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Kinetic and spectroscopic study of slow-binding inhibition processes in aldolase†

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ABSTRACT: Compounds similar in structure to reactants, intermediates and products of the aldolase-catalysed reaction were synthesized and their affinities for the enzyme determined. The best situations were found with β-dicarbonyl phosphorylated compounds which are a good mimics of the incoming groups in the bond-forming process; the corresponding binding is characterized by slow-binding inhibition type, the inhibitors forming stabilized iminium ions and enamines with the enzyme; similar effects were obtained with an aromatic aldehyde, also capable of forming a stabilized iminium ion. The use of aldolase mutants allows one to characterize the lysyl group involved in the process and also to suggest a proton transfer mechanism for the iminium ion formation with the enzyme natural substrate. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: aldolase; slow-binding inhibition; enzyme affinity

INTRODUCTION

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) reversibly catalyses the cleavage of fructose 1,6-bisphosphate (FDP) into triose phosphates D-glyceraldehyde phosphate (GAP) and dihydroxyacetone phosphate (DHAP).[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 cofactor. Class I aldolases can be classified into three isoenzyme forms, distinguishable on the basis of immunological reactivity and of turnover with respect to FDP and fructose 1-phosphate substrates.[3] Isoenzyme A, from rabbit muscle, has been the most extensively studied of class I aldolases.[4,5] Over 14 different isoenzymes have been sequenced[6] and three aldolase isoenzymes 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 aldolase 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. The aldol condensation proceeds by several ordered steps (Scheme 1): (i) iminium ion (or Schiff base) formation between the carbonyl of DHAP and the ε-amino group of an essential Lys residue (Lys 229); (ii) enamine formation after *pro-S* proton abstraction at C-3 in the iminium ion; (iii) enamine reaction with the carbonyl of GAP to form a new C—C bond and a second Schiff base; and (iv) hydrolysis of the latter iminium ion leading to FDP and free enzyme.

Owing to the analogy between the reaction catalysed by aldolase and the chemical aldolization in acidic conditions, the intermediate enzymatic enamine being the equivalent of the enol, this enzyme offers an interesting situation in considering how evolution has arrived at an efficient catalytic process.

One of the significant aspects of the parallel between the two situations is the energy profile of the two reactions: whereas that of the chemical reaction is the formation of an enol intermediate either in a fast pre-equilibrium or in a steady state followed by the addition slow step, the profile for the enzymatic reaction is more complex. It implies a larger number of intermediates and another important feature is that product release in either direction becomes the slow step.[10] This situation is outlined in Fig. 1.

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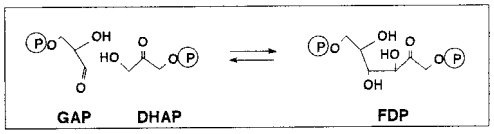
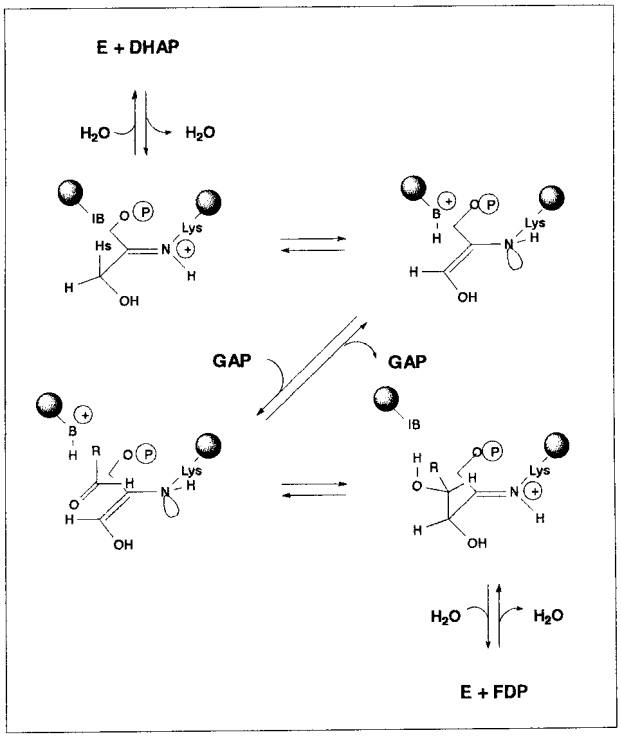




Scheme 1. Reaction catalysed by aldolase and mechanistic pathway.

To gain a better insight into the mechanistic importance of the different intermediates evidenced on the aldolase pathway, compounds similar in structure to three representatives of these intermediates, early, medium and late on the energy profile, were designed and their affinities to the enzyme determined. For one of them, the binding site to the enzyme was determined using different mutants. This strategy also allowed additional information on proton transfer at the aldolase active site.

RESULTS

The compounds under study are presented in Scheme 2. Compounds **1a–e** are DHAP analogues. Although it is known[11] that only slight modifications at C-1 in the DHAP structures are accepted, these compounds were assayed since they possibly correspond to intermediates of the left part of the energy profile in Fig. 1.

