Aldolase A Ins(1,4,5)P_3-binding domains as determined by site-directed mutagenesis

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We substituted neutral amino acids for some positively charged residues (R42, K107, K146, R148 and K229) that line the active site of aldolase A in an effort to determine binding sites for inositol 1,4,5-trisphosphate. In addition, D33 (involved in carbon–carbon bond cleavage) was mutated. K229A and D33S aldolases showed almost no catalytic activity, but Ins(1,4,5)P_3 binding was similar to that determined with the use of wild-type aldolase A. R42A, K107A, K146R and R148A had markedly decreased affinities for Ins(1,4,5)P_3 binding, increased EC_{50} values for Fru(1,6)P_2-evoked release of bound Ins(1,4,5)P_3 and increased K_i values for Ins(1,4,5)P_3-evoked inhibition of aldolase activity. K146Q (positive charge removal) had essentially no catalytic activity and could not bind Ins(1,4,5)P_3. Computer-simulated docking of Ins(1,4,5)P_3 in the aldolase A structure was consistent with electrostatic binding of Ins(1,4,5)P_3 to K107, K146, R148, R42, R303 and backbone nitrogens, as has been reported for Fru(1,6)P_2 binding. Results indicate that Ins(1,4,5)P_3 binding occurs at the active site and is not dependent on having a catalytically active enzyme; they also suggest that there is competition between Ins(1,4,5)P_3 and Fru(1,6)P_2 for binding. Although Ins(1,4,5)P_3 binding to aldolase involved electrostatic interactions, the aldolase A Ins(1,4,5)P_3-binding domain did not show other similarities to pleckstrin homology domains or phosphotyrosine-binding domains known to bind Ins(1,4,5)P_3 in other proteins.

Key words: competition, fructose 1,6-bisphosphate, inositol phosphates, ligand, recombinant.

INTRODUCTION

Ins(1,4,5)P_3 can bind to the A, B and C isoforms of fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) [1,2]. The interaction of aldolase isoforms with its substrate Fru(1,6)P_2 decreased the affinity for Ins(1,4,5)P_3 binding [1–3]. Ins(1,4,5)P_3 inhibited the hydrolysis of Fru(1,6)P_2, in a partly competitive manner [1]. The affinity of Ins(1,4,5)P_3 binding to aldolase C was also decreased by the products of the reaction, D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP) [2,3]. The significance of binding of Ins(1,4,5)P_3 to aldolase A has not been yet determined; however, there is evidence that Ins(1,4,5)P_3 binding to aldolase C in smooth muscle is physiologically significant under both unstimulated and agonist-stimulated conditions [3]. It was computed for pig trachealis smooth muscle that the displacement of aldolase C-bound Ins(1,4,5)P_3 could double free [Ins(1,4,5)P_3] to levels greater than those required to release Ca^{2+} from the sarcoplasmic reticulum.

The molecular site for Ins(1,4,5)P_3 binding to aldolase isoforms is unknown. The mechanisms for the mutual antagonism of Ins(1,4,5)P_3 binding and Fru(1,6)P_2 cleavage are unknown. One ligand might bind to aldolase and evoke conformational changes that interfere with binding of the other ligand. Alternatively, the binding of Ins(1,4,5)P_3 might involve the same surface charge generated by positively charged amino acids, or amino acids that are important for substrate binding. In the present study we have undertaken to investigate the second possibility by using recombinant wild-type (WTr) and mutant aldolase A. Inositol phosphates, including Ins(1,4,5)P_3, have been reported to bind to pleckstrin homology (PH) [4–6] and phosphotyrosine-binding (PTB) [7] domains. A goal of our study was to start to define the Ins(1,4,5)P_3-binding domain in aldolase A.

The class I aldolases exist as homotetramers or heterotetramers in vertebrates [8]. Rabbit skeletal-muscle tetrameric aldolase contains four A monomers [9,10]. Structures of rabbit tetrameric skeletal-muscle aldolase, both unliganded [9] and liganded with DHAP bound to two of the monomeric units [10], have been determined by X-ray crystallography. Multiple amino acid residues have been implicated in the catalytic mechanism [10–17], in substrate binding [9,18–20] and in the actin-binding [17,21] properties of aldolase A. These results served as an important start for our study, in which we used mutant aldolase A altered in residues located near the active site. We mutated single positively charged amino acids (Lys and Arg) that might bind Ins(1,4,5)P_3 via salt bridges, as well as amino acids that were implicated in proton abstraction from the C-4-hydroxy group, which initiates carbon–carbon bond cleavage. K_d values and the number of Ins(1,4,5)P_3 molecules bound per tetramer for each mutant and for WTr were determined. We also studied, with the use of various mutants, the effects of Fru(1,6)P_2 on Ins(1,4,5)P_3 binding and the effects of Ins(1,4,5)P_3 on catalytic activity. In addition, we employed computer simulation to study the docking of Ins(1,4,5)P_3 to aldolase A.

MATERIALS AND METHODS

Mutants of rabbit skeletal-muscle aldolase A

The mutants were obtained by site-directed mutagenesis [22]. Details of construction of the expression vector and subsequent expression in *Escherichia coli* have been described previously [11,23]. The mutations used were: D33 changed to Ser (D33S), K107 to Ala (K107A), K229 to Ala (K229A), K146 to Gln

Abbreviations used: DHAP, dihydroxyacetone phosphate; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; PEG, poly(ethylene glycol); PH, pleckstrin homology; PLC, phospholipase C; PTB, phosphotyrosine binding; WTr, wild-type recombinant enzyme.

