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KINETIC STUDIES WITH ACETATE KINASE

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SUMMARY

1. Acetate kinase (EC 2.1.2.7) is relatively unstable at 25°.

2. A kinetic study, at 4°, of the acetate kinase catalysed transfer of phosphate between acetyl phosphate (acetyl-*P*) and ADP in the presence of Mg^{2+}, suggests that the complex ADP–Mg^{2+} is taken up by the active site, but that ADP and Mg^{2+} separately are not.

3. Acetyl-*P* also interacts with the active site and the overall reaction probably involves a synchronous transfer of phosphate between the two adsorbed species. At least two roles played by the Mg^{2+} are identified.

4. Under conditions of enzyme saturation the pH profile between pH 6.4 and 8.7 closely resembles the dissociation curve of a group with $pK_a \approx 6.25$. This result, which is contrary to previous findings, is given a simple interpretation.

5. The association constant between Mg^{2+} and ADP at 4° has been determined.

INTRODUCTION

Acetate kinase (EC 2.1.2.7) catalyses Reaction 1. Rather few studies of this process exist and virtually all are due to either Lipmann or Rose and their co-workers. These studies have been summarised by ROSE[1]. Slightly more recent work[2], using possibly the same

$$\text{ATP} + \text{acetate} \rightleftharpoons \text{ADP} + \text{acetyl-}P \qquad (1)$$

enzyme from a different source, is in general agreement with the earlier studies. The principle conclusions which have accumulated are: (i) the presence in the reaction mixture of Mg^{2+} (or a similar ion) is essential; (ii) the pH profile exhibits a sharp maximum at pH 7.4 (or perhaps at a somewhat lower pH); (iii) at pH $\simeq$ 7.4 the equilibrium position lies well towards ATP and acetate; (iv) of several carboxylate ions tested only propionate appears able to replace acetate, and is phosphorylated about 10-fold more slowly; (v) ITP and IDP can replace ATP and ADP, respectively, and are about equally efficient; (vi) successful inhibitors indicate that sulphydryl groups are important for the catalytic activity; (vii) experiments with isotopes suggest that enzyme–phosphate intermediates are not formed and that a synchronous transfer of phosphate between the substrates is involved.

ATP and ADP are known to form complexes with Mg^{2+} and these complexes have been implicated in several related enzymatic phosphorylations[4–10]. No analysis of the acetate kinase system in these terms exists and we here report a kinetic study of this aspect of the reaction. Some of our findings are not in agreement with previous work.

MATERIALS AND METHODS

Chemicals

Acetate kinase was Boehringer's product, isolated from *Escherichia coli*, and supplied as a suspension in 2.4 M $(NH_4)_2SO_4$ at pH 6. The sodium salts of ADP and ATP, and the lithium, potassium salt of acetyl-*P* were also Boehringer products. $FeCl_3$, 8-hydroxyquinoline, and aqueous HCl were AnalaR grade reagents. The remaining chemicals were the purest available commerical samples. Tetraethylammonium chloride was recrystallised from ethanol and dried under vacuum.

Association between Mg^{2+} and ADP and acetyl-P

Values of K_{ADP}, the equilibrium constant of Reaction 2, referring to a variety of conditions are available[3]. None, however, refer to pH 8.15,

$$[ADP\text{–}Mg]^- \rightleftharpoons ADP^{3-} + Mg^{2+} \qquad (2)$$

4°, and $I = 1.0$, the conditions obtaining in our main kinetic studies. We have therefore determined the dissociation constant K_{ADP} under these conditions. The value of $K_{acetyl\text{-}P}$ for Reaction 3 under the same conditions, is known from our previous

TABLE I

RATE AND DISSOCIATION CONSTANTS

$I = 1.0$; pH 8.15 (Tris buffer); temp., 4.0 ± 0.1°.

Dissociation constants for the complexes ADP–Mg^{2+} and acetyl-P–Mg^{2+}	
K_{PDP}	$= [ADP^{3-}]\,[Mg^{2+}]/[ADP\text{–}Mg^-] = 3.0 \pm 0.3$ mM
$K_{acetyl\text{-}P}$	$= [acetyl\text{-}P^{2-}]\,[Mg^{2+}]/[acetyl\text{-}P\text{–}Mg] = 33.0 \pm 3.0$ mM
Derived constants for Eqn. 7	
K_2	$= 5.0 \pm 0.5$ mM
k	$= 0.125 \pm 0.01$ mM·min^{-1}·(μg·ml^{-1})$^{-1}$

work[11]. Our results are in Table I. We used the spectrophotometric method of BURTON[12], which involves a competition between 8-hydroxy-

$$Acetyl\text{-}P\text{–}Mg \rightleftharpoons Acetyl\text{-}P^{2-} + Mg^{2+} \qquad (3)$$

quinoline and the phosphate derivative for the metal ion. At pH ≃ 8.15 ADP and acetyl-*P* can be taken to be fully ionised, as indicated in Reactions 2 and 3. The measurements were made using a Unicam SP 500 spectrophotometer fitted with a thermo-regulated cell holder suitable for cells (quartz, ground-glass stoppered) of 1-cm path. At 4° condensation on the cell windows can be prevented by passage over them of a current of dry air.

