Inhibition of rabbit muscle aldolase by phosphorylated aromatic compounds

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The interactions of the phosphorylated derivatives of hydroquinone (HQN- P_2), resorcinol (RSN- P_2), 4-hydroxybenzaldehyde (HBA- P) and 2,4-dihydroxybenzaldehyde (DHBA- P ; phosphate group at position 4) with fructose biphosphate aldolase were analysed by enzyme kinetics, UV/visible difference spectroscopy and site-directed mutagenesis. Enzyme activity was competitively inhibited in the presence of HQN- P_2 , RSN- P_2 and HBA- P , whereas DHBA- P exhibited slow-binding inhibition. Inhibition by DHBA- P involved active-site Schiff-base formation and required a phenol group *ortho* to the aldehyde moiety. Rates of enzyme inactivation and of Schiff-base formation by DHBA- P were identical, and corresponded to 3.2–3.5 DHBA- P

molecules covalently bound per aldolase tetramer at maximal inactivation. Site-directed mutagenesis of the active-site lysine residues at positions 107, 146 and 229 was found to be consistent with Schiff-base formation between DHBA- P and Lys-146, and this was promoted by Lys-229. Mutation of Glu-187, located vicinally between Lys-146 and Lys-229 in the active site, perturbed the rate of Schiff-base formation, suggesting a functional role for Glu-187 in Schiff-base formation and stabilization. The decreased cleavage activity of the active-site mutants towards fructose 1,6-bisphosphate is consistent with a proton-transfer mechanism involving Lys-229, Glu-187 and Lys-146.

INTRODUCTION

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) reversibly catalyses the cleavage of fructose 1,6-bisphosphate [Fru(1,6) P_2] into the triose phosphates D-glyceraldehyde phosphate and dihydroxyacetone phosphate [1]. Aldolases exist in two distinct classes [2]; class I aldolases are found in animals and higher plants, and catalyse Schiff-base formation with substrate, whereas class II aldolases are found in algae, bacteria and yeasts, and require a bivalent metal ion as a cofactor. Class I aldolases can be classified into three isoenzyme forms, distinguishable on the basis of immunological reactivity as well as turnover with respect to Fru(1,6) P_2 and fructose 1-phosphate substrates [3]. Isoenzyme A, from rabbit muscle, has been the most extensively studied of the class I aldolases [4,5]. Over 14 different isoenzymes have been sequenced [6] and three aldolase isoenzyme structures have been determined, including those from rabbit muscle [7], human muscle [8] and *Drosophila* [9]. With the exception of the 20 amino acid residues comprising the C-terminal region, the molecular architecture of these isoenzymes has been highly conserved. The polypeptide fold of each aldolase subunit of the homotetramer corresponds to that of a β -barrel, with the active site located in the centre of the β -barrel [7]. Unlike other β -barrel isoenzymes, the active site is composed of a substantial number of charged amino acid residues, i.e. Asp-33, Lys-107, Lys-146, Glu-187 and Lys-229.

Of these residues, the role of Lys-229 has been clearly established; it is responsible for Schiff-base formation with the substrate [10]. Site-directed mutagenesis of Asp-33 [11] and Lys-146 [12] results in significant decreases in enzymic activity. Chemical modification of active-site lysine residues has also been shown to result in the loss of enzyme activity [13–15]. In contrast, the role of Glu-187, located between Lys-146 and Lys-229 in the active site, has not yet been examined.

With the exception of pyridoxal phosphate [15], D-erythrulose phosphate [16] and dihydroxyacetone phosphate analogues [17], few slow-binding inhibitors of aldolase have been developed, despite interest in such compounds [18]. In a previous study examining the interaction of bisphosphorylated aromatic compounds with aldolase [19], several analogues exhibited strong competitive inhibition and suggested a design for a novel slow-binding inhibitor of the enzyme.

In the present paper we describe the synthesis of four compounds, resorcinol bisphosphate (RSN- P_2), hydroquinone bisphosphate (HQN- P_2), 4-hydroxybenzaldehyde phosphate (HBA- P) and 2,4-dihydroxybenzaldehyde 4-phosphate (DHBA- P), and establish the mechanisms by which these compounds inhibit rabbit muscle aldolase. Unlike the first three compounds, which are competitive inhibitors, inhibition observed in the presence of DHBA- P is only slowly reversed and involves the formation of a Schiff base with a lysine residue at the active site. The mode of interaction of this compound with rabbit muscle aldolase was examined by several methods, namely enzyme kinetics, UV/visible difference spectroscopy and site-directed mutagenesis of the active-site residues that are potentially implicated in the mechanism of inhibition.

