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KINETIC STUDIES WITH PHOSPHOTRANSACETYLASE

II. THE ACETYLATION OF ARSENATE BY ACETYL COENZYME A

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SUMMARY

1. Phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) catalyses the reaction

Arsenate + acetyl-CoA → acetyl arsenate + CoA

We have studied the kinetics of this process, using a range of Tris buffers, at 25 °C.

2. The kinetic form of the acylation shows that both substrates are adsorbed by the enzyme, probably under Michaelis–Menten conditions. Arsenate is found to be taken up much more strongly than previously believed. Kinetic analysis shows the outline mechanism to be random bimolecular in type, and leads to the values of the various Michaelis parameters as a function of pH.

3. The arsenate dianion ($HAsO_4^{2-}$) is bound by an acidic group(s) in the enzyme, $pK_a > 9$. This group engages at least two of the arsenate oxygen atoms since the monoanion ($H_2AsO_4^-$) is not taken up by the enzyme.

4. Binding of acetyl-CoA involves a group, $pK_a \simeq 8.5$ in the free enzyme, active in its protonated form. The group, of $pK_a \simeq 7.2$ in the enzyme–substrate complex, which assists CoA binding in the forward process of the phosphotransacetylase catalysed equilibrium

CoA + acetyl phosphate ⇌ acetyl-CoA + phosphate

is not detected in the present arsenate reaction. This suggests that the enzyme–CoA and enzyme–acetyl-CoA adducts have different conformations.

5. The rate of unimolecular transformation, k, of the enzyme–substrate ternary complex is affected by at least two acid–base groups, both of which probably originate in the enzyme. One, active when protonated, has $pK_a \simeq 9.0$, and another, active in its basic form, has $pK_a \simeq 7.0$. These groups are probably identical with those of similar pK_a identified for the forward step of the acetyl phosphate equilibrium above.

6. The acetylation of arsenate appears likely to follow a detailed mechanism which is essentially the microscopic reverse of that of the CoA–acetyl phosphate reaction previously studied. It follows that the acetylation of phosphate should display all the features here revealed for arsenate.

INTRODUCTION

Much of the early work with phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8), which catalyses equilibrium (1), was by Stadtman and co-workers[1-5].

$$\text{CoA} + \text{acetyl phosphate} \overset{\text{Enzyme}}{\rightleftharpoons} \text{acetyl-CoA} + \text{phosphate} \quad (1)$$

They normally studied this system in the presence of added arsenate ions, when the overall process is then the hydrolysis of acetyl phosphate. Reactions 1, 2 and 3 were proposed to account for this, phosphotransacetylase catalysis of reaction 2 also being postulated.

$$\text{Acetyl-CoA} + \text{arsenate} \overset{\text{Enzyme}}{\rightleftharpoons} \text{acetyl arsenate} + \text{CoA} \quad (2)$$

$$\text{Acetyl arsenate} + H_2O \rightarrow \text{acetic acid} + \text{arsenate} \quad (3)$$

Stadtman[2] found that the rate of acetyl phosphate hydrolysis was rectilinearly dependent on the arsenate concentration up to at least 0.1 M and concluded that the Michaelis constant for arsenate must be large. Stadtman and co-workers' experiments were mostly qualitative.

We have recently reported[6] a quantitative kinetic study of the forward process of equilibrium 1 in the absence of arsenate. In this direction we showed that the reaction probably follows a random bimolecular scheme[7], and we made an analysis of the effects on the Michaelis parameters of changes in pH and in the structure of the acyl phosphate. We reached certain conclusions about the detailed reaction mechanism on the enzyme surface. If Stadtman's suppositions are correct, the forward process of equilibrium 2 might be expected to be mechanistically analogous to the reverse process of equilibrium 1, and will be easier to study since reaction 3 prevents the reversal of 2, whereas 1 lies well to the right. We have accordingly examined the acetylation of arsenate by acetyl-CoA in some detail.

MATERIALS AND METHODS

Chemicals

Acetyl-CoA was the Boehringer product. The remaining chemicals were obtained from the sources previously listed[6], or were B.D.H. AnalaR or Reagent grade materials. Arsenate was added as KH_2AsO_4. The pH values of the various Tris buffer solutions were determined with a Radiometer N26 pH meter. Tris is known to have no deleterious effects on the behaviour of the enzyme.