Compounds **2a, 2b** and **3** correspond to a central situation in the profile; they can be considered as mimics of the two approaching insaturated groups in the carbon–carbon bond formation step; compounds **6–8** are FDP analogues and therefore susceptible to forming intermediates analogous to those in the right part of the profile.

Compounds **4** and **8** are able to form iminium ion intermediates stabilized by the neighbouring OH group. Compound **9**, an FDP analogue, was synthesized as a possible inhibitor in the α-diketo open form. Finally, compound **10** was studied on the grounds of the high

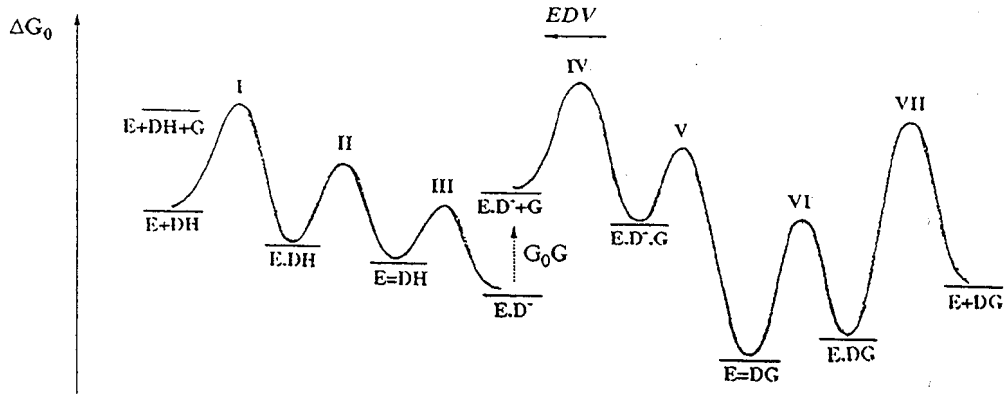
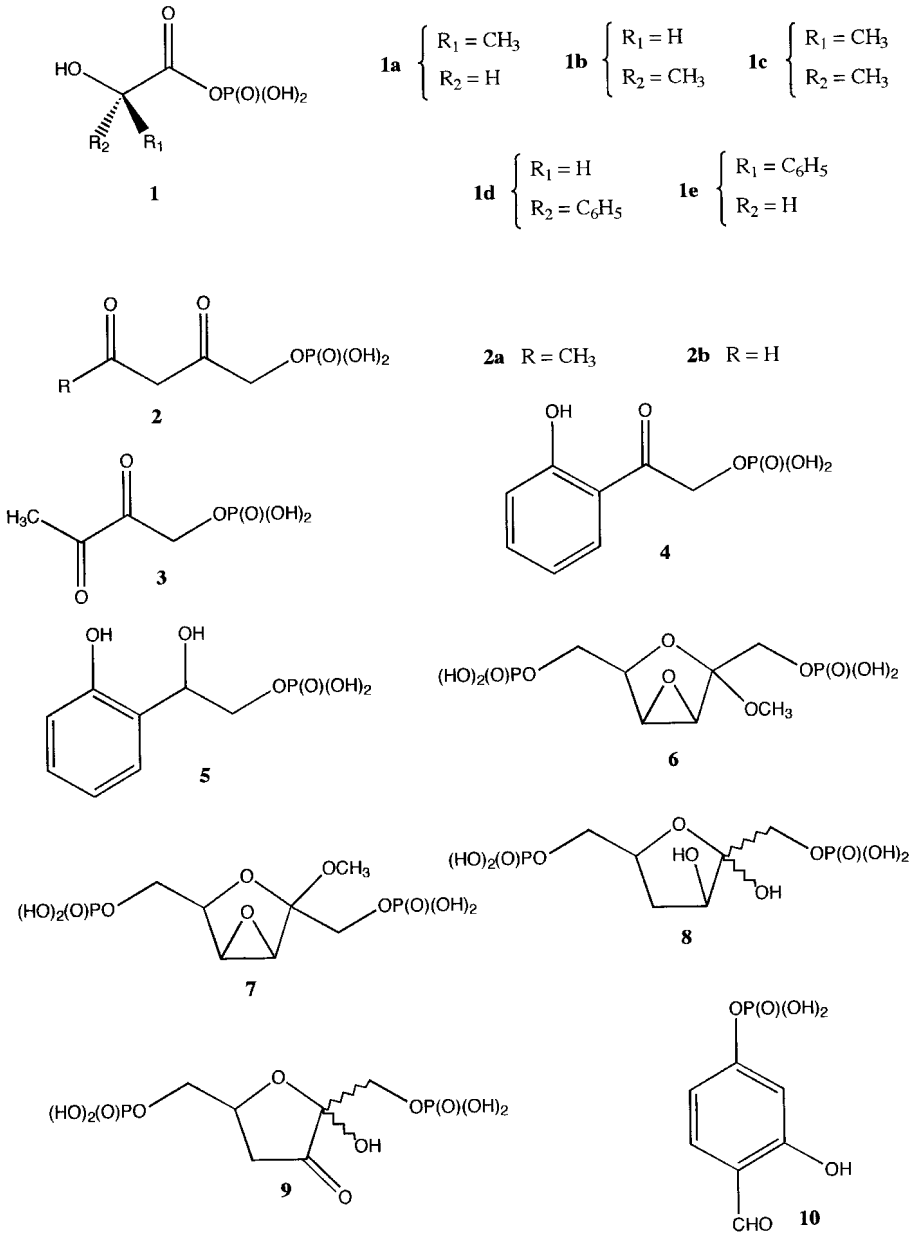


