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SUBSTRATE-DEPENDENT INHIBITION OF YEAST ENOLASE BY FLUORIDE

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ABSTRACT: A possibly physiologically significant inhibition of yeast enolase by fluoride occurs in the absence of inorganic phosphate. The inhibition increases with time, is strongly dependent on fluoride concentration and requires substrate and "catalytic" Mg^{2+}. The inhibition increases more slowly in the presence of product (phosphoenolpyruvate) than substrate (2-phosphoglycerate) The dependence on fluoride concentration and the spans of substrate analogue displacement titrations suggest the inhibition is produced by two moles of fluoride per active site.

INTRODUCTION:

Yeast enolase (2-phospho-D-glycerate hydrolyase, E.C. 4.2.1.11) binds one mole of "conformational" Mg^{2+} per subunit (1,2). This permits substrate binding, but an additional mole of "catalytic" Mg^{2+} must then bind for catalysis to occur (3,4).

Fluoride causes a physiologically significant inhibition of enolases (5,6). This was originally attributed to formation of a complex between Mg^{2+}, fluoride and inorganic phosphate (P_i) (5). However, recent NMR studies by Maurer and Nowak (7,8) show that the fluoride binds to the metal ion in the Mn^{2+}-yeast enolase complex at 8 Å away from the P_i binding site.

Fluoride produces 50% competitive inhibition of enolases from diverse organisms with the free ion product (Mg^{2+}) (F^-) (P_i) near 10^{-10} M^3 (6,9). Since under *in vivo* conditions the P_i concentration is probably well below millimolar levels (10), strong inhibition of enolases may occur in the absence of P_i if fluoride toxicity is manifest at millimolar levels.

Literature sources agree that fluoride inhibition occurs in the absence of P_i and is weaker than in its presence, but disagree as to its exact type and extent (9,11). The characteristics of the inhibition seem to depend upon what portions of enolase reaction time courses are studied.

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MATERIALS AND METHODS

Yeast enolase was isolated using a modification of the method of Westhead and McLain (12). The enzyme was deionized, concentrated and assayed for residual "conformational" metal ion as described (1,13). Protein concentrations were calculated assuming the molecular weight is 93,300 and the extinction coefficient at 280 nm is 0.895 cm^2/mg (1).

Solutions were prepared from deionized water (Continental Deionized Water Service) and "Ultrapure" potassium chloride (Alfa Chemical Co.) or twice-recrystallized tris or AR Reagent Grade chemicals, where these were available. Plasticware was used whereever possible. 2-phosphoglycerate and phosphoenolpyruvate were obtained as the sodium salts from Calbiochem. The chromophoric substrate analogues, 3-aminoenolpyruvate-2-phosphate (AEP) and D-tartronate semialdehyde-2-phosphate (TSP) were synthesized as described by Hartman and Wold (14) and Spring and Wold (15).

Absorbance readings were made using a Bausch and Lomb Spectronic 200 spectrophotometer equipped with a digital readout. Measurements of pH were made using a Corning Model 10 pH meter.

RESULTS

The inhibition of enolase by fluoride in the absence of P_i increases with time. The time-courses of control and fluoride-inhibited substrate dehydrations are shown in Figure 1. The lower half of the Figure shows the percent inhibition as a function of time. These values were obtained from relative slopes of the curves in the upper half of the Figure, taken at equivalent midpoint values of OD_{230}. This must be done because the rate of the control enolase reaction, expressed as ΔOD_{230}/minute, decreases with time as the solution approaches equilibrium. The inhibition by 10-20 mM fluoride increases rapidly, then remains essentially constant. The final level of inhibition and the rate of attainment of the final level are highly dependent on fluoride concentration. There is also some dependence on Mg^{2+} concentration. The reverse reaction (not shown) is affected more slowly, with 10 mM fluoride producing half of its maximum inhibition (80-90%) in 10 minutes. Control experiments have shown that incubation of the enzyme with any one or two of Mg^{2+}, fluoride or substrate is not sufficient for inhibition; all three must be present.

Use of extrapolated very early (0-10 seconds) reaction rates indicate that fluoride is initially a weak (K_I about 15 mM) inhibitor (16), competitive with substrate or product (not shown). If velocities of the forward and reverse reactions are taken at times beyond one minute of reaction, the inhibition appears to be more noncompetitive (16), as Wang and Himoe found (11).

