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Expression, Purification, and Characterization of Natural Mutants of Human Aldolase B

ROLE OF QUATERNARY STRUCTURE IN CATALYSIS*

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Fructaldolases (EC 4.1.2.13) are ancient enzymes of glycolysis that catalyze the reversible cleavage of phosphofructose esters into cognate triose (phosphates). Three vertebrate isozymes of Class I aldolase have arisen by gene duplication and display distinct activity profiles with fructose 1,6-bisphosphate and with fructose 1-phosphate. We describe the biochemical and biophysical characterization of seven natural human aldolase B variants, identified in patients suffering from hereditary fructose intolerance and expressed as recombinant proteins in *E. coli*, from which they were purified to homogeneity. The mutant aldolases were all missense variants and could be classified into two principal groups: *catalytic* mutants, with retained tetrameric structure but altered kinetic properties (W147R, R303W, and A337V), and *structural* mutants, in which the homotetramers readily dissociate into subunits with greatly impaired enzymatic activity (A149P, A174D, L256P, and N334K). Investigation of these two classes of mutant enzyme suggests that the integrity of the quaternary structure of aldolase B is critical for maintaining its full catalytic function.

Catalytic deficiency of the hepatic isozyme of fructose-1,6-aldolase (EC 4.1.2.13) (aldolase B) causes the disease, hereditary fructose intolerance (online Mendelian Inheritance in Man (OMIM) catalogue no. 22960) (1–3). This inborn error of metabolism is transmitted as an autosomal recessive character and occurs with a frequency of at least 1 in 20,000 live births (4). The condition is characterized by severe abdominal symptoms and a metabolic disturbance, including hypoglycemia, that follows ingestion of fructose and related sugars. During challenge with fructose, deficiency of fructose 1-phosphate-splitting activity attributable to liver aldolase leads to inhibition of glycogenolysis and gluconeogenesis, as well as nucleotide breakdown (2, 3, 5). Aldolase B, unlike aldolase A, the constitutive glycolytic isozyme, has equal activity toward fructose 1-phosphate and fructose 1,6-bisphosphate (6).

Molecular analysis of the aldolase B gene in patients suffering from hereditary fructose intolerance has identified multiple defects, including at least eight missense mutations, that segregate with the disease phenotype in affected pedigrees and are of diagnostic significance (2, 3, 7). Because these natural variants are associated with disturbed catalytic activity of the aldolase B molecule, they are likely to be informative about the identity of key residues that contribute to its functional integrity. We report here the overexpression, purification, and novel properties of seven natural human aldolase B variants and discuss their characteristics in relation to the emerging high resolution crystal structures of their mammalian homologues.

EXPERIMENTAL PROCEDURES

Materials

DNA restriction endonucleases were purchased from Roche Molecular Biochemicals or New England Biolabs. *Taq* DNA polymerase was obtained from Promega. *Pfu* DNA polymerase and the *Escherichia coli* strain BL21(DE3) were obtained from Stratagene. The protease inhibitors phenylmethylsulphonylfluoride, leupeptin, and pepstatin A and the substrate fructose 1,6-bisphosphate were purchased from Roche Molecular Biochemicals. The reagents for dye terminator sequencing were purchased from Amersham Pharmacia Biotech. The *E. coli* strain INVαF+ was purchased from Invitrogen. The pET-21(+) expression vector was purchased from NBL Gene Sciences (Northumberland, United Kingdom). Ampicillin, isopropyl-1-thio-β-D-galactopyranoside, and the aldolase substrates fructose 1-phosphate, dihydroxyacetone phosphate, and D-glyceraldehyde, as well as other chemicals and reagents, were from Sigma. Blue Sepharose CL-6B, DEAE-Sepharose and CM-Sepharose, mono-S, desalting PD-10, and Superose 12 columns and resins were obtained from Amersham Pharmacia Biotech.

Construction of Aldolase B Overexpression Clones and Mutagenesis

The aldolase B cDNA gene from plasmid pΔXB was amplified by polymerase chain reaction so that a unique *Hin*dIII site was introduced downstream of the stop codon of the structural genes, thereby removing the polyadenylation signal by incorporating the primer HP (5′-GGAGCTAAGCTTGCGGGCA-3′) (8). A unique *Eco*RI site was introduced 18 bases upstream of the first codon of the aldolase B structural gene by polymerase chain reaction using the primer ER1 (5′-CGGATAACGAATTCATAAGGAGGAAACAG-3′). Incorporation of this primer also resulted in the introduction of a consensus *E. coli* ribosome binding site with an ideal spacing of 9 bases immediately preceding the start codon of the structural gene. The amplified DNA was digested with the restriction enzymes *Hin*dIII and *Eco*RI and ligated into the T7 DNA polymerase expression plasmid pET21(+) generating the plasmid pEBR+ (8).

Mutations were introduced into the structural gene using the "two primer" method developed by Stratagene Inc. Overlapping mutagenic primers were hybridized onto the pEBR+ plasmid DNA, and mutagenesis was performed with 16 cycles of polymerase chain reaction under conditions of 30 s at 95 °C, 1 min at 55 °C, and 13.5 min at 68 °C. The amplified DNA was digested with 10 units of *Dpn*I and transformed without further treatment. Initial mutagenesis trials indicated that >95% of transformants contained the mutation as detected by colony hybridization analysis (data not shown).