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(K146Q), K146 to Arg (K146R), R42 to Ala (R42A) and R148 to Ala (R148A). WTr was expressed in the same manner as the mutants. Three of the positively charged amino acids mutated in this study, K107, K146 and R148, are known, or are likely candidates, to be involved in the binding of Fru(1,6)P_2 at the active site [9,18–20,24]. Studies with these mutants tested a postulate that the binding of Ins(1,4,5)P_3 and Fru(1,6)P_2 involved the same positively charged amino acids. The positive charge of K146 has also been implicated as being essential for catalysis [13–15,25]. We also mutated an amino acid known to be involved in the formation of Schiff-base intermediates (K229) [12] and an amino acid implicated in carbon–carbon bond cleavage (D33) [11]. The study of K229A and D33S aldolase A tested a hypothesis that Ins(1,4,5)P_3 binding involved amino acids participating in Schiff-base intermediate formation and cleavage, and not amino acids involved in substrate binding. The study of K229 and D33 mutants also tested the hypothesis that the Fru(1,6)P_2-evoked release of bound Ins(1,4,5)P_3 required a catalytically active enzyme. CD studies failed to show differences between WTr and the various mutants, evidence that these single point mutations did not evoke gross changes in protein structure [11,14,15,21].

Enzyme preparation

Protein expression and purification were performed as described previously [11]. For binding studies, 300 μg of enzyme [80 %-satd. $(NH_4)_2SO_4$ precipitates stored at −70 °C] was pelleted by centrifugation at 15000 ***g*** for 5 min. The pellet was resuspended in 200 μl of 15 % (w/v) poly(ethylene glycol) (PEG), pelleted (15000 ***g***, 15 min) and dissolved in 20 mM HepesNa (pH 7.0)/20 mM KCl/1 mM *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA)/0.1 mM dithiothreitol/20 % (v/v) glycerol. Protein concentration was determined by the micro bicinchoninic acid method.

Binding of Ins(1,4,5)P_3 to aldolase

Binding was assayed in a manner similar to the PEG method described previously [2], except that the volume of the initial mixture was decreased to 25 μl and a low ionic strength was maintained to maximize binding [3]. The mixtures (incubated for 20 min at 0 °C) contained approx. 2 μg of aldolase, 30 mM imidazolepropanoic acid, pH 7.0, 1 mM EDTA, 30 μg of γ-globulins, 10 mM KCl, approx. 6700 c.p.m. (for K_d) or 3600 c.p.m. (for EC_{50}) of [^{3}H]Ins(1,4,5)P_3. PEG (25 μl) (30 %, w/v) was added and the mixture was incubated for at least an additional 20 min at 0 °C before centrifugation [2]. Pellets were dissolved in 100 μl of 0.1 M NaOH and transferred to scintillation vials, along with a 200 μl water wash of each tube, and counted for radioactivity. Assays used to determine K_d values contained added non-radioactive Ins(1,4,5)P_3 (concentration range from 12 nM to 100 μM). Although the sensitivity of the assay was greatest for aldolase mutants with the highest affinity (WTr, D33S and K229A), measurements on R148A, which had the lowest affinity, were still possible. The sensitivity of the assay is reflected in the ratio of radioactivity in the sample pellet to radioactivity in the blank pellet [e.g. for Ins(1,4,5)P_3 concentrations from 0.012 to 0.8 μM, the values (±S.D.) for WTr and R148A were 8.5±1.0 and 2.3±0.1 respectively, which fell progressively until at an Ins(1,4,5)P_3 concentration of 100 μM the values for both were 1.6±0.2]. Assays performed for the determination of EC_{50} for the displacement of Fru(1,6)P_2 from Ins(1,4,5)P_3 contained from 7 to 10 nM Ins(1,4,5)P_3. Blanks, run at each concentration tested, were identical with the above mixture but contained no aldolase. Pellets contained all aldolase activity originally in the reaction mixture with both WTr and mutant aldolases [2] [determined by using WTr (104±6 %) and R42A (103±6 %) (±S.D.)].

Aldolase activity

The 1 ml assay contained 30 mM imidazolepropanoate, pH 7.0, 1 mM EDTA, 10 mM KCl, 2 mM Na_2HAsO_4, 2 mM NAD^+, 1 unit of glyceraldehyde-3-phosphate dehydrogenase, 10 units of triose-phosphate isomerase, 5–1000 μM Fru(1,6)P_2 and enzyme. K_i was determined in the presence of 10 or 25 μM Ins(1,4,5)P_3. The assay was done at 30 °C. This assay was modified from that previously used [2], to obtain activity under ionic and pH conditions similar to binding.

Curve fitting

Constants and standard errors were obtained by using non-linear curve fitting with the program SYSTAT (version 3.0) with the use of data at all concentrations. This program gave the estimates±S.E.M. for each constant in the equation used for the fit.

Values of K_d and the number of Ins(1,4,5)P_3-binding sites (n) were generated by using all data points and were fitted to a single-site model:

$$r = (nC_s/K_d)/(1 + C_s/K_d)$$

where r is the molar ratio of the amount of ligand bound to the total amount of acceptor (molecular mass of aldolase A, 172 kDa [26]), n is the respective number of acceptor sites, C_s is the concentration of free ligand and K_d is the intrinsic dissociation constant [27].

Sigmoidal curves (for the determination of EC_{50} values) were fitted to the general equation:

$$y = (a - c)/[1 + (x/k)^b] + c$$

where a was set equal to 100 % (i.e. maximum binding) and c, if less than 0, was set equal to 0, $k = EC_{50}$ and b is the steepness factor [28].

K_i was determined by fitting all data determined to the equation for competitive inhibition:

$$v = V_{max}[Fru(1,6)P_2]/(K_m\{1 + [Ins(1,4,5)P_3]/K_i\} + [Fru(1,6)P_2])$$

where [Fru(1,6)P_2] was varied from 5 to 1000 μM and [Ins(1,4,5)P_3] was either 10 or 25 μM. The V_{max} values were identical with and without inhibitor.