Preliminary experiments

Preliminary questions concerning reactions 2 and 3 were:
(i) How fast is acetyl-CoA hydrolysis in the absence of both enzyme and arsenate? (ii) Is this non-enzymatic hydrolysis catalysed by arsenate or is enzyme necessary to the arsenate catalysis? (iii) Is reaction 3 fast compared with reaction 2? To answer questions (i) and (ii) we have examined the rate of hydrolysis of acetyl-CoA (4) in Tris buffers of various pH values, both in the presence and the absence of added arsenate ions. Our results are in Table I.

TABLE I

EFFECT OF pH AND ARSENATE IONS ON THE SPONTANEOUS HYDROLYSIS OF ACETYL-CoA AT 25 °C
$[\text{Acetyl-CoA}]_0 \simeq 5 \cdot 10^{-5}$ M; Tris–HCl buffers; for k_{obs} see text.

(i) Effect of arsenate ions at pH 8.32

$[KH_2AsO_4] \times 10^3$ (M)	0.00	4.40	43.7
$k_{obs} \times 10^4$ (min^{-1})	4.24	3.98	3.00

(ii) Effect of pH at constant arsenate concentration

$[KH_2AsO_4] = 5.15 \cdot 10^{-2}$ M; $[NH_4^+] = 1.6 \cdot 10^{-3}$ M; ionic strength = 0.24 M.

pH	8.60	8.90	9.25	9.59	9.90
$k_{obs} \times 10^3$ (min^{-1})	0.60	1.27	3.19	8.27	17.6

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

The hydrolysis was followed spectrophotometrically at 235 nm where CoA and acetyl-CoA have significantly different extinction coefficients[6], and where the absorptions of arsenate ions and of acetic acid do not interfere. Measurements were made with either a Unicam SP 500 or SP 800 spectrophotometer, fitted with a thermostatted cell compartment. The loss of acetyl-CoA in an excess of buffer is an accurately first-order process over more than three half-lives. Values of k_{obs}, the observed first-order rate constant, were calculated from plots of log [acetyl-CoA] against time and were reproducible to within $\pm$ 3% (Fig. 1).

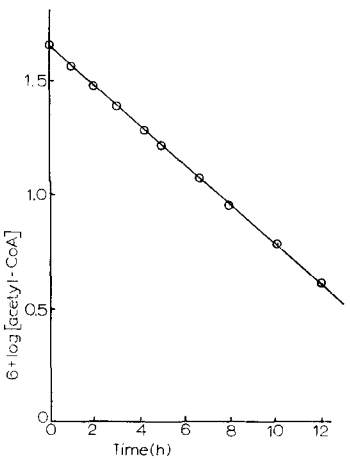


Fig. 1. Spontaneous hydrolysis of acetyl-CoA. pH 8.90; $[KH_2AsO_4] = 5.15 \cdot 10^{-2}$ M; $[NH_4^+] = 1.6 \cdot 10^{-3}$ M; ionic strength = 0.24 M.

Table I shows that the non-enzymatic hydrolysis is base catalysed. The complete rate equation for the present system is probably[8,9] (5) in which, in the pH range and buffer (RNH_2) used, the term in $[OH^-]$ is dominant. Added arsenate ions ob-

$$-\mathrm{d[acetyl\text{-}CoA]}/\mathrm{d}t = \{k_{H_2O}[H_2O]^2 + k_{OH^-}[OH^-][H_2O] + k_{RNH_2}[H_2O][RNH_2]\} = [\text{acetyl-CoA}] = k_{obs}[\text{acetyl-CoA}] \quad (5)$$

viously produce negligible catalysis, but lead to a negative salt effect. It is clear that, as Stadtman and coworkers assumed, the presence of the enzyme is essential for arsenate to catalyze the hydrolysis of acetyl-CoA. In our enzymatic studies, detailed below, the non-enzymatic hydrolysis rate was negligible compared with the enzymatic rate under almost all conditions. However, for the slowest enzymatic rates (Table II (iii), sampling technique) a correction was necessary at pH $\gtrsim$ 8.9.

