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Structural and functional analysis of aldolase B mutants related to hereditary fructose intolerance

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Abstract Hereditary fructose intolerance (HFI) is a recessively inherited disorder of carbohydrate metabolism caused by impaired function of human liver aldolase (B isoform). 25 enzyme-impairing mutations have been identified in the aldolase B gene. We have studied the HFI-related mutant recombinant proteins W147R, A149P, A174D, L256P, N334K and Δ6ex6 in relation to aldolase B function and structure using kinetic assays and molecular graphics analysis. We found that these mutations affect aldolase B function by decreasing substrate affinity, maximal velocity and/or enzyme stability. Finally, the functional and structural analyses of the non-natural mutant Q354E provide insight into the catalytic role of Arg^{303}, whose natural mutants are associated to HFI.

Key words: Aldolase B; Hereditary fructose intolerance; Site-directed mutagenesis; Protein expression; Kinetic analysis; Homology-modeling technique

1. Introduction

Aldolase B, the hepatic isozyme of fructose-1,6-aldolase (EC 4.1.2.13), catalyzes the specific and reversible cleavage of fructose-1,6-bisphosphate (FBP) and fructose-1-phosphate (F1P) into dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate, or D-glyceraldehyde, respectively. Aldolase B is equally active with FBP and F1P, whereas aldolase A and aldolase C, the other two vertebrate isozymes, are more active with FBP than with F1P [1]. In humans, aldolase B deficiency causes hereditary fructose intolerance (HFI; OMIM 229600), a recessively inherited disorder of carbohydrate metabolism whose frequency is estimated at 1 in 20 000 live births [2]. The human aldolase B gene is 14 500 bp long; it consists of nine exons and encodes a 364 amino acid polypeptide (type B monomer). The functional enzyme is a homotetramer of about 160 kDa [1]. Missense and nonsense mutations, large and small deletions and mutations in the splicing region have been identified in the aldolase B gene of HFI patients [3–6].

The effects of missense mutations R134C [7], R303Q and R303W [8], and A337V [9] on aldolase B function have previously been investigated by in vitro expression and characterization of the recombinant enzymes [7–9]. Here we have expressed and characterized the natural mutants W147R, A149P, A174D, L256P, N334K and Δ6ex6 in an attempt to evaluate the effect of structural perturbations on the three-dimensional structure and on the consequent functional activity of the enzyme. Crystallographic studies on rabbit aldolase A showed that Arg^{303} and Glu^{354} side chains form a strong salt bridge, which may be important in the release of the enzyme product [10]. Consequently, we investigated the role played by Arg^{303} in aldolase B enzymatic activity by characterizing the Q354E mutant. We then analyzed the structural perturbations induced by this and the above-mentioned natural mutations using the recently solved structure of human aldolase B [11].

2. Materials and methods

2.1. Materials

Restriction endonucleases were purchased from New England Biolabs. *Taq* polymerase, T4 DNA ligase, isopropyl-1-thio-β-D-galactopyranoside, ampicillin, phenylmethanesulfonyl fluoride, and α-glycerol-phosphate dehydrogenase/triose phosphate isomerase (GDH/TIM) were purchased from Roche Molecular Biochemicals. Imidazole, FBP, F1P, NADH were purchased from Sigma-Aldrich. The pET-16b vector and the *Escherichia coli* strain BL21(DE3) were purchased from Novagen.

2.2. Construction and expression of mutant aldolase B

The full-length wild type human aldolase B cDNA [12] was cloned into the His-tag bacterial expression vector pET-16b thereby elongating the N-terminus of the protein by 10 histidine residues [8,9]. The pET16b/cDNA construct was used as a template to generate the aldolase B missense mutant forms (see Table 1). The QuickChange™ site-directed mutagenesis kit (Stratagene) and specific primers were used for this procedure. Mutagenesis reactions were carried out with a Perkin-Elmer 9600 thermal cycler. The parental, methylated DNA was digested with *Dpn*I, and the newly synthesized DNA was used to transform the *E. coli* XL1-blue competent cells, according to the manufacturer's instructions. Recombinant mutated plasmids were pu-

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Abbreviations: FBP, fructose-1,6-bisphosphate; F1P, fructose-1-phosphate; HFI, hereditary fructose intolerance; GDH/TIM, α-glycerol-phosphate dehydrogenase/triose phosphate isomerase

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rified over Qiagen columns (Qiagen, Valencia, CA, USA) and their full sequence verified by automated sequencing. Recombinant aldolases B were expressed in the *E. coli* B strain BL21(DE3). Wild type, A149P, and Q354E proteins were recovered in soluble form in bacteria grown at 37°C, while W147R, L256P and N334K mutants were recovered in soluble form at 22°C, 16 h after induction. Recombinant proteins corresponding to the A174D and Δ6ex6 enzymes precipitated in the *E. coli* inclusion bodies at both 37°C and 22°C. Neither urea extraction nor guanidine–HCl methods ensured correct refolding of these latter proteins.