The mutations that were introduced into the aldolase B expression system were originally discovered by us in the screening of patients with hereditary fructose intolerance. These natural human aldolase B missense variants are W147R (9), A149P (7), A174D (10), L256P (11), R303W (12, 13), N334K (14), and A337V (8). The corresponding primers used for the site specific mutagenesis and the resultant mutant plasmid

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constructs are as follows: GTTGACTTTGGGAAGCGGCGTGCTGTGCTG and CAGCACAGCACGCCGCTTCCCAAAGTCAAC for pEBR147R (W147R), CACCGTAACAGCTCCCCACCGTACTGTTC and GAACAGTACGGTGGGGAGCTGTTACGGTG for pEBR256P (L256P), GTTTCTCTTATGGATGGGCCCTGCAGGCC and GGCCTGCAGGGCCCATCCATAAGAGAAAC for pEBR303W (R303W), and CGGGCCATGGCTAAGTGCCAGGCGGCCAA and TTGGCCGCCTGGCACTTAGCCATGGCCCG for pEBR334K (N334K).

The generation of the A337V expression clone pEBR337V has recently been reported (8). The A149P expression clone pEBR149P was generated by subcloning the cDNA from a polymerase chain reaction-amplified fragment bearing the A149P mutation from the plasmid pA149P (a pΔXB derivative) using the HP and ER1 primers into pET21+. The sequence of plasmid constructs was verified by dideoxy nucleotide sequencing using the dye terminator method (Applied Biosystems Inc.).

Purification of Recombinant Wild-type and Mutant Aldolases

Preparation of Lysates—Expression of recombinant human aldolases was carried out in the protease-deficient *E. coli* host strain, BL21(DE3), as previously reported (8). 250–500-ml cultures of mutant and wild-type aldolase B bacterial clones were grown in 2YT ampicillin media inoculated from overnight cultures that were grown in this medium supplemented with 0.2% (w/v) glucose. The cells were grown to mid-log phase at either 37 °C (wild-type, W147R, and R303W) or 23 °C (other mutant proteins), and expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 1 mM. Cells were chilled on ice following a further 2.5 h of growth at 37 °C or approximately 16 h if grown at 23 °C. All subsequent steps were rapidly conducted at 4 °C where possible. The bacteria were harvested by centrifugation and lysed with a mortar and pestle, with alumina added at twice the estimated wet weight of cells. The bacteria were ground for 6–8 periods of 1 min with the addition of DNase and RNase (1 mg/ml) for the last three grindings. The lysed material and alumina mixture was resuspended in 15 ml of 50 mM Tris, 1 mM EDTA, pH 7.6, 10 mM $MgCl_2$, 5% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml pepstatin, and 20 mg/ml leupeptin A, and the alumina was removed by centrifugation at 3000 × *g*.

Bacterial cultures expressing N334K and A337V aldolase variants were lysed by sonication at 15 μm amplitude in the previously described lysis buffer. The lysates were cleared by centrifugation at 30,000 × *g* for 1 h at 4 °C for all samples. Lysates containing recombinant wild-type aldolase B and the W147R and R303W mutant aldolases were acid-precipitated by the addition of cold acetic acid to a final pH of about 5.9 and incubated on ice for 15 min. Cell debris rendered insoluble by acid precipitation was removed by centrifugation at 20,000 × *g* for 20 min at 4 °C. The recombinant proteins A149P, A174D, L256P, N334K, and A337V were found to be unstable, and it was necessary to develop a rapid purification procedure for the isolation: these recombinant proteins were therefore not precipitated under acidic conditions before chromatographic separation.

Chromatography of Recombinant Wild-type, W147R, A174D, L256P, and R303W Aldolase—DEAE-Sepharose equilibrated with 10 mM Tris·HCl, 1 mM EDTA, pH 7.5, was used as a primary separation step to remove soluble acidic *E. coli* proteins immediately after generation of cleared bacterial lysates: under these conditions, the recombinant aldolases were not bound to the matrix. Partially purified wild-type and all recombinant mutant proteins were dialyzed against 10 mM MOPS,[1] pH 7.0, where the first dialysis buffer included 20 mM NaCl. The dialysate was applied to CM-Sepharose matrix in columns equilibrated with 10 mM MOPS, pH 7.0. The matrix was washed with 10 mM MOPS, pH 7.9, and aldolase protein was eluted with 2 mM fructose 1,6-bisphosphate (FBP) in the same buffer; affinity elution was found to release aldolase protein at greater than 90% purity. The recombinant aldolase R303W partly eluted at a pH of 7.9 without addition of FBP. All samples of purified recombinant human aldolase were rapidly concentrated by ultrafiltration in the presence of 100 mM NaCl using a Millipore ultrafiltration device (30-kDa exclusion). The protein concentrates were finally fractionated on a Superose 12 fast protein liquid chromatography gel exclusion column in 50 mM Tris·HCl, pH 7.6, 100 mM NaCl. Recombinant wild-type and mutant proteins were recovered at a region corresponding to a molecular mass of ~150 kDa as predicted for homotetrameric class I aldolases.

The recombinant proteins were obtained at greater than 98% purity as indicated by SDS-polyacrylamide gel electrophoresis. They were stored in 50 mM Tris·HCl, 10 mM EDTA, pH 7.6, 100 mM NaCl, 1 mM dithiothreitol on ice, and under these conditions, most remained stable for several days. The wild-type, W147R, and R303W proteins remained stable while stored unfrozen for several months on ice.

The A174D protein could not be recovered in an active form and bound very weakly to the CM-Sepharose matrix following DEAE-Sepharose passage and equilibration against 10 mM MOPS, pH 7.0. The A174D protein was found to be extremely labile and could be stabilized neither by the presence of 1 mM fructose 1,6-diphosphate, reducing conditions, high ionic strength, nor by being bound either by cation exchange or Blue Sepharose matrices. The protein aggregated rapidly, even when dilute. Unlike the other recombinant aldolase variants, this mutant protein was found to be at low abundance in the host bacterial cells, suggesting that it is rapidly degraded *in situ* as a result of denaturation.