MATERIALS AND METHODS

Materials

Fru(1,6) P_2 , NADH, glycerol-3-phosphate dehydrogenase and triose-phosphate isomerase were purchased from Boehringer Mannheim, dihydroxyacetone phosphate (lithium salt) was from Sigma, and other chemicals were from Aldrich. Hexitol bisphosphate (hexitol- P_2) was prepared from Fru(1,6) P_2 by the procedure of Ginsburg and Mehler [20].

Abbreviations used: Fru(1,6) P_2 , fructose 1,6-bisphosphate; RSN- P_2 , resorcinol bisphosphate; HQN- P_2 , hydroquinone bisphosphate; HBA- P , 4-hydroxybenzaldehyde phosphate; DHBA- P , 2,4-dihydroxybenzaldehyde 4-phosphate; hexitol- P_2 , hexitol bisphosphate; TEA, triethylamine.

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Activity assays

Aldolase cleavage activity was measured using a coupled assay system by following NADH oxidation at 340 nm, with detection by a Perkin–Elmer Lambda 2 spectrophotometer thermostatted at 25 °C [21]. Assays were initiated by the addition of substrate [Fru(1,6) P_2 ; 1 mM final concentration] to a final volume of 1 ml of solution containing aldolase made up in triethylamine (TEA) buffer (100 mM TEA/HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA), 0.42 mM NADH and coupling enzymes (10 μ g/ml glycerol-3-phosphate dehydrogenase; 1 μ g/ml triose-phosphate isomerase). The aldolase concentration was determined spectrophotometrically from $\epsilon_{280} = 0.91 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ [22] or with a BCA protein concentration determination kit (Pierce Chemical Co.).

Inhibition constants (K_i) were determined on the basis of double-reciprocal plots; concentrations used in the kinetic inhibition assays were: 2 μ g/ml aldolase, 10–100 μ M Fru(1,6) P_2 , 10–100 μ M HQN- P_2 , 40–500 μ M RSN- P_2 and 0.1–1.0 mM HBA- P .

Reduction by sodium borohydride

Sodium borohydride treatment of the enzyme–inhibitor complex at equilibrium (pH 7.0) was performed using a previously described technique [17].

Kinetic methods

Slow-binding inhibition [18] involves rapid equilibrium formation between enzyme E and inhibitor I, followed by the initial complex EI undergoing a slow and reversible isomerization to a kinetically more stable complex form EI*, as shown in Scheme 1.

Scheme 1

For this sequence, K_1^* is the overall dissociation constant, K_i is the dissociation constant for the rapidly formed EI complex, K_d is the dissociation constant for the EI* complex and K is the equilibrium constant between EI and EI*. This latter enzyme–ligand form is assumed to be inactive towards the enzyme substrate.

Characterization of slow-binding inhibition

In the absence of substrate, the apparent first-order rate constant (k_{app}) describing the formation of EI* is defined by eqn. (1) [18]. This constant exhibits saturating first-order kinetics with increasing concentration of inhibitor ([I]). At high inhibitor concentrations, and whenever $k_2 > k_{-2}$, eqn. (2) can be used [23,24]. At chemical equilibrium, eqn. (3) is obtained, where R represents the

residual fraction of active enzyme [23]. In cases where $[\text{EI}^*] > [\text{EI}]$, eqn. (4) (taken from Segel [25]) can be used, where $[\text{E}]_i$ and $[\text{I}]_i$ represent the initial concentrations of free enzyme and inhibitor respectively.

$$k_{\text{app}} = k_{-2} + \left[k_2 \frac{[\text{I}]/K_i}{1 + [\text{I}]/K_i} \right] \quad (1)$$

$$\frac{1}{k_{\text{app}}} = \frac{1}{[\text{I}]} \frac{k_{-1}}{k_1 k_2} + \frac{1}{k_2} \quad (2)$$

$$\frac{1}{[\text{I}]} = \frac{k_1 k_2}{k_{-1} k_{-2}} \frac{1}{1 - R} - \frac{1 + k_2}{k_{-2}} \frac{k_1}{k_{-1}} \quad (3)$$

$$[\text{EI}^*] = \frac{([\text{E}]_i + [\text{I}]_i + K_d) - \sqrt{([\text{E}]_i + [\text{I}]_i + K_d)^2 - 4([\text{E}]_i \cdot [\text{I}]_i)}}{2} \quad (4)$$

UV/visible difference spectroscopy

Absorbance spectra were measured using a Cary 1E Varian spectrophotometer at a constant temperature of 25 °C. The buffers used for titrations and enzymic kinetics were identical, i.e. TEA buffer. Absorbance spectra were measured using a variation of the method of Santi et al. [26]. In method A, absorption spectra were scanned either between 250 and 500 nm or at wavelengths corresponding to the maximum and minimum absorption, and recorded as a function of time. Measurements were initiated by the addition of DHBA- P at various final concentrations to TEA buffer containing a fixed concentration of aldolase (10 μ M subunit). The measured absorption spectra of the enzyme complex were corrected for absorption by buffer and enzyme alone. The resultant difference absorption spectra were used for determination of the dissociation constant for the aldolase–DHBA- P complex and of the rate constants describing its formation. Method B was used for the titration of DHBA- P and aminocaproic acid against enzyme. In each assay, enzyme (or aminocaproic acid) was added at different final concentrations in the presence of a fixed DHBA- P concentration (10 or 50 μ M). Spectra were recorded at timed intervals. Difference absorption spectra corresponding to complex-formation were corrected for absorption by buffer and DHBA- P alone.

