mutation in the proband’s family. DNA samples were amplified in two reactions containing 0.2 μg of genomic DNA and 1 unit Taq polymerase (PerkinElmer/Cetus). After an initial denaturing step of 1 min at 94°C, samples were amplified for 32 cycles at 94°C for 20 s, 55°C for 20 s and 72°C for 30 s. At a final volume of 50 μl, each reaction mixture contained 200 μM of each dNTP, 50 mM KCl, 10 mM Tris/HCl, pH 8.8, 2.5 mM MgCl₂, 0.1% (w/v) gelatin, the appropriate forward ARMS primer, a common reverse primer and an additional primer pair that amplified a β-globin gene fragment, which is an internal control of amplification conditions (Table 1). The results were analysed on a 2% (w/v) agarose gel.

Total RNA extracted from the patient’s peripheral blood by the QIAamp RNA Blood Mini Kit (Qiagen Inc.) was first analysed by automated sequencing. The amplified cDNA was subjected to RT (reverse transcriptase)-PCR using specific primers and the SuperScript™ III One-Step RT-PCR System (Invitrogen, Milan, Italy). The amplified fragment, corresponding to the patient’s aldolase A cDNA, was subjected to automated sequencing.

Construction and expression of wild-type and mutant aldolase A

The full-length wild-type human aldolase A cDNA [16] was amplified with the forward primer containing a NdeI site (shown underlined) at the 5′-end (plus primer: 5′-GGAAATTCCATATG-CCCTACAAATATCCAGGC-3′), and the reverse primer containing the aldolase A termination codon (UAA) followed by a BamHI site (also shown underlined) (minus primer: 5′-GGAAATTCCGATCCCCTATAGGCGTGGTAG-3′). The cDNA sequence was verified by automated sequencing. The amplified cDNA was cloned into the NdeI and BamHI sites of the bacterial expression vector pET-16b, downstream of a His-tag coding sequence. To select recombinant clones, *E. coli* XL1-Blu host cells were transformed with the vector containing the target gene. Recombinant plasmid (pET-16b/cDNA) was purified over a Qiagen column (Qiagen).

The QuickChange™ site-directed mutagenesis kit (Stratagene) was used to introduce mutations into the pET-16b/cDNA construct. Mutagenesis reactions were carried out with a PerkinElmer 9600 thermal cycler. To change the normal Glu206 into a lysine residue, primers 5′-CAGTATGTGACCAAGAAGGGTGG-3′ (sense) and 5′-CCAGCACCTTCTTTGATCATATCTGTG-3′ (antisense) were synthesized. To change Gly346 into a serine, the sense primer was 5′-CAGCTCGACGAGTCGCTGG-3′ and the antisense primer was 5′-CCAGCTCTAGCTCTGGAGTGTG-3′. The parental methylated DNA was digested with DpnI, and the newly synthesized DNA was used to transform the *E. coli* XL1-Blu competent cells, according to the manufacturer’s instructions. Recombinant mutant plasmids were purified over Qiagen columns, and their full sequences were verified by automated sequencing. Recombinant aldolase A proteins were expressed in the *E. coli* B strain BL21(DE3). All the wild-type, Glu206 → Lys and Gly346 → Ser proteins were recovered in soluble form in bacteria grown at 37°C. Recombinant enzymes were purified on Ni²⁺/nitrilotriacetate resin (Qiagen), as described elsewhere [7,17]. Fractions containing the bulk of the enzyme were pooled, dialysed against 20 mM Tris/HCl, pH 7.4/50% (v/v) glycerol, and stored at −20°C. All purification steps were performed at 4°C. The purity degree of each recombinant enzyme was analysed by SDS/PAGE [10% (v/v) gel].

Molecular mass determination, denaturation profile and CD spectra

Enzyme molecular masses were determined at 25°C by co-fractionation (on FPLC) of the purified proteins with β-amylose (200 kDa), chicken ovalbumin (44 kDa) and carbonic anhydrase (29 kDa) on a 50 cm x 1.6 cm Sephadex G-200 column (Amer sham Pharmacia Biotech.) in 20 mM Tris/HCl, pH 7.4/20% (v/v) glycerol, with a flow rate of 0.2 ml/min. CD spectra were recorded at 20°C using a Jasco J-810 spectropolarimeter equipped with a cuvette with a 0.1 cm path length. The molar ellipticity ([θ] in deg cm² dmol⁻¹) was calculated from the equation [θ] = [θ]obs · mrw/10·1·C, where [θ]obs is the measured ellipticity in degrees, mrw is the mean residue molecular weight, l is the optical path length of the cuvette in cm, and C is the protein concentration expressed in g·cm⁻¹. Spectra were recorded from 190–250 nm, with a scanning speed of 20 nm/min. Thermal denaturation data on the recombinant proteins were collected using a scan rate of 1°C/min in the range 20–80°C. CD experiments were performed with a protein concentration of 1 mg/ml in 20 mM Tris/HCl, pH 7.2.