Chromatography of Recombinant N334K and A337V Aldolases B—Bacterial lysates containing the expressed N334K and A337V aldolase variants were passaged through DEAE-Sepharose and the eluate was immediately fractionated on Cibacron Blue Sepharose CL-6B affinity matrix (instead of CM-Sepharose). The Blue Sepharose matrix was washed with 50 mM Tris·HCl, 10 mM EDTA, pH 7.6, buffer, and the recombinant proteins were affinity eluted with a pulse of 2–5 mM fructose 1,6-bisphosphate in 50 mM Tris, 5 mM EDTA, pH 8.6, to effect purification by affinity chromatography. The A337V mutant was exchanged immediately after the affinity elution step from the Blue Sepharose affinity matrix into 50 mM MOPS buffer solution at pH 7.0 and applied to an fast protein liquid chromatography mono-S Sepharose column. The mutant protein was eluted between 50 and 100 mM NaCl.

Assays of Enzyme Activity—The original tetrameric isolates of the recombinant mutant aldolases were used for all subsequent determinations of enzymatic activity. Aldolase cleavage activity toward the substrates fructose 1-phosphate and fructose 1,6-bisphosphate was assayed by the spectrophotometric measurement of NADH oxidation at 340 nm by the method of Racker (15). Enzymatic assays were conducted at 22 °C in a final volume of 1 ml by the addition of aldolase protein to reaction mixtures containing substrate, 0.15 mM NADH, 50 mM Tris-acetate, 10 mM EDTA, pH 7.6, 100 mg/ml bovine serum albumin, and 20 μl of α-glycerophosphate dehydrogenase/triose phosphate isomerase (2 mg/ml). The reactions were monitored for 4 min following an initial 1-min lag phase to ensure linearity. Protein concentration of pure preparations of aldolase was determined spectroscopically by determining the optical absorbance at 280 nm and by using the BCA protein determining method (Pierce) and the DC method (Bio-Rad). All cleavage specific activity and K_m assays were done within 1 day of purification. The kinetic estimates were determined by least squares analysis of at least two experiments from two purifications using the Leonora program (16).

Fructose 1-phosphate synthesis activities at 22 °C were measured by end point assays from 0 up to 20 min, sampling at 1–5-min intervals, in the previous Tris-acetate buffer containing 100 mg of bovine serum albumin and 45 mM each of D-glyceraldehyde and dihydroxyacetone phosphate. All mutants demonstrated linear kinetics in the synthesis reaction over this time period (data not shown). Fructose 1-phosphate generated in the synthesis was quantified by the method of Berthiaume *et al.* (17). Absorbances were read at 380 and 412 nm, and the differences obtained between A_{412} and A_{380} were compared with a standard curve using dilutions of fructose 1-phosphate and appropriate blank solutions.

Generation of the carbanion intermediates in the aldolase reaction was determined by monitoring the formation of carbanion-enamine enzyme-substrate intermediates when oxidized in the presence of hexacyanoferrate (III) to yield hydroxypyruvaldehyde phosphate (18). The reaction was monitored at 420 nm using 50 mg/ml aldolase protein in 200 mM Tris-glycine, pH 7.3, 1.43 mM $K_3Fe(CN)_6$, 100 mg/ml bovine serum albumin, and 1 mM of either fructose 1,6-diphosphate or dihydroxyacetone-P or 50 mM fructose 1-phosphate. The initial rate of the reaction over 24 s was used to determine the rate of reduction for the preparations of mutant aldolases B; generation of carbanions by wild-type aldolase B was followed at the initial reaction rate for 18 s. The amount of hexacyanoferrate (III) reduced to hexacyanoferrous ion (II) was determined by calibrating the optical absorbance of diluted standard solutions of potassium ferrocyanide at 420 nm.

[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; F-1-P, fructose 1-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate.

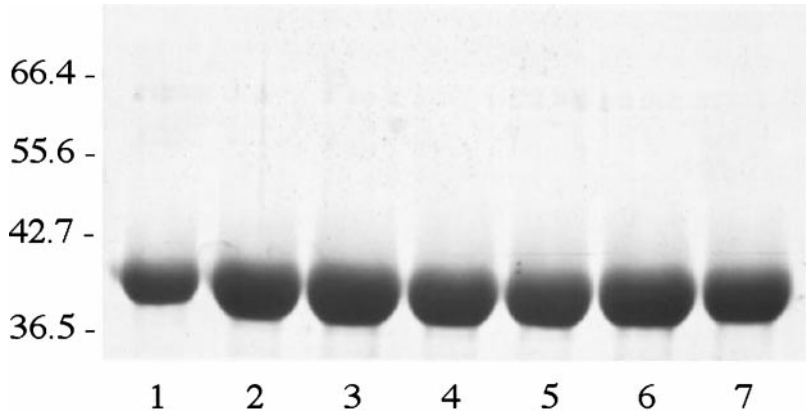

FIG. 1. **SDS-polyacrylamide gel electrophoresis of the recombinant wild-type and natural variants of human aldolase B expressed as recombinant proteins in *E. coli*.** Recombinant purified aldolases B stained with Coomassie Blue G250 after separation on a SDS-polyacrylamide gel electrophoresis gel under denaturing conditions using 10% (w/v) resloving gel. The positions of molecular mass markers are indicated in kDa at the *left*. *Lane 1,* Pro[149]; *lane 2,* Arg [147]; *lane 3,* Pro[256]; *lane 4,* Arg[303]; *lane 5,* Lys[334]; *lane 6,* Val[337]; *lane 7,* wild-type. Pro[149], Pro[256], Lys[334], and Val[337] were expressed at 22 °C. The recombinant wild-type, Arg[147], and Arg[303] variants were expressed at 37 °C. All proteins were purified by affinity elution and gel filtration as described under "Experimental Procedures."