Figure 1. Possible energy profile for aldolase. The left part represents the relative energy levels of the complexes formed between aldolase and DHAP and the right part those of FDP (noted DG). The gap between the two parts corresponds to the standard free enthalpy difference due to the contribution of GAP (referred to as G_0G). The different complexes are indicated as follows: E.DH, non-covalent complex between DHAP and aldolase; E = DH, iminium for DHAP–enzyme; ED^-, enamine–enzyme; ED^-.G, ternary complex enamine–GAP–enzyme; E = DG, iminium for FDP–enzyme; E.DG, non-covalent complex between FDP and enzyme; E + DH/E + DG/ED^- + G, aldolase + substrates. The relative energy levels of the ground states and intermediates are fixed according to the radioactivity distribution starting from DHA32P or FD32P, under equilibrium conditions. The activation barriers are fixed, based on the following considerations: (i) from left to right (FDP synthesis direction), DHAP iminium formation (step II) and enamine formation (step III) are faster than the aldolization step (step IV). Also, addition of GAP to the ED^- complex is followed by the fast formation of FDP (steps IV, V and VI) and slow release of FDP (step VII); (ii) from right to left (FDP cleavage), isotope effects indicate that the rate-determining step is either C3—C4 bond cleavage (step V) or GAP release (step IV). Complementary evidence indicated that the slow step is IV (see Ref. 5).



Scheme 2. Compounds in the study.

affinity of aldolase for aromatic phosphorylated compounds.[12]

Compounds 1a–1e

With compounds **1a–e**, different situations are observed: **1a** and **b** give a time-dependent inactivation of aldolase, meaning that they have a significant affinity for the enzyme. This inactivation is protected by DHAP and is reversed by addition of FDP. Such time-dependant and reversible inactivation corresponds to a slow-binding process as defined by Morrisson and co-workers:[13] the effect of an intermediate analogue of high affinity on the enzyme activity and/or conformational change of the enzyme (see Scheme 4). The fact that this inhibition can no longer be reversed after treatment with sodium borohydride indicates that an iminium ion intermediate has been formed between aldolase and **1a** or **b**. A full analysis of the process given elsewhere[14] allows the determination of the slow-binding parameters for these two compounds (Table 1).

Conversely, **1c** only gives a competitive inhibition process. Double reciprocal plots[15] allow the determination of the inhibition constant K_i = 4.0 mM which corresponds to a weak affinity since that of the substrate DHAP is 50 μM. Finally, **1d** and **e**, with a bulkier substituant at C-3, produce only a poor effect ($K_i > 10$ mM).

Table 1. Kinetic parameters for aldolase inhibition by **1a, 1b, 2a** and **2b**

Constant	**1a**	**1b**	**2a**	**2b**
K_i (μM)	800	240	1250	900
K	0.95	0.45	0.120	0.0047
k_2 (min^{-1})	0.105	0.245	0.620	0.70
k_{-2} (min^{-1})	0.10	0.11	0.074	0.0033
K_i* (μM)	390	74.5	130	3.5

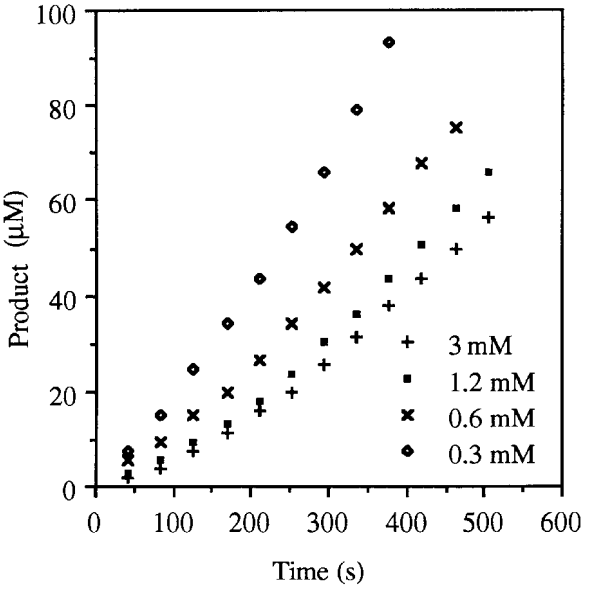


Figure 2. Reversion of aldolase inhibition by **2a** in the presence of FDP. Aldolase (0.20 mg ml^{-1} TEA buffer, pH 7.6) was inhibited by **2a** at the indicated concentrations. Reversal of inhibition was determined on 10 μl aliquots diluted in 1 ml of assay solution containing 1 mM FDP. The rate value was extrapolated to infinite using Eqn (1) (see Experimental section) and found to be identical with the reference assay (made with the aldolase alone).

Compounds 2a–4

Although these four compounds are liable to form stabilized iminium ion structures, they exhibit different behaviour patterns.