Recombinant enzymes expressed at 37°C (wild type, A149P, and Q354E) were purified on Ni-nitriloacetic resin (Qiagen) as described elsewhere [8]. Enzymes expressed at 22°C (W147R, L256P and N334K) were purified with the same procedure [8], except that each of the purification buffers was supplemented with 20% (v/v) glycerol to allow enzyme solubilization. Fractions containing the bulk of the enzyme were pooled, dialyzed against 20 mM Tris–HCl pH 7.5, 50% (v/v) glycerol and stored at −20°C. All purification steps were carried out at 4°C. The degree of purification of each recombinant enzyme was analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.3. Molecular mass determination and denaturation profile analysis

Enzyme molecular weights were determined at 25°C by co-fractionation (fast protein liquid chromatography, FPLC) of the purified proteins with β-amylase (200 kDa), chicken ovalbumin (44 kDa), and carbonic anhydrase (29 kDa) on a 50 cm×1.6 cm Sephadex G-200 column (Amersham-Pharmacia Biotech.) in 20 mM Tris–HCl pH 7.4, 20% (v/v) glycerol, with a flow rate of 0.2 ml/min. By FPLC, we also separated the tetrameric form of the recombinant enzymes from the monomeric form, if present (Fig. 1 and Table 2). The thermal stability of the recombinant enzymes was monitored with an ultraviolet (UV) melting curve [13] and by measuring the difference of absorbance at 286 and 274 nm versus temperature in the range of 15–75°C, in 20 mM Tris–HCl pH 7.4, 20% (v/v) glycerol. The same procedure was used for the previously characterized R303Q and R303W mutants [8].

2.4. Aldolase B 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, and FBP or F1P as substrates [9]. Assays were conducted at 22°C as described previously [8]. One unit of activity corresponds to the cleavage of 1 μmol of hexose substrate/min. For each recombinant enzyme, we ran three assays with at least two different enzyme preparations and evaluated the K_m and k_{cat} from double reciprocal plots using a least-square method.

2.5. Molecular graphics analyses

We evaluated mutation-induced structural alterations by comparison with the structure of aldolase B recently refined by Littlechild and co-workers (PDB code 1QO5) [11]. The crystal form used for this crystallographic study contains 18 independent molecules in the asymmetric unit. We used the tetramer constituted by subunits A, B, C and D of the PDB entry in the present analysis, and programs PROMOTIF [14], O [15], and PROCHECK [16]. Figs. 2 and 3 were generated with MOLSCRIPT [17] and Raster3D [18].

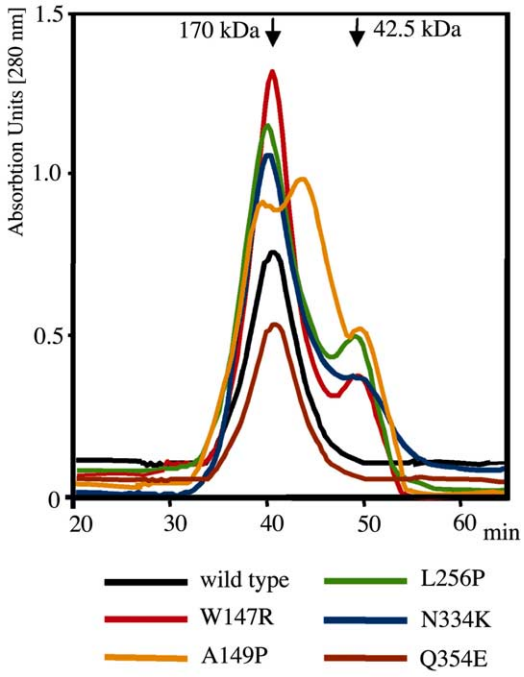

Fig. 1. FPLC profiles of the aldolase B wild type and missense mutants. The arrows indicate the molecular mass corresponding to the tetrameric (170 kDa) and monomeric form (42.5 kDa), as expected for the histidine-tagged aldolase B. Molecular masses were derived by the protein standards used in the calibration (see Section 2). Each recombinant enzyme is marked by a different color (see legend). All purified aldolase B enzymes (mutants and wild type) were eluted at 22°C.