RESULTS AND DISCUSSION

Overexpression and Purification of Human Aldolases B—As judged by SDS-gel electrophoresis and densitometric scanning, the recombinant wild-type and mutant proteins were all expressed to about 30% of soluble cellular protein levels in *E. coli* after induction for 2.5 h at 37 °C (16 h induction at 22 °C for A149P, A174D, L256P, N334K, and A337V). The purity of the recombinant proteins was close to homogeneity as detected by SDS-polyacrylamide gel electrophoresis (Fig. 1). When analyzed by gel exclusion chromatography on Superose-12, the wild-type, W147R, R303W, and A337V enzymes had molecular masses of about 158 kDa, which corresponds to the native tetrameric molecular mass of the aldolase B purified from human liver (19) (Fig. 2). The mutant protein L256P eluted from the molecular exclusion column as two peaks with molecular masses estimated to be 158 and 60–40 kDa, indicating that the mutant tetramer partly dissociated into its constituent subunits during purification and storage. The A149P and N334K aldolase variants were isolated originally as a polydisperse mixture of protein conformers that encompassed all molecular sizes between tetramer and monomer. The mutant proteins A149P, L256P, N334K, and A337V dissociated to produce monomeric proteins after several days of storage (Fig. 2*B*).

Stability of Purified Aldolase B Variants—In this study, aldolase activity was used as a measure of enzyme integrity. Once purified, recombinant wild-type human aldolase B was stable and maintained a high specific activity that declined slowly by about 50% after 3 months storage. Over this period, a progressive increase in the Michaelis constant for both hexose substrates and in the hydrodynamic radius of the protein was observed. In contrast, the mutant aldolases all had accelerated loss of activity and reduced stability (Table I). The aldolase mutants were not stabilized by the presence of substrate (FBP), reducing agents, high ionic strength, or binding to cation exchange or affinity dye resins. Once dissociated, the mutant aldolases aggregated freely, even when dilute. For instance, the mutant A149P aldolase when purified as a monomer aggregated rapidly as shown by its increasing hydrodynamic radius in solution; within 30 min of sampling, its molecular weight exceeded the technical limit of estimation (Fig. 3).

The recombinant A174D, L256P, and N334K aldolases proved difficult to recover when expressed at 37 °C, suggesting that they undergo rapid proteolysis when expressed in *E. coli*. It is likely that they are partly denatured *in situ* at this temperature, thus precluding formation of stable tetramers, because correct subunit folding will be critical for tetrameric assembly and cohesion. Accordingly, subsequent purification of the recombinant A149P, A174D, L256P, and N334K aldolases were carried out after expression at 22–23 °C. The induction period was extended to 16 h, and all subsequent purification procedures were carried out rapidly and at temperatures below 10 °C.

Thermostability Studies of Recombinant Proteins—A model of the rabbit aldolase B subunit structure determined from the high order crystal structure onto which the disease mutations have been mapped is presented in Fig. 4.[2] On the basis of their behavior during purification, we assigned the mutant aldolases to two classes: catalytic mutants that can be expressed in an intact tetrameric form that retained activity at 37 °C (W147R and R303W) and structural mutants that generate catalytically active enzymes only after expression at 22–23 °C (A149P, L256P, and N334K). The A337V variant was intermediate in character because it can be expressed as a tetramer with residual activity at 37 °C but appears more prone to proteolysis in *E. coli* at this temperature (8).

The wild-type and all mutant proteins except for A149P and L256P had similar thermostability profiles (Table I). All of the mutants except A149P and L256P had $t_{1/2}$ temperatures close to 50 °C, similar to the native human liver aldolase B (19). That thermal inactivation was associated with denaturation was evident from the precipitates that formed at the $t_{1/2}$ temperature for all the proteins examined. The mutant A149P and L256P proteins were particularly prone to thermal inactivation, as demonstrated by their reduced $t_{1/2}$ temperatures of 25 °C and 45 °C, respectively. This temperature sensitivity indicates that the structural integrity of these proteins is profoundly disrupted. A149P affects an internal residue in the active site region and probably distorts each subunit of the tetramer. This subunit distortion is likely to be communicated to the subunit contact sites by long range conformational changes; unfavorable subunit contacts thus formed would predispose the protein to dissociation and inactivation. This was demonstrated by the tendency of the A149P variant to aggregate when expressed at 37 °C, a temperature at which the purified protein was found to be unstable *in vitro* (Fig. 3).

The L256P mutation affects the interface between the aldolase subunits; it thereby impairs assembly of the stable tetrameric molecule. It was notable that this protein variant showed a slight decrease in thermal stability, indicating that

[2] N. S. Blom, A. White, and J. Sygusch, manuscript in preparation.