Compound **4** demonstrates a competitive inhibition which is prevented by addition of DHAP and therefore occurs at the active site; this inhibition is not changed following sodium borohydride treatment and therefore probably involves no iminium ion intermediate; double reciprocal treatment allows the determination of the inhibition constant, K_i = 650 μM. It is noteworthy that the corresponding alcohol **5** (in the racemic form) also behaves as a competitive inhibitor and with a better affinity, since the corresponding inhibition constant is 165 μM. Results obtained with **4** and **5** indicate that, contrary to what might be concluded from assays with **1d** and **e**, compounds bearing an aromatic ring can be accommodated in the active site of aldolase with a significant affinity although no iminium structure is formed.

Compound **3** should be able to form an iminium ion possibly stabilized by the second enolizable carbonyl group. In fact, a different situation is observed since **3** induces a time-dependent irreversible inactivation of aldolase. The classical treatment of this type of inhibition (k_{-2} = 0 in Scheme 4)[15] allows the determination of the corresponding parameters: K_i = 3.6 mM and k_2 = 0.03 min^{-1}. These values correspond to a compound of weak affinity (see below, compound **2a**).

With **2a**, a different pattern was obtained, since this compound gives a slow-binding inhibition process. From the study of the interaction between aldolase and **2a** (see Fig. 2 as an example and the Experimental section), the different parameters for **2a** could be determined (Table 1).[16]

As for **2b**, incubation of the enzyme with this compound leads to a first-order loss of enzyme activity. Under the same conditions as those used for **2a** (Fig. 2), no significant restoration of enzyme activity was detected, allowing this compound to be considered and analysed as an irreversible inhibitor (Table 1, K_i and k_2 values). However, the inhibition could be slowly reversed in presence of a competitive inhibitor of high affinity such as phenyl 1,4-diphosphate and therefore demonstrated the slow-binding process (Table 1).[16]

For both **2a** and **b**, the slow-binding inhibition was associated with iminium ion formation (or enamine), possibly stabilized through enolization of the second cabonyl group; this could not be clearly proved by sodium borohydride treatment, but by following the absorbance change in UV difference spectroscopy observed by mixing **2a** or **b** and aldolase. The same experiment was carried out with **2a** or **b** and aminocaproic acid as reference for such iminium ion formation. Full analysis of this parallel has been given elsewhere[16] with additional proof for the binding of **2a** or **b** at the active site Lys 229 residue.

Compounds 6–9

Two of these compounds, **8** and **9**, exist in closed and open forms, whereas **6** and **7** can only be cyclic. Compound **8** can form an iminium ion similar to that of FDP in the retroaldolization process (Scheme 1), but the reaction cannot go any further owing to the lack of an OH group at the C-4 position. The same is true with **9**, but it can alternatively bind to an arginine through the α-diketo structure. Finally, the epoxide group in **6** and **7** is liable to promote covalent binding with the active site Asp 33 residue as nucleophile, this residue being considered as interacting with the OH group at the C-4 position.[17]

The results were as follows: **8** does not give any time-dependent effect, but behaves as a competitive inhibitor with an affinity constant of 70 μM, slightly higher than

Table 2. Kinetic parameters for the interaction of **10** with aldolase in TEA buffer: (a) based on aldolase activity and (b) based on UV–visible difference spectroscopy

Constant	Method a	Method b
K_i (μM)	200 ± 100[a] 300 ± 100[b]	500 ± 25
K_d (μM)	21 ± 10[b]	30 ± 5
k_2 (min^{-1})	0.8 ± 0.2[a]	1.10 ± 0.05
k_{-2} (min^{-1})	0.13 ± 0.05[b]	0.080 ± 0.003
K_i* (μM)	35 ± 10	34 ± 5
$\Delta\varepsilon_{386}$ ($l\ mol^{-1}cm^{-1}$)		4700 ± 300[c]

[a] Using Eqn (3).
[b] Using Eqn (4).
[c] $\Delta\varepsilon_{386}$ was calculated assuming that one molecule of **10** binds per aldolase subunit.

Table 3. Interaction of **10** with lysine and Glu 187 mutant aldolases[a]

Aldolase[b]	Specific activity ($U\ mg^{-1}$)	Residual specific activity (%)	k_{app} (min^{-1})	Relative ΔA at 386 nm (%)
Wild type	12.5	25	0.216	100
			0.0102	30
K107M	0.610	25	0.228	98
			0.009	25
K146M	1.8×10^{-3}	95	0.0095	30
K229M	0.020	40	0.100	30
			0.0102	40
K229A	nd[c]	nd[c]	0.090	30
			0.0098	35
E187Q	4.8×10^{-3}	20	0.03	135
E187D	7.9×10^{-2}	30	0.140	70
			0.010	30

[a] Assays were performed in TEA buffer (pH 7.0) at fixed aldolase (10 μM subunits) and **10** (100 μM) concentrations. Rates of Schiff base formation were monitored at 386 nm.
[b] K = Lys; M = Met; A = Ala; Q = Glu; D = Asp.
[c] Not determined.

that of FDP (15 μM). Under conditions identical with those used to characterize the iminium of DHAP by sodium borohydride addition, and where at least 70% inactivation is observed, only 20% inactivation is observed with **8**. With this compound, the corresponding iminium should not be stabilized. A similar situation is observed with **9**, which gives no time-dependent effect and therefore neither covalent binding with an arginine nor a slow-binding effect. Only a competitive effect is observed, with an inhibition constant of *ca* 150 μM. As for **6** and **7**, no time-dependent effect is noted, indicating that epoxide ring opening does not occur. These two compounds, which cannot form iminium ion intermediates owing to the protection of the OH group at position 2, behave as competitive inhibitors, with inhibition constants of 330 and 925 μM, respectively.