Apparent first-order rate constants (k_{app}) and limiting maximal absorption differences (ΔA_{max}) were obtained for each assay by fitting the time-dependent absorption data to a first-order kinetic equation (or the sum of two first-order kinetic processes). The dissociation constant (K_d) was obtained from experimentally determined maximal absorption differences using eqn. (4). The dissociation constant K_i for the rapidly formed aldolase–DHBA- P complex and the rate constants k_2 and k_{-2} , corresponding respectively to the formation and dissociation of the slow-reacting aldolase–inhibitor complex, were derived from analysis of apparent rate constants using eqn. (1).

The first-order rate constant k_{-2} was also derived independently from absorbance data corresponding to the displacement of DHBA- P from the enzymic complex by 10 mM hexitol- P_2 (method A).

All UV/visible difference spectroscopy experiments using aldolase mutants (10 μ M subunit) were performed using method A.

Aldolases

The construction, overexpression and purification of native recombinant rabbit muscle aldolase and site-directed mutants was performed as described elsewhere [11,27].

Syntheses

NMR spectra were recorded on a Bruker AC200 apparatus (200 MHz ^1H NMR, 80 MHz ^{31}P NMR and 50 MHz ^{13}C NMR); chemical shifts are reported in p.p.m. with respect to trimethylsilane for ^1H and ^{13}C spectra. For ^{31}P spectra, H_3PO_4 was used as the internal standard.

RSN- P_2

This compound was prepared as described previously [19].

HQN- P_2

The hydrolysis of hydroquinone bisphosphodichloride (2 g; 5.8 mmol), obtained as described previously [28], yielded under mild conditions [19] the acid form of HQN- P_2 as a white solid (1.56 g; 99.2% pure); this was converted into its sodium salt with NaOH. ^1H NMR (200 MHz, $^2\text{H}_2\text{O}$), δ 7.03 (s); ^{31}P NMR (80 MHz, $^2\text{H}_2\text{O}$), δ -3.78 (s). $\lambda_{\text{max}} = 275 \text{ nm}$ ($\epsilon = 1384 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

HBA- P

This compound was prepared using the method of Friedman and Seligman [29]. To a mixture of 4-hydroxybenzaldehyde (0.1 g; 0.82 mmol) and phosphorus oxychloride (1 equiv.) heated in dry benzene (10 ml) under reflux, dry pyridine (1 equiv.) was added dropwise for 15 min. The mixture was then cooled to room temperature. The white precipitate (pyridinium chloride) was removed by filtration and washed with a small amount of dry benzene. The filtrate and washings were pooled and the solvent was removed by evaporation. The remaining residue was neutralized (pH 7–8) with a solution of sodium bicarbonate (10%, w/v). The resulting solution (diluted with water to 2 ml final volume) was purified by anion-exchange chromatography using a DEAE-Sephadex A25 resin column (30 cm $\times$ 1 cm) developed with a linear gradient of triethylammonium bicarbonate buffer, pH 7.8 (0.01–0.5 M in 4 h; flow rate 0.5 ml/min). Column fractions (3 ml) were monitored at 280 nm and assayed for phosphate content by ^{31}P NMR. Column fractions containing the required product were combined and lyophilized to yield HBA- P as the triethylammonium salt (99 mg; 39.8% pure). ^1H NMR (200 MHz, $^2\text{H}_2\text{O}$): δ 9.73 (s, 1H, CHO), 7.63 (d degenerated, J 9.12 Hz, 2H), 7.30 (d, J 8.3 Hz, 2H), 3.09 (q, J 7.3 Hz, 6H), 1.16 (t, J 7.3 Hz, 9H). ^{13}C NMR (50 MHz, $^2\text{H}_2\text{O}$): δ 197.7 (s, CHO), 161.4 (d, $^2J_{\text{CP}}$ 6.0 Hz), 134.6, 133.5, 123.1 (d, $^3J_{\text{CP}}$ 5.2 Hz), 49.1 (CH_2), 10.6 (CH_3). ^{31}P NMR (80 MHz, $^2\text{H}_2\text{O}$): δ -2.66. High-resolution MS (FAB $^-$), (M-H) $^-$: calculated, 200.9953; found, 200.9936 (elemental composition $\text{C}_7\text{H}_6\text{O}_5\text{P}$).