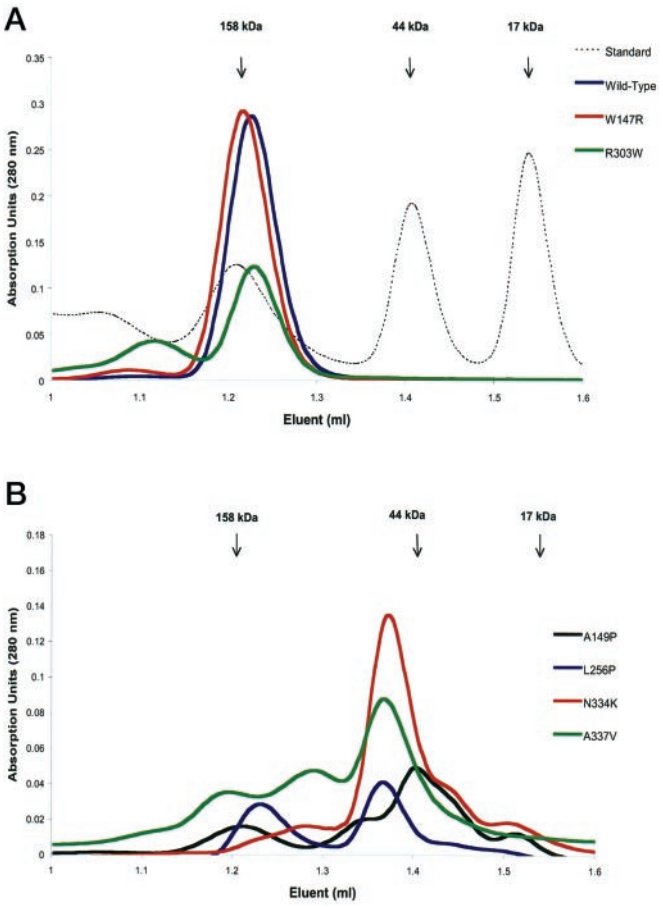

FIG. 2. **Molecular exclusion chromatography of recombinant human aldolase B and natural human variants.** Chromatograms of recombinant aldolase B and natural variants were obtained by fractionating protein samples 2 days after initial purification through a Superose 12 gel exclusion column using an Amersham Pharmacia Biotech SMART™ system. The protein was resolved by elution at 22 °C in 50 mM Tris·HCl, 100 mM NaCl. *Panel A* depicts the elution profile of wild-type and mutants that maintained a tetrameric quaternary structure after 2 days of incubation in 50 mM Tris·HCl, 100 mM NaCl. The molecular masses of the protein standards used in the calibration are as follows: bovine γ globulin, 158 kDa; chicken ovalbumin, 44 kDa; equine myoglobin; 17 kDa. *Panel B* depicts the elution profile of mutants that dissociated into monomeric subunits after 2 days of storage at 4 °C.

TABLE I
Properties of recombinant aldolase B variants

Protein	Isolated molecular form[a]	Long term stability[b]	Activities of monomers[c]	$t_{1/2}$[d]
			%	°C
Wild-type	T	~3 months	<5	50
W147R	T	~3 months	<5	53
A149P	T and M	~2 days	<5	25
L256P	T and M	~2 days	<5	45
R303W	T	~3 months	<5	52
N334K	T and M	~30 min	ND[e]	50
A337V	T	~2 weeks	<5	50

[a] Tetramer (T) or monomer (M) when expressed at 37 °C for wild-type, W147R, and R303W and 22 °C for all other mutants.
[b] As stored in 50 mM Tris·HCl, pH 7.6, 100 mM NaCl at 4 °C.
[c] As compared with wild-type.
[d] Temperature at which half activity is retained after 15 min of incubation.
[e] ND, not determined.

the integrity of the tetramer contributes to the overall protein stability (21). Surprisingly, the thermal stability profile of the N334K mutant aldolase B did not greatly differ from wild-type, even though it could only be purified after expression at the

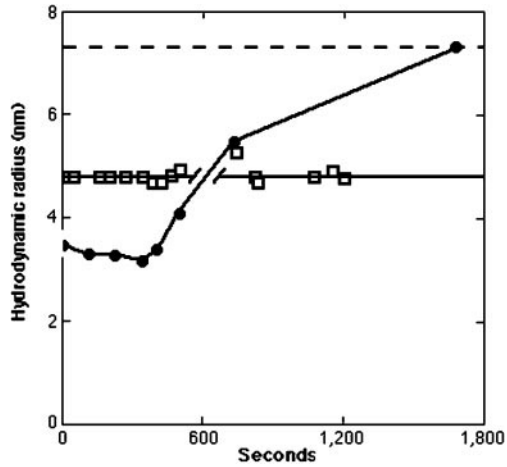

FIG. 3. **Dynamic light scattering analysis of aldolase B aggregation.** Dynamic light scattering measurements of recombinant wild-type and A149P aldolase B were made using a Dyna Pro-801 TC Molecular sizing instrument using approximately 150 μl of 1 mg/ml protein solutions in 50 mM Tris·HCl, pH 7.6, 100 mM NaCl. Consecutive readings of samples were taken at 22 °C. Recombinant A149P generated at 37 °C was isolated as a monomeric subunit that aggregated to complex multimeric mixtures within 30 min of sampling, as shown. ●, A149P variant; □, recombinant wild-type human aldolase B. The *dashed line* represents the upper limit of size detection as estimated by monochromatic light scattering.

