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KINETIC STUDIES WITH PHOSPHOTRANSACETYLASE

III. THE ACYLATION OF PHOSPHATE IONS BY ACETYL COENZYME A

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SUMMARY

1. The kinetics of the phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) catalysed reaction:

Orthophosphate + acetyl-CoA → acetyl phosphate + CoA

have been studied using Tris buffers at 25 °C.

2. The kinetic form shows the outline mechanism to be random bimolecular in type. The Michaelis parameters have been determined as functions of pH.

3. The various results strongly suggest that the reaction is mechanistically analogous both in outline and in detail with the corresponding process:

Arsenate + acetyl-CoA → acetyl arsenate + CoA

studied earlier. The results also support the mechanistic conclusions drawn originally for the phosphate–acetyl phosphate interconversion from the reverse process:

CoA + acetyl phosphate → phosphate + acetyl-CoA.

INTRODUCTION

We have previously[1] shown that Reaction 1, catalysed by phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8), probably follows a random

$$RCO{\cdot}OPO_3^{2-} + CoASH \rightarrow CoASCOR + HPO_4^{2-} \quad (1)$$

bimolecular scheme[2]. We there gave an analysis of the effects on the Michaelis parameters of changes in R and in pH, and reached certain conclusions about the detailed reaction mechanism on the enzyme surface. We have recently[3] provided a similar analysis for the phosphotransacetylase catalysed acetylation of arsenate by acetyl coenzyme A (Reaction 2).

Acetyl-CoA + arsenate → CoA + acetyl arsenate (2)

A random bimolecular scheme was again observed, and the pH analysis revealed most of the catalytic groups found active in Reaction 1, and no others. These results for Reaction 2 are clearly relevant to any study of Reaction 3, the reverse of Reaction 1. We now present such a study. This aspect of phosphotransacetylase catalysis does not appear to have been examined previously.

$$\text{Acetyl-CoA} + \text{phosphate} \xrightarrow{\text{enzyme}} \text{acetyl phosphate} + \text{CoA} \qquad (3)$$

MATERIALS AND METHODS

(1) Chemicals

These were obtained from sources previously[1,3] listed, or were B.D.H. AnalaR or Reagent grade materials. pH values were determined with a Radiometer N 26 pH meter.

(2) Equilibrium position and kinetic complications

One difficulty in studying Reaction 3 is that the equilibrium constant[1]

$$K = [\text{acetyl-CoA}]\,[\text{phosphate}]/[\text{CoA}]\,[\text{acetyl phosphate}] = 10^2.$$

To obtain an appreciable conversion of acetyl-CoA to CoA it is therefore necessary to use a large excess of phosphate (and *vice versa*). We always chose concentration conditions which ensured a $> 50\%$ (and usually $> 80\%$) conversion of acetyl-CoA to CoA at equilibrium. Another difficulty, largely absent from the study of Reaction 1, is that the products of the reverse process 3, CoA and acetyl phosphate, are more strongly adsorbed by the enzyme than are the reactants, acetyl-CoA and phosphate. Inhibition by products is therefore very evident, and the extended first-order kinetic behaviour, found under appropriate conditions[1], for Reaction 1 are not observed for its reverse. We have accordingly measured initial rates only in the present case.

A final complication in studies of enzymatic acylation by acetyl-CoA (3) is the need to know the rate of the spontaneous hydrolysis of acetyl-CoA (4) in the relevant media, in order to be able to allow for this contribution to the observed total rate of disappearance of acetyl-CoA. Relevant results for this hydrolysis are presented in Table I and in Part II[3], Table I. We were able

$$\text{Acetyl-CoA} + H_2O \rightarrow \text{acetic acid} + \text{CoA} \qquad (4)$$

to arrange our concentration conditions for the enzymatic reaction so that the concommitant loss of acetyl-CoA *via* spontaneous hydrolysis was negligible at pH $\lesssim 8.9$; for the slowest enzymatic reactions at higher pH values we corrected the observed initial velocities in the light of Table I.