Computer modelling of Ins(1,4,5)P_3 binding to aldolase A

Models were generated with the X-ray crystallographic structure of the rabbit aldolase A (Brookhaven Protein Data Bank accession code 1ADO) and the co-ordinates of Ins(1,4,5)P_3 (ligand extracted from Brookhaven Protein Data Bank accession code 1MAI). The docking program AutoDock 3.0 [29] (Scripps Research Institute, La Jolla, CA, U.S.A.) was used to generate a lowest-energy structure. The solvent molecules and DHAP were removed from the aldolase A structure; only residues 1–344 of one monomer (A from 1ADO) were used, because the last 19 residues are in widely different conformations in each monomer. Partial charges for the protein atoms (including polar hydrogen atoms) were assigned by using QUANTA97 (Molecular Simulations, San Diego, CA, U.S.A.). The input parameters for AutoDock were those described in the user guide. Ten runs of 50 cycles each were performed in the simulated annealing. Each cycle of the simulation consisted of 25000 steps. The simulation was started at high temperature (RT = 8400 J/mol) and was

Table 1 Catalytic constants for aldolase mutants

Mutant	K_m (μM)	V_{max} (units/mg)	Reference
WTr	14.3	20.8	[11]
K229A	$> 10^5$*	< 0.0002	[14]
D33S	36.5	0.0056	[11]
K146R	13.3	0.039	[15]
R42A	45	10.2	[20]
K107A	166	0.35	[20]
R148A	141	0.31	[20]
K146Q	$> 10^5$*	< 0.0002	[14]

* Could not be determined.

decreased by a factor of 0.95 in each cycle. The final docking structure shown had the lowest binding energy of 60 docking runs (−216 kJ/mol).

Molecular structures

Figures of molecular structures were produced by employing RasMol (RasWin Molecular Graphics, Windows Version 2.6) [30] [the software (rw32b2a.exe for Windows 95) was obtained from the RasMol Home Page, http://www.umass.edu/microbio/rasmol]. The data for the X-ray crystal structures were obtained from the Protein Data Bank of the Brookhaven National Laboratory (http://www.pdb.bnl.gov/) [31,32]. Each structure used in this paper cites the original reference for the structural 'PDB' file. Aldolase molecular structures shown in this paper are for rabbit skeletal-muscle aldolase A [10].

Materials

[^{3}H]Ins(1,4,5)P_3 (21.0 Ci/mmol; at 40 % counter efficiency this is equivalent to 18480 c.p.m./pmol) was purchased from Dupont–NEN (Wilmington, DE, U.S.A.). Non-radioactive Ins(1,4,5)P_3 was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.) or Alexis Corp. (San Diego, CA, U.S.A.). Hepes,

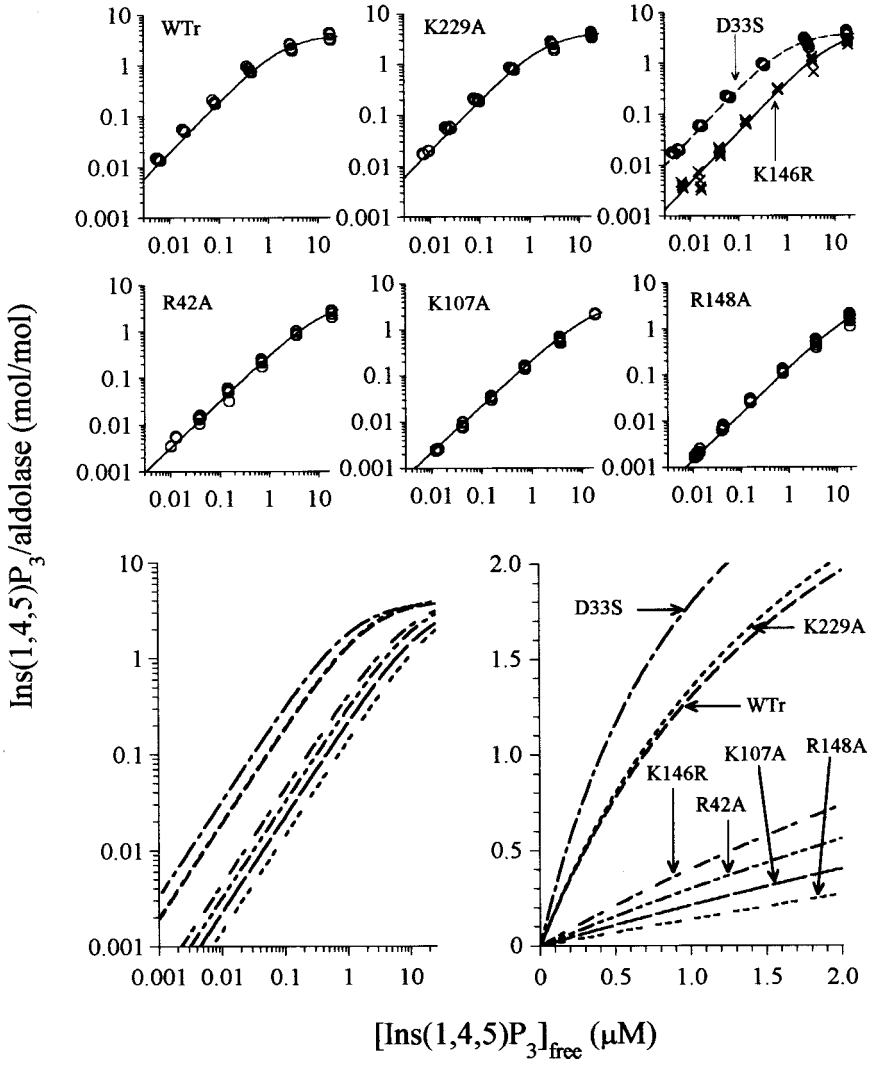

Figure 1 Ins(1,4,5)P_3 binding to WTr and aldolase A mutants

Top six panels: data are shown in log–log form for ease of visualization of all data points. All data were fitted to a single-site model (see the Materials and methods section). The K_d and number of binding sites are listed in Table 2. Bottom two panels: comparison of binding curves for WTr and aldolase A mutants. The fitted data for WTr and aldolase mutants are shown in both log–log form (left) and linear form (right; up to 2 μM).

imidazole, PEG-8000, γ-globulins, EDTA, HEDTA, NAD, arsenic acid (disodium salt) and Fru(1,6)P_2 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rabbit skeletal muscle glyceraldehyde-3-phosphate dehydrogenase and triose-phosphate isomerase were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The micro bicinchoninic acid protein assay reagent was from Pierce (Rockford, IL, U.S.A.). All other chemicals were of reagent grade.