Aldolase A activity assays and kinetic studies

The substrate cleavage rate was determined spectrophotometrically by measuring NADH oxidation at 340 nm in a coupled assay using GDH/TIM, with PFPB as the substrate [18]. Assays were conducted at 30°C, as described previously [7]. One unit of activity corresponds to the cleavage of 1 μmol of hexose substrate/min. Specific activity was obtained by normalizing for mg of protein. For each recombinant enzyme, we ran three assays with at least two different enzyme preparations, and evaluated the *Kₐ* and *kₐ* values from double-reciprocal plots using a least-squares method. The kinetic data that were fitted directly to the Michaelis–Menten equation gave the same *kₐ* and *Kₐ* values calculated from the double-reciprocal plot.

Molecular graphics analyses

The alterations induced by mutations in the aldolase A structure were evaluated by molecular graphics using the crystallographic structures of human and rabbit aldolase A reported in the PDB.
state in the patient. No other mutations were detected in the patient’s aldolase A mRNA.

Expression and characterization of recombinant aldolase A proteins

We expressed the wild-type aldolase A protein, the Glu206 → Lys mutant and the new aldolase A Gly346 → Ser mutant in E. coli. The recombinant His-tagged proteins were purified to > 95% homogeneity by Ni²⁺-affinity chromatography, as verified by SDS/PAGE (results not shown). The recombinant enzymes were expressed in bacteria grown at 37 °C, and recovered from the soluble fraction of the bacterial lysate. The purification rate was 10–12 mg/l of culture for all recombinant proteins. The molecular mass of the recombinant enzymes, determined by FPLC, was approx. 170 kDa (Figure 2A). The enzymes remained stable at 4 °C in 20 mM Tris/HCl, pH 7.4, for at least 2 weeks, and for at least 1 month at −20 °C in 50% (v/v) glycerol/20 mM Tris/HCl, pH 7.4. CD spectra of wild-type, Glu206 → Lys and Gly346 → Ser recombinant aldolases revealed nearly identical curves within experimental error, suggesting that the three enzymes share very similar secondary structures (Figure 2B), and therefore that the mutations did not cause major perturbations in the enzyme’s secondary structure.

The analysis of the thermal stability (Table 2, first column, and Figure 2C) of these recombinant forms of aldolase A reveals that the Tm (melting temperature) of the Gly346 → Ser mutant (54.8 °C) is virtually identical with that of the wild-type enzyme (54.4 °C). On the other hand, the replacement of a glutamic acid in position 206 with a lysine significantly reduces the thermal stability of the enzyme (Tm = 48.0 °C).

Analysis of the kinetic properties of aldolase A recombinant forms (Table 2) demonstrates that the Glu206 → Lys and Gly346 → Ser proteins display a reduced activity towards FBP, the physiological substrate of the enzyme. In particular, the reduction in specific activity was greater in the case of the Gly346 → Ser mutant than Glu206 → Lys. Although the catalytic efficiency of the two mutants was reduced by the same extent (i.e. to at least one-third lower compared with the wild-type enzyme), the Glu206 → Lys and Gly346 → Ser proteins have different kcat and Km values. In the case of the Gly346 → Ser mutant, the decreased kcat/Km ratio is mostly due to the decreased kcat value, whereas in the case of Glu206 → Lys, it is due mainly to the increase in Km. The intracellular FBP concentration of approx. 14 µM [31], lower than the Km value of the recombinant wild-type enzyme, is not sufficient to saturate the Glu206 → Lys mutant enzyme (see Table 2), which hence would be less active than normal in vivo. This observation explains, at the molecular level, the severe disease phenotype of haemolytic anaemia and myopathy in the patient carrying the Glu206 → Lys mutation in the homozygous state [11].

