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 approximately[1] 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 ($k_{cat} = V_{max}/[E]$) and since k_3 need not necessarily equal k_{cat}.

$$E\text{–}H + \underset{(S)}{ROP_1} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH\sim ROP_1 \xrightarrow{k_2} E\text{–}P_1 + ROH$$

$$E\text{–}P_1 \xrightarrow{k_3.[H_2O]} E\text{–}H + HOP_1$$

(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 ($2—5 \times 10^{-4}$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 shown[4] that $P/[E] = [S.k_2/(k_2 + k_3)(S + K_m)]^2$. 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 [ROH]; all conditions except the first are known to be fulfilled ($K_m = 4·2 \times 10^{-4}$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 $\times$ sec.$^{-1}$ under the same conditions so that $k_3.(k_2 + k_3)/k_2 = 3·47 \times 10^{-2}$sec.$^{-1}$ Values for k_{cat} drawn from the literature[6–8] 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}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 ($S = 5·1 \times 10^{-4}$M) reduced the intercept 0·7-fold and the steady state rate 0·9-fold. The enzyme is phosphorylated by inorganic phosphate at low pH[3] (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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