Stadtman's assumption that the hydrolysis of acetyl arsenate is fast has proved impossible to test rigorously. Acetyl arsenate has not been prepared previously, and we have been unable to make it using methods suitable for acetyl phosphate[10,11]. This fact certainly suggests that acetyl arsenate decomposes rapidly in aqueous solution, but the evidence is only circumstantial. Acetyl phosphate hydrolyses comparitively slowly[12] ($t_{\frac{1}{2}} \simeq 14$ h at pH 7) and there is no immediately obvious reason for acetyl arsenate to behave differently. However, $HAsO_4^{2-}$ is somewhat more basic[13,14] than is HPO_4^{2-}; if the same is true for their acetyl derivatives a greater fraction of acetyl arsenate will exist in solution at any pH as the (presumably) more reactive monoanion than for acetyl phosphate. This may account for at least some of the apparent difference in reactivity. In what follows we have, like Stadtman, assumed reaction (3) is fast.

Kinetics of the enzymatic reaction

The forward process of equilibrium (2) was followed, like the non-enzymatic hydrolysis (4), by spectrophotometric analysis for acetyl-CoA at 235 nm. A difficulty with this reaction, which is absent from the forward process of equilibrium (1) studied previously[6], is that the products CoA and acetyl arsenate are likely to be more strongly bound to the enzyme than the reactants. Inhibition by CoA is, indeed, found and the extended first-order behaviour observed previously[6] is not found here. We have accordingly measured initial rates only. The first 15–20% of a run were normally monitored accurately and the initial rate, v_0 (moles·l^{-1}·min^{-1}), calculated from a plot of [acetyl-CoA] against time (*e.g.* Fig. 2). Runs were conducted essentially as

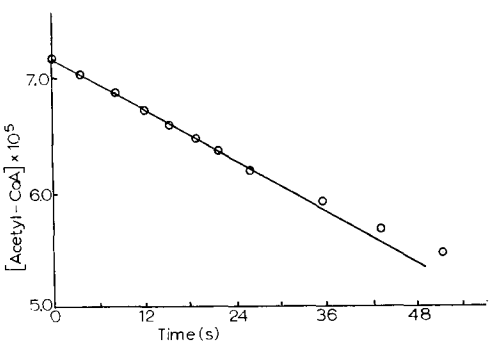


Fig. 2. Determination of v_0. pH 5.37; $[KH_2AsO_4] = 5.15 \cdot 10^{-2}$ M; $[NH_4^+] = 2.9 \cdot 10^{-2}$ M; [enzyme] = 10^{-7} M; ionic strength = 0.41 M.

previously described[6]. When $[\text{acetyl-CoA}]_0 \gtrsim 2 \cdot 10^{-4}$ M, a sampling technique was used; at lower concentrations the spectrophotometer cell served as the reaction vessel. To minimise the effects of metal ions[6], runs were, so far as possible, carried out at

constant individual ion concentrations (and therefore at constant ionic strength); always $[NH_4^+] \geqslant 7 \cdot 10^{-3}$ M.

If we assume that the reaction being studied is mechanistically analogous to the reverse process of (1), then it follows from our study[6] of the forward process of (1), that one of the following two schemes should obtain, Michaelis–Menten conditions (very) probably prevailing*:

(i) E + acetyl-CoA $\rightleftharpoons$ E–acetyl-CoA — fast, K_{ACA} (6)

E–acetyl-CoA + arsenate $\rightarrow E\begin{<}^{\text{CoA}}_{\text{acetyl arsenate}}$ — slow, k (7)

(ii) E + acetyl-CoA $\rightleftharpoons$ E–acetyl-CoA — fast, K_{ACA} (8)

E + arsenate $\rightleftharpoons$ E–arsenate — fast, K_{As} (9)

E–acetyl-CoA + arsenate $\rightleftharpoons E\begin{<}^{\text{acetyl-CoA}}_{\text{arsenate}}$ — fast, K'_{As} (10)

E–arsenate + acetyl-CoA $\rightleftharpoons E\begin{<}^{\text{acetyl-CoA}}_{\text{arsenate}}$ — fast, K'_{ACA} (11)

$E\begin{<}^{\text{acetyl-CoA}}_{\text{arsenate}} \rightarrow E\begin{<}^{\text{CoA}}_{\text{acetyl arsenate}}$ — slow, k (12)

Paths (i) or (ii) will be followed by the steps (13)–(17).