TABLE I

EFFECT OF pH ON THE SPONTANEOUS HYDROLYSIS OF ACETYL-CoA IN THE PRESENCE OF PHOSPHATE IONS AT 25 °C

$[\text{Acetyl-CoA}]_0 \simeq 5 \cdot 10^{-5}$ M; Tris–HCl buffers (total concentration 0.1 M); $[K^+] = 0.40$ M; [phosphate] = 0.40 M; $[Na^+] = 0.80$ M; for k_{obs} see text.

pH	8.90	9.25	9.59	9.62	9.90
$k_{obs} \times 10^3$ (min^{-1})	2.32	5.50	15.1	16.2	32.1

(3) Kinetics of Reaction 3

The enzymatic process (3) was followed, as in our previous studies of related systems[1,3], by spectroscopic analysis for acetyl-CoA at 235 nm. The same considerations noted in the earlier work, in conjunction with the points noted in section (2) above, controlled the composition of reaction mixtures. Details are given in the figures and tables. We used Tris buffers throughout. For the present initial rate measurements the first 5–10% of a run was (accurately) monitored and the initial velocity, v_0 (mole·l^{-1}·min^{-1}), calculated from a plot of [acetyl-CoA] against time.

The known[1,3] kinetics of Reactions 1 and 2 strongly suggest the following random bimolecular scheme for (3), Michaelis–Menten conditions prevailing*. Assuming $K_{ACA} = K'_{ACA}$ and $K_P = K'_P$

$$\text{Acetyl-CoA} + E \rightleftharpoons E\text{-acetyl-CoA} \qquad \text{fast } K_{ACA} \qquad (5)$$

$$\text{Phosphate} + E \rightleftharpoons E\text{-phosphate} \qquad \text{fast } K_P \qquad (6)$$

$$E\text{-acetyl-CoA} + \text{phosphate} \rightleftharpoons \begin{matrix} \text{acetyl-CoA} \\ E \\ \text{phosphate} \end{matrix} \qquad \text{fast } K'_P \qquad (7)$$

$$E\text{-phosphate} + \text{acetyl-CoA} \rightleftharpoons \begin{matrix} \text{acetyl-CoA} \\ E \\ \text{phosphate} \end{matrix} \qquad \text{fast } K'_{ACA} \qquad (8)$$

$$\begin{matrix} \text{acetyl-CoA} \\ E \\ \text{phosphate} \end{matrix} \rightarrow \begin{matrix} \text{CoA} \\ E \\ \text{acetyl phosphate} \end{matrix} \qquad \text{slow } k \qquad (9)$$

the initial rate equation for scheme (5)–(9) is Eqn 10, in which $[E_0]$ is the total enzyme concentration. Our present results are

$$v_0 = -\frac{d}{dt}[\text{acetyl-CoA}]_0 = \frac{k[E]_0\,[\text{acetyl-CoA}]_0\,[\text{phosphate}]_0}{([\text{acetyl-CoA}]_0 + K_{ACA})\,([\text{phosphate}]_0 + K_P)} \qquad (10)$$

indeed compatible with this equation. Thus, at fixed pH, with $[\text{phosphate}]_0$ constant, simple Michaelis–Menten behaviour should be found for changes in $[\text{acetyl-CoA}]_0$ (and *vice versa*). Results of this type are in Fig. 1. It is evident that phosphate ions are weakly adsorbed; that the adsorption is real is attested by the competitive inhibition of Reaction 1 brought about by appropriate concentrations of phosphate[1,4]. The data in Fig. 1 lead to good reciprocal plots. The Michaelis parameters were calculated from such plots or by Wilkinson's method[5]. The observed strength of the acetyl-CoA binding ($K_{ACA} = (1.2 \pm 0.1)\cdot 10^{-3}$ M) is in excellent agreement with the result found[3] for the corresponding reaction with arsenate (2).

The weakness of the phosphate binding ($K_P = 0.24 \pm 0.07$ M at pH 8.3) makes it impractical to saturate the enzyme with phosphate. This has two important consequences: (i) a complete pH analysis, along the lines[1,3] used for Reactions 1 and 2

* This conclusion is confirmed by studies[4] of inhibition by products and by sodium ions: examples of purely non-competitive inhibition are found, a fact which argues strongly against any steady-state mechanism obtaining.