Molecular graphics analyses

Aldolase A is physiologically active as a tetramer composed of four monomers each characterized by an eight-membered αβ-barrel structure. The N-terminal helix and the C-terminal region are located at opposite ends of the central cavity of the barrel structure. The Gly346 residue, conserved among vertebrate aldolase A sequences, is located in the C-terminal region of the enzyme. In aldolase A, this region is particularly flexible, and is thus frequently disordered in the various reported three-dimensional structures of the enzyme. However, a reliable tracing of this region has been obtained for human aldolase A complexed with FBP [22] and for rabbit aldolase A complexed with the reaction product DHAP [21]. Interestingly, in the AldA–DHAP complex, the C-terminus assumes different conformations in the different chains.
Aldolase A obtained by monitoring molar ellipticity at 222 nm. and Gly346

Figure 2 Characterization of recombinant wild-type and mutant aldolases A

(A) FPLC profiles of the wild-type and mutant aldolase A enzymes. The elution profiles indicate that all the recombinant enzymes have a molecular mass of approx. 170 kDa, as expected for the His-tagged homo-tetrameric form of aldolase A, purified under native conditions. The dotted line and arrows indicate the molecular masses of the protein standards used in calibration and apply to all three panels (see the Experimental section). (B) CD spectra of wild-type, Glu206 → Lys and Gly346 → Ser aldolase A recorded at a protein concentration of 0.4 mg/ml, at 20 °C as a function of wavelength. (C) Melting curves of wild-type, Glu206 → Lys and Gly346 → Ser aldolase A obtained by monitoring molar ellipticity at 222 nm.

of the tetramer, depending on the binding state of each subunit (Figure 3). In particular, when a DHAP molecule is bound to the subunit, the C-terminus folds over the surface of its own subunit [21]. On the other hand, in subunits with no DHAP bound to the active site, the C-terminus protrudes towards an intersubunit cleft of an adjacent tetramer, thereby leaving the active site fully exposed. In both the AldA–FBP and AldA–DHAP complexes, a serine residue could replace the glycine without any steric overlap, because residue 346 is exposed to the solvent. However, we underline that the main chain dihedral angles of Gly346 in the AldA–FBP and AldA–DHAP complexes indicate that this residue assumes, in some cases, conformations that are usually unfavourable for non-glycine residues (Table 3).

The Glu206 residue is located on the polar side of the amphipathic helix E (residues 197–219). This residue forms a strong intrasubunit salt bridge with Arg258, which is the C-terminal residue of helix F (residues 244–258). Interaction between Glu206 and Arg258 may affect the tetramer stability, since the Arg258 side chain is also involved in an intersubunit salt bridge with Glu206 of an adjacent subunit (Figures 4A and B). The aldolase A tetramer is stabilized by four Glu206/Arg258/Glu224 clusters of charged residues (Figure 4A). Interestingly, Glu206, Glu224, and Arg258 are strictly conserved residues in vertebrate aldolase isoenzymes. These residues are also present in the sequences of insects and plants, such as Drosophila melanogaster and rice. The Glu206/Arg258/Glu224 cluster (Figure 4B) has been detected in all vertebrate aldolase structures reported in the PDB, independently of the protein source, the binding state and the occurrence of mutations. In this framework, the replacement of Glu206 with a positively charged lysine residue may severely perturb the structural integrity of the enzyme.

DISCUSSION

Class I aldolases are important enzymes in glycolysis. Aldolase deficiencies may lead to severe genetic diseases. In particular, aldolase B deficiency in humans is linked to the widespread HFI, which affects 1 in 2000 newborns.

Mutations in the aldolase A gene are very rare in vivo. Thus far, only two naturally occurring mutations (Asn128 → Lys and Gly346 → Ser) have been reported, both of which are associated with non-spherocytic haemolytic anaemia and myopathic symptoms [10,11]. Whereas a large body of biochemical and thermodynamic data is available for Asn128 → Lys [10], the Glu206 → Lys mutant has never been expressed and characterized.

Here, we report a novel naturally occurring mutation of aldolase A (Gly346 → Ser), which has been found, in the heterozygous state, in an infant in whom an aldolase deficit was suspected, because of severe muscle weakness and presumed haemolytic anaemia. We have expressed and characterized the Gly346 → Ser and Glu206 → Lys mutants.

Analysis of the thermal stability of the two mutants indicates that the Gly346 → Ser enzyme is as stable as the wild-type, whereas replacement of Glu206 with a lysine residue reduces the enzyme Tm by 6.4 °C.