Compound 10

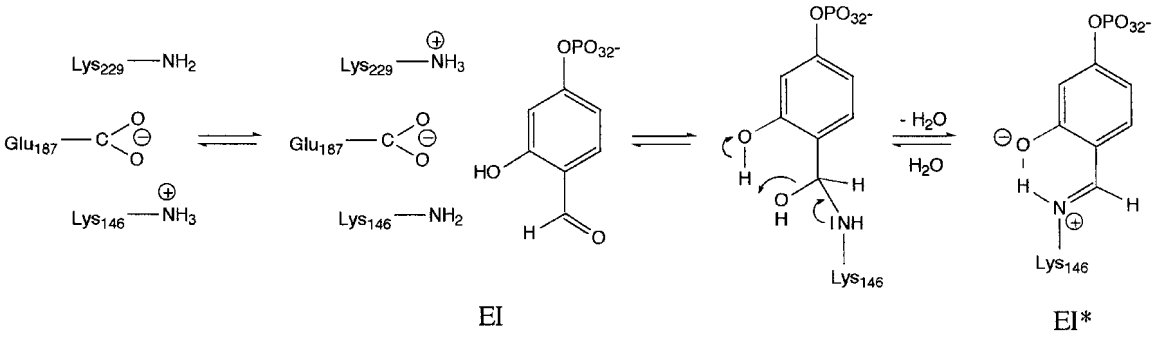
Whereas phenyl phosphate has a weak affinity for aldolase (K_i = 2.3 mM),[10] incorporation of an aldehyde group at the *para* position improves it since K_i drops to 500 μM;[18] however, the inhibition process remains competitive. Adding an OH group on the aromatic ring *ortho* with respect to the aldehyde moiety (**10**) changes the process: a reversible time-dependent inhibition is obtained with Schiff base formation as evidenced by sodium borohydride treatment. Further analysis of the data [using Eqns (3) and (4); see Experimental section] provides the corresponding inhibition parameter values (Table 2, method a).[18]

Using UV–visible difference spectroscopy, this slow-binding effect could be paralleled to the formation of an iminium ion between **10** and the enzyme. The interaction of aldolase (10 μM subunits) with **10** (25–600 μM) resulted in UV–visible difference 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; slight wavelength shifts for the enzyme–**10** complex with respect to the model reaction adduct (**10** plus aminocaproic acid) were observed. Molar absorption coefficients for bound **10** were calculated by assuming that 1 mol of **10** binds per aldolase subunit at saturation (checked by electrospray mass spectrometry; $\Delta\varepsilon_{386}$ was $4700 \pm 300\ l\ mol^{-1}\ cm^{-1}$. The difference absorbance spectra were consistent with two successive first-order processes. Only the rapid kinetic phase displaying a significantly larger absorbance change which has a saturating behaviour at high inhibitor concentrations was correlated with loss of enzyme activity and this was used in subsequent analyses.

From these kinetics, the different inhibition parameters could also be determined (Table 2, method b). The overall dissociation constant K_i* calculated from these results corresponds to 34 ± 5 μM. The good agreement between the two sets of values obtained by entirely independent methods allowed the correlation between the slow-binding process and iminium ion formation. A complete description of these results has been given elsewhere.[18]

Lysine mutants. In collaboration with Professor J. Sygusch (University of Montreal), it was possible to ascertain which lysyl residue at the active site (107, 146 or 229) was responsible for the differential absorbance (λ_{max} = 386 nm) from several point mutations of these active site lysyl residues. 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 **10** (100 μM) was examined for each mutant. The results are shown in Table 3.

The difference spectra observed for the mutant K107M



Scheme 3. Proposed mechanism for Schiff base formation between **10** and Lys 146.

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 from the faster rate process, the inhibition of the enzyme activity and EI* complex formation, were identical with those of the native enzyme and this rules out Lys 107 as being the residue implicated in the inactivation. The reaction of K146M with **10** gave rise, conversely, to only a slow kinetic phase whose rate constant was similar to the value observed with the native recombinant enzyme. The absence of the rapid phase strongly suggests that Lys 146 is responsible for the observed slow-binding inhibition. The inhibition kinetics for the K229M and K229A mutants were in accordance with the occurrence of the two processes observed with the recombinant native enzyme, although the capacity of these mutants to form the EI* complex was reduced: Lys 229 is not required for EI* complex formation, but merely facilitates it.