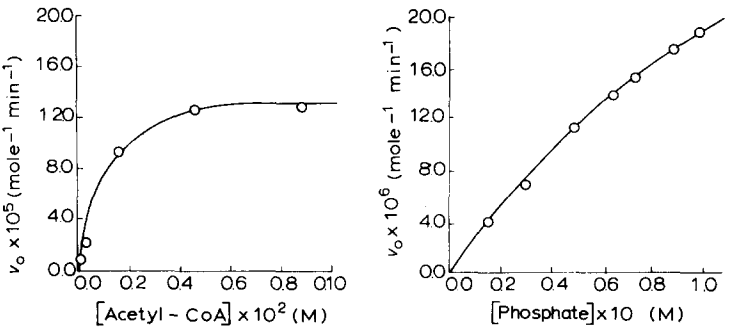

Fig. 1. (a) Effect of added acetyl-CoA at constant phosphate concentration. $[Na_2HPO_4] = 0.25$ M; $[NH_4^+] = 1 \cdot 10^{-2}$ M; [enzyme] $= 3.5 \cdot 10^{-8}$ M; ionic strength = 0.85; Tris buffer at pH 8.32; v_0 = initial rate; temperature = 25 °C.
(b) Effect of added phosphate at constant acetyl-CoA concentration. [Acetyl-CoA] $= 4.8 \cdot 10^{-5}$ M; $[Na^+] = 0.20$ M; $[NH_4^+] = 7.2 \cdot 10^{-3}$ M; [enzyme] $= 2.5 \cdot 10^{-8}$ M; ionic strength = 0.4; Tris buffer at pH 8.32; v_0 = initial rate; temperature = 25 °C; phosphate added as Na_2HPO_4.

is impossible and (ii) even directly determined values of K_P, like that from Fig. 1(b), inevitably involve appreciable error. We therefore considered it not worthwhile to directly determine K_P at every pH, especially since there is good reason for believing (see below) that the value of K_P increases still further at pH $\gtrsim$ 7.0. We have therefore constructed a partial pH analysis.

(4) pH analysis

When $[\text{acetyl-CoA}]_0 \ll K_{ACA}$ and $[\text{phosphate}]_0 \ll K_P$, Eqn 10 reduces to (11), and when $[\text{acetyl-CoA}]_0 \gg K_{ACA}$ and $[\text{phosphate}]_0 \lesssim K_P$ it becomes (12). Results of experiments using these

$$v_0 = k[E]_0 \, [\text{phosphate}]_0 \, [\text{acetyl-CoA}]_0 / K_P K_{ACA} \qquad (11)$$

TABLE II

pH EFFECTS IN THE ENZYMATIC REACTION AT 25 °C

v_0 = initial velocity (mole·l^{-1}·min^{-1}); Tris buffers (total concentration usually 0.1 M).

(i) $[\text{Acetyl-CoA}]_0 \ll K_{ACA}$; [phosphate] $\ll K_P$

$[\text{Acetyl-CoA}]_0 = 7.2 \cdot 10^{-5}$ M; [phosphate] = 0.01 M; $[Na^+] = 0.020$ M; $[NH_4^+] = 3.6 \cdot 10^{-2}$ M; [enzyme] $= 1.25 \cdot 10^{-7}$ M; ionic strength = 0.17.

pH	5.28	5.87	6.16	6.27	6.45	6.64	6.75	6.86
$v_0 \times 10^5$	0.20	0.57	0.73	1.73	2.75	3.52	4.71	5.50
pH	6.96	7.26	7.87	8.17	8.26	8.74	9.19	9.50
$v_0 \times 10^5$	5.56	6.03	6.55	5.27	5.04	3.00	0.92	0.20

(ii) $[\text{Acetyl-CoA}]_0 \gg K_{ACA}$; [phosphate] $\lesssim K_P$

$[\text{Acetyl-CoA}]_0 = 5 \cdot 10^{-3}$ M; [phosphate] = 0.4 M; $[Na^+] = 0.8$ M; $[K^+] = 0.4$ M; $[NH_4^+] = 7.2 \cdot 10^{-3}$ M; [enzyme] $= 2.5 \cdot 10^{-8}$ M; ionic strength = 1.5.

pH	5.52	5.83	6.40	6.70	7.10	8.00	8.59	8.70
$v_0 \times 10^5$	0.6	1.0	1.9	4.3	6.3	8.0	7.4	8.0*
pH	9.30	9.59	9.90					
$v_0 \times 10^5$	5.5*	3.3*	1.8*					

* Corrected for spontaneous hydrolysis.

$$v_0 = k[E]_0\,[\text{phosphate}]_0/(K_P + [\text{phosphate}]_0) \quad (12)$$

concentration conditions, at a series of pH values between 5.3 and 9.9, are in Table II.