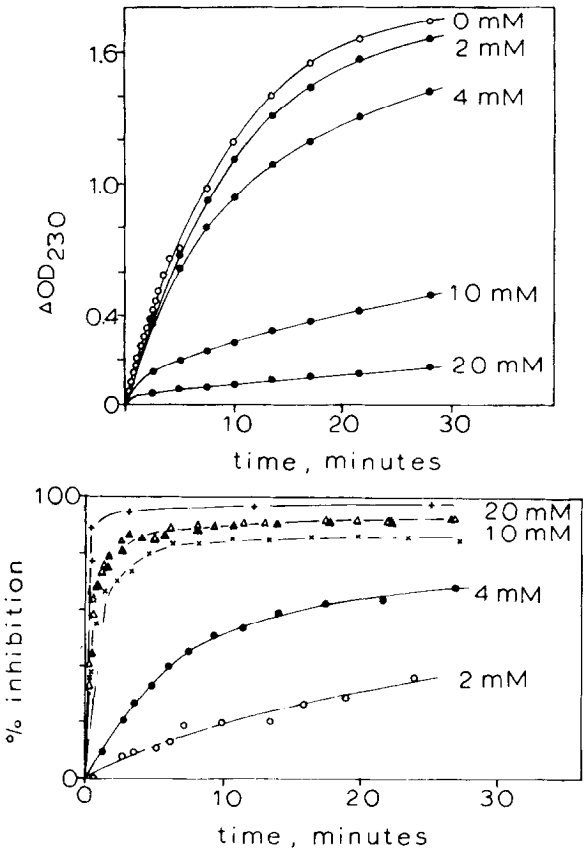

Figure 1. (Upper) Time-courses of the forward reaction of yeast enolase in the presence of various concentrations of fluoride. The absorbancies at 230 nm of several assay mixtures were followed simultaneously. Reaction volumes were 2.5 ml of 0.05 ionic strength tris-HCl, pH 7.6, with 1 mM substrate and $MgCl_2$ and KF as shown. Reactions were carried out at 22° and were initiated by addition of yeast enolase to 5 nM and mixing by inversion. (Lower) Time-dependence of inhibition produced by fluoride. Rates of reactions in the presence of fluoride were taken from tangents to the time-courses at various times of reaction and compared to control (no fluoride) rates over identical values of ΔOD_{230}. Some data is from time courses which are not shown. The solutions used contained: 20 mM KF(+); 10 mM KF with 1.0 (Δ) or 0.1 mM (▲) substrate and 5 or 2.5 nM enzyme or 0.1 mM substrate and 0.1 mM $MgCl_2$ (X); or 4 mM KF (●) or 2 mM KF (0) and 1 mM substrate and $MgCl_2$.

Dilution of concentrated enzyme that had become maximally inhibited by 10 mM KF into fresh assay solution with 10 mM KF showed less than 10% of normal activity. If the dilution is into assay medium without fluoride, there was a very low initial activity followed by a time-dependent increase in reaction velocity until complete recovery of enzymatic activity had occurred. The half-time of recovery is about one minute. Addition of excess (2 mM) EDTA to maximally inhibited concentrated enzyme followed by assay without fluoride present also shows a measurably time-dependent but, after five minutes, complete

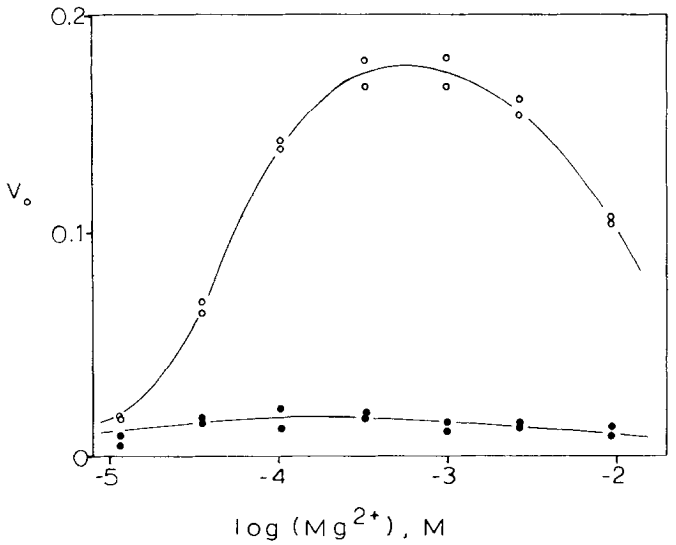

Figure 2. The dependence of enzyme activity on Mg^{2+} concentration in the presence and absence of fluoride. Enzyme activities (V_o) are in ΔOD_{230} over the first minute of reaction. The assay solutions were 2.5 ml of 0.05 ionic strength tris-HCl, pH 7.6, with 1 mM substrate, $MgCl_2$ as shown and either 0.017 M KCl (open circles) as 0.017 M KF (filled circles). The reactions were started by adding enzyme to 5 nM and mixing by inversion.

recovery of enzymatic activity. We conclude that the inhibition is fully reversible, does not depend (in final extent) on the ratio of 2-PGA and PEP present and is due to formation of a complex with the enzyme which involves Mg^{2+}.