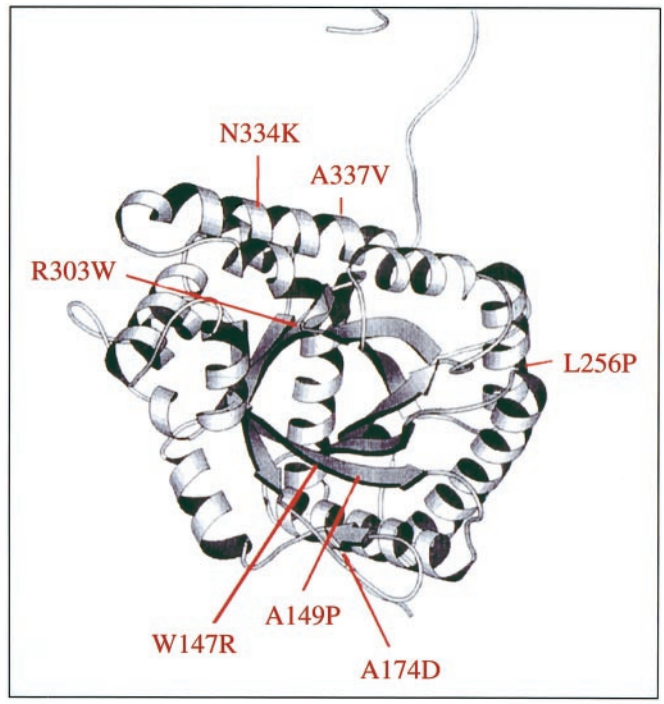

FIG. 4. **Model of the rabbit liver aldolase structure depicting the location of mutated residues in natural variants of human aldolase B.** This model represents the rabbit liver aldolase B subunit using the program Rasmol (20); the coordinates were derived by Blom *et al.*[2] The relative positions of the mutated residues in the natural human missense variants of aldolase B are indicated.

lower temperature. It is likely this protein variant forms two distinct conformers at 22 and at 37 °C and that the conformer generated at physiological temperatures may be partially unfolded while still maintaining sufficient structural integrity to retain catalytic activity. The N334K conformer produced at 37 °C is likely to be more vulnerable to proteolytic cleavage in *E. coli* than the conformer generated at 22 °C; indeed, this phenomenon has been observed with the A337V variant (8).

TABLE II
Specific activities and K_m estimations of recombinant aldolase B (at 22 °C)

Protein	FDP cleavage		F-1-P cleavage		FDP/F-1-P	F-1-P synthesis[c]	S/C[d]
	SA[a]	K_m[b]	SA[a]	K_m[b]			
	μmol/min/mg	*μM*	*μmol/min/mg*	*mM*		*μmol/min/mg*	
Wild-type	4.787	4.0 ± 0.6	4.507	2.4 ± 0.24	1.062	12.02	2.67
W147R	0.750	47 ± 0.004	0.889	26.6 ± 2.7	0.844	5.19	5.84
A149P	0.760	27 ± 0.002	0.658	9.8 ± 1.7	1.155	1.18	1.79
L256P	2.062	5.9 ± .05	1.902	3.3 ± 0.37	1.084	2.15	1.13
R303W	0.232	330 ± 110	0.115	NMM[e]	2.022	8.95	77.83
N334K	0.622	ND	0.518	ND	1.201	0.10	0.19
A337V	4.174	22 ± 0.003	2.557	24 ± 5	1.632	0.25	0.10

[a] Specific activities determined from a minimum of two preparations, where each preparation was assayed over a minimum of two separate experiments with triplicate samplings.
[b] K_m estimated from a minimum of two preparations, where each preparation was assayed from a minimum of two experiments.
[c] F-1-P synthesis using 45 mM each of DHAP and D-glyceraldehyde.
[d] F-1-P synthesis specific activity/F-1-P cleavage specific activity.
[e] NMM, not Michaelis-Menten kinetics; ND, not determined.

The A337V and N334K residues are situated in a region of the protein that is immediately proximal to the C terminus. The A337V variant of aldolase B was isolated as a partly truncated protein at 37 °C, indicating that it also forms a distinct conformer at this temperature that is sensitive to proteolysis in *E. coli*, probably because it is also partly denatured. No proteolysis was detected when the A337V protein was expressed at 22 °C, indicating that the conformer produced at the lower temperature is fully intact (8).

Aldol Cleavage and Synthesis Reactions—The specific activities and Michaelis constants of the aldolase cleavage and condensation reactions of the enzyme variants are presented in Table II. The structural mutants all consistently dissociated into minimally active monomers (Table I). All the mutant aldolases had reduced catalytic activity toward the substrates FBP and F-1-P as expected from their causal association with the disease hereditary fructose intolerance. Those variants possessing appreciable residual catalytic activity were those that could be isolated by gel filtration chromatography as homotetrameric conformers. The catalytic activity of all the mutants was predominantly associated with the tetrameric fraction isolated by gel filtration.

The W147R aldolase is a catalytic mutant affecting a tryptophan residue that is adjacent to the Lys^{146} and Arg^{148} residues which participate in carbon-6-phosphate binding and proton abstraction (22). The mutant protein possesses 16–20% of wild-type activity toward the substrates FBP and F-1-P, respectively and had a similar FBP to F-1-P ratio to wild-type. Mutation of the large tryptophan residue in the W147R variant affects the binding of both substrates similarly: the Michaelis constant for FBP was 14-fold and for F-1-P about 16-fold greater than wild-type. The effect on substrate binding probably results from distortion of the phosphate binding residues within the active site.

The A149P mutation is also in proximity to the substrate-binding residues; this variant possesses 16 and 15% of the activity of wild-type on FBP and F-1-P, respectively. The A149P aldolase has a 6-fold decrease in affinity for FBP and a 9-fold decrease in affinity for F-1-P. It possesses catalytic properties that resemble those of the W147R variant, suggesting that it similarly affects substrate binding. However, the A149P protein was unstable when expressed at 37 °C, and therefore it is also a structural mutant (24). Mutation of the invariant alanine to proline had a severe effect on tertiary structure, presumably as a result of distortion of the polypeptide chain by the rigid pyrroline ring of this imino acid; this effect was not observed with the W147R variant (7).

