The Roles of Univalent Cations during Catalysis by Phosphate Acetyltransferase Derived from *Clostridium kluyveri*

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We recently reported (Hibbert *et al.*, 1971; Kyrtopoulos & Satchell, 1972*a,b*) on the detailed kinetics of the phosphate acetyltransferase (EC 2.3.1.8)-catalysed equilibrium (1), including, for each direction (1*a*) and (1*b*), an analysis of the effects of inhibition by products:

These studies were made with moderately high fixed concentrations of NH_4^+ and other ions, since previous work had suggested that a certain concentration of NH_4^+ (or K^+) was necessary for the maximum efficiency of these systems, although no detailed study of the effects of metal ions had been made (Bergmeyer *et al.*, 1963; Stadtman, 1957). We now report such a study.

Our experimental methods followed those used previously (Hibbert *et al.*, 1971), the initial velocities, v_0 , of reactions (1*a*) and (1*b*) being obtained by observing acetyl-CoA spectrophotometrically at 235 nm. All solutions were made up in carefully de-ionized distilled water. Tris-HCl buffers (pH 8.32; final concentration 0.20–0.28 M) were used throughout. Tetramethylammonium chloride was used to maintain a constant ionic strength of 0.75 M. The enzyme from *Clostridium kluyveri* (Boehringer Corp., Mannheim, W. Germany) is supplied as a stable suspension in 3 M-(NH_4)₂SO₄. Samples largely freed (≥ 0.5 mM) from NH_4^+ were obtained by centrifugation and dissolution of the residue in tris buffer (containing 38 mM-dithiothreitol to stabilize the dissolved enzyme). Such enzyme samples lost activity only slowly and were always used within 2 h. Previous work (Stadtman, 1957) has shown that Li^+ has a very small effect on the initial velocity in the present systems compared with Na^+ , K^+ and NH_4^+ ; reactants were chosen accordingly. CoA and acetyl-CoA were obtained (Boehringer Corp.) as the free acid and as the trilitium salt respectively. These compounds were present in very low concentration (approx. 0.1 mM) throughout. For reaction (1*b*) phosphate was added as H_3PO_4 neutralized with tris, and for reaction (1*a*) acetyl phosphate was added as the dilithium salt. The concentration of phosphate species never exceeded 20 mM. The effects of chosen metal ions were studied by adding known concentrations of their A.R. chlorides. All experiments were carried out at 25°C.

In the absence of deliberately added metal ions both enzymic reactions (1*a*) and (1*b*) are relatively very slow, the residual velocity very probably being due to traces of NH_4^+ left in the enzyme sample. Both reactions (1*a*) and (1*b*) are accelerated by the addition of Na^+ , K^+ and NH_4^+ , the sequence of effectiveness being $\text{NH}_4^+ \gg \text{K}^+ \gg \text{Na}^+$. As the concentration of added ion, M^+ , rises, the velocity passes through a maximum value; the maximum occurs at $[\text{M}^+]$ 0.1–0.2 M. The observed effects with Na^+ are relatively small, but for NH_4^+ and K^+ the regions where the velocity is rising with added M^+ can be subjected to kinetic activation analysis (Dixon & Webb, 1964; Rabin & Crook, 1956). Plots of $[\text{M}^+]/v_0$ against $[\text{M}^+]$ for a constant nucleotide concentration and for various fixed phosphate (or acetyl phosphate) concentrations lead to results typified in Fig. 1. This particular behaviour, taken in conjunction with the negligible reaction observed in the absence of M^+ , can be shown to be compatible only with a scheme such as reactions (2)–(4) for the principal path leading to products:

Fig. 1. Activation by NH_4^+ of reaction (1*b*) catalysed by *Cl. kluyveri* phosphate acetyltransferase

Experiments were carried out as indicated in the text, with [acetyl-CoA] 63 μM and [phosphate] 4.70, 9.40 and 18.9 mM for curves A, B and C respectively.

