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# SLOW REVERSIBLE INHIBITION OF RABBIT MUSCLE ALDOLASE BY D-ERYTHRULOSE 1-PHOSPHATE

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**Summary:** Rabbit muscle aldolase was found to be inactivated in a slow, reversible manner by D-erythrulose 1-phosphate. This compound combined rapidly and reversibly with the enzyme to form an initial complex, which then only slowly ($k_i$ = 0.28 $min^{-1}$) converted to a kinetically more stable form. This stable enzyme-ligand form was inactive toward the normal substrate of aldolase, fructose 1,6-bisphosphate. The inactive enzyme-ligand complex, however, could be decomposed ($k_r$ = 0.0041 $min^{-1}$) to yield active enzyme once again by incubation in a solution devoid of D-erythrulose 1-phosphate. 

Rabbit muscle aldolase (4.1.2.13) catalyzes the reversible cleavage of D-fructose 1,6-bisphosphate to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. The enzyme can also use D-fructose 1-phosphate (1), D-xylulose 1-phosphate (2) and L-erythrulose 1-phosphate (3) as substrates. These molecules are split into dihydroxyacetone phosphate plus D-glyceraldehyde, glycolaldehyde, and formaldehyde, respectively. These findings have shown that aldolase prefers substrates which have the S-configuration at carbon-3. D-Erythrulose 1-phosphate, then, having the R-configuration at this carbon, would not be expected to be a substrate for the enzyme. We have found that, in fact, this compound is not a substrate of aldolase, but, surprisingly, it is a slow-binding, reversible inhibitor (a class of inhibitor defined by Williams and Morrison (4)) of the enzyme.

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## MATERIALS AND METHODS

D-Fructose 1,6-bisphosphate tetrakis(cyclohexylammonium)-salt, dihydroxyacetone phosphate dimethylketal bis(cyclohexylammonium)salt, α-glycerol phosphate dehydrogenase, triosephosphate isomerase, yeast alcohol dehydrogenase, and β-NADH were purchased from Sigma Chemical Co. Dimethylketal bis(cyclohexylammonium) salts of D- and L-erythrulose 1-phosphate were obtained from C. E. Ballou. Hexitol 1,6-bisphosphate was synthesized by the procedure of Ginsburg and Mehler (5). All other reagents and solvents were reagent grade materials. The buffer solution used was 50 mM triethanolamine hydrochloride, 0.2 mM EDTA, pH 7.4.

Aldolase was isolated from rabbit muscle as described by Penhoet et al. (6). Aldolase activity (9.5-14 U/mg) was routinely measured at room temperature in triethanolamine buffer according to Blostein and Rutter (7) using fructose 1,6-bisphosphate as substrate.

The rate of cleavage of L-erythrulose 1-phosphate was determined by the rate of production of dihydroxyacetone phosphate as described above (7) and also by measurement of the rate of formaldehyde reduction in the presence of excess alcohol dehydrogenase and NADH. The latter assay was performed since both D- and L-erythrulose 1-phosphates are substrates for α-glycerol phosphate dehydrogenase, the coupling enzyme used in the assay of Blostein and Rutter (7). In experiments containing 0.11 mM L-erythrulose 1-phosphate, the cleavage rate given by the alcohol dehydrogenase assay was 94% of that measured in the presence of α-glycerol phosphate dehydrogenase.

Rates of inactivation of aldolase by D-erythrulose 1-phosphate were determined as follows. D-Erythrulose 1-phosphate (50-300 μM) in 1.0 ml of TEA buffer was preequilibrated for 10 minutes at 25° C. Aldolase was added to give a final concentration of 0.17 mg/ml, and 10 μl aliquots were removed at various times for determination of enzyme activity. Control experiments contained no D-erythrulose 1-phosphate. To determine rates of reactivation upon dilution, aldolase (50 μM subunits) was inactivated by incubation with 200 μM D-erythrulose 1-phosphate for 2 hours at room temperature, then diluted 10-fold in TEA buffer containing hexitol 1,6-bisphosphate (30-200 μM). Aliquots (10 μl) were removed at various times for determination of aldolase activity.