DHBA- P

The synthesis of DHBA- P from 2,4-dihydroxybenzaldehyde was essentially as for HBA- P , and a yield of 20% was obtained for the bis-triethylammonium salt. ^1H NMR (200 MHz, $^2\text{H}_2\text{O}$): δ 9.66 (s, 1H, CHO), 7.57 (d, 1H, J 8.6 Hz, ^6H), 6.79 (dd, 1H, J 8.6 Hz, $^4J_{\text{HP}}$ 0.8 Hz, ^5H), 6.70 (d, 1H, $^4J_{\text{HP}}$ 1.6 Hz, ^3H), 3.07 (q, 12H, J 7.3 Hz, 1.16 (t, 18H, J 7.3 Hz). ^{13}C NMR (50 MHz, $^2\text{H}_2\text{O}$): δ 198.5, 164.4, 163.8 (d, $^2J_{\text{CP}}$ 5.9 Hz), 138.0, 119.2, 115.5 (d, $^3J_{\text{CP}}$ 5.4 Hz), 109.7 (d, $^3J_{\text{CP}}$ 5.4 Hz), 49.1, 10.8. ^{31}P NMR (80 MHz, $^2\text{H}_2\text{O}$): δ -1.62. High-resolution MS (FAB $^-$) (M-H) $^-$: calculated, 216.9902; found, 216.9884 (elemental composition $\text{C}_7\text{H}_6\text{O}_6\text{P}$). $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ (pH 6.5): 230 nm (ϵ 7800 $\text{M}^{-1} \cdot \text{cm}^{-1}$), 276 nm (ϵ 13970 $\text{M}^{-1} \cdot \text{cm}^{-1}$), 317 nm (ϵ 6170 $\text{M}^{-1} \cdot \text{cm}^{-1}$). The pK_a of the phenol group (= 8.30) was determined by spectrophotometric

titration using the apparent molar absorption coefficient value of the phenate form at 370 nm.

RESULTS

Competitive inhibition of rabbit muscle aldolase by RSN- P_2 , HQN- P_2 and HBA- P

Rabbit muscle aldolase activity was competitively inhibited in the presence of the aromatic phosphorylated derivatives RSN- P_2 , HQN- P_2 and HBA- P , with K_i values of 40 ± 10 , 135 ± 15 and $500 \pm 60 \mu\text{M}$ respectively. The Michaelis constant (K_m) determined for Fru(1,6) P_2 as substrate was $15 \pm 5 \mu\text{M}$. Addition of sodium borohydride to aldolase in the presence of HBA- P did not inactivate the enzyme irreversibly, indicating that the competitive inhibition observed does not involve Schiff-base formation.

Reversible inhibition of aldolase by DHBA- P

Incubation of aldolase with DHBA- P resulted in a first-order rate loss of enzyme activity (kinetics not shown). Both the rate and extent of inactivation were saturating with respect to inhibitor concentration, with a half-life of $1.0 \pm 0.2 \text{ min}$ and maximum inhibition of $92 \pm 5\%$ (Table 1). DHBA at $200 \mu\text{M}$ inhibited only 5% of total activity (results not shown), compared with the 80% inhibition observed in the presence of DHBA- P at the same concentration. The mode of inhibition by DHBA- P is consistent with slow-binding inhibition [18], since complete reversal of inhibition by DHBA- P occurred in the presence of saturating concentrations (10 mM) of the competitive inhibitor hexitol- P_2 (Table 1). The recovery of enzymic activity obeyed first-order kinetics, with a rate constant k_{-2} of $0.13 \pm 0.05 \text{ min}^{-1}$. By contrast, activity could not be restored after treatment with sodium borohydride, suggesting that the mechanism of inhibition in the case of DHBA- P involves Schiff-base formation.

Values of kinetic parameters were obtained from both residual activities and rates of inhibitor–enzyme complex-formation at different inhibitor concentrations (shown in Table 1), according to eqn. (2) and eqn. (3) respectively (Table 2). Based on these values, K_i^* was estimated to be $35 \pm 20 \mu\text{M}$.

