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Phosphate ion inactivation of rabbit skeletal muscle aldolase in the crystalline state

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SUMMARY: Catalytically active crystals of rabbit skeletal muscle aldolase are inactivated by phosphate ion and D-glyceraldehyde-3-phosphate. Four moles of phosphate are incorporated per mole of tetrameric enzyme. The inactivation rates are first order in time and demonstrate saturation behaviour. Competition inactivation experiments are consistent with the two substrates competing for the same site on the enzyme. Protection is afforded by substrates binding to the active site on the enzyme. No phosphate inactivation is observed in solution under identical experimental conditions and D-glyceraldehyde-3-phosphate inactivation in solution is unaffected by phosphate ion concentrations. Inactivation by phosphate is apparently due to an unique enzyme conformation stabilized upon protein crystallization.

The packing of a soluble enzyme into a highly condensed state such as a crystalline lattice is expected to produce pertubations in the kinetic behaviour of the enzyme. Provided the enzyme is not inhibited by the crystallization conditions, differences in kinetics between the soluble and crystalline state is attributed to the conformational contraints imposed by packing into a crystal lattice. Aldolase from rabbit skeletal muscle which retains much of its catalytic activity in the crystalline state (1) was probed for conformational changes about its active site upon crystallization by examining the highly specific inactivation by one of its cleavage products D-glyceraldehyde-3-P (2). In the course of these inactivation studies, controls using dihydroxyacetone-P in protection studies showed significant inactivation in the crystalline state. The inactivation was subsequently traced to the presence of phosphate ion impurities in the dihydroxyacetone-P.

Abbreviations: Fru-P_2, fructose-1,6-diphosphate; Fru-1-P, fructose-1-phosphate; D-glyceraldehyde-3-P, D-glyceraldehyde-3-phosphate; dihydroxyacetone-P, dihydroxyacetone phosphate.

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Phosphate ion similar to D-glyceraldehyde-3-P is an irreversible inactivator of crystalline rabbit muscle aldolase binding with a stoichiometry of 4 moles of phosphate per mole of tetrameric enzyme. The inactivation rates are first order with respect to time and variation in substrate concentration displays saturation behavior. Prior inactivation with D-glyceraldehyde-3-P prevents phosphate ion binding to the crystallized enzyme. In the presence of D-glyceraldehyde-3-P, phosphate ion inactivation rates display competitive behaviour similar to competitive inhibition. The substrates Fru-P_2 and Fru-1-P in the presence of excess dihydroxyacetone-P protect against phosphate ion inactivation. Dihydroxyacetone-P alone protects only against D-glyceraldehyde-3-P inactivation suggesting that the phosphate ion binds to the C_6 phosphate binding locus at the active site.

In contrast to the crystalline state, no phosphate ion inactivation of aldolase is observed under the same conditions in the soluble state. The inactivation by D-glyceraldehyde-3-P in the soluble state is the same as in the crystalline state but independent of phosphate ion concentration. The packing of the soluble enzyme into a crystalline lattice has thus apparently stabilized at the active site a unique protein conformation capable of binding and reacting with phosphate ions.

MATERIAL AND METHODS

All chemicals were at least reagent grade and obtained from commercial sources. D-glyceraldehyde-3-P was prepared from the dicyclohexylammonium salt of the D-glyceraldehyde-3-phosphate-diethylacetal derivative (3) which was purchased from Boehringer Mannheim Ltd. The lithium salt of dihydroxyacetone-P was purchased from Sigma Chemical Co. and according to the company contained approximately 5 mole % inorganic phosphate. Rabbit skeletal muscle aldolase, triose phosphate isomerase and α-glycerol phosphate dehydrogenase were purchased from Boehringer Mannheim Ltd. Enzyme purity was verified on SDS polyacrylamide (12%) gels using the protein silver staining kit available from Bio-Rad Laboratories. Aldolase activity was monitored by the loss of NADH using a coupled assay system (4) or by the continuous formation of hydrazone at 240nm (1). All glassware and plasticware utilized in the assays and inactivations was siliconized.