These results may be interpreted in view of the location of the mutated sites in the three-dimensional structure of the enzyme. Indeed, Gly346 is located in the proximity of the flexible C-terminus, whereas Glu206 is involved in strong electrostatic interactions at the subunit interfaces of the aldolase A tetramer. Remarkably, the destabilization of the Glu206 → Lys mutant is comparable with that reported for the disease-causing mutation, Asn128 → Gly [10].

From the biochemical viewpoint, both Glu206 → Lys and Gly346 → Ser mutants have a lower catalytic efficiency than wild-type aldolase A. However, a closer evaluation of the kinetic parameters revealed that the kcat/Km ratio is reduced by different mechanisms in the two mutants. In the case of Glu206 → Lys, both Kcat and kcat are significantly decreased, despite the distance [larger than 20 Å (1 Å ≡ 0.1 nm)] between the mutation site and the active site. This finding indicates that structural modifications
Table 2  Functional enzymic parameters of the recombinant human aldolase A enzymes analysed

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$T_m$ (°C)</th>
<th>Specific activity ($\mu$mol·min$^{-1}$·mg$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>54.4</td>
<td>22.3 ± 2.3</td>
<td>51.9 ± 6.5</td>
<td>16.7 ± 1.8</td>
<td>0.32 ± 0.07</td>
<td>–</td>
</tr>
<tr>
<td>Mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly$^{346}$→ Ser</td>
<td>54.8</td>
<td>6.1 ± 0.5</td>
<td>56.6 ± 5.2</td>
<td>4.7 ± 0.5</td>
<td>0.08 ± 0.01</td>
<td>4.0</td>
</tr>
<tr>
<td>Gln$^{206}$→ Lys</td>
<td>48.0</td>
<td>15.7 ± 3.4</td>
<td>105.0 ± 15.0</td>
<td>10.7 ± 1.7</td>
<td>0.10 ± 0.03</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Table 3  Dihedral angles of Gly$^{346}$ in aldolase A structures

<table>
<thead>
<tr>
<th>Structure</th>
<th>Subunit</th>
<th>$\phi$ (°)</th>
<th>$\psi$ (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ADO</td>
<td>A</td>
<td>68.6</td>
<td>−162.0</td>
</tr>
<tr>
<td>1ADO</td>
<td>B</td>
<td>130.0</td>
<td>−31.5</td>
</tr>
<tr>
<td>1ADO</td>
<td>C</td>
<td>53.7</td>
<td>28.3</td>
</tr>
<tr>
<td>1ADO</td>
<td>D</td>
<td>119.9</td>
<td>−1.5</td>
</tr>
<tr>
<td>4ALD</td>
<td>A,B,C,D*</td>
<td>113.1</td>
<td>−138.1</td>
</tr>
</tbody>
</table>

* The four chains of 4ALD present identical structures, since the asymmetric unit contains a single monomer.

Figure 3  Superimposition of the four subunits of aldolase A found in the complex with DHAP [21]

Given the location of Gly$^{346}$ in each chain (black circles), this residue is clearly important for the flexibility of the C-terminal segment of the aldolase A protein.

Figure 4  Ion pairs involving residues Glu$^{206}$, Glu$^{224}$ and Arg$^{258}$ at monomer–monomer interfaces within the tetramer structure of aldolase A [21]

(A) Location of the four clusters within the tetramer. (B) Close-up of two of the four charged clusters, showing in greater detail the positions of residues Arg$^{258}$, Glu$^{224}$, Glu$^{206}$ at the monomer–monomer interface.
aldolase A structures, Gly^{346} often adopts conformations that are energetically unfavourable for non-glycine residues, such as serine. Therefore the flexibility of the C-terminal region is important for the efficiency of aldolase A function.

In conclusion, our study was prompted by the discovery of a novel in vivo mutation of human aldolase A. Not only does our investigation lend additional support to the role of the flexible C-terminal region in the enzyme function [21,22], but it also provides a strong indication that Gly^{346}, a residue embodied within a hinge fragment at the C-terminus, is critical for the enzyme’s activity.

We thank Dr Vincenzo Granata for help in recording CD spectra and Jean Ann Gilder for text editing. This work was supported by grants from the Ministero dell’Istruzione, dell’Universitá e della Ricerca (MIUR, Rome, Italy) and Consiglio Nazionale delle Ricerche (CNR) Target Project “Biotecnologie”, from Regione Campania “Ricerca Sanitaria Finalizzata” and Regione Molise (POP 1994–1999), Italy.

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Received 16 December 2003/4 February 2004; accepted 6 February 2004
Published as BJ Immediate Publication 6 February 2004, DOI 10.1042/BJ20031941

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