Role of the OH group in compound 10. As indicated above, the OH group in **10** is required for the slow-binding process and therefore iminium ion formation. To clarify if this OH group is involved in a proton transfer to the intermediate carbinolamine and/or participates as an anion in the stabilization of the iminium ion (Scheme 3), several experiments were carried out at pH values close to the p*K* of this phenol group, determined as being 8.3 in solution. The results are given in Table 4. They first show that the fast equilibrium K_i is more displaced towards the complex at higher pH values, with therefore a better affinity not only through the phenol group but also the phosphate; second, for the slowly formed equilibrium, the corresponding rate constants k_2 and k_{-2} are decreased at higher pH values, close to the p*K* of the phenol group, this effect being more pronounced for k_{-2} than for k_2. As for the slow-binding affinity constant K_i^*, some compensation between the two main terms K_i and k_2 (see Scheme 4) operates, since this value can be considered as roughly constant.

Glutamate 187 mutants. To establish whether one of the acid residues of the active site close to Lys 146 responsible for the binding of **10** namely Glu 187, could be also involved, different mutants of this residue were assayed. The results are given in Table 3 and correspond to experiments carried out at pH 7.0. They show that the replacement of this acid residue by an amide significantly reduces the rate of iminium ion formation, although the final amount formed is the same. With aspartate, where the acid group is borne by a chain shorter than that of glutamate, the rate is a also reduced but to a lesser extent.

Table 4. Effect of pH on the interaction of **10** with aldolase

	pH		
Constant	6.0	7.0	8.0
k_2 (min^{-1})	5.40	1.06	0.211
k_{-2} (min^{-1})	0.10	0.090	0.025
K_i (μM)	1020	450	115
K_i^* (μM)	15	30	13
K_d (μM)	16	30	13

DISCUSSION

Concerning first DHAP analogues, the situation exhibited by **1a** and **b** indicates that these compounds have a significant affinity for aldolase, since particularly for **1b** the K_i^* value is in the range of the Michaelis constant for DHAP. As shown by the nonrestoration of activity following sodium borohydride reduction, this affinity corresponds to the formation of an iminium ion with the enzyme in a slow-binding process. The affinity of **1a** and **b** for aldolase depends on chirality at C-3. It is noteworthy that although this enzyme only transforms compounds of *S*-configuration at C-3, **1e** of *R*-configuration has a better affinity for aldolase than **1a**, this better affinity being under the control of both constants K_i and k_2.

For compounds in the second set, **2a**–**4**, they were expected to give stabilized iminium ions and therefore compounds of high affinities. Whereas this is true for **2a** and **2b, 3** and **4** give different patterns: irreversible in the case of **3**, competitive with **4**. It is likely that the

irreversible inactivation observed with **3** is similar to that obtained with other diketo compounds, such as butanedione, a specific reagent for arginine. As several arginine residues are present at the active site (Arg 42 and 148), one of them should covalently bind **3**. However, irreversible inactivation of aldolase by butanedione is only observed in the presence of borax as buffer, which stabilizes the adduct formed between the arginine residue and butanedione.[19] For **3**, as to a lesser extent with hydroxypyruvaldehyde phosphate,[20] the presence of a phosphate group stabilizes the adduct since irreversible inactivation is observed without borax in the triethanolamine buffer.

Compounds **4** and **5** confirm that aromatic group-bearing compounds can interact at the active site of aldolase. Stacking interactions with the aromatic tryptophane residue at position 147 and also with tyrosine 363 of the *C*-terminus which closes one side of the active site barrel[21] are to be considered. These interactions can account for reasonably good affinities, particularly for **5**.

Compounds **2a** and **b** give the best affinities for aldolase through a slow-binding process, with K_i^* values of 130 and 3.5 μM, respectively; several pieces of evidence indicate that these compounds bind to aldolase to form stabilized iminium ion or eneamine intermediates, particularly in the case of **2b** since the k_{-2} value is dramatically reduced, thus indicating that this compound binds irreversibly. The two compounds can be considered as good mimics of the two approaching groups in the bond-forming process catalysed by aldolase. This proposal is confirmed by a result from the literature[22] where a β-diketo moiety borne by an aromatic ring was used as a hapten to produce antibodies, which reveal class I aldolase activity. Moreover, the UV spectra of these selected antibodies are similar to that of **2a** with aldolase[16] and also to the adduct formed between acetopyruvate and acetoacetate decarboxylase,[23] an enzyme which also operates through an iminium intermediate. As for the difference observed between **2a** and **b**, two factors should be considered: the higher steric hindrance with the ketone **2a** and also the difference in p*K* of the CH_2 group inserted between the two carbonyl groups, more acidic for the aldehyde, and therefore more prone to lead to the corresponding stabilized enolate (p*K*5, 9 for acetylpropanal against 9 for acetylacetone).

Turning to FDP analogues **6**–**9**, it is noteworthy that none of them exhibits slow-binding behaviour. This is related to the fact that no iminium ion is formed or only to a weak extent with **8**. Concerning **6** and **7**, neither forms a covalent bond with the enzyme, although other epoxides such as phosphomycin[24] and pentalenolactone[25] inactivate enzymes by covalent binding. These two compounds are recognized at the active site since both have typical competitive inhibition, but there is not likely to be electrophilic assistance to promote the ring opening by a nucleophile. The better affinity of **6** versus the isomer **7** bears similarity to the observation made by Hartman and Barker[26] in comparing 2,5-anhydro-D-glycitol and 2,5-anhydro-D-mannitol, the former having an affinity for aldolase three times higher than that of the latter.