Table 1 Interaction of DHBA- P with rabbit muscle aldolase

Native recombinant aldolase (12.5 units/mg; 10 μM subunits) was incubated in the presence of DHBA- P (25–600 μM) in TEA buffer (1 ml final volume; pH 7.0). Aliquots were analysed at various times for enzymic activity (see the Materials and methods section). Maximal degrees of inactivation are shown. The control corresponded to a reaction performed with aldolase alone. The values in parentheses for residual activity at 100 μM DHBA- P represent: *activity present after addition of hexitol- P_2 following inactivation by DHBA- P , and †residual activity of the same solution treated with sodium borohydride prior to addition of hexitol- P_2 . Aldolase was inactivated with DHBA- P (100 μM) as described above. Hexitol- P_2 was then added at a final concentration of 10 mM to a solution containing inactivated aldolase that had previously been incubated either with or without sodium borohydride (see the Materials and methods section). Aliquots were analysed at various times for enzymic activity. Controls consisted of identical protocols without DHBA- P . No activity loss (3%) was detected with a sodium borohydride-treated control that had previously been incubated without DHBA- P . Residual activities have an error of $\pm 5\%$.

DHBA- P (μM)	Residual activity (%)
0	100
25	56
50	41
100	28 (98*; 40†)
200	20
300	13
600	8

Table 2 Kinetic parameters describing the interaction of DHBA-*P* with aldolase in TEA buffer (pH 7.0)

Parameter	Value
K_i (μM)	$200 \pm 100^*$; $300 \pm 100^\dagger$
k_2 (min^{-1})	$0.8 \pm 0.2^*$
k_{-2} (min^{-1})	$0.13 \pm 0.05^\ddagger$
K	$0.06 \pm 0.01^\dagger$
K_d (μM)	$21 \pm 10^\ddagger$

* Calculated using eqn. (2).

† Calculated using eqn. (3).

‡ Calculated from data corresponding to activity recovered (see Table 1).

Figure 1 Interaction of DHBA-*P* with aminocaproic acid and with native recombinant rabbit muscle aldolase

Upper panel: UV/visible difference spectra of a mixture of $50 \mu\text{M}$ DHBA-*P* and 50 mM aminocaproic acid in TEA buffer (1 ml; pH 7.0) with time (interval of 5 min). Lower panel: UV/visible difference spectra of $10 \mu\text{M}$ aldolase subunits and $100 \mu\text{M}$ DHBA-*P* with time (interval of 2 min).

Interaction of DHBA-*P* with aminocaproic acid

The reaction of DHBA-*P* with the lysine residues of aldolase was modelled based on the reaction of DHBA-*P* ($50 \mu\text{M}$) with aminocaproic acid ($10\text{--}300 \text{ mM}$) [26]. The model reaction, shown in Figure 1 (upper panel), is characterized by changes in UV/visible absorbances corresponding to maxima at 296 nm ($\Delta\epsilon$ $7140 \pm 400 \text{ M}^{-1}\cdot\text{cm}^{-1}$) and 376 nm ($\Delta\epsilon$ $5400 \pm 300 \text{ M}^{-1}\cdot\text{cm}^{-1}$) which are ascribed to Schiff-base formation, and two minima at 269 nm ($\Delta\epsilon$ $-5230 \pm 300 \text{ M}^{-1}\cdot\text{cm}^{-1}$) and 323 nm ($\Delta\epsilon$ $-2000 \pm 100 \text{ M}^{-1}\cdot\text{cm}^{-1}$) resulting from loss of DHBA-*P*. The presence of isosbestic points at 255 , 282 , 313 and 337 nm indicates that there was no accumulation of intermediates. Formation of the DHBA-*P*-aminocaproic acid adduct occurred with a K_d of $80 \pm 5 \text{ mM}$ (see, e.g., Figure 2)

Figure 2 Change in absorbance as a function of aminocaproic acid concentration

Absorbance was measured at 376 nm in TEA buffer (pH 7.0). The DHBA-*P* concentration was $50 \mu\text{M}$. Data points are experimental, and the line represents the best fit of the data to eqn. (4). The K_d value determined was $80 \pm 5 \text{ mM}$.

Table 3 Characterization of the interaction of DHBA-*P* with aldolase in TEA buffer (pH 7.0) by UV/visible difference spectroscopy

The results obtained using methods A and B are compared (see the Materials and methods section). $\Delta\epsilon_{386}$ was calculated assuming that 1 molecule of DHBA-*P* binds per aldolase subunit.

Parameter	Method A	Method B
K_d (μM)	40 ± 5	30 ± 5
K_i (μM)	450 ± 25	500 ± 25
k_2 (min^{-1})	1.06 ± 0.05	1.10 ± 0.05
k_{-2} (min^{-1})	0.078 ± 0.003	0.080 ± 0.003
K_i^* (μM)	30 ± 5	34 ± 5
$\Delta\epsilon_{386}$ ($\text{M}^{-1}\cdot\text{cm}^{-1}$)	4700 ± 300	4500 ± 300