$E\begin{<}^{\text{CoA}}_{\text{acetyl arsenate}} \rightleftharpoons E$–acetyl arsenate + CoA — fast (13)

$E\begin{<}^{\text{CoA}}_{\text{acetyl arsenate}} \rightleftharpoons E$–CoA + acetyl arsenate — fast (14)

E–acetyl arsenate $\rightleftharpoons$ E + acetyl arsenate — fast (15)

E–CoA $\rightleftharpoons$ E + CoA — fast (16)

Acetyl arsenate + $H_2O \rightarrow$ acetic acid + arsenate — fast (17)

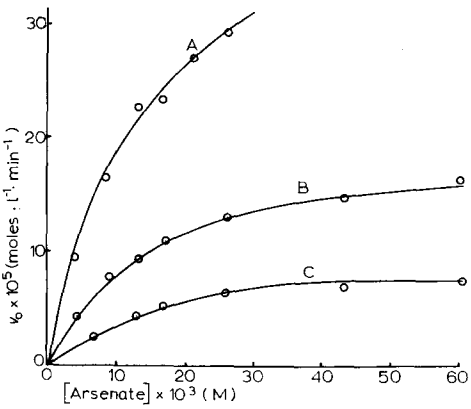
In equations (6)–(17), E represents enzyme. Scheme (6)–(7) leads to the initial rate equation (18). Assuming $K_{ACA} = K'_{ACA}$ and $K_{As} = K'_{As}$, the corresponding equation for scheme (8)–(12), the random bimolecular mechanism, is (19).

$$v_0 = -\mathrm{d}[\text{acetyl-CoA}]_0/\mathrm{d}t = k\,E_0[\text{acetyl-CoA}]_0\,[\text{arsenate}]_0/(K_{ACA} + [\text{acetyl-CoA}]_0) \quad (18)$$

$$v_0 = -\mathrm{d}[\text{acetyl-CoA}]_0/\mathrm{d}t = k\,E_0[\text{acetyl-CoA}]_0\,[\text{arsenate}]_0/(K_{As} + [\text{arsenate}]_0)\,(K_{ACA} + [\text{acetyl-CoA}]_0) \quad (19)$$

* The reported kinetics of the forward process[6] satisfy either a random bimolecular mechanism occurring under Michaelis–Menten conditions, or an ordered bimolecular scheme occurring under Briggs–Haldane conditions, the evidence rather favouring the former. It would therefore seem reasonable to assume that the very similar compounds involved in the present reverse reaction will also undergo fast (pre-equilibrium) adsorption. Briggs–Haldane conditions (and therefore all steady-state mechanisms) have, in fact, been independently excluded with some certainty[15,16] for reactions (1) and (2) by the finding that cases of purely non-competitive inhibition occur during inhibition by products and by Na^+. (This work is being reported in detail elsewhere[17,18].)

In equations (18) and (19), E_0 is the total enzyme concentration. Stadtman's results for the effect of arsenate[2], although not definite, suggest scheme (6)–(7). It is clear from our experiments, however, that arsenate is more strongly adsorbed than Stadtman believed and that scheme (6)–(7) cannot obtain. Data in Fig. 3, which give good reciprocal plots, lead to $K_{As} = 1.4 \cdot 10^{-2} \pm 0.1 \cdot 10^{-2}$ M. Similar experiments, at the same pH, concerning acetyl-CoA adsorption (Fig. 4) give $K_{ACA} = 6.9 \cdot 10^{-4}$ M. (This



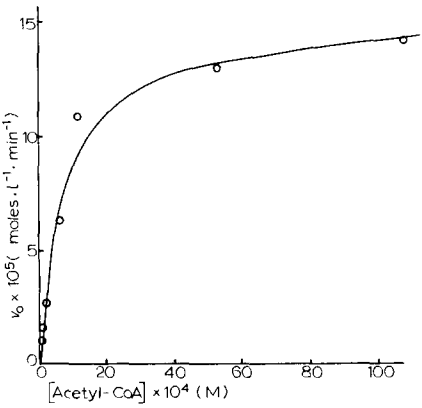


Fig. 3. Adsorption of arsenate. [Enzyme] = $6.25 \cdot 10^{-8}$ M (A), $4.17 \cdot 10^{-8}$ M (B), $2.08 \cdot 10^{-8}$ M (C); pH 8.32; [acetyl-CoA] = $1.2 \cdot 10^{-4}$ M; [K^+] = $6.06 \cdot 10^{-2}$ M.