With **10**, advantage is taken of the high affinity of aldolase for phosphorylated aromatic derivatives.[12] An aldehyde group fixed in the *para* position with respect to the phosphate improves the affinity from 2.3 to 0.5 mM but the binding remains competitive. An extra OH group in the vicinity of the aldehyde moiety allows the formation and/or stabilization of the corresponding iminium ion. Following (i) the slow-binding inhibition and (ii) the iminium ion formation, showing that the two processes are correlated, the extents of enzyme inactivation and of iminium ion formation are very similar. Displacement of **10** from the enzyme complex by a competitive inhibitor, hexitoldiphosphate, suggests that iminium ion formation most probably occurs with a lysine residue in the active site. Site-directed mutagenesis of the active site lysine residues shows that Lys 146 is involved in the process: with the mutant K146M, no inactivation occurs and no iminium formation is detected. The mutations K229M and K229A corresponding to the lysyl group implicated in the bonding with the substrates indicate that this residue is indirectly involved in the inactivation process since iminium ion formation by Lys 146 is reduced, but not abolished, in these two mutants. Compound **10** represents a specific probe of Lys 146.

As for the experiments performed at different pH values, they show that rate constants for formation and hydrolysis of the iminium ion intermediate are higher at lower pH values, and therefore under conditions where the phenol group is in the protonated form. However, these two reactions can also be promoted by the protons of the medium and therefore complementary experiments are needed by changing the buffer concentration to discriminate clearly between the two possible contributions. In any case, as this OH group is required for the slow-binding effect, it must intervene in the iminium ion stabilization, probably as a phenate. Moreover, point mutations of Glu 187 indicate that this residue is also involved in the process of iminium ion formation between aldolase and **10**. The replacement of this residue by an amide or by an acid borne by a shorter chain clearly slows the reaction. Although Glu 187 can also contribute to a proton transfer in the intermediate carbinolamine and thus to iminium ion formation, a slow step at these pH values[27] (see Scheme 3), it seems more likely that this residue mediates a proton transfer from Lys 146 to Lys 229, allowing as iminium ion between lys 146 and **10** to form. Point mutations on Lys 229 (Table 3) revealed a contribution of this residue to the iminium ion formation, in accordance with the present proposal. On these grounds, it can be suggested that the reverse transfer from Lys 229 to Lys 146 again mediated by Glu 187 may operate during DHAP or FDP iminium ion formation in the reaction catalysed by aldolase.

Considering now the whole set of the compounds in

the study, it can be concluded that those having the best affinities for the enzyme are those able to form a stabilized iminium ion and should correspond to early or central situations along the reaction coordinates when defined in the FDP synthesis direction. Compound **2b**, which corresponds to a central intermediate, exhibits the best affinity in the series. Surprisingly, compounds such as **8** and **9**, although of significant affinities and close in structure to FDP, are unable to form an iminium ion, at least to an appreciable extent. This bears some relationship to the fact that the FDP iminium ion is formed to a weak extent since it is not capable of being assayed, under conditions similar to those in which DHAP iminium ion was characterized.[28] In addition, the enzyme itself should contribute differently to the stabilization of the iminium ion formed with different substrate analogues; in that respect, it is significant that Heyduck *et al.*[29] have shown that monophosphorylated compounds induce larger conformational changes than those resulting from binding of diphosphorylated compounds. Slow-binding effects are observed with the monophosphorylated compounds **1a, 1b, 2a, 2b** and **10** and also with D-erythrulose-1-phosphate, described earlier,[30] whereas compounds **8, 9** and D-ribulose-1,5 diphosphate[31] produce competitive inhibition.

In other words, compounds of high affinity for aldolase through a slow-binding process are monophosphorylated compounds able to form stabilized iminium ion intermediates, a conformational change of the enzyme contributing to this stabilization.

Besides their interest in the deeper understanding of aldolase and as specific residue probes, such compounds are also of interest in the design of biologically active compounds against damaging parasites such as trypanosome and leishmania, responsible for widepread diseases such as sleeping sickness, and whose energy source is exclusively supplied by glucose metabolism. This strategy has been described elsewhere.[32] By taking advantage of enzyme sequence differences between those of the host and the parasite, slow-binding inhibitors which only interact with the latter can be designed. Promising results have been obtained along these lines.

EXPERIMENTAL

Enzymes and reagents. FDP sodium salt, NADH, DHAP lithium salt, glycerol phosphate dehydrogenase, triose phosphate isomerase and rabbit muscle aldolase were purchased from Boehringer Mannheim. All other chemicals were purchased from Aldrich and were used without further purification.

Synthesis. Compounds **1a–2b** have been described previously.[14,16] Compounds **2–9** are described in Ref. 33 and will be published elsewhere.[34] Compound **10** is described in Ref. 18.