3. Results

3.1. Expression and characterization of mutant aldolase B proteins

The aldolase B mutants studied are listed in Table 1. All the His-tagged recombinant proteins were purified to a >95% homogeneity by Ni^{2+} affinity chromatography as verified by SDS–PAGE, and each preparation, except N334K expressed at 37°C, resulted in a single band (data not shown). The wild type enzyme and Q354E, expressed at 37°C, were recovered from the soluble fraction of the bacterial lysate. After purification, about 10–12 mg of recombinant enzyme per liter of culture was obtained. A single peak corresponding to 170 kDa (tetrameric form) was obtained with FPLC. The wild type

Table 1
Positioning of aldolase B missense mutations and corresponding amino acid change within the enzyme protein

Name	Exon	Nucleotide modification	Amino acid change
R134C [7]	5	**T**GT→**C**GT	Arg^{134}→Cys
W147R [24]	5	**T**GG→**C**GG	Trp^{147}→Arg
A149P [4]	5	**G**CT→**C**CT	Ala^{149}→Pro
A174D [4]	5	GCC→G**A**C	Ala^{174}→Asp
Δ6ex6 [6]	6	delCTGGTA	del Val^{182}–Leu^{183}
L256P [25]	7	C**T**C→C**C**C	Leu^{256}→Pro
R303W [5]	8	**C**GG→**T**GG	Arg^{303}→Trp
R303Q [8]	8	CGG→C**A**G	Arg^{303}→Gln
N334K [26]	9	AA**C**→AA**G**	Asn^{334}→Lys
A337V [9]	9	GCG→G**T**G	Ala^{337}→Val
Q354E[a]	9	**C**AG→**G**AG	Gln^{354}→Glu

[a]Not a naturally occurring HFI mutation.

Table 2
Enzyme activity parameters of various recombinant human aldolase B mutants

Enzyme	Specific activity (μmol min^{-1} mg^{-1})		Specific activity (ratio FBP/F1P)	T_m (°C)
	FBP	F1P		
Wild type	0.97 ± 0.05	0.94 ± 0.02	1.032	47.0
W147R	0.64 ± 0.06	0.38 ± 0.08	1.72	40.0
A149P	N.D.	N.D.	N.D.	37.5
L256P	0.81 ± 0.04	0.58 ± 0.14	1.3	40.0
N334K	0.28 ± 0.07	0.13 ± 0.01	1.05	45.0
Q354E	0.86 ± 0.02	0.88 ± 0.06	1	46.5
R303W[a]	0.10 ± 0.04	N.D.	N.D.	46.5
R303Q[a]	0.47 ± 0.10	0.25 ± 0.1	2.44	49.0

[a]Data from [8].

enzyme and Q354E remained stable at −20°C in a 50% (v/v) glycerol solution for at least 1 month.

W147R and L256P were recovered from the soluble fraction of the bacterial lysate only after growth at 22°C and with 20% (v/v) glycerol. In fact, both mutants completely precipitated in the *E. coli* inclusion bodies when bacteria grew at 37°C. Furthermore, the addition of glycerol to the purification buffer was necessary for enzyme solubilization. The purification yield ranged from 2 to 5 mg of recombinant proteins per liter of culture: most of the enzyme remained in the bacteria inclusion bodies. In addition to the principal peak corresponding to the tetrameric form of the enzyme, a minor peak corresponding to the 42.5-kDa monomeric form of aldolase B appeared with these two mutants after FPLC (Fig. 1). Fractions containing monomers did not have catalytic activity.

Table 2 lists the specific activity of the wild type and the other mutant enzymes and their thermal stability determined as T_m. Table 3 shows the kinetic profile of the purified recombinant enzymes. As expected for HFI-causing enzymes, all the mutant aldolases had reduced catalytic efficiency versus the substrates.

The kinetics of Q354E resembled that of the wild type enzyme except for a slight variation in K_m versus both FBP and F1P; the specific activity ratio and thermal stability were as the wild type. However, there was a global functional effect with a fold variance of 4 and 2.6 versus wild type catalytic efficiency, for FBP and F1P respectively (last two columns in Table 3).