The R303W residue is situated on the surface of the active site and participates in the binding of the carbon-1-phosphate of the substrates (23).[2] The catalytic mutant R303W had only 5% of the activity toward FBP and 3% of the activity toward F-1-P, when compared with wild-type. The ratio of FBP to F-1-P was about 2, in contrast to the ratio of 1.06 observed for wild-type. The substitution of a large tryptophan residue likely impedes the binding of substrate, as indicated by the reduced enzyme activity and the greatly increased Michaelis constant for FBP.

Once purified as a native tetrameric protein, the L256P aldolase B variant proved to be one of the most active of the recombinant proteins examined. L256P possesses more than 40% of the activity of wild-type toward both FBP and F-1-P substrates. The Michaelis constant for FBP did not differ from wild-type, and likewise, the affinity for F-1-P was only slightly reduced. Because this mutation affects a residue on the subunit interface, it presumably disrupts the contacts of subunits necessary for tetramer formation. The tetrameric form of the variant enzyme was unstable and dissociated into mixtures of tetramer and monomer within days of storage. The main characteristic of the L256P protein was that it readily dissociated into minimally active monomers. This strongly suggests that maintenance of the tetrameric conformation is necessary for catalytic activity, a factor that accounts for the causal association of L256P with a disease due to human aldolase B deficiency.

The N334K aldolase B was found to be the most unstable of the recoverable recombinant proteins; it readily dissociated into inactive monomers, precluding reliable determination of its catalytic properties. Thus, the main evident effect of the N334K residue was to destabilise the quaternary structure of the mutant protein.

Under saturating conditions, the A337V variant of aldolase B maintained near wild-type cleavage rates with FBP and had 57% of the wild-type activity toward F-1-P. However, mutation of this invariant alanine greatly affects the affinity for F-1-P and also partly denatures the protein, as evidenced by its reduced stability upon long term storage and when expressed in *E. coli* at 37 °C. Confirmation of the partial unfolding of the purified recombinant A337V aldolase B was recently provided by detailed circular dichroism measurements (8). The alanine residue is in proximity to Asn^{334} on the preterminal α-helix, and the presence of the mutations in this segment will affect the conformation of the critical C-terminal tail segment of the enzyme and distort subunit integrity, thus also promoting tetramer dissociation (23). The incorrectly aligned C terminus postulated to result from the A337V residue causes a change in substrate specificity and affects affinity for the specific substrate of aldolase B, F-1-P. This confirms the role of the C

TABLE III
Aldolase B carbanion intermediate detection assays
Estimated (at 22 °C) from a minimum of two preparations using a minimum of six samplings.

Protein	1 mM FBP	50 mM F-1-P	1 mM DHAP	DHAP/FBP	DHAP/F-1-P
		μmol/min/mg			
Wild-type	1.077 ± 0.06	1.360 ± 0.27	1.620 ± 0.10	1.50	1.19
W147R	0.778 ± 0.08	0.703 ± 0.05	6.524 ± 0.19	8.39	9.28
A149P	0.974 ± 0.13	0.636 ± 0.10	2.100 ± 0.09	2.16	3.30
L256P	0.470 ± 0.10	0.250 ± 0.08	0.330 ± 0.07	0.70	1.32
R303W	0.553 ± 0.07	0.524 ± 0.26	0.116 ± 0.05	0.21	0.22
N334K	0.044 ± 0.02	0.086 ± 0.01	0.226 ± 0.11	5.14	2.63
A337V	0.330 ± 0.05	0.280 ± 0.09	0.554 ± 0.08	1.68	1.98

terminus in modulating the substrate specificity of the aldolase isozymes (25).

Condensation rates of the fructose 1-phosphate synthesis reactions catalyzed by wild-type and mutant aldolases are shown in Table III. Each mutant had reduced activity in the enzymatic formation of hexose phosphates from cognate triose substrates. R303W and W147R were found to be the most active mutant adolases with 75 and 43% of the activity of wild-type, respectively. The other variants were unable to synthesize F-1-P at rates comparable to wild-type aldolase B, indicating they have markedly reduced affinity for the triose sugars. The mutant L256P aldolase was more disabled in the direction of hexose synthesis than in the direction of aldolase cleavage. This clearly supports the observation that an intact quaternary structure of aldolase B is important for maintaining the retro-aldol catalytic activity of the enzyme for gluconeogenesis (condensation of triose phosphates), as well as the aldol cleavage reaction in the direction of glycolysis.

Carbanion Intermediate Activities—The kinetic mechanism of the class I aldolase reduction of hexacyanferrate (III) is represented as an ordered uni-bi scheme as shown in Scheme I (25),

$$\mathbf{E} + \text{Fru-P} \rightleftharpoons \mathbf{E}{=}\text{Fru-P} + \text{D-GH} \rightarrow \mathbf{E}{=}\text{D}^- \rightleftharpoons \mathbf{E}{=}\text{DH} \rightleftharpoons \mathbf{E} + \text{DH}$$
$$\Downarrow\Uparrow$$
$$\mathbf{E} + \text{HP} \leftarrow \mathbf{E}{=}\text{DH}$$

SCHEME I

where **E** is the enzyme, Fru-P is fructose 1-phosphate, D-GH is D-glyceraldehyde, D^- is the carbanion-enamine enzyme intermediate, DH is the protonated form of the carbanion (dihydroxyacetone-P), and HP is hydroxypyruvaldehyde-P (kinetic constants are not represented). The specific activities of hexacyanoferrate (III) reduction for each of the aldolase B variants are presented in Table III. Each mutant with disrupted quaternary structure, except for A149P, had reduced ability to generate carbanion intermediates in both directions of catalysis. This supports our observation that the intact quaternary structure is critical for constraining the tertiary structure of each subunit in a cooperative and catalytically active conformation.