Fig. 4. Adsorption of acetyl-CoA. [Enzyme] = $6.7 \cdot 10^{-8}$ M; pH 8.32; [KH_2AsO_4] = $6.5 \cdot 10^{-4}$ M; [NH_4^+] = $1.9 \cdot 10^{-2}$ M.

value is larger[6] than K_{CA}, hence the marked product inhibition already noted.) The data in Figs. 3 and 4 can be used to test the adequacy of equation (19). When [acetyl-CoA]$_0 \gg K_{ACA}$ then (19) reduces to (20); when [arsenate]$_0 \gg K_{As}$ it becomes (21). Values of k calculated

$$v_0 = k\, E_0[\text{arsenate}]_0/(K_{As} + [\text{arsenate}]_0) \quad (20)$$

$$v_0 = k\, E_0[\text{acetyl-CoA}]_0/(K_{ACA} + [\text{acetyl-CoA}]_0) \quad (21)$$

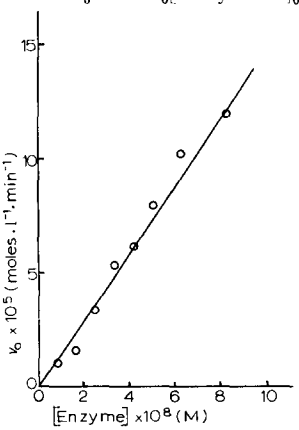


Fig. 5. Effect of enzyme concentration. [Acetyl-CoA] = $1.76 \cdot 10^{-4}$ M; [KH_2AsO_4] = $2.1 \cdot 10^{-3}$ M; [NH_4^+] = $4.8 \cdot 10^{-2}$ M; pH 8.32; ionic strength = 0.18 M.

from the two sets of data and from equations (20) and (21) respectively agree within experimental error ($k = (4.5 \cdot 10^4 \pm 1.0 \cdot 10^4\ \text{min}^{-1}$ at pH 8.32). This shows that equation (19) correctly represents the overall kinetics and that scheme (8)–(12) is a real possibility. Finally, results in Fig. 5 show the reaction to be, as expected, first order in enzyme concentration.

pH analysis

When $[\text{acetyl-CoA}]_0 \ll K_{ACA}$ and $[\text{arsenate}]_0 \ll K_{As}$, equation (19) reduces to (22), when $[\text{acetyl-CoA}]_0 \ll K_{ACA}$ but $[\text{arsenate}]_0 \gg K_{As}$ it reduces to (23), and when $[\text{acetyl-CoA}]_0 \gg K_{ACA}$ and $[\text{arsenate}]_0 \gg K_{As}$ it reduces to (24).

$$v_0 = k\, E_0[\text{arsenate}]_0\, [\text{acetyl-CoA}]_0 / K_{ACA} K_{As} \tag{22}$$

$$v_0 = k\, E_0[\text{acetyl-CoA}]_0 / K_{ACA} \tag{23}$$

$$v_0 = k\, E_0 \tag{24}$$

Hence, determination of initial velocities, at a series of pH values, under each of the three concentration conditions specified, can lead to a knowledge of the variation of k, K_{ACA}, and K_{As} with pH. Details of such experiments, conducted at constant ionic strength, are in Table II. The derived pH dependencies are plotted

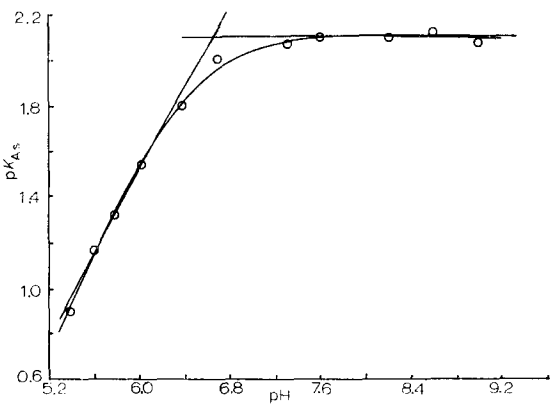


Fig. 6. Effect of pH on K_{As}.

in Figs. 6–8. We covered the pH range 5.4–9.4. The values of the Michaelis parameters at pH 8.32 arising from these experiments are in good agreement with those obtained independently from the complete Michaelis curves at this pH (Figs. 3 and 4). This agreement lends further support to the adequacy of equation (19).