Interaction of DHBA-*P* with aldolase

The interaction of aldolase ($10 \mu\text{M}$ subunits) with DHBA-*P* over a 25-fold concentration range ($25\text{--}600 \mu\text{M}$) resulted in difference UV/visible spectra characterized by maxima at 311 and 386 nm , minima at 274 and 337 nm , and isosbestic points at 255 , 290 , 334 and 341 nm (Figure 1, lower panel). Slight wavelength shifts for the enzyme-DHBA-*P* complex with respect to the model reaction adduct (Figure 1, upper panel) are probably due to differences in the environment of the Schiff base. Molar absorption coefficients for bound DHBA-*P* were calculated by assuming that 1 mol of DHBA-*P* binds per mol of aldolase subunit at saturation; the values were $\Delta\epsilon_{311} = 9680 \pm 500 \text{ M}^{-1}\cdot\text{cm}^{-1}$, $\Delta\epsilon_{386} = 4700 \pm 300 \text{ M}^{-1}\cdot\text{cm}^{-1}$, $\Delta\epsilon_{274} = -8960 \pm 500 \text{ M}^{-1}\cdot\text{cm}^{-1}$ and $\Delta\epsilon_{337} = -300 \pm 20 \text{ M}^{-1}\cdot\text{cm}^{-1}$. Difference absorbance spectra were consistent with two successive first-order rate processes. Only the rapid kinetic phase displaying a significantly larger absorbance change and saturating behaviour at high inhibitor concentrations was correlated with loss of enzyme activity, and this was used in subsequent analyses. Formation of the aldolase-DHBA-*P* complex, shown in Figure 1 (lower panel), occurred with a K_d of $40 \pm 5 \mu\text{M}$ (obtained as described for Figure 2).

Results from the kinetic analysis of data describing EI* complex-formation according to eqn. (1) are reported in Table 3.

Table 4 Interaction of DHBA-*P* with recombinant mutant aldolases generated by site-directed mutagenesis of active-site lysine residues and Glu-187

Assays were performed in TEA buffer (pH 7.0) according to method A (see the Materials and methods section) at fixed aldolase (10 μ M subunits) and DHBA-*P* (100 μ M) concentrations. Rates of Schiff-base formation were monitored at 386 nm. nd, not determined.

Aldolase	Specific activity (units/mg)	Residual specific activity (%)	k_{app} (min ⁻¹)	Relative ΔA at 368 nm (%)
Wild type	12.5	25	0.21 $\pm$ 0.01	100
			0.010 $\pm$ 0.001	30
K107M	0.610	25	0.22 $\pm$ 0.01	98
			0.009 $\pm$ 0.001	25
K146M	1.8 $\times 10^{-3}$	95	0.009 $\pm$ 0.001	30
K229M	2 $\times 10^{-2}$	40	0.10 $\pm$ 0.01	30
			0.010 $\pm$ 0.001	40
K229A	nd	nd	0.09 $\pm$ 0.01	30
			0.010 $\pm$ 0.001	35
E187Q	4.8 $\times 10^{-3}$	20	0.031 $\pm$ 0.003	135

The overall dissociation constant K_i^* calculated from these results corresponds to $30 \pm 5 \mu$ M. Analysis of the data recorded at 386 nm according to method B (see the Materials and methods section) yielded the values shown in Table 3, which are in good agreement with those obtained by method A.

Dissociation of the DHBA-*P*-enzyme complex, monitored at 386 nm, was observed at saturating concentrations (10 mM) of the competitive inhibitor hexitol- P_2 (results not shown), and displacement of DHBA-*P* from the enzymic complex was characterized by a first-order rate constant k_{-2} of $0.09 \pm 0.005 \text{ min}^{-1}$, a value in agreement with that determined by methods A and B. Although the hexitol- P_2 chase fully restored enzyme activity, a fraction of DHBA-*P* complexed with the enzyme (approx. 25%) could not be displaced and represents a secondary, non-specific, reaction. The relative concentration of this enzyme fraction is consistent with the amplitude observed for the slow phase in Schiff-base formation.

The stoichiometry of DHBA-*P* binding to aldolase, calculated on the basis of the differential molecular absorbance of the aminocaproic-acid-DHBA-*P* complex, corresponds at maximal inhibition to an EI* complex of 3.2–3.5 mol of DHBA-*P* bound per mol of tetrameric aldolase.

Interactions of site-directed aldolase mutants with DHBA-*P*

Lysine mutants

To ascertain which lysine residue (107, 146 or 229) in the active site is responsible for the differential absorbance ($\lambda_{max} = 386 \text{ nm}$) observed in the presence of inhibitor, several point mutations of active-site lysine residues were constructed. The active-site mutants K107M, K146M, K229M and K229A possess no charge, are virtually isosteric (except for K229A) and are unable to participate in Schiff-base formation. Complex-formation in the presence of DHBA-*P* (100 μ M) was examined for each mutant by UV/visible difference spectroscopy (method A), and analysis of the results is shown in Table 4.