RESULTS

General characteristics of WTr and mutated aldolase A

We confirmed the effects of various mutations on the catalytic activity that had been previously reported [11,14,15,21]. We give published data in Table 1. With K229A, D33S or K146Q there was almost no measurable catalytic activity, indicating that these amino acids were essential for catalytic activity. K107A, R148A, K146R and R42A aldolase A had catalytic activity, but there were decreases in V_{max} and increases in K_m (except for the K_m of K146R, which was the same as that of WTr) [18,21].

Ins(1,4,5)P_3 binding curves are shown in Figure 1 (top six panels); the computed K_d values and number of sites (n) are listed in Table 2. In the mutants and the WTr enzyme, there were four identical binding sites per tetramer. Figure 1 (bottom two panels) compares data found for the various mutants. The K_d for WTr aldolase A was approx. twice the value of 1.1 μM (by direct binding) originally reported for this enzyme [1] obtained under different ionic and pH conditions. Fru(1,6)P_2 released bound Ins(1,4,5)P_3 from WTr with an EC_{50} of 22 μM (Table 2). This is close to the K_m reported for this enzyme, 14.3 μM [11]. Ins(1,4,5)P_3 competitively inhibited the rate of Fru(1,6)P_2 catalysis with a K_i of 4.1 μM.

Amino acids involved in Schiff-base intermediate formation and proton abstraction from immonium ion

K229A and D33S aldolase A bound Ins(1,4,5)P_3 equally well as or better than WTr. Therefore K229 and D33 do not participate in binding Ins(1,4,5)P_3. K_d values determined with K229A and D33S aldolase A were 1.2 and 2.2 μM respectively (Table 2). The inhibition by Fru(1,6)P_2 of Ins(1,4,5)P_3 binding to K229A aldolase A (Figure 2 and Table 2) was unchanged from results obtained with WTr aldolase A. With D33S aldolase A, there was a significant increase in the EC_{50} for the Fru(1,6)P_2-evoked displacement of bound Ins(1,4,5)P_3 (Table 2). We did not study Ins(1,4,5)P_3-evoked inhibition of catalytic activity because the activity in these mutants was extremely low.

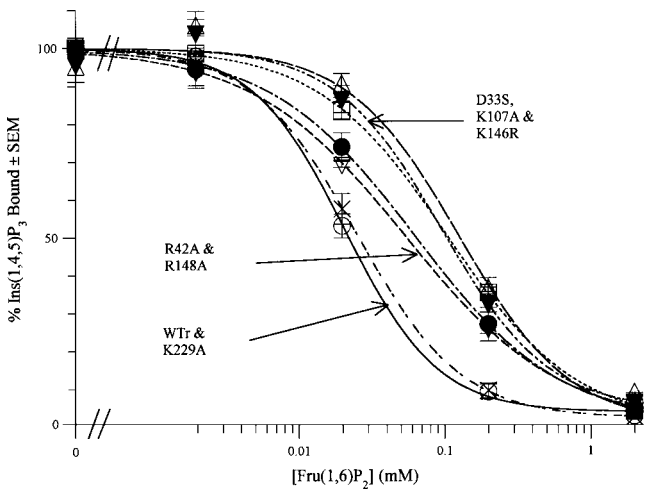

Figure 2 Displacement of Ins(1,4,5)P_3 by Fru(1,6)P_2

Ins(1,4,5)P_3 binding to WTr and mutant aldolases was performed in the presence of 7–10 nM Ins(1,4,5)P_3 as described in the Materials and methods section. Symbols: ○, WTr; ×, K229A; □, D33S; △, K146R; ▽, R42A; ▼, K107A; ●, R148A. The EC_{50} values are listed in Table 2. The maximum amount of Ins(1,4,5)P_3 bound to each aldolase species was set equal to 100%. The comparative order of binding at 8.5 nM Ins(1,4,5)P_3 (the mean level used to obtain these data) in the absence of Fru(1,6)P_2 was: D33S (171%) K229A (103%) = WTr (100%) K146R (23%) R42A (17%) K107A (12%) R148A (8%) K146Q (0%; results not shown).

R42, K107, K146 and R148 mutants

K107A and R148A aldolase A showed 8- and 14-fold increases respectively in their K_d values for Ins(1,4,5)P_3 binding compared with WTr aldolase A (Figure 1 and Table 2). The EC_{50} for the inhibition of Ins(1,4,5)P_3 binding by Fru(1,6)P_2 increased 3-fold and 4-fold (R148A and K107A respectively) from that determined with WTr aldolase A (Figure 2). The inhibition by Ins(1,4,5)P_3 of K107A aldolase activity was less effective (17-fold increase in K_i) than with WTr aldolase A.

Table 2 Ins(1,4,5)P_3 binding constants, Fru(1,6)P_2 inhibition of Ins(1,4,5)P_3 binding and Ins(1,4,5)P_3 inhibition of aldolase activity

Numbers in parentheses are the numbers of data points. Abbreviation: n.t., not testable.

	Ins(1,4,5)P_3 binding			
Mutant	K_d (μM)	n	Fru(1,6)P_2 inhibition, EC_{50} (μM)	Ins(1,4,5)P_3 inhibition K_i (μM)
WTr	2.1 ± 0.2	4.0 ± 0.1 (42)	22 ± 2 (45)	4.1 ± 0.7*
K229A	2.2 ± 0.2	4.2 ± 0.1 (40)	25 ± 2 (29)	†
D33S	1.2 ± 0.3	3.9 ± 0.2 (39)	106 ± 9 (29)	†
K107A	17.3 ± 1.9	3.9 ± 0.1 (41)	96 ± 11 (30)	71 ± 2‡
R148A	30.2 ± 2.9	4.3 ± 0.2 (61)	63 ± 10 (30)	§
R42A	13.9 ± 1.2	4.5 ± 0.1 (40)	54 ± 5 (30)	30 ± 1*
K146R	9.7 ± 0.9	4.3 ± 0.1 (46)	127 ± 10 (30)	12 ± 2*
K146Q	0		n.t.	§

* Performed with 10 μM Ins(1,4,5)P_3.
† Aldolase activity too low to test.
‡ Performed with 25 μM Ins(1,4,5)P_3.
§ Ins(1,4,5)P_3 binding too low to test.