## RESULTS

Cleavage of D- and L-erythrulose 1-phosphates by aldolase:

Kinetic parameters of the aldolase catalyzed cleavage of L-erythrulose 1-phosphate at 24° C were determined from an Eadie-Hofstee plot (8) of initial rates of reaction of substrate in the concentration range 0.0505-2.52 mM. The Km value (0.19 mM) was similar to that (0.56 mM) determined by Gillett and Ballou (3) and the maximal activity was consistent with their observation that L-erythrulose 1-phosphate was cleaved only 2.1% as rapidly as fructose 1,6-bisphosphate. D-Erythrulose 1-phosphate was tested as a substrate of aldolase by incubating the compound (0.3 mM) in the presence of aldolase (0.4 U), alcohol dehydrogenase (1800 U) and NADH (0.2 mg). No significant change of absorbance

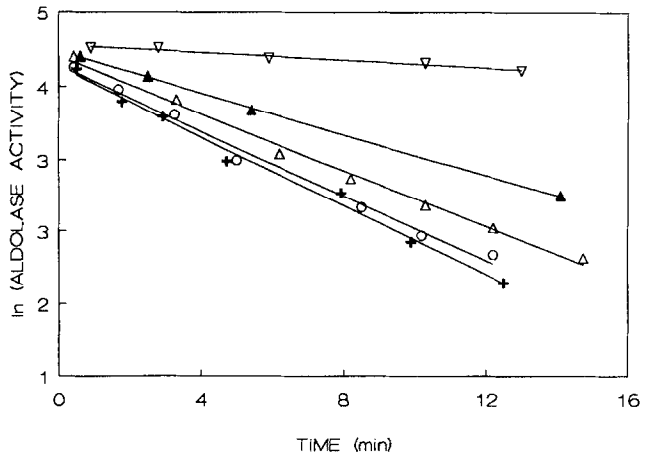


Figure 1. Inactivation of aldolase by D-erythrulose 1-phosphate. Aldolase (0.17 mg/ml) was incubated at pH 7.4, 25°C with D-erythrulose 1-phosphate at the following concentrations (μM): 53(▲); 105 (Δ); 196 (O); 291 (+). The enzyme was protected in the presence of 100 μM dihydroxyacetone phosphate (▽).

was seen during a 40-minute period in which the limits of detectable absorbance change was 0.02. The rate constant of cleavage ($k_{cat}$/Km) was estimated to be less than 0.1% that of the L-enantiomer.

Reactions of D-erythrulose 1-phosphate with aldolase: Incubation of aldolase with D-erythrulose 1-phosphate led to a first order loss of enzyme activity (fig. 1). Dihydroxyacetone phosphate protected the enzyme against inactivation, supporting the assump-

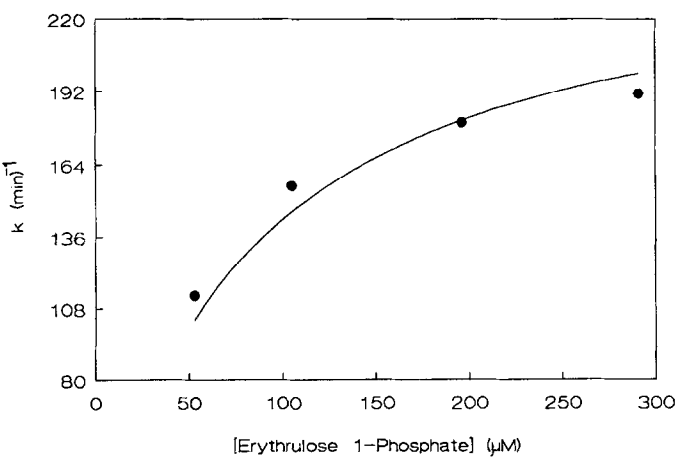


Figure 2. Saturation kinetics of the D-erythrulose 1-phosphate inactivation of aldolase. Observed first-order rate constants of inactivation were obtained from slopes of the linear (determined by linear regression analysis) plots of the data shown in figure 1 at four concentrations of D-erythrulose 1-phosphate, 53, 105, 196, and 291 μM.

$$E + D \underset{K_D}{\rightleftharpoons} ED \xrightarrow[k_i]{} ED^*$$

Scheme 1

tion that the inactivation involved a reaction occurring at the active site. Observed first-order rate constants exhibited saturation kinetics with increasing concentrations of D-erythrulose 1-phosphate (fig. 2), suggesting reaction scheme 1. The dissociation constant of the rapidly formed ED complex ($K_D$ = 75 $\mu$M) and a calculated first-order rate constant for formation of the ED* complex ($k_i$ = 0.25 $min^{-1}$) were determined from the slope and intercept, respectively, of a linearized plot of the data (not shown).