UV/visible difference spectra observed for the mutant K107M were identical with those observed with the native recombinant enzyme. Schiff-base formation could be accounted for in terms of two distinct first-order rate processes. The kinetic parameters derived for the faster rate process, the inhibition of enzyme activity and EI* complex-formation were identical with those of

the native enzyme, and this rules out Lys-107 as being the lysine residue implicated in the observed inactivation.

The reaction of the K146M mutant enzyme with DHBA-*P* gave rise to only a slow kinetic phase whose rate constant was similar to the value observed for the native recombinant enzyme. Although catalytic activity is highly compromised by the mutation of Lys-146 to Met, inhibition of the residual catalytic activity was slight. The absence of a rapid phase strongly suggests that Lys-146 is responsible for the observed slow-binding inhibition (Table 4).

The inhibition kinetics for the K229M and K229A mutants were in accordance with the occurrence of two distinct first-order rate processes, similar to those observed for the recombinant native enzyme (Table 4). The substitution of Lys-229 with Met diminished (but did not abolish) the capacity of this mutant to form the EI* complex, compared with wild-type aldolase. Based on these data, Lys-229 is not required for EI* complex-formation, but merely facilitates it.

Glu-187 mutant

The positioning of active-site residues in rabbit muscle aldolase [7] places Glu-187 within potential hydrogen-bonding distance of both Lys-146 and Lys-229, suggesting that this residue may participate in Schiff-base formation with DHBA-*P*. To examine this hypothesis, an isosteric and electrically neutral mutant, E187Q, was constructed.

The mutant E187Q displayed considerably lowered activity towards Fru(1,6) P_2 , indicating a functional role for Glu-187 in catalysis (Table 4). Reaction of this mutant with DHBA-*P* resulted in an overall absorbance change comparable at equilibrium with that observed for the native recombinant enzyme. The inactivation kinetics differed from those observed for the native recombinant enzyme, and are consistent with a first-order rate process corresponding to very slow Schiff-base formation ($\lambda_{max} = 386 \text{ nm}$) that cannot be resolved into two distinct rate processes. The significant decrease in the rate of Schiff-base formation suggests that Glu-187 most probably participates in a proton transfer step of the reaction mechanism.

DISCUSSION

Phosphorylated substrate analogues of mammalian aldolases possessing an aromatic moiety inhibit aldolase activity. These aromatic substrate analogues bind at the active site and interact with amino acid residues essential for catalytic activity. The inhibition constants obtained for the bisphosphorylated compounds RSN- P_2 and HQN- P_2 (40 μ M and 135 μ M respectively) compare quite favourably with the inhibition constant of 25 μ M for bisphosphorylated diols synthesized from C_6 alkanes [30]. The rigid structure imposed by an aromatic ring compared with a compound that is not conformationally restricted presumably would tend to favour a smaller entropy loss upon binding, and may be responsible for the observed affinity differences in enzyme-inhibitor complex-formation.

Monophosphorylated analogues of the same class of compounds exhibit considerably poorer affinity for the enzyme (K_i for phenyl phosphate = 2.3 mM [19]). Incorporation of an aldehyde group into these monophosphorylated analogues in the *para* position with respect to the phosphate improves affinity (e.g. HBA-*P*; $K_i = 500 \mu$ M). Further incorporation of a hydroxy group in a position *ortho* to the aldehyde group (DHBA-*P*) leads to time-dependent inhibition resulting in a maximum 92% loss of activity that is slowly reversed and fully restored by hexitol- P_2 under saturating conditions. The inactivation kinetics for this compound correspond to slow-binding inhibition [18]. Irrevers-

Scheme 2 Proposed mechanism for Schiff-base formation between DHBA-*P* and Lys-146

Proton transfer between Lys-229 and Lys-146 is mediated by Glu-187. Lys-146 in the neutral form reacts with the aldehyde group of DHBA-*P*, resulting in the carbinolamine intermediate. The dehydration is made possible by protonation of this intermediate by Glu-187 in the case of wild-type aldolase, or less efficiently by the phenol moiety in the case of the E187Q mutant aldolase.

ible inactivation was observed upon treatment of the DHBA-*P*-enzyme complex with sodium borohydride. By contrast, treatment of the enzyme in the presence of HBA-*P* with sodium borohydride did not result in irreversible inactivation, in accordance with the absence of detectable Schiff-base formation. This finding indicates that the hydroxy group is required for inactivation by DHBA-*P* through a mechanism involving Schiff-base formation, since this latter species is not observed in the presence of HBA-*P*. This group can act as an acid catalyst for the formation of the carbinolamine intermediate. When the immonium species is thus formed, the negative charge of the phenate contributes additional stabilization by acting as an internal counter-ion.