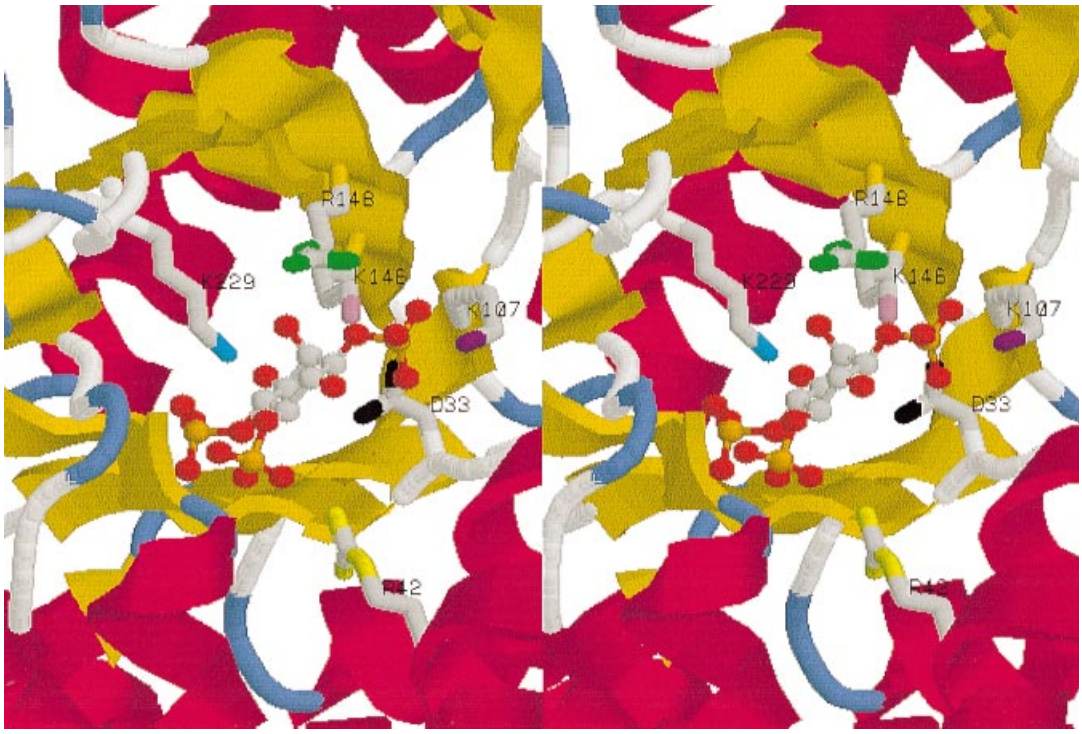

Figure 3 Modelling of Ins(1,4,5)P_3 bound to aldolase A

Stereo representation of the molecular structure of a portion of Ins(1,4,5)P_3 (shown in ball-and-stick form) bound to aldolase A as produced by AutoDock [29] (described in the Materials and methods section). The ribbon model shows the centre of the eight-membered β-barrel involved in the formation of the active site. Also illustrated is the position of residues (shown in stick form) mutated in this study. K229, K146, R148 and D33 lie deep within the cavity. R42 is located on the opposite side of the active site from R148 and is closer to the outer surface. Intermolecular distances between mutated residues are given in Table 3. Intermolecular distances between the phosphate groups of Ins(1,4,5)P_3 are: C-1 to C-4, 7.2 Å; C-1 to C-5, 8.2 Å; C-4 to C-5, 5.0 Å. Colour codes for aldolase A: two carboxy O on D33, black; guanidino nitrogen on R42, bright yellow; guanidino nitrogen on R148, green; ϵ-amino nitrogen on K107, purple; ϵ-amino nitrogen on K146, violet; ϵ-amino nitrogen on K229, cyan; α-helices, magenta; β-sheets, mustard yellow; turns, blue; other structures, greyish white. For Ins(1,4,5)P_3: phosphates, orange; oxygen, red. All carbons are whitish-grey; hydrogens are not shown. This Figure was produced by RasMol [30].

R42A aldolase A showed an approx. 85% decrease in Ins(1,4,5)P_3 affinity (Figure 1 and Table 2). This mutant still possessed considerable aldolase activity, with a V_{max} approx. half that of WTr and a K_m approx. 3-fold higher. The EC_{50} for Fru(1,6)P_2 inhibition of Ins(1,4,5)P_3 binding was double that determined with WTr aldolase A (Figure 1 and Table 2). The K_i for the inhibition of aldolase activity by Ins(1,4,5)P_3 increased 7-fold compared with results obtained with WTr aldolase A (Table 2).

Binding of Ins(1,4,5)P_3 was entirely absent for K146Q aldolase A when tested with 0.009–5 μM Ins(1,4,5)P_3. As indicated above, catalytic activity is almost completely absent for this mutant (Table 1), although the secondary structure and ability to form a Schiff base (i.e. at K229) are still intact [15]. Some Ins(1,4,5)P_3 binding could be retrieved by substituting Arg for Lys (K146R), thus maintaining a positive charge at this residue. However, Ins(1,4,5)P_3 binding was not returned to the level seen in WTr [there was a 5-fold increase in K_d (Figure 1 and Table 2)]. Re-establishing a positive charge at this site in the molecule partly restored aldolase activity (from 0.001% to 0.2% of the V_{max} for WTr), showing the importance of the charge for enzymic activity, as shown previously [15].