All aldolase variants examined oxidized the aldol sugars at a lower rate than wild-type aldolase B. The DHAP oxidation rate was also reduced for all the mutants except for W147R and A149P, which had higher activities than wild-type. These two residues reside in a region of the protein that is involved in binding of phosphate moieties. It is likely that the positive charge in the vicinity of the putative carbon-6-phosphate binding region present in the W147R mutant contributes to the reduction of the hexacyanoferrate (III) indicator. However, as shown in the table, the uncharged substitution present in A149P also induced a higher specific activity for carbanion oxidation than wild-type aldolase. It is likely that both mutations distort the phosphate-binding region of the enzyme so that access and binding of the DHAP triose moiety is favored. These mutant aldolases may allow incorrect binding of substrate molecules because of inappropriate docking of the C1-phosphate moiety into the C6-phosphate-binding site

The N334K and A337V proteins were more active in the direction of DHAP oxidation than in hexose sugar oxidation. We postulate that these mutants indirectly affect the positioning of the C terminus (8). C terminus-mediated protonation of the DHAP-enzyme complex is impaired less severely for these mutant proteins. However, the reduced oxidation in the direction of aldol cleavage suggests that the C terminus promotes entry of the hexose substrates into the active site and favors catalytic protonation of the enamine intermediate.

The lowest carbanion oxidation activity with respect to all substrates was observed with the R303W variant. The carbon-1-phosphate is an analogous moiety on F-1-P and DHAP and the common enamine intermediate binds to the Arg^{303} residue (23).[2] The R303W residue could have an indirect effect on the C-terminal tyrosine. The release of DHAP by the C terminus is a limiting step in the class 1 aldolase cleavage reaction (26). The C terminus in the rabbit liver aldolase binds to a triad of anionic surface residues adjacent to the active site that includes Arg^{303}; these residues serve as part of a docking site for the C-terminal tyrosine when substrate molecules are absent (23). When aldol substrate is present in the active site, the carbon-1-phosphate interacts with the Arg^{303} residue and thus displaces the C terminus (23). Hence, the Arg^{303} residue is implicated in maintaining the catalytically important C-terminal tyrosine in proximity to the active site: the large apolar tryptophan residue at position 303 would disturb the binding of the carbon-1-phosphate moiety (including its derivative DHAP) and the subsequent displacement of the C terminus.

Relationship between Tetrameric Structure and Aldolase Function—The natural mutants of aldolase B A149P, W147R, L256P, and N334K were unstable and dissociated readily. The L256P mutation induces distortion at the subunit interface so that subunit binding is weakened. It is probable that the proline at position 256 would prevent correct alignment of the overlapping prolines found at the turns of α-helices 245–260. These residues form a hydrophobic pocket across adjacent subunits that aids their attachment and the stabilization of the tetrameric protein.[2]

The A149P and A174D mutations alter buried residues that are distal to the subunit interface boundary; however, native A149P protein was found unexpectedly to bind substrate, showing that the active site was at least partly intact. Nonetheless, A149P and A174D affect the β-strand that spans residues 145–153, extending into the active site, and contains the invariant active site phosphate-binding residues Lys^{146} and Arg^{148} (27).[2] This β-strand is hydrogen bonded to the adjacent β-strand comprising residues 185–190. The mutated residues

would disrupt critical interactions between the 145–153 and 185–190 β-strands. Transmission of these local distortions within each subunit to the interface boundaries would weaken cohesion of the constituent subunits of the tetrameric molecule without necessarily inactivating their active sites.

The overall result of the adjustments necessary to accommodate these structural mutations is that tetramer association is impaired. It appears that the quaternary structure not only stabilizes the molecule but also constrains the configuration that each subunit adopts and thereby promotes cooperative interactions in an active tetramer. Sygusch and Beaudry (28) have recently demonstrated cooperativity in the rabbit A isozyme. This may be a general phenomenon relating to all the multimeric class I aldolases. Beernink and Tolan (21) have generated a catalytically active monomer of aldolase A; however, the tertiary structure of this monomeric mutant protein was not determined. We suspect that this mutation may simply stabilize the subunit or induce a substantial change in its structure that mimics the conformation adopted normally upon assembly into the active tetramer.

Many glycolytic enzymes adopt a α/β-barrel structure and are isolated as homomeric tetramers. The class I aldolases are no exception, but the role of their tetrameric structure has hitherto not been explained. It remains unclear as to whether the quaternary structure of class I aldolases is necessary solely for maintenance of thermodynamic stability or for enzymatic catalysis. Certainly, in this study, the monomeric subunits of wild-type aldolase B are only minimally active, and the greatest enzymatic activities were observed only in those mutant aldolases that were isolated as intact tetramers, as exemplified by the L256P variant. The observation that the mutants A149P and L256P had reduced heat stability strongly suggests that assembly into the active tetramer confers increased resistance to denaturation. It is notable, however, that the integrity of the aldolase monomer and tetramer are interdependent; clearly, disruption of the molecular structure at either level will affect overall protein stability *in vitro* or in any cellular environment.

We have obtained diffracting crystals of wild-type human aldolase B and its natural mutants, including R303W. The solution of these structures should elucidate the role of the C terminus and the contribution of quaternary structure to class I aldolase function.

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Expression, Purification, and Characterization of Natural Mutants of Human Aldolase B: ROLE OF QUATERNARY STRUCTURE IN CATALYSIS

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