The inactivated enzyme (ED*) was slowly but completely reactivated by incubation in the presence of the competitive inhibitor, hexitol 1,6-bisphosphate (fig. 3). The bisphosphate

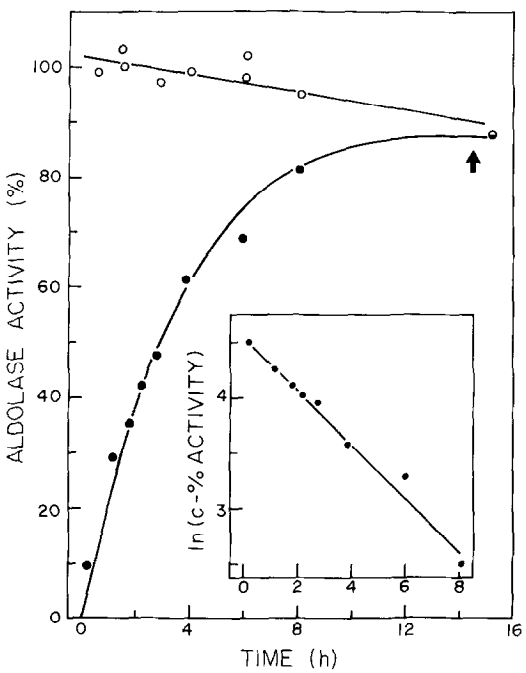


Figure 3. Reactivation of aldolase after inactivation by D-erythrulose 1-phosphate. Samples of normally active aldolase (○), and aldolase which was 90% inactivated by treatment with D-erythrulose 1-phosphate (●), were incubated in the presence of 33 $\mu$M hexitol 1,6-bisphosphate at pH 7.4, 25°C. The rate of activity loss in control samples ($k_o$ = 0.815% $h^{-1}$) was determined by linear regression analysis. The reactivation process was treated as a first-order reaction approaching a declining end-point ($c$ = 100 - 0.815$t$), yielding a rate constant of reactivation, $k_r$ = 0.244 $hour^{-1}$. Values of $k_o$ and $k_r$ were used to calculate a theoretical progress curve of the reactivation reaction, and to predict the time required for appearance of maximal activity (arrow).

$$ED^{*} \xrightarrow[k_r]{} E + \text{Products}$$

Scheme 2

inhibitor was included to stabilize the enzyme and to protect the active site from reaction with D-erythrulose 1-phosphate or its potential decomposition products. The rate of reactivation of aldolase was independent of the concentration of hexitol 1,6-bisphosphate (30-200 $\mu$M) present. Correcting for a slow loss of enzyme activity in the control, a rate constant, $k_r = 4.1 \times 10^{-3}$ $min^{-1}$, was calculated for the reactivation reaction. The chemical nature of the product(s) (scheme 2) of the reaction has not yet been determined.

**DISCUSSION**

Previous data suggest that aldolase prefers the S-configuration at carbon-3 for its substrates (1,2,3). Consistent with this, we have found that D-erythrulose 1-phosphate is not a substrate of the enzyme. Interestingly, however, D-erythrulose 1-phosphate was found to be a slow-binding, reversible inhibitor of aldolase. The half-life for the first order inactivation of aldolase by D-erythrulose 1-phosphate was 2.8 minutes, and the half-life for reactivation was 2.8 hours. Whereas "slow-binding inhibition is commonly associated with analogs of intermediates of enzymic reactions" (9), there seems to be no apparent similarity between the structure of D-erythrulose 1-phosphate and any of the intermediates proposed (10) for the aldolase reaction.

When the natural substrate of aldolase, dihydroxyacetone phosphate, is incubated with the enzyme in the absence of an aldehyde acceptor, the compound rapidly forms both imine and enamine intermediates. These intermediates have been detected chemically by borohydride reduction of the imine (11) and by ferricyanide oxidation of the enzyme bound enamine complex (12). Preliminary data obtained by us (not shown) indicate that D-erythrulose 1-phosphate also forms an imine intermediate with aldolase and that this intermediate is a member of the $ED^{*}$ complex. Thus, although D-erythrulose 1-phosphate reacts at the active site of aldolase in a manner similar to that of dihydroxyacetone phosphate, the speed of the D-erythrulose 1-phosphate binding reaction is limited by the rate of one (or more) unusually slow step(s). Apparently the D-configuration not only pre-

vents D-erythrulose 1-phosphate from being a substrate of the enzyme, it also increases the kinetic stability of one or more enzyme-bound complexes.

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