where E is enzyme and S is a substrate [in the present systems acetyl phosphate (or phosphate)] whose

where k_{+2} represents the rate constant for the surface process leading to products and K represents a dissociation constant with $K_{\text{CA}} \simeq K'_{\text{CA}} \simeq K_{\text{CA}}$, $K_{\text{AP}} \simeq K'_{\text{AP}}$ and $K_{\text{M}} \simeq K'_{\text{M}}$. Such a scheme leads to the rate eqn. (13) for v_0 :

$$v_0 = \frac{k_{+2} [\text{E}]_0 [\text{acetyl phosphate}] [\text{CoA}]}{([\text{acetyl phosphate}] + K_{\text{AP}}) \left\{ [\text{CoA}] + K_{\text{CA}} \left(1 + \frac{K_{\text{M}} K_{\text{AP}}}{[\text{M}^+] (K_{\text{AP}} + [\text{acetyl phosphate}])} \right) \right\}} \quad (13)$$

adsorption depends on the prior adsorption of M^+ (represented by M). At this stage of the argument

Runs at different nucleotide concentrations show that M^+ does not affect the adsorption of the nucleotide substrates. We have previously shown (Hibbert *et al.*, 1971; Kyrtopoulos & Satchell, 1972a,b) that equilibrium (1) involves a random non-interacting adsorption of substrates under Michaelis-Menten conditions. The outline mechanism for reaction (1a) can therefore be written as eqns. (5)–(12):

An analogous equation applies for reaction (1b). These equations correspond to the various kinetic observations. For the behaviour illustrated in Fig. 1 the point of intersection can be shown to be $(-K_{\text{M}}, -K_{\text{M}}([\text{CoA}] + K_{\text{CA}})/k_{+2}[\text{E}]_0[\text{CoA}])$. Values of K_{M} and k_{+2} for the different metal ions and reaction directions are given in Table 1. The fact that K_{M} is found to be independent of the reaction direction supports the conclusion that M^+ activates by interaction with the enzyme rather than with a substrate. Indeed, the decreasing extent of activation observed at $[\text{M}^+] > 0.2 \text{ M}$ is almost certainly due to the formation of (inactive) phosphate- (or acetyl phosphate-) metal ion complexes (e.g. MHPO_4^-). (At pH 8.32 the predominant phosphate and acetyl phosphate species are HPO_4^{2-} and $\text{CH}_3\text{-CO-PO}_4^{2-}$ respectively.) These complexes have dissociation constants of about 0.3 M (Sillén & Martell, 1961). They begin to be formed in significant amounts when $[\text{M}^+] > 0.2 \text{ M}$, and we know from our previous studies that mononegative phosphate ions are not adsorbed by the enzyme. This interpretation of the fall in activation is supported by the finding that, if the decrease in v_0 at high $[\text{M}^+]$ is treated as an inhibition by M^+ and Dixon plots of $1/v_0$ against $[\text{M}^+]$ are constructed at fixed phosphate (or acetyl phosphate) concentrations, such plots curve upwards, as expected (Webb, 1963) for an inhibitor that combines with a substrate to form an inactive complex.

The conclusion therefore is that the metal ion controls the adsorption of phosphate or acetyl phosphate, although metal ion-phosphate complexes are not usefully adsorbed. The clear implication is that metal ion and phosphate species occupy separate sites and that for phosphate or acetyl phosphate only becomes available after adsorption of M^+ . The reason that M^+ and phosphate are likely to occupy distinct sites is that if adsorbed M^+ were to bind phosphate directly it would certainly be via the phosphate oxygen atom(s), so that a co-ordination site, or sites, on M^+ , plus steric access, would have to be available after any occupation of co-ordination sites on M^+ by the enzyme. If such a process could occur it is chemically difficult to conceive how M^+ -phosphate complexes could fail to be adsorbed by the enzyme, for M-O co-ordination is also involved in these complexes and

Table 1. *Michaelis parameters from metal ion activation and inhibition of Cl. kluyveri phosphate acetyltransferase*

Experiments were carried out at pH 8.32 (tris buffers) and at 25°C, as indicated in the text. $K_{AP}=7.8 \times 10^{-4}$ M; $K_{CA}=1.5 \times 10^{-4}$ M; $K_P=0.03$ M; $K_{ACA}=1.0 \times 10^{-3}$ M (AP, P, CA and ACA represent acetyl phosphate, phosphate, CoA and acetyl-CoA respectively). Errors were computed by drawing extreme slopes.