Protein absorbances were measured on a Cary Model 20 double beam spectrophotometer. Rabbit muscle aldolase concentrations were calculated using an extinction $E^{1\%}_{1cm} = 0.91$.

Aldolase crystals were grown by ammonium sulfate precipitation as described previously (1). The crystallization buffer (100mM triethanolamine - HCl pH 7.4, 1mM EDTA, 1mM DTT) was saturated to 45% ammonium sulfate (v/v) and made up to a final protein concentration of 5 mg/ml.

For crystalline state kinetic and inactivation studies, precisely cut crystals (~2 µg aldolase) were crushed to small size to eliminate diffusional effects (1). Substrate concentrations employed were always utilized in great excess with respect to protein concentrations. For the inactivation studies in the crystalline state, crystalline aldolase was incubated with substrate in the crystallizing buffer at room temperature. For the soluble enzyme, inactivations were carried out in crystallization buffer with and

without the presence of 45% saturating ammonium sulfate and the loss of aldolase activity was followed by assaying for enzyme activity at saturating concentrations of Fru-P_2 (10mM). After enzyme incubation with substrate, soluble or crystalline enzyme was dialyzed against several changes of assaying buffer, prior to activity assaying.

The loss of enzyme activity in the presence of substrate was analyzed in terms of rapid equilibrium binding by the substrate to a single site on each subunit of the enzyme followed by a slow covalent reaction of the substrate with the binding site. Assuming first order reactions for each of the elementary steps, the loss of enzyme activity as a function of time and substrate concentration then has the following analytical form

$$\log (e_r/e_o) = -k\ t/(1+K_m/[S]_o)$$

where e_o represents the initial enzyme concentration, e_r the concentration of active enzyme remaining, t the incubation time of enzyme with substrate, $[S]_o$ the initial substrate concentration and K_m a dissociation constant defined from the assumptions above. A reciprocal plot of the primary inactivation rates relating the slope of the rate of inactivation and the initial substrate concentration will be linear. The intercept of this plot determines the maximal rate of inactivation and together with the slope the dissociation constant of the substrate with its site on the enzyme.

Carrier-free acid-free [^{32}P] labelled orthophosphate (10mCi/ml, radioactive concentration) for assaying phosphate incorporation was purchased from Amersham Radiochemical Co. and employed at a specific activity 16,500 dpm/ nanomole on reference day 0. Radiolabelled samples were dissolved in Aquasol (New England Nuclear) and counted by a Beckman Liquid Scintillation counter LS 8000. All labelling experiments were carried out in triplicate utilizing approximately 100 µg of aldolase per experiment. Labelled samples were dialyzed against buffer until no further changes in counts could be detected between successive buffer changes.

RESULTS

D-glyceraldehyde-3-P inactivation of soluble aldolase is highly specific and irreversible (2). Incubations with dihydroxyacetone-P at equal concentrations protects against inactivation by D-glyceraldehyde-3-P (2). Similar studies with crystalline aldolase showed that in control experiments dihydroxyacetone-P alone inactivated the crystallized enzyme. Binding constants of dihydroxyacetone-P to aldolase in the crystalline state are of the order of 15 µM (1) and are consistent with one mole of dihydroxyacetone-P bound per mole of aldolase subunit (5, 6). Inactivation rates in the presence of high concentrations of dihydroxyacetone-P ($\geq$ 500 µM) did not display saturation behaviour suggesting possibly that impurities such as inorganic phosphate in dihydroxyacetone-P were implicated in the loss of enzymatic activity of crystalline aldolase. Overnight incubation of crystalline aldolase with high concentrations (10 mM) of various phosphate salts (lithium, sodium, potassium) were equally efficient in inactivating aldolase. Dissolution and extensive dialysis of inactivated crystalline aldolase did not restore activity. Pyrophosphates did not inactivate crystalline aldolase.