With the W147R tetramer there was a 3.25- and 2-fold variance of catalytic efficiency with respect to the wild type, for FBP and F1P respectively (see Table 3). A similar effect (fold variance 3.88 and 1.30 for FBP and F1P) was observed with the L256P mutation. Melting temperature was 40°C for enzymes W147R and L256P versus 47°C for the wild type (Table 2). Furthermore, these mutants aggregated after 15 days of storage at −20°C in a 50% glycerol solution, suggesting structural instability.

N334K was recovered from the soluble fraction of the lysate after bacterial growth at 37°C and 22°C. SDS–PAGE analysis of the recombinant enzyme expressed at 37°C showed, in addition to the main 40-kDa band, two minor bands of lower molecular mass that were not observed when the enzyme was expressed at 22°C (data not shown). A similar finding has been reported for the V337A recombinant enzyme [9]. Like the other mutant recombinant enzymes that we expressed at 22°C, also the N334K mutant expressed at 22°C showed two FPLC peaks corresponding to the tetrameric and monomeric forms (Fig. 1). Melting temperature was the same as the wild type. When stored at −20°C in 50% glycerol, N334K was stable for about 1 month. There was a fold variance of 37.1 and 8.8 for FBP and F1P respectively (Table 3).

The A149P enzyme was soluble in bacteria grown at 37°C. After purification, FPLC analysis of the A149P aldolase showed, together with tetrameric and monomeric forms, other species with molecular masses intermediate between those corresponding to the tetramer and monomer. Its T_m of 37.5°C was the lowest measured among all other mutated and wild type proteins. This enzyme did not show any activity for either FBP or F1P.

Finally, recombinant aldolase B enzymes corresponding to A174D and Δ6ex6 mutants were retained in the *E. coli* inclusion bodies after growth at 37°C or 22°C.

3.2. Molecular graphics analyses

Structurally, the aldolase B missense mutations we have

Table 3
Kinetic parameters of various recombinant human aldolase B mutants

Enzyme	K_m		k_{cat} (s^{-1})		Catalytic efficiency			
	FBP	F1P	FBP	F1P	k_{cat}/K_m		Decrease in k_{cat}/K_m	
					FBP	F1P	FBP	F1P
Wild type	2.3 ± 0.3 μM	2.2 ± 0.2 mM	0.61 ± 0.03	0.67 ± 0.03	0.26 ± 0.01	0.3 ± 0.002	–	–
W147R	5.5 ± 0.7 μM	1.70 ± 0.14 mM	0.43 ± 0.04	0.25 ± 0.05	0.077 ± 0.005	0.16 ± 0.03	3.25	2
L256P	7.3 ± 0.6 μM	1.73 ± 0.25 mM	0.54 ± 0.03	0.38 ± 0.09	0.077 ± 0.004	0.23 ± 0.04	3.88	1.30
N334K	32.4 ± 2.0 μM	22.0 ± 3.0 mM	0.19 ± 0.05	0.085 ± 0.005	0.006 ± 0.001	0.004 ± 0.001	37.1	8.8
Q354E	9.1 ± 0.45 μM	5.0 ± 0.43 mM	0.57 ± 0.01	0.59 ± 0.04	0.064 ± 0.004	0.116 ± 0.001	4.0	2.6
R303W[a]	1.6 ± 0.6 mM	N.D.	0.08 ± 0.02	N.D.	$(5.2 \pm 0.7) \times 10^{-5}$	N.D.	4800	N.D.
R303Q[a]	147 ± 15 μM	57.5 ± 11 mM	0.39 ± 0.04	0.15 ± 0.02	$(2.7 \pm 0.2) \times 10^{-3}$	$(2.6 \pm 0.2) \times 10^{-3}$	93	119

[a]Data from [8].