Modelling Ins(1,4,5)P_3 binding in the active site of aldolase A

The hypothesis that Ins(1,4,5)P_3 is held in the active site by positive charges requires that the molecule fit into the cavity and that the geometry of Ins(1,4,5)P_3 negative charges match the geometry of positive surface charges. To determine whether Ins(1,4,5)P_3 might bind in the active site of aldolase A in an analogous manner to that of Fru(1,6)P_2, the inhibitor was docked into a monomer of rabbit aldolase A structure by using AutoDock 3.0 [29]. The same parameters used for docking Ins(1,4,5)P_3 were used to dock a ring-closed model of Fru(1,6)P_2, which showed that it bound to a site similar to what was found by X-ray crystallography [20]. The Ins(1,4,5)P_3 molecule fits well, with the phosphate-binding sites shown in crystal structures of aldolase complexed with either Fru(1,6)P_2 [20] or DHAP [10]. The Ins(1,4,5)P_3 C-1 phosphate was found 3.2–4.8 Å (1Å ≡ 0.1 nm) from K107, K146, R148 and S38. The C-4 phosphate was located 3.4, 3.1, 2.9 and 3.2 Å from the S271, G272, G302 and R303 backbone nitrogens respectively, and 2.6 Å from the S271 hydroxy group. The C-5 phosphate-binding site was found between 2.6 and 3.9 Å from guanidino nitrogens of R42 and R303 and was 3.1 and 3.6 Å from the backbone nitrogens of R303 and G273 respectively. Figure 3 illustrates the results of the computer-simulated docking and shows the proximity of Ins(1,4,5)P_3 phosphates to residues mutated in this study.

DISCUSSION

Ins(1,4,5)P_3 binding to aldolase A

Mutation of some of the positively charged amino acid residues that line the active site of aldolase A diminished or abolished

Table 3 Intermolecular distances between residues mutated in the present study and other polar residues that are close enough to be candidates to function in Ins(1,4,5)P_3 binding

Values are means for each monomeric unit of the tetrameric rabbit aldolase A model [10], measured between guanidino (R), ϵ-amino (K) or end of acyl-chain oxygens (D, E or S).

Residues mutated in this study	Distance (Å)	Other polar residues	Distance (Å)
K229 to R148	9.5	R148 to E189	2.7
K229 to K146	4.9	R42 to R303	5.4
K229 to K107	8.5	R42 to E34	3.6
K229 to R42	10.7	R42 to K41	8.7
K229 to D33	5.5	R42 to S45	6.9
R148 to K146	6.7	K107 to D109	5.1
R148 to K107	7.5	K107 to S35	5.7
R148 to R42	10.1	K107 to S38	6.7
R148 to D33	8.8	K146 to D109	4.9
K146 to K107	4.1	K229 to E187	4.2
K146 to R42	10.0	K229 to S300	2.9
K146 to D33	2.9	K229 to S271	8.1
K107 to R42	9.5		
K107 to D33	2.9		
R42 to D33	8.2		

Ins(1,4,5)P_3 binding. Different mutations effected different decreases in Ins(1,4,5)P_3 binding affinities. R42A or K107A mutations caused an approx. 7-fold increase in the binding K_d. The R148A mutation increased the K_d approx. 15-fold. Of the mutations that inhibited Ins(1,4,5)P_3 binding, only the K146Q mutation entirely abolished binding. When K146 was mutated to Arg, restoring a charged amino acid at this position in the molecule, Ins(1,4,5)P_3 binding was detected, although at a somewhat lower affinity than seen with WTr aldolase A. Of the amino acid residues studied, K146 is located closest to K229, which is a site of Schiff-base formation, and it is likely that the positive charge at this site is essential for Ins(1,4,5)P_3 binding. However, the positive charge of K229 was not used in binding Ins(1,4,5)P_3. Taken together, these results suggest that Ins(1,4,5)P_3 binding occurred within the active site of aldolase A and that binding involved the formation of salt bridges with positively charged amino acids. An electrostatic mechanism for the binding of Ins(1,4,5)P_3 within the active site is supported by our previous finding that binding was suppressed with increasing ionic strength [3]. Because the removal of a single charge decreased the affinity for Ins(1,4,5)P_3 binding (with the exception of the K146Q, for which no binding could be detected), it seems that multiple positively charged amino acids are involved in generating surface charge used for Ins(1,4,5)P_3 binding. We cannot exclude the possibility that the removal of a single positive charge at the active site evoked allosteric changes in the molecule that influenced our findings. The single point mutations used in our study did not alter CD spectra [11,14,15,21]. However, CD measurements might not reveal small changes in structure that are important in protein function.

Data obtained with the AutoDock program were consistent with those obtained with mutant aldolase, indicating the importance of the positive charges on R42, K107, K146 and R148 in binding Ins(1,4,5)P_3 in the catalytic cavity. These data extend the mutant-derived data in suggesting that K107, K146 and R148 are involved in binding the C-1 phosphate of Ins(1,4,5)P_3 and that R42 is involved in binding the C-5 phosphate of this compound. Studies with inositol phosphate isomers indicate the importance of C-1 and C-5 phosphates in Ins(1,4,5)P_3 binding [1]. There might be additional residues involved in the docking of Ins(1,4,5)P_3 in the aldolase A catalytic cavity. AutoDock data suggest C-1 is also in close proximity to S38. C-4 is in close proximity to S271, G272 and R303. C-5 is in close proximity to R303 and G273. Additional residues that are close enough for potential involvement in Ins(1,4,5)P_3 binding are listed in Table 3. A number of residues in this list have been shown to participate in the binding of DHAP [10] and Fru(1,6)P_2 [20]. The modelling of Ins(1,4,5)P_3 into the aldolase active site resulted in phosphate–cationic residue interactive distances of 2.5–5.0 Å, comparable with those found in the interaction of Ins(1,4,5)P_3 with PH domains of phospholipase C-δ_1 (PLC-δ_1) [5] and β-spectrin [4].