The similarity of the UV/visible difference spectra of the enzyme-DHBA-*P* complex with those of the complex formed on reaction of DHBA-*P* with aminocaproic acid, a model reaction used for studying Schiff-base formation by lysine residues [26], further suggests Schiff-base formation during enzyme inactivation. Although the kinetics observed are first-order for both the model and enzyme reactions, a single kinetic process is observed in the case of the model reaction, whereas for the enzymic reaction two distinct kinetic processes are present. The faster kinetic process is associated with the larger absorbance change and results in inactivation. Comparison of K_d values indicates that the DHBA-*P*-enzyme complex formed has substantially greater stability than the aminocaproic-acid-DHBA-*P* complex. Moreover, the significant inhibition of aldolase by DHBA-*P* compared with DHBA, which is not phosphorylated, underscores the importance of the phosphate group of DHBA-*P* for binding at the aldolase active site.

The extents of enzyme inactivation and of Schiff-base formation are very similar, and both are maximal when 3.2–3.5 molecules of DHBA-*P* have been covalently bound by an aldolase tetramer. The presence of residual catalytic activity is consistent with the model used to describe the inactivation kinetics, shown in Scheme 1, which involves rapid equilibration of the Michaelis complex EI with the unliganded enzyme.

Displacement of DHBA-*P* from the enzymic complex by hexitol-*P*₂, a competitive inhibitor, suggests that, in the native enzyme, Schiff-base formation most probably occurs with lysine residues in the active site. Site-directed mutagenesis of the active-site lysine residues 146 and 229 to neutral amino acids yielded mutant recombinant proteins (K146M, K229M and K229A) that displayed not only diminished catalytic activity but also modified inactivation kinetics. The most significant change in

DHBA-*P* inactivation was for mutant K146M, for which Schiff-base formation was not detected and enzyme inactivation did not occur. The identification of Lys-146 as the amino acid essential for inactivation by DHBA-*P* is consistent with inactivation results obtained for the other mutants. The inactivation kinetics for the mutant K107M were very similar to those of the wild type, and thus Lys-107 cannot be the amino acid implicated in inactivation. The mutations K229M and K229A of the lysine residue responsible for Schiff-base formation with the substrate diminished, but did not eliminate, the capacity of the enzyme to promote Schiff-base formation with DHBA-*P*. Modulation of inactivation, however, by these two mutations suggests that Lys-229 must participate in the reaction with DHBA-*P*. A functional interaction between these two lysine residues is not inconsistent with the decreased rate of Schiff-base formation observed with substrate by mutant aldolases in which Lys-229 and Lys-146 have been altered [12]. From the three-dimensional structure, Lys-229 and Lys-146 are proximal, thereby lowering their respective pK_a values and consistent with the alkylation of Lys-146 by *N*-bromoacetyethanolamine phosphate that occurs at pH 8.5 [13].

The rate of inactivation by DHBA-*P* was greatly diminished for the mutant E187Q, yet the amount of Schiff base formed was not significantly different compared with the native enzyme, suggesting a functional role for Glu-187. Although the carboxamide moiety of Gln-187 is a weaker base than the carboxylate function of Glu-187, its capacity to participate in hydrogen-bond formation is very similar [31]. The carboxamide could thus contribute through hydrogen bonding to stabilization of the immonium species, but would not participate efficiently in proton transfer.

The postulated mechanistic role of Glu-187 during Schiff-base formation between DHBA-*P* and Lys-146 appears to be twofold: (i) as a base, Glu-187 could deprotonate Lys-146, and (ii) then, as a general acid, it could transfer a proton to the carbinolamine intermediate, the proton being required for the rate-limiting dehydration step at neutral pH [32,33]. These roles are consistent with the occurrence of immonium ion formation in the mutant E187Q similar to the wild-type enzyme, but at a considerably lower rate. The lower rate can be attributed to protonation of the carbinolamine intermediate by the immediate phenolic group, a much weaker acid ($pK \sim 10$ [34]) than the protonated form of Glu-187 (Scheme 2).

The results obtained in the present study are also of significance with respect to Schiff-base formation with substrate: the sub-

stantially compromised catalytic activity of the E187Q mutant obviously implies a functional role for Glu-187 in the normal catalysed reaction of Fru(1,6) P_2 cleavage. From the present study, it can be concluded that Lys-229 and Glu-187 mediate the reaction of DHBA-*P* with Lys-146. The positioning of Lys-146, Glu-187 and Lys-229 in the active site of rabbit muscle aldolase indicates that the mechanisms of Schiff-base formation between Lys-229 and Fru(1,6) P_2 , and between Lys-146 and DHBA-*P*, are similar and involve proton transfer mediated by Glu-187.

From a pharmacological perspective, DHBA-*P* represents a novel example of a slow-binding inhibitor for aldolase and represents both: (i) a specific probe for Lys-146, and (ii) a lead compound for the development of a new class of aldolase inhibitors.

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