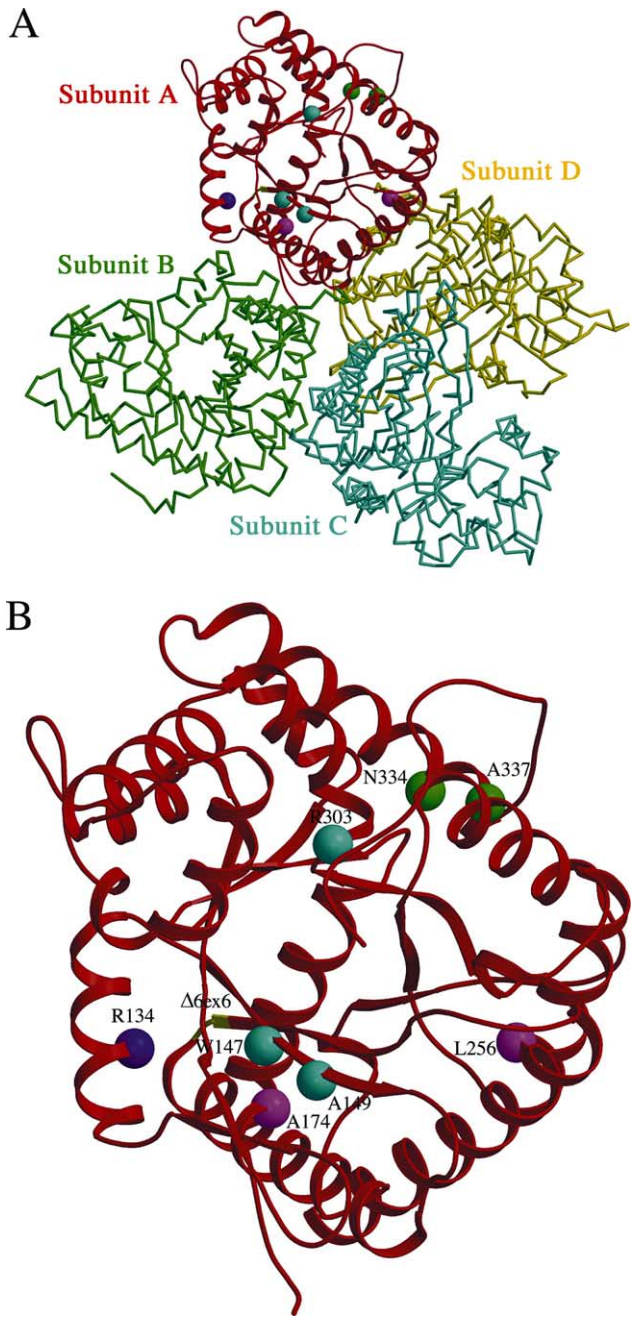

Fig. 2. Distribution of HFI-related missense mutations in the aldolase B structure. A: The mutation sites are marked as balls only in the subunit A of the homotetramer. B: Close-up of subunit A structure. The color code for the mutation sites is: cyan for the active site, purple for the tetramer interface, green for the C-terminus and blue for the 134 site. The deletion Δ6ex6 site is also shown (in yellow).

studied fall into three types (Fig. 2A, B). Firstly, Trp^{147} and Ala^{149}, together with the previously analyzed Arg^{303} [8], belong to the active site of the enzyme (active site mutations). Secondly, Ala^{174} and Leu^{256} belong to helices involved in tetramer interface (interface mutations); however, these residues are not directly involved in interactions with residues of other subunits because their side chains protrude inwards. Thirdly, Asn^{334}, like the previously characterized Val^{337} (C-terminal mutations) [9], is located near the C-terminal region, which plays an important catalytic role [10]. Interestingly, all these mutation sites involve residues belonging to secondary structure elements of aldolase B. Indeed, Trp^{147} and Ala^{149} belong to β-strands, and Ala^{174}, Leu^{256} and Asn^{334} belong to α-helices. This analysis shows that these mutations alter the structural and the functional integrity of the enzyme by means of a variety of mechanisms. In particular, enzyme destabilization may be ascribed to: (a) the introduction of a polar or charged residue into a hydrophobic environment (W147R and A174D); (b) the substitution of small with bulkier side chains (N334R); and (c) the insertion of proline residues in α-helices or β-strands (L256P and A149P).

4. Discussion

Here we report the biochemical characterization of several aldolase B mutations related to HFI. In all cases, the catalytic efficiency of the mutated enzymes was significantly reduced compared with the wild type. Bacterial growth at 22°C is necessary for the expression and purification, in soluble form, of mutants W147R, L256P, and N334K: under these conditions both tetrameric and inactive monomeric forms were recovered. W147R and L256P tetramers were partially active and differed slightly from the wild type in K_m and specific activity towards FBP and F1P (Table 2). Also the N334K tetramer was partially active, and had a more pronounced effect than W147R and L256P on specific activity and K_m for both substrates. Functional measurements suggested that W147R and L256P have little effect on substrate binding, but they significantly reduce catalytic efficiency (Table 3). These two mutations probably affect the enzyme folding process since we were unable to recover them in soluble form from bacteria grown at physiological temperature. It is noteworthy that most of the produced enzyme remained in the *E. coli* inclusion bodies also after growth at 22°C.