DISCUSSION

Outline mechanism

We conclude that the present and previous[6] experiments are both compatible with the outline mechanism (8)–(16) for the phosphotransacetylase catalysed acylation of arsenate by acetyl-CoA (and its reverse). It follows that the reverse of equili-

TABLE II

pH EFFECTS IN THE ENZYMATIC REACTION AT 25 °C

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

(i) $[\text{Acetyl-CoA}]_0 \ll K_{ACA}$; $[\text{arsenate}]_0 \ll K_{As}$; $[\text{acetyl-CoA}]_0 = 7.2 \cdot 10^{-5}$ M; $[\text{arsenate}]_0 = 1.03 \cdot 10^{-3}$ M; [enzyme] = 10^{-7} M; $[NH_4^+] = 2.9 \cdot 10^{-2}$ M; $[K^+] = 5.15 \cdot 10^{-2}$ M; ionic strength = 0.34 M

pH	5.54	5.78	6.02	6.31	6.66	7.08	7.29	7.74	8.29	8.63	8.80	9.36
$v_0 \times 10^6$	0.86	2.15	7.90	10.4	25.5	29.7	31.4	28.5	18.9	14.2	7.9	2.0

(ii) $[\text{Acetyl-CoA}]_0 \ll K_{ACA}$; $[\text{arsenate}]_0 \gg K_{As}$; $[\text{acetyl-CoA}]_0 = 7.2 \cdot 10^{-5}$ M; $[\text{arsenate}]_0 = 5.15 \cdot 10^{-2}$ M; [enzyme] = 10^{-7} M; $[NH_4^+] = 2.9 \cdot 10^{-2}$ M; $[K^+] = 5.15 \cdot 10^{-2}$ M; ionic strength = 0.41 M

pH	5.37	5.56	5.89	6.15	6.35	6.70	7.26	7.81	8.17	8.55	8.94	9.29
$v_0 \times 10^6$	23.4	55.3	107	164	159	238	270	225	164	103	56.4	28.1

(iii) $[\text{Acetyl-CoA}]_0 \gg K_{ACA}$; $[\text{arsenate}]_0 \gg K_{As}$; $[\text{acetyl-CoA}]_0 = 5 \cdot 10^{-3}$ M; $[\text{arsenate}]_0 = 5.15 \cdot 10^{-2}$ M; [enzyme] = $5.5 \cdot 10^{-9}$ M; $[NH_4^+] = 1.6 \cdot 10^{-3}$ M; $[K^+] = 5.15 \cdot 10^{-2}$ M; ionic strength = 0.24 M

pH	5.02	5.62	6.42	7.27	8.03	8.51	8.85	9.36	9.68
$v_0 \times 10^5$	3.6	6.3	13.0	22.6	22.7	19.3	14.4*	10.5*	6.2*

* Corrected for spontaneous hydrolysis.

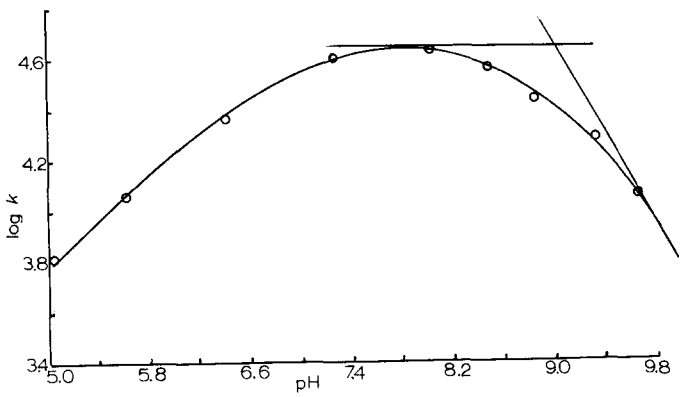


Fig. 7. Effect of pH on k.