Division of Eqn 11 by (12) leads, at fixed pH, to Eqn 13, in which the primes refer to the concentration conditions of Eqn 12. K_P is known at pH 8.32 from

$$\frac{v_0}{v_0'} = \frac{[E]_0}{[E]_0'} \cdot \frac{[\text{acetyl-CoA}]_0}{K_{ACA}} \cdot \frac{[\text{phosphate}]_0}{[\text{phosphate}]_0'} \cdot \frac{(K_P + [\text{phosphate}]_0')}{K_P} \quad (13)$$

direct measurement (Fig. 1 (b)) so that K_{ACA} can be estimated from Eqn 13 at this pH. The value obtained ($K_{ACA} = 0.5 \cdot 10^{-3}$ M) is in only fair agreement with that determined directly at this pH (Fig. 1 (a)). The discrepancy very probably arises from the inevitable large differences in Na^+ and K^+ concentrations obtaining between the two sets of data in Table II. Such large differences will be expected[6] to have an effect on the ratio v_0/v_0'.

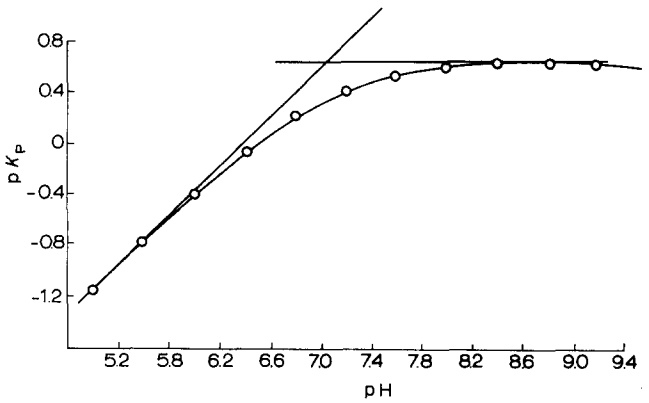

Fig. 2. Effect of pH on pK_P at 25 °C.

If it is tentatively assumed that the behaviour of the phosphate ions in the present system is essentially analogous to that of the arsenate ions in Reaction 2, then the effect of pH on K_P can be represented as in Fig. 2. This profile is determined by the measured K_P value at pH 8.32 and by the pK_a value ($\simeq$ 7.0*) of $H_2PO_4^-$, since our study[3] of Reaction 2 showed (i) that only $HAsO_4^{2-}$ (and not $H_2AsO_4^-$) is adsorbed, and (ii) that K_{As} for $HAsO_4^{2-}$ is unaffected by pH at values $\lesssim$ 5. Using

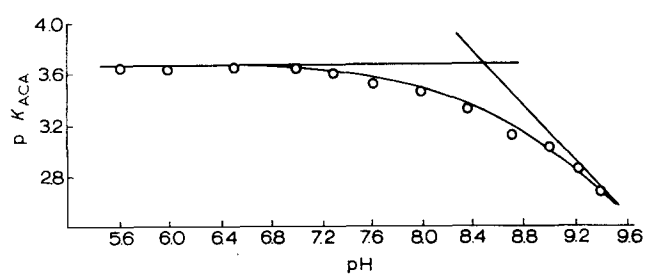

Fig. 3. Effect of pH on pK_{ACA} at 25 °C.

* Value appropriate at moderate ionic strength[7].

K_P values from Fig. 2, K_{ACA} can be calculated as a function of pH from results in Table II. The resulting profile (Fig. 3) is strikingly similar to that obtained for acetyl-CoA in the arsenate reaction[3].

Fig. 4 shows a plot of log k as a function of pH. The calculations are based on Eqn 12, Fig. 2 and results in Table II. The values of K_{ACA} and k calculated from the pH analysis will only be approximate; their trends, however, are probably reliable.

