Effective Charge in the Controlling Transition-state in the Phosphorylation of *Escherichia coli* Alkaline Phosphatase

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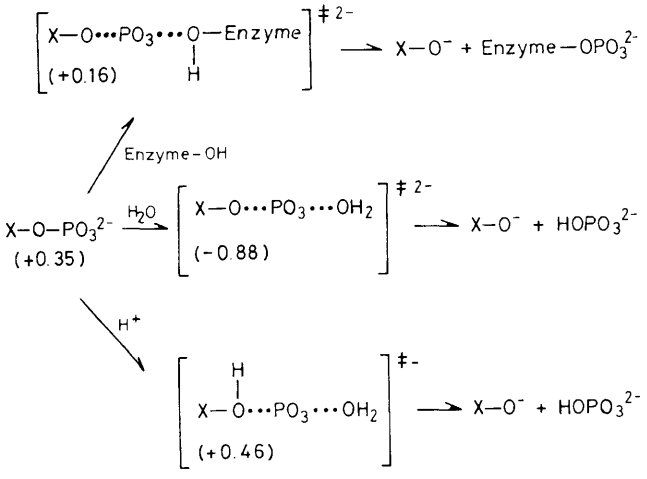
The Brønsted relationship for the leaving hydroxy group in the phosphorylation of alkaline phosphatase has a β value of −0.19; by comparison with $\beta_{eq.}$ for the overall equilibrium (−1.35) there is little change in the charge on the leaving oxygen atom between the ground- and transition-states.

Effective charge on an atom in a transition-state may be measured from the effect of substituents on the rate and equilibrium constants.[1] Measurement of effective charge is only possible for those enzymes (for example *Escherichia coli* alkaline phosphatase)[2] with wide specificity where a sufficient number of substrates can be studied in order to exclude non-electronic effects such as a special mode of binding or steric exclusion from binding sites.[1] The substituent effect on the equilibrium for hydrolysis of monophosphate esters ($\beta_{eq.}$ −1.35) has recently been reported;[3] we can therefore investigate the change in effective charge on the leaving oxygen species in the enzyme-catalysed hydrolysis of monophosphates.

The values of $k_{cat.}$ and K_m have been measured for 29 monophosphate esters; in a Brønsted plot, the parameter $k_{cat.}/K_m$ falls essentially on two linear regression lines when plotted against the pK of the leaving hydroxy group (Figure 1). Both lines have $\beta_{lg.} = -0.19$ but are separated by about two orders of magnitude. The low negative value of β agrees with data previously obtained for a smaller pK range and sample.[4] The single value of β indicates a single mechanism and the same rate limiting step over the wide range of pK studied.

The kinetic scheme for the enzymatic reaction involves a minimum of four steps including binding of substrate, phosphorylation, dephosphorylation, and phosphate dissociation.[2] The ratio $k_{cat.}/K_m$ is a direct measure of the change in free energy in going from unbound ester and enzyme to the transition-state of the rate limiting step in phosphorylation, regardless of the intervening steps.[4] The change in the effective charge on oxygen as measured from $\beta_{lg.}$ and $\beta_{eq.}$ refers to the charge 'seen' on the leaving oxygen atom by the substituents in the above two states. There are +0.16 units of effective charge on the oxygen in the transition-state for phosphorylation of the enzyme compared with +0.35 and −1 in ground- and product-states respectively. The small change in charge requires either extensive P–O fission compensated for by substantial electrophilic participation on the oxygen or little P–O fission in the transition-state. Chemical hydrolysis of the monophosphate dianion involves −0.88 units of effective charge on the oxygen in the transition state indicating substantial P–O fission.[3] The electronic requirements of the enzymatic phosphorylation are closer to those for the hydrolysis of the monophosphate monoanion; the small change in effective charge in the transition-state of this reaction comes from extensive fission of the P–O bond compensated for by protonation of the oxygen (Scheme 1).[5]



Scheme 1

The greater reactivity of the aryl monophosphate compared with that of the alkyl analogues (by about two orders of magnitude) may be due to the existence of a lipophilic site preferentially binding the aromatic substrates. The boundary of this site is presumably such as to exclude partially the bulky 2,6-dimethyl and (4-phenyl)phenyl esters. Addition of phenyl

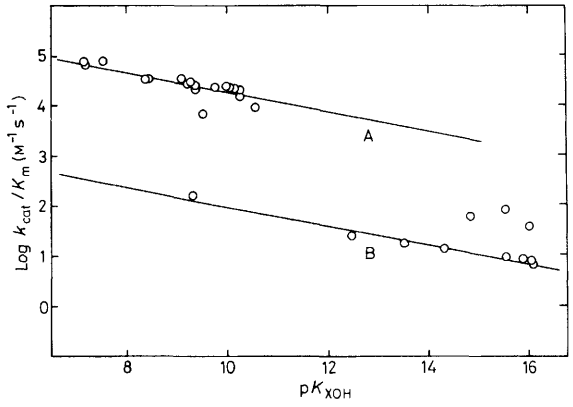


Figure 1. Dependence of $k_{cat.}/K_m$ on the pK of the leaving hydroxy group for the alkaline phosphatase-catalysed hydrolysis of monophosphates. Conditions: 25 °C, ionic strength maintained at 0.25 M with KCl, pH 8.00, tris(hydroxymethyl)aminomethane buffer at 0.05 M. The alkaline phosphatase was Sigma grade III and had a specific activity of between 30 and 40 units per mg protein. The monoesters were prepared as the sodium or cyclohexylammonium salts or as the free acids. They had satisfactory analytical properties and were judged to be free of impurities by chromatographic and n.m.r. spectroscopic procedures. Individual $k_{cat.}$ and K_m values agree with those from previous work;[4] the $k_{cat.}$ values employed here are *relative* to that for 4-nitrophenyl phosphate (defined as unity) under the above conditions. Substituted phenyl esters in increasing order of pK of the hydroxy group: 4-nitro-, 2-nitro-, 3,4,5-trichloro-, 3-nitro-, 2-chloro-, 3-chloro-, 1-naphthyl phosphate, 2-naphthyl phosphate, 4-chloro-, 2-carboxylato- (because of the intramolecular interaction in the ionisation of salicylic acid not seen in nucleophilic attack at salicylate esters the pK used is that for the 4-carboxylic acid), 4-phenyl-, 3-carboxylato-, unsubstituted ester, 3-methyl-, 3,5-dimethyl-, 4-methyl-, 2-methyl-, 2,6-dimethyl-substituted alkyl esters in increasing order of pK of the leaving hydroxy: 1,1,1,3,3,3-hexafluoropropyl-, 2,2,2-trifluoroethyl-, propargyl-, 2-chloroethyl-, benzyl-, 2-phenylethyl-, methyl-, ethyl-, 3-phenylpropyl-, n-propyl-, n-butyl-. The lines are calculated from the equations: A $\log k_{cat.}/K_m = -0.19 \pm 0.02$ pK(ArOH) + (6.2 ± 0.15), $r = 0.950$ (all except 4-phenyl- and 2,6-dimethyl points); B $\log k_{cat.}/K_m = -0.19 \pm 0.01$ pK(ROH) + (3.95 ± 0.17), $r = 0.989$ (all except benzyl-, 2-phenylethyl-, and 3-phenylpropyl points).

groups as substituents on the alkyl monophosphates gives rise to increased reactivity over that predicted from the Brønsted plot for alkyl phosphates; this may arise from interaction of the aryl side chain with the possible lipophilic site. Since results have been obtained for a large number of ester substrates it is unlikely that the existence of two correlations is fortuitous.

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