Inactivation rates in the crystalline state for phosphate ion, figure 1, were first order in time and displayed saturation behavior with respect to substrate

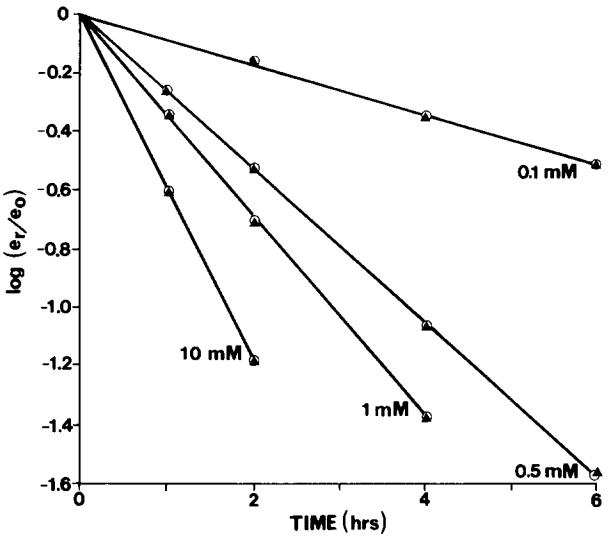

Figure 1: Specific loss of aldolase activity in the crystalline state as a function of incubation time for different phosphate ion concentrations in the absence of dihydroxyacetone-P (▲) and in the presence of 10 mM dihydroxyacetone-P (○).

concentration. Reciprocal plot of the phosphate inactivation rates was a linear function of initial substrate concentration, figure 2, with maximal rate of inactivation $k = 1.36\ min^{-1}$ and dissociation constant, $K_m = 0.64$ mM. Results of experiments with [^{32}P] phosphate incorporation by soluble and crystalline aldolase is shown in table I. Phosphate incorporation for crystalline enzyme is stoichiometric with 4 moles of phosphate incorporated per mole of tetrameric enzyme. Dihydroxyacetone-P did not

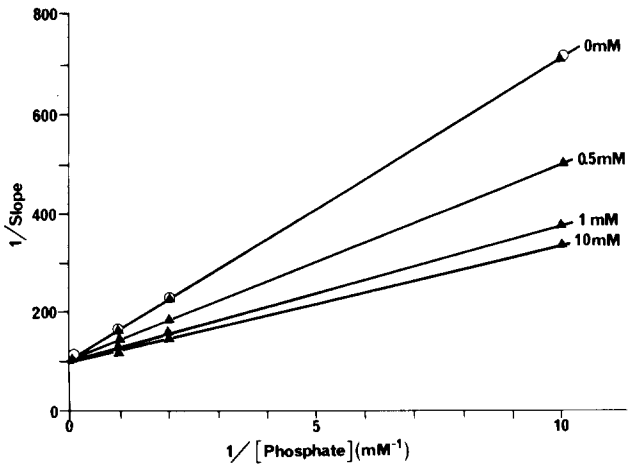

Figure 2: Reciprocal plot of activity loss data as a function of phosphate ion concentration in the presence of varying concentrations of D-glyceraldehyde-3-P (▲) or in the presence of 10 mM dihydroxyacetone-P (○).

Table I
Incorporation of phosphate into aldolase

	Radioactivity incorporated cpm/μg protein	Phosphate incorporated equiv./mole of protein
soluble aldolase	4	0
crystalline aldolase	430[a]	4.2
crystalline aldolase but prior inactivation with D-glyceraldehyde-3-P	1	0

[a] Average of two experiments carried out at different times with different lots of crystallized protein.

alter the rates of inactivation by phosphate in the crystalline state (figure 1). No phosphate incorporation was detected for the soluble enzyme nor any loss in enzymatic activity.

In the presence of D-glyceraldehyde-3-P, as observed for phosphate ions, inactivation rates of crystalline aldolase were first order in time and displayed saturation behaviour with respect to substrate concentration (data not shown). Prior inactivation of crystalline aldolase with D-glyceraldehyde-3-P also prevents phosphate incorporation. In the presence of D-glyceraldehyde-3-P, reciprocal plots of the phosphate inactivation rates display behaviour analogous to competitive inhibition (figure 2). Rates of inactivation by D-glyceraldehyde-3-P of soluble aldolase was virtually identical to that found for crystalline aldolase (figure 2) and independent of phosphate ion concentration. From the reciprocal plot the maximal rates of inactivation and dissociation constant for D-glyceraldehyde-3-P were 0.38 min^{-1} and 1.20mM respectively.