M ⁺	10 ² K _M (M)		10 ⁻⁴ k ₊₂ (min ⁻¹)	
	Reaction (1a)	Reaction (1b)	Reaction (1a)	Reaction (1b)
NH ₄ ⁺ *	1.74 ± 0.18	2.07 ± 0.18	1.3 ± 0.1	2.7 ± 0.3
NH ₄ ⁺ †				6.5 ± 0.6
NH ₄ ⁺ ‡				5.8 ± 0.6
NH ₄ ⁺ §			1.4 ± 0.2	5.3 ± 0.8
K ⁺ *	2.50 ± 0.21	2.50 ± 0.21	0.86 ± 0.10	1.4 ± 0.2
K ⁺				1.6 ± 0.2
Na ⁺ †		13 ± 1		
Na ⁺ ‡		12 ± 1		
Na ⁺		13 ± 1		

* Activation experiments.

† Competitive inhibition by Na⁺ with respect to NH₄⁺.

‡ Competitive inhibition by Na⁺ with respect to phosphate in the presence of NH₄⁺.

§ From Michaelis curves for substrates in the presence of NH₄⁺ (Hibbert *et al.*, 1971; Kyrtopoulos & Satchell, 1972a,b).

|| Competitive inhibition by Na⁺ with respect to K⁺.

space would be available on the surface. To find that M⁺-phosphate complexes are unacceptable to the enzyme, as we do, is effectively equivalent to establishing that any bonding between adsorbed M⁺ and phosphate species will lead to the removal of M⁺ from the surface and not to the binding of phosphate to the surface. The facilitation of phosphate binding by adsorbed M⁺ probably therefore involves an indirect effect, such as a conformational change. There is some independent evidence from protection studies (Shimizu *et al.*, 1969) that the presence of (NH₄)₂SO₄ leads to a conformational change. It is noteworthy that a similar mechanism of activation may obtain for phosphate acetyltransferase derived from *Escherichia coli* B, except that for this enzyme pyruvate takes the role of M⁺ here (Suzuki, 1969). Our study supports previous tentative suggestions that univalent cations activate enzymes by inducing conformational changes (Evans & Sorger, 1966) but is incompatible with the generalizations by Suelter (1970) about activation mechanisms involving such cations.

As noted above, Na⁺ activates the enzyme, but much less effectively than does either NH₄⁺ or K⁺. As would be expected, Na⁺ acts as an inhibitor in the presence of these latter ions. We have shown that such inhibition is competitive with respect to NH₄⁺ (or K⁺), and with respect to phosphate (or acetyl phosphate), but non-competitive with respect to the nucleotide substrates. A mechanism based on eqns. (5)–(12) in which Na⁺, K⁺ and NH₄⁺, when present

together, compete for the same site, leads to eqn. (14), of the appropriate form:

$$v_0 = k'_{+2}[E]_0 [M^+] / \left\{ [M^+] + K'_M \left(1 + \frac{[Na^+]}{K_{Na}} \right) \right\} \quad (14)$$

where M⁺ represents K⁺ or NH₄⁺, $k'_{+2} = k_{+2}[\text{acetyl phosphate}][\text{CoA}] / ([\text{acetyl phosphate}] + K_{AP}) ([\text{CoA}] + K_{CA})$ and $K'_M = K_{AP}K_M / ([\text{acetyl phosphate}] + K_{AP})$. Thus a Dixon plot of 1/v₀ against [Na⁺] at different fixed values of [NH₄⁺], but at constant [CoA] and [acetyl phosphate], displays for [Na⁺] < 0.2 M the competitive inhibition between these two univalent ions, the various straight lines intersecting, according to eqn. (14), at the point (−K_{Na}, 1/k'_{+2}[E]₀). In this and similar ways were obtained the remainder of the values given in Table 1. When [Na⁺] > 0.2 M the Dixon plots begin to curve upwards. This is because, in this concentration region, Na⁺ is not only competing with NH₄⁺ (or K⁺) for the enzyme, but is also inactivating significant amounts of phosphate species (see above).

The general self-consistency of our various results will be evident. That the same value of K_{Na} is obtained regardless of the species being inhibited is strong support for our overall picture. It now appears that our previous work (Hibbert *et al.*, 1971; Kyrtopoulos & Satchell, 1972a,b) was, in some instances, performed at less than the optimum metal ion concentrations. The K_m values for the nucleotide and phosphate species quoted here supersede our previously

reported values and have been calculated in the light of eqn. (14). None of our earlier general conclusions is affected.

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