The fact that we could not recover a soluble form of A174D and Δ6ex6 may indicate that the structural perturbations produced by the mutations affect the overall integrity of the enzyme. It is noteworthy that also Cox and co-workers, in an independent characterization carried out in slightly different conditions of some of the mutants (W147R, A149P, A174D, L256P and N334K), failed to recover the A174D enzyme [19]. Ala^{174} is located on the apolar side of an amphipatic helix. Therefore, it is not surprising that the insertion of a charged and bulkier side chain in this environment affects the overall structure of the protein.

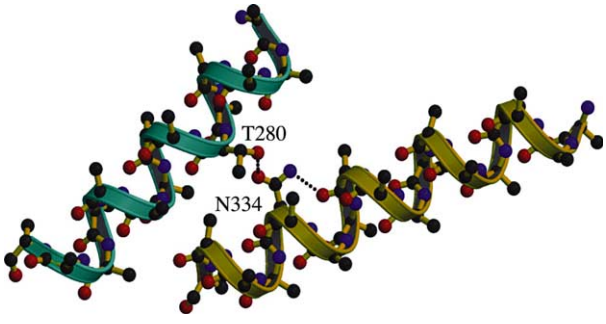

Fig. 3. Intra- and inter-helical interactions of the Asn^{334} side chain in the aldolase B structure. The $O^{\delta 1}$ and $N^{\delta 2}$ atoms of Asn^{334} form hydrogen bonds with Thr^{280} O^{γ} and Arg^{330} O atoms, respectively [13]. For clarity, only the side chains of Asn^{334} and Thr^{280} are shown.

The alterations induced by the other aldolase B mutations appear to have a less dramatic effect on the enzyme structure. As reported by Cox and co-workers [19], W147R, A149P, L256P and N334K affect the integrity of the tetrameric structure of aldolase B even though most of them (W147R, A149P and N334K) are distant from the tetramer interface.

In agreement with Cox and co-workers [19] we found that all monomeric forms of aldolase B are inactive. Differently, monomeric aldolase A mutants were reported to retain significant activity [20]. Consequently, some functional aspects of these two enzymes may be strikingly different despite the high sequence homology (69%). In contrast with Cox and co-workers [19] we obtained a rather stable mutant N334K. However, the activity of this mutated enzyme was significantly reduced. As suggested by Dalby et al. [11], this may be ascribed to the structural destabilization of the enzyme caused by the loss of a hydrogen bond of the Asn^{334} side chain with the Thr^{280} side chain (Fig. 3). It is noteworthy that this interaction is preserved in aldolase A, even though in this protein Thr^{280} is replaced by a Ser.

Although recovered as a soluble tetramer, we were unable to detect any activity for the active site mutant A149P after growth at either 37°C or 22°C – a finding probably due to the tendency of this mutant to aggregate rapidly [19]. The high instability of A149P is supported by the low T_m (Table 2) and by the FPLC forms with a molecular mass intermediate between those of tetramer and monomer. Interestingly, the other active site mutant (W147R) retains a significant portion of wild type aldolase B activity. Taken together, these data indicate that the perturbation of enzyme function produced by the insertion of proline in the β-sheet is greater than that induced by a dramatic variation of the size and the charge of the side chain in position 147.

Studies of aldolase A showed that the Arg^{303} mutation interacts in the various catalytic steps with either the C6 or the C1 moiety of FBP [21–23]. In addition, Arg^{303} forms a salt bridge with Glu^{354} [10], which is located in the flexible C-terminal region of aldolase A. This finding suggests that Arg^{303} promotes the C-terminus conformation which facilitates substrate release. The finding that Glu^{354} is not conserved in aldolase B or in other class I aldolases, suggested that this interaction plays an insignificant role in these enzymes [8]. To address this aspect, we characterized the aldolase B mutant Q354E and found that the k_{cat} is practically unaffected by the mutation. The small increase in K_m may be ascribed to the extra negative charge of the enzyme. Accordingly, the increase in K_m was greater for FBP than for F1P, which carries a smaller negative charge. These findings show that the formation of a salt bridge 303–354, which is believed to be tucked in the C-terminus in aldolase A, does not significantly affect the catalytic properties of aldolase B.

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