Assays of equilibrated fluoride-containing reaction mixtures for P_i (17) showed none - there is no substrate or product hydrolase activity detectable. Chromatographic experiments indicated that no fluorolactic acid phosphate had been formed (18).

The dependence of the reaction velocity (0-1 minute) on Mg^{2+} concentration and presence or absence of fluoride is shown in Fig. 2. The approach to the activity optimum between 10^{-4} and 10^{-3} M Mg^{2+} reflects "catalytic" metal ion binding (1,4). The decrease in activity observed above 1 mM Mg^{2+} is attributed to "inhibitory" metal ion binding (4,19). The inhibition by fluoride increased with Mg^{2+} concentration up to 10^{-3} M. This suggests participation of "catalytic" Mg^{2+} in the inhibition.

Rough calculations from kinetic data suggest the inhibition is second-order in fluoride (not shown). The "transition state analogue" D-tartronate semialdehyde-2-phosphate (TSP) undergoes large changes in its absorption spectrum

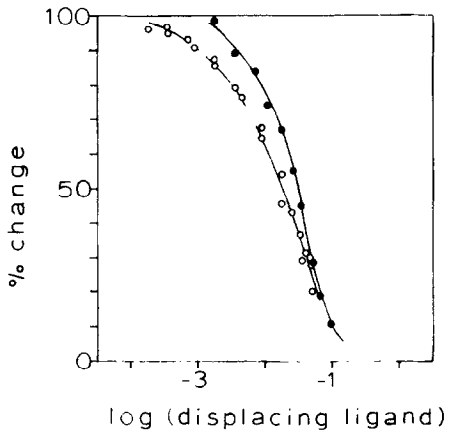

Figure 3. Displacement of bound TSP from Mg^{2+}-enzyme by fluoride or phosphate. Solutions (2.5 ml) of 0.01 M HEPES-NaOH, pH 7.6, at room temperature (22°) contained 10 μM enzyme, 20 μM TSP and 1 mM $MgCl_2$. Microliter amounts of 1 M KF (closed circles) or phosphate (open circles), pH 6.1 or 8.2, were added, mixed and the changes in absorbance at 285 nm recorded once a stable reading was obtained (usually after a few seconds).

when bound to the Mg^{2+}-enzyme (20). Excess phosphate or fluoride can displace TSP from the enzyme, resulting in a decrease in absorbance at 285 nm (Figure 3). These titrations were done within 20 minutes, as TSP is unstable in the solutions employed. (The half life of unbound TSP is 100 minutes; when bound to the Mg^{2+}-enzyme, it is much more stable.) The slopes of the curves suggest that two equivalents of fluoride or one of phosphate were involved in displacing TSP. Similar results were obtained using the analogue AEP (J. I. Elliott and J. M. Brewer, unpublished observations). Arsenate behaves like P_i (not shown).

An abstract (21) mentions fluoride electrode measurements indicating 1.5 moles of fluoride bind per subunit (P_i present). Those authors also noted that substrate and fluoride binding were competitive.

DISCUSSION

It is clear that the interaction of fluoride with enolase is more extensive and complex than originally believed. The displacement titrations indicate an essentially competitive interaction between the substrate analogue and fluoride. The role of "catalytic" Mg^{2+} is unclear; since "catalytic" binding does not occur without substrate binding (3,4), it is hard to see how fluoride binding to "catalytic" Mg^{2+} would result in substrate analogue displacement. "Catalytic" Mg^{2+} may simply change the conformation around the active site so a second

fluoride binding site appears, perhaps also on "conformational" Mg^{2+}. Since the rate of inhibition is much lower if the active site contains product than substrate, the configuration of the active site appears to be important.

We believe the inhibition of this and presumably other enolases (6,9,22) in the absence of P_i may be an important mechanism of fluoride poisoning. Measurement of P_i in resting barnacle muscle cells using ^{31}P-NMR showed none detectable, which suggested an upper limit of 100 μM (10). If the P_i level in cells is indeed on the micromolar level, the high dependence of inhibition on fluoride concentration would add an unsuspected safety factor to water fluoridation, since low concentrations would have less effect.

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