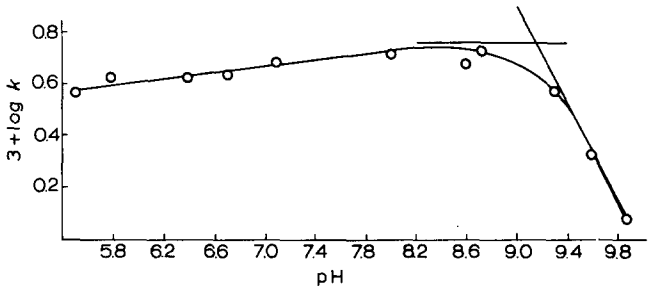

Fig. 4. Effect of pH on k at 25 °C.

DISCUSSION

(1) Effects of pH

Always assuming that, as found for the arsenate Reaction 2, only the dianion (HPO_4^{2-}) and not the monoanion ($H_2PO_4^-$) is taken up by the enzyme (Fig. 2), the essential conclusions following from Dixon's rules[8] for the present pH analysis are:

(i) adsorption of acetyl-CoA (Fig. 3) is affected by the ionisation of a group of $pK_a \simeq 8.5$ in either the free enzyme or acetyl-CoA. This group must be protonated for effective adsorption and is clearly the same group detected previously[1,3] in both Reactions 1 and 2 in connection with CoA and acetyl-CoA binding. As for the arsenate acylation[3], no other acid-base group ionising in the pH range 5–9.6 appears to affect acetyl-CoA binding.

(ii) The rate (k) of transformation of the ternary enzyme–substrate complex into the product complex is certainly affected by a group in this complex of $pK_a \simeq 9.1$, active in its protonated form. This again is a group previously identified[1,3]. At pH < 9, k appears largely independent of the hydrogen ion concentration. This result is similar to that obtained for the arsenate reaction where there is evidence that it arises from the operation of two groups of similar pK_a ($\simeq$ 6–7), one effective in its basic and the other in its protonated form. The shape of the present profile lends support to that idea.

(2) Mechanism

Our kinetic studies of Reactions 1, 2 and 3 are entirely self-consistent so far as their outline mechanisms are concerned: phosphotransacetylase catalysed equilibria are probably random bimolecular schemes in both directions. A more detailed picture can be constructed from the pH analyses. As we have seen, these are also mostly self-consistent and to that extent reinforce each other. The present reaction must be the microscopic reverse of Reaction 1, and it is evident that the mechanism

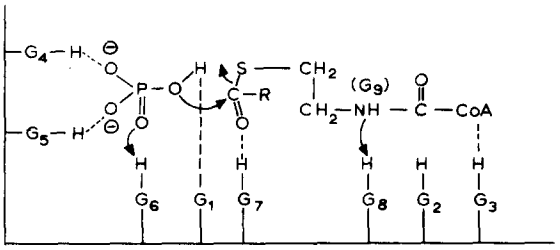

Fig. 5. Groups G_1–G_9 numbered as in ref. 1. G_1, $pK_a<6$; G_2, $pK_a\simeq 7.2$; G_3, $pK_a = 8.5$–8.7; G_4, G_5, $pK_a>9$; G_6, $pK_a = 6.4$–6.9; G_7, $pK_a>9$; G_8, $pK_a = 8.9$–9.1; G_9, $pK_a\simeq 7.4$.

originally[1] suggested for the later requires but little modification in the light of the pH analyses now available for Reactions 2 and 3. For (3) it can be represented as in Fig. 5.

The essential differences between the influence of groups in Reaction 1, and in (2) and (3), are: (i) That G_2 is only detected for CoA adsorption, not for acetyl-CoA. A conformational difference between *E*-CoA and *E*-acetyl-CoA could underlie this result. (ii) That, for Reactions 2 and 3, while G_8 is evident, G9 is partly masked by the ionisation of another group, perhaps G_6. In Fig. 5 the curved arrows represent the transfer of electron pairs in the slow step; the dots are hydrogen bonds involved in adsorption. Our model provides, as it stands, a reasonable rationalisation of the difference between the K_m values of phosphate and acyl phosphate, and also between those of CoA and acetyl-CoA. Much further interaction between substrate and enzyme is, however, likely for the nucleotides. Whatever the deficiencies of Fig. 5 (and alternative transition states can be conceived[9]) the true picture is undoubtedly of at least equal complexity.

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