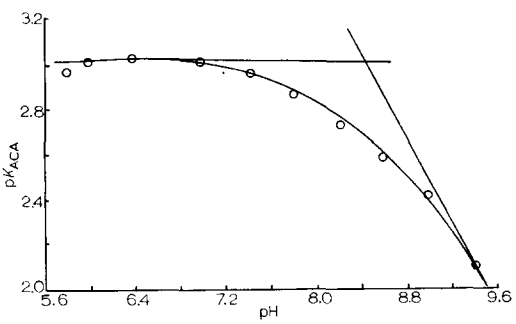


Fig. 8. Effect of pH on K_{ACA}.

brium (1), a process not yet studied, is likely to have a mechanism analogous to equations (8)–(12). In all these related processes the two substrates appear to be adsorbed on independent sites ($K_{ACA} = K'_{ACA}$, *etc.*).

Effects of pH on the arsenate reaction

Interpretation of Figs. 6–8, using Dixon's[15] rules, leads to the following conclusions:

(i) Arsenate binding. A group situated either in the free enzyme or free arsenate, with $pK_a \simeq 6.7$, influences the binding. When this group is protonated binding is greatly reduced (and possibly absent). Since the pK_a for the species $H_2AsO_4^-$ is probably[13] close to 6.7, our results strongly suggest that only the dianion is adsorbed.

$$H_2AsO_4^- + H_2O \rightleftharpoons HAsO_4^{2-} + H_3O^+ \qquad (25)$$

An analogous situation will probably obtain for the corresponding phosphate reaction. It follows too that, for reactions in the other direction, the enzyme only adsorbs the acyl phosphate and acyl arsenate dianions.

The group, $pK_a \simeq 6.4$ in the free enzyme and $pK_a \simeq 6.9$ in the enzyme–substrate complex*, which affects[6] acetyl phosphate binding, is not detected in arsenate binding and therefore presumably influences mainly the acyl portion of acetyl phosphate. As for acetyl phosphate[6], the group(s) which actually bind(s) arsenate does not respond to pH changes in the region studied. It may be an ammonium or (possibly) a metal grouping. It seems, however, that it engages two negative charges.

(ii) The effect on k. Fig. 7 shows that at least two acid–base groups in the enzyme–substrate ternary complex affect its reactivity. One, $pK_a \simeq 9.0$, active when protonated, is probably that identified in the same context for the forward process of equilibrium (1). There $pK_a \simeq 8.7$. The function of this group in the surface reaction has been previously suggested[6]**. In reaction (1) this group is associated with another group, $pK_a \simeq 7.3$, which must be in its basic form. This latter group would sensibly be expected to appear also in the present reaction. A group of this type is, indeed, suggested by Fig. 7, but the slope of the rectilinear part of the plot differs appreciably from the expected unit slope[15]. The reason for this is unknown. Possibly a further group, which assists reaction at low pH, is implicated; perhaps the group $pK_a \simeq 6.9$ in the enzyme–substrate complex which affects acetyl phosphate (and acetyl arsenate?) binding.

(iii) Acetyl-CoA binding. Binding of acetyl-CoA is enhanced by the protonated form of a group $pK_a \simeq 8.5$ in either the free enzyme or acetyl-CoA. The same group affects CoA binding in reaction (1). It is probably an NH_3^+ group in the free enzyme[6]. No other groups responsive to pH between 5.6 and 9 appear to affect acetyl-CoA adsorption. This contrasts with the position for CoA which employs another protonated group, $pK_a \simeq 7.2$, which is, however, only detected in the enzyme–substrate

* The assignment of these pK_a values was accidentally reversed in ref. 6.

** Other functions are, of course, possible since transition states for the surface reaction other than that suggested in ref. 6 can be imagined[19].

ternary complex, a fact which suggested a conformational change on CoA adsorption[6]. If CoA and acetyl-CoA enjoy different surface conformations, this could be the reason that the group of $pK_a \simeq 7.2$ does not influence the latter species.

General conclusions

The kinetic form and the pH dependencies of the Michaelis parameters of the present reaction are compatible with its detailed mechanism being the arsenate analogue of the microscopic reverse of the acyl phosphate–CoA reaction previously studied.

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