Overnight incubations of soluble and crystalline enzyme in the presence of saturating concentrations of inactivating substrate and of substrates known to bind at the active site on the enzyme were assayed for activity. The results are presented in table II. Saturating concentrations (10 mM) of Fru-P_2 results in enzyme inactivation overnight. As both soluble and crystalline enzyme turnover in the crystallization buffer, it is likely that the cleavage product D-glyceraldehyde-3-P is responsable for inactivation. Co-incubation in the presence of hydrazine sulfate (20 mM) which rapidly reacts with glyceraldehyde-3-P prevents inactivation is consistent with this

Table II
Protection experiments against overnight inactivation by phosphate ion and D-glyceraldehyde-3-P substrates

Substrates		% Activity remaining	
Inactivator	Protector	Soluble aldolase	Crystalline aldolase
Fru-P_2, 10mM	---	0	0
	Hydrazine sulfate, 20 mM	100	100
Phosphate, 10 mM	---	100	0
	Dihydroxyacetone-P, 10mM	---[a]	0
	Fru-P_2, 10mM and Dihydroxyacetone-P, 10mM	---[a]	100
	Fru-1-P, 50mM and Dihydroxyacetone-P, 10mM	---[a]	100
D-glyceraldehyde-3P, 1 mM	---	0	0
	Dihydroxyacetone-P, 1mM	100	100

[a] Experiments not carried out.

interpretation. To ensure that the entire active site remained saturated with substrate both Fru-P_2 and Fru-1-P in saturating concentrations in separate experiments were incubated with dihydroxyacetone-P also present in saturating concentrations (10 mM). Since the thermodynamic equilibrium is strongly in favor of aldol condensation (7) the equilibrium concentration of D-glyceraldehyde-3-P is approximately 10 μM. Under these conditions, no inactivations were observed both in the absence and presence of saturating concentrations (10 mM) of phosphate after overnight incubations with crystalline aldolase. Protection was afforded against D-glyceraldehyde-3-P inactivation only when dihydroxyacetone-P was incubated simultaneously and at a concentration identical with D-glyceraldehyde-3-P.

DISCUSSION

The inactivation of aldolase by phosphate ion in the crystalline state is both highly specific and apparently irreversible. Crystalline aldolase incorporates phosphate at the active site locus at a stoichiometry of one mole of phosphate per aldolase subunit. Surprisingly no phosphate ions are incorporated in the soluble state of the enzyme. In contrast, D-glyceraldehyde-3-P does inactivate specifically at the active

site locus in solution (2) and packing of the soluble enzyme into the crystalline state apparently does not alter the D-glyceraldehyde-3-P inactivation rates nor the protection afforded by dihydroxyacetone-P.

The enzymatic inactivation is consistant with a rapid equilibrium binding of substrate to enzyme followed by a slow and apparently irreversible reaction under the experimental conditions. Analysis of the maximal rates of inactivation and dissociation constants reveals that the phosphate ion binds tighter to the enzyme and reacts more rapidly than does D-glyceraldehyde-3-P. Given that prior incubation of aldolase crystals with D-glyceraldehyde-3-P prevents phosphate incorporation (table I) reciprocal plots of the phosphate ion inactivation data in the presence of different glyceraldehyde-3-P concentrations should display competitive behaviour analogous to competitive inhibition observed for substrates binding to the same locus on an enzyme. This interpretation is consistent with the reciprocal plot shown in figure 2.

Soluble aldolase is not inactivated by phosphate ions under the experimental conditions. However phosphate ions are known to bind to aldolase in solution under somewhat similar experimental conditions (8). The existence of a phosphate binding site at the same locus where D-glyceraldehyde-3-P covalently binds in solution should result in inhibition of the inactivation rates. The absence of any effect by phosphate ion on the glyceraldehyde-3-P reaction in solution suggests that packing of soluble aldolase into a crystalline lattice has stabilized an unique protein conformation capable of binding and reacting with phosphate ions.

It is intriguing to speculate that the phosphate binding site on the crystalline enzyme may represent a potential phosphorylation site.

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