

The Mechanism of Action of Alkaline Phosphatase

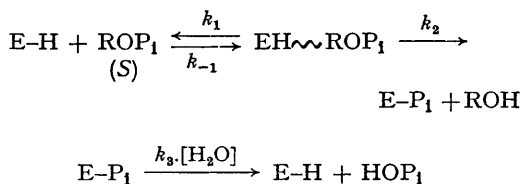
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THERE is much indirect evidence supporting a three-step mechanism for the hydrolysis of organic phosphates catalysed by the alkaline phosphatase of *Escherichia coli*.¹⁻³ The identity

of V_{\max} for different organic monophosphates^{2,3} supports this and the view that k_3 is less than k_2 ; however, this identity could result from a rate-determining conformational change in the enzyme

or from a two-step mechanism where V_{\max} , the catalytic step, is insensitive to a change in the leaving group ROH. The hydrolytic rate constant of a phosphorylated alkaline phosphatase coincides approximately¹ with k_{cat} . This identity can be used as an argument for the existence of a phosphoryl intermediate but the power of this is reduced by the uncertainty of the enzyme concentration ($k_{\text{cat}} = V_{\max}/[E]$) and since k_3 need not necessarily equal k_{cat} .



(K_m and k_{cat} are the Michaelis-Menten parameters)

This Communication presents the first direct evidence for the existence of a phosphoryl-enzyme intermediate in the hydrolysis of organic phosphates catalysed by alkaline phosphatase.

The hydrolysis of *p*-nitrophenyl phosphate ($2-5 \times 10^{-4}\text{M}$) catalysed by enzyme (10^{-5}M) was done in acetate buffer (0.1 M) at pH 3.6 and 25°. The production of *p*-nitrophenol, followed at 3500 Å in a recording spectrophotometer, was zero-order for the initial 30 sec. of trace. Extrapolation to zero time (usually 2 or 3 sec. from the addition of substrate) gave an intercept P on the product axis.

These results can only be explained by the existence of a phosphoryl-enzyme intermediate

and the three-step mechanism is the simplest consistent with this. It can be shown⁴ that $P/[E] = [S.k_3/(k_2 + k_3)(S + K_m)]^2$. P is observable if $k_3 \sim k_2$, $S \sim K_m$, S is greater than $[E]$ and $[E]$ is greater than the error in $[\text{ROH}]$; all conditions except the first are known to be fulfilled ($K_m = 4.2 \times 10^{-4}\text{M}$).⁵ The data fit the above equation and give $[E].[k_2/k_2 + k_3]^2 = 0.64$ absorbance units; $V_{\max} = [E].k_2.k_3/(k_2 + k_3) = 2.22 \times 10^{-2}$ absorbance units $\times \text{sec.}^{-1}$ under the same conditions so that $k_3.(k_2 + k_3)/k_2 = 3.47 \times 10^{-2}\text{sec.}^{-1}$. Values for k_{cat} drawn from the literature⁶⁻⁸ corrected to pH 3.6 using the known pH-dependence of k_{cat} ($pK_a = 7$)^{3,5} vary from 1.1 to $1.5 \times 10^{-2}\text{sec.}^{-1}$. Thus k_2/k_3 ranges from 1.3 to 1.9 M^{-1} . These ratios are almost certainly low owing to over estimation of the enzyme concentration in determining k_{cat} .

Experiments with added phosphate confirm these results. The presence of inorganic phosphate (to give 10^{-2}M) in the enzyme solution ($S = 5.1 \times 10^{-4}\text{M}$) reduced the intercept 0.7-fold and the steady state rate 0.9-fold. The enzyme is phosphorylated by inorganic phosphate at low pH³ (probably at a serine); the smaller intercept arises because the substrate need phosphorylate fewer active sites to reach the steady-state concentration of intermediate.

These experiments, while definitive, give no clue to the site of phosphorylation during catalysis nor do they exclude the possibility of a concurrent mechanism involving no covalently-bound intermediate.

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