Interactions between Ins(1,4,5)P_3 and Fru(1,6)P_2

As indicated in the Introduction section, previous results indicate that there are interactions between the binding of Ins(1,4,5)P_3 to aldolase A and C and activation by Fru(1,6)P_2. Ins(1,4,5)P_3 is a partial competitive inhibitor for Fru(1,6)P_2 hydrolysis [1]. Fru(1,6)P_2-evoked activation of aldolase A and aldolase C decreased the affinity for Ins(1,4,5)P_3 binding [1–3]. Ins(1,4,5)P_3 binding inhibited Fru(1,6)P_2-evoked activity in aldolase C [2].

Mutations of K229 and D33, to Ala and Ser respectively [mutations known to almost abolish Fru(1,6)P_2-evoked enzyme activity (Table 1)], had no effect on (or evoked an increase in) Ins(1,4,5)P_3 binding. This dissociation of enzyme activity from Ins(1,4,5)P_3 binding indicates that the mechanism by which Fru(1,6)P_2 caused a decreased affinity for Ins(1,4,5)P_3 binding did not involve a conformational change triggered by the hydrolysis of Fru(1,6)P_2.

The finding that the Fru(1,6)P_2-evoked displacement of Ins(1,4,5)P_3 was attenuated in the K107, K146, R148 and R42 mutants is consistent with an interaction between the binding of Ins(1,4,5)P_3 and Fru(1,6)P_2. The Ins(1,4,5)P_3-evoked inhibition of aldolase activity was decreased in K107A, R42A and K146R aldolase, a finding consistent with an interaction between binding of the two ligands. K107, K146 and R148, shown in the present study to be involved in Ins(1,4,5)P_3 binding to aldolase A, have been implicated in the binding of substrate Fru(1,6)P_2 to aldolase A [9,18,19,33]. The computer-simulated docking of Ins(1,4,5)P_3 to aldolase A showed that all three Ins(1,4,5)P_3 phosphates were easily accommodated and bound near known phosphate-binding sites for Fru(1,6)P_2. The C-1 phosphate of Ins(1,4,5)P_3 was found 3.7–5.0 Å from K107, K146 and R148, which have been shown to comprise one of the C-6 phosphate-binding sites for Fru(1,6)P_2 [20]. The Ins(1,4,5)P_3 C-4 phosphate-binding site was found 2.6–3.4 Å from S271 and S272 backbone nitrogens, which have been shown to comprise the C-1 phosphate-binding site for DHAP [10] and Fru(1,6)P_2 [20]. The Ins(1,4,5)P_3 C-5 phosphate-binding site was found between 2.6–3.9 Å from R42 and R303 which has been shown to comprise part of another Fru(1,6)P_2 C-6 phosphate-binding site in crystals where Fru(1,6)P_2 was bound in an alternative binding mode in a mutant K146A-aldolase A (K. H. Choi and D. R. Tolan, unpublished work). Taken together, the above results suggest that the same positively charged amino acid residues are involved in the binding of Ins(1,4,5)P_3 and Fru(1,6)P_2. Thus interactions between Ins(1,4,5)P_3 and Fru(1,6)P_2 phosphates might be explained by competitive binding and overlapping sites. It is still possible that these residues might interact with different types of atom in the two ligands.

The finding that Fru(1,6)P_2 released bound Ins(1,4,5)P_3 in the K229 mutant with an EC_{50} identical with that determined in the WTr supports the concept that K229A aldolase A could still bind