Assay methods. Aldolase activity (10 units mg^{-1} at 25 °C) was measured using a coupled assay system by following NADH oxidation at 340 nm, with detection by a Perkin-Elmer Lambda 2 spectrophotometer thermostated at 25 °C.[35] Assays were initiated by the addition of substrate (FDP; 1 mM final concentration) to a final volume of 1 ml of solution containing aldolase made up in triethanolamine (TEA) buffer (100 mM TEA–HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA), 0.42 mM NADH and coupling enzymes (10 μg ml^{-1} glycerol phosphate dehydrogenase, 1 μg ml^{-1} triose phosphate isomerase). The aldolase concentration was determined spectrometrically from $\varepsilon_{280} = 0.91$ ml mg^{-1} cm^{-1} or with a BCA protein determination kit (Pierce Chemical). Inhibition constants (K_i) were determined on the basis of double-reciprocal plots of the initial velocities of aldolase (2 μg ml^{-1}) for different FDP and inhibitor concentrations.[15]

Reduction by sodium borohydride. Sodium borohydride treatment of the enzyme–inhibitor complexes at equilibrium (TEA buffer, pH 7.0) was performed using a previously described technique.[14]

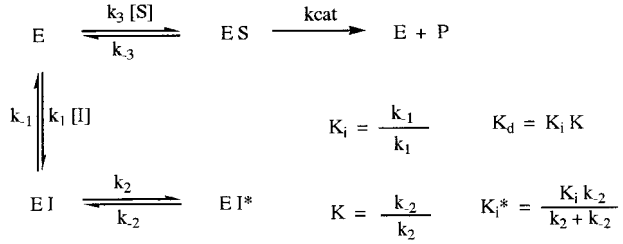
Inhibition study. Aldolase (0.2 mg ml^{-1} in 0.2 ml of TEA buffer) was incubated in the presence of the compound under study at the appropriate concentration. The enzymatic activity was assayed as a function of time with 10 μl aliquots. Control experiments were run without inhibitor and all measurements were made in triplicate.

Kinetic methods. Slow-binding inhibition[13] involves rapid equilibrium formation between enzyme E and inhibitor I, followed by the initial complex EI undergoing a slow reversible isomerization to a kinetically more stable complex form EI*, as shown in Scheme 4.

For this general system, K_i* is the overall dissociation constant, K_i is the dissociation constant for the EI* complex, K_d is the dissociation constant for the EI* complex and K is the equilibrium constant between EI and EI*. P production (concentration P) versus time is given by the following equation:

$$P = V_s t + (V_0 - V_s)(1 - e^{-k_{app}\, t})/k_{app} \qquad (1)$$

where V_0, V_s and k_{app} represent the initial velocity, the



Scheme 4. Inhibition parameters for a slow-binding process.

steady-state rate and the apparent first-order rate constant for reaching the equilibrium between EI and EI*, respectively. The apparent first-order rate constant (k_{app}) describing the formation of EI* is defined by Eqn (2), where K_s is the dissociation constant for the ES complex.[13] This constant exhibits saturating first-order kinetics with increasing concentration of inhibitor [I] with lower and upper limits k_{-2} and $k_{-2} + k_2$, respectively (after dilution, for inhibitor concentrations close to zero, $k_{app} = k_{-2}$). At high inhibitor concentrations, and whenever $k_2 > k_{-2}$, Eqn (3) can be used.[36,37] At chemical equilibrium, Eqn (4) is obtained, where R represents the residual fraction of active enzyme.[36] In cases where [EI*] > [EI], Eqn (5) (taken from Segel[15]) can be used, where $[E]_t$ and $[I]_t$ represent the initial concentrations of free enzyme and inhibitor, respectively.

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

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

$$\frac{1}{[I]} = \frac{k_1 k_2}{k_{-1}k_{-2}}\frac{1}{1-R} - \frac{1+k_2}{k_2}\frac{k_1}{k_{-1}} \quad (4)$$

$$[EI^*] = \frac{([E]_t + [I]_t + K_d) - \sqrt{([E]_t + [I]_t + K_d)^2 - 4([E]_t[I]_t)}}{2} \quad (5)$$

UV–visible difference spectroscopy. Absorbance spectra were measured using a Varian Cary 1E 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.*[38] 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 **10** at various final concentrations to TEA buffer containing a fixed concentration of aldolase (10 μM 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–**10** complex and for the rate constants describing its formation. Method B was used for the titration of **10** and aminocaproic acid against enzyme. In each assay, enzyme (or aminocaproic acid) was added at different final concentrations in the presence of a fixed **10** concentration (10 or 50 μM). Spectra were recorded at timed intervals. Difference absorption spectra corresponding to complex formation were corrected for absorption by buffer and **10** alone. Apparent first-order rate constants (k_{app}) and limiting maximum 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 maximum absorption differences using Eqn (4). The dissociation constant K_i for the rapidly formed aldolase–**10** complex and the rate constants k_2 and k_{-2}, corresponding to the formation and dissociation respectively, of the slow-reacting aldolase–inhibitor complex, were derived from analysis of apparent rate constants using Eqn (2). The first-order rate constant k_{-2} was also derived independently from absorbance data corresponding to the displacement of **10** from the enzymic complex by 10 mM hexitol bisphosphate (method A).

All UV–visible difference spectroscopic experiments using aldolase mutants (10 μM subunit) were performed using method A.

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