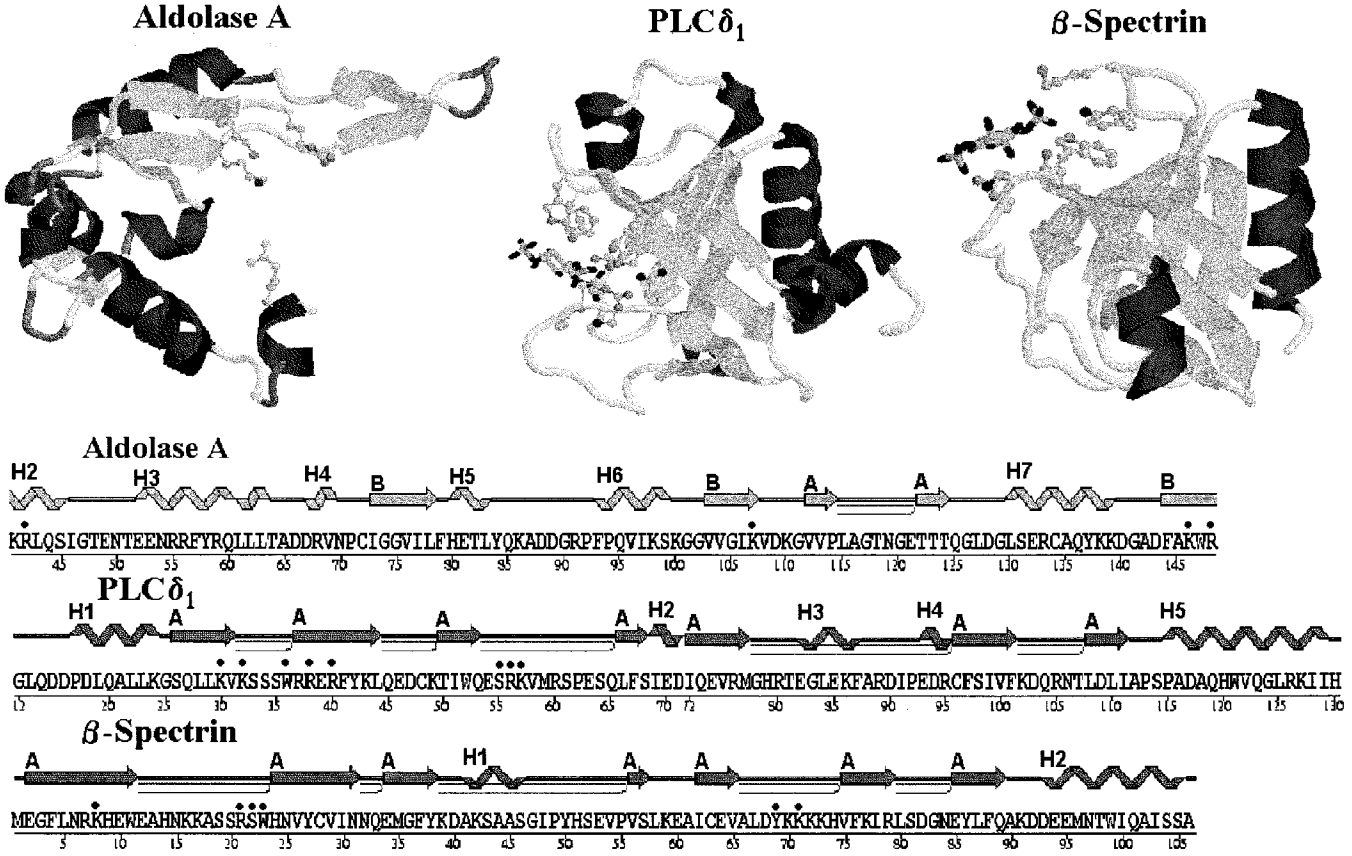

Figure 4 Structure of the residues 41–148 region of aldolase A that includes the negatively charged amino acids mutated in this study shown to be involved in Ins(1,4,5)P_3 binding (extracted from Brookhaven Protein Data Bank accession code 1ADO)

Upper panels: this aldolase A domain is compared with PH Ins(1,4,5)P_3-binding domains in PLC-δ_1 (Brookhaven Protein Data Bank accession code 1MAI) and β-spectrin (Brookhaven Protein Data Bank accession code 1BTN). In structures, β-sheets are given in grey and α-helices in black. For aldolase A, the orientation was designed for the optimal visualization of β-sheets and α-helices. The amino acids shown to be involved in Ins(1,4,5)P_3 binding are indicated in ball-and-stick form. There are multiple α-helices between R42 and K107 and a single α-helix between K107 and R148. K107, K146 and R148 are present in β-sheets, whereas R42 is present in an α-helix. For the PH domain of PLC-δ_1, the bound Ins(1,4,5)P_3 is shown in stick form and the binding-site amino acids are shown in ball-and-stick form [5]. For the PH domain of β-spectrin, the bound Ins(1,4,5)P_3 and the binding-site amino acids are shown as above [34]. These Figures were produced by RasMol [30]. Lower panel: the linear amino acid sequences and the secondary structures for the aldolase A domain that includes R42, K107, K146 and R148, and the PH domains of PLC-δ_1 [5] and β-spectrin [34]. For aldolase, the numbers under residues indicate the sequence number starting at the N-terminus of the protein. For PLC-δ_1 and β-spectrin, the numbers under residues indicate the sequence number starting at the N-terminal end of the PH domain constructs. Horizontal arrows indicate β-sheets (A, anti-parallel sheet; B, parallel sheet). H indicates an α-helix. Thin straight lines connected at one end indicate β-hairpins [consisting of two β-strands that are anti-parallel and hydrogen-bonded together (connected by at least one bridge)]. Black dots indicate residues shown to be involved in the binding of Ins(1,4,5)P_3 to the three different proteins.

Fru(1,6)P_2 and that Fru(1,6)P_2 and Ins(1,4,5)P_3 compete for binding sites even under conditions in which activity was absent. However, it will require direct Fru(1,6)P_2 binding measurements to prove that the Fru(1,6)P_2-evoked displacement of Ins(1,4,5)P_3 bound to K229A aldolase A was triggered by Fru(1,6)P_2 binding.

With D33S, the EC_{50} for the inhibition by Fru(1,6)P_2 of Ins(1,4,5)P_3 binding was increased, a finding that might not be consistent with the 'competition for binding' hypothesis given above. This result could be explained if the removal of the negative charge, which is only 2.9 Å from both K107 and K146, resulted in alterations of their positions so that Fru(1,6)P_2 binding was diminished.

Ins(1,4,5)P_3-binding domain is not a PH or PTB domain

Before the present study, Ins(1,4,5)P_3 and other inositol phosphates were known to bind only to PH or PTB domains; no other Ins(1,4,5)P_3-binding domain had been identified. PH domains are characterized by seven anti-parallel β-sheets with a strong bend that results in an orthogonal structure. An α-helix is present at the C-terminal end of the domain; other α-helices vary in position within the PH domain. There is a region made up of clustered positively charged amino acids, usually located in connecting links between β-sheets, giving strong electrostatic polarization at the site of binding of phosphate groups in inositol phosphates and PtdIns(4,5)P_2 via salt bridges and hydrogen bonds [4]. PTB domains contain two orthogonal β-sheets and connecting loops and a C-terminal amphipathic α-helix capping one end of the β-sandwich [7]. Figure 4 compares the PH domains of PLC-δ_1 and β-spectrin with the portion of the Ins(1,4,5)P_3-binding domain in aldolase A identified in the current study that includes R42, K107, R146 and R148 (residues 41–148). This domain includes five β-sheets (one pair is anti-parallel) and six α-helices. K107 and K146 (and R148) are present in different β-sheets; R42 is located in an α-helix. It is evident that the portion of the aldolase A Ins(1,4,5)P_3-binding domain that includes the positively charged amino acids mutated in our study does not have the three-dimensional structure of a

PH domain. PTB domains also show little similarity to the structure of the aldolase A Ins(1,4,5)P_3-binding domain. We conclude that the Ins(1,4,5)P_3-binding domain in aldolase A is neither a PH nor a PTB domain.

This work was supported by grants from the National Heart, Lung and Blood Institutes (Bethesda, MD, U.S.A.), contract grant numbers HL 137498 and HL 505320 (R.F.C. and C.B.B.), and by National Institutes of Health grant number DK43521 (D.R.T. and K.H.C.).

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Received 2 December 1998/21 April 1999; accepted 19 May 1999