Kinetic experiments

The progress of the reaction was followed by measuring the changes in acetyl-*P* concentration. Acetyl-*P* was determined by the method of LIPMANN AND TUTTLE[14] as modified by CAPONY AND PECHÈRE[13]. For reasons explained below most of the runs were conducted at pH 8.15 and 4°. The ionic strength was always maintained at 1.0 by appropriate additions of tetraethylammonium chloride. The general procedure was as follows. Fresh 'stock' solutions of ADP and acetyl-*P* (or of ATP and sodium acetate) in Tris buffer at pH 8.15 were made up for each day's work. Suitable volumes of these solutions, and of stock solutions of $MgCl_2$ and of tetraethylammonium chloride in buffer, were added to a volumetric flask (5 ml) and the flask made up to the mark with more buffer solution. Without delay the flask and contents were cooled to 4° and reaction started by addition of a small (5–10 μl) sample of the enzyme suspension, using a Hamilton μl syringe. The enzyme was kept at 4° throughout. Samples (1 ml, 2–4 samples per run) were taken at 3-min intervals, each sample being added to 0.1 ml of 1 mM *p*-chloromercuribenzoate solution to stop the reaction[15]. The resulting solution was analysed for acetyl-*P*. Plots of [acetyl-*P*] against time covering the first 10% of the reaction were normally rectilinear and led to values of the initial velocity (v_0) expressed as mole·l^{-1}·min^{-1} of acetyl-*P* lost (or formed). At pH 8.15 Tris approaches the limit of its useful buffering region; it was shown by direct measurements on samples of the reaction mixtures that there was a negligible pH change during a run. All pH measurements were made, at 4°, with a Cambridge pH meter using a glass electrode and a calomel reference electrode. In determing the pH value at the start of a reaction it was essential to carry out the measurement on a separate sample of the reaction mixture, otherwise the subsequent reaction is inhibited owing to the brief contact of the solution with the electrodes. Tetraethylammonium chloride was chosen in preference to $NaClO_4$, or other salts, for use as the inert electrolyte since it shows little tendency to form complexes with ADP or ATP[16].

A few preliminary studies, at 25°, examined both the forward and the reverse directions of Reaction 1. Other experiments examined the dependence of v_0 on pH under fixed conditions. The main experiments, at pH 8.15 and 4°, involved the reverse direction of Reaction 1 and were organised along lines described by MORRISON *et al.*[9] in their studies of creatine phosphotransferase (see below). Experimentally this involves the determination of v_0 for a series of free Mg^{2+} concentrations at constant

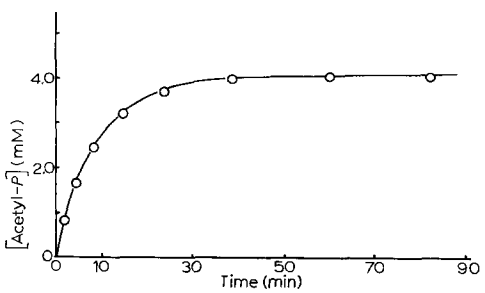


Fig. 1. Position of equilibrium of Reaction 1. Conditions: Temp., 4°; I = 1.0; pH 8.15; $[MgCl_2]$ = 12 mM; $[CH_3CO_2^-]$ = 0.7 M; $[ATP]_{total}$ = 10 mM; acetate kinase = 7.5 μg/ml. Equilibrium concentration of acetyl-*P* corresponds to $K = 5 \cdot 10^{-3}$.

$[ADP]_{free}$ and $[acetyl\text{-}P]_{free}$. This procedure is repeated at various values of $[ADP]_{free}$. The necessary total concentrations of Mg^{2+}, ADP, and acetyl-*P* to provide the desired concentrations of free species are calculated using the *K* values in Table I.

Position of equilibrium in Reaction 1

Experiments at 25° using ATP and sodium acetate in the presence of 12 mM. $MgCl_2$ led to results such as those in Fig. 1. The acetyl-*P* formed at infinite time (approx. 60 min) corresponded to an apparent value of $K = [ADP][acetyl\text{-}P]/[ATP][acetate] = 5 \cdot 10^{-3}$. This value is in reasonable agreement with that of ROSE[1,15]. In the kinetic experiments on the reverse direction at 4°, runs allowed to continue for the whole course of the reaction revealed that very little acetyl-*P* finally remained. Under these conditions also therefore the position of equilibrium lies well towards ATP and acetate. The forward reaction can be safely neglected in studying the initial stages of the reverse reaction.

Concomitant hydrolysis of acetyl-P

The known[11] velocity of hydrolysis of acetyl-*P*, in the presence of Mg^{2+}, at different pH values shows that our various experiments with acetate kinase were negligibly affected by this concomitant hydrolysis.

RESULTS

Previous kinetic work[2,15] with acetate kinase has been conducted at 25–30°. Our preliminary experiments at 25° showed that acetate kinase as supplied in 2.4 M $(NH_4)_2SO_4$ is relatively unstable at this temperature, and that even if the enzyme is stored at 4°, and only briefly raised to 25° once or twice a day, it is not possible to obtain reproducible results on successive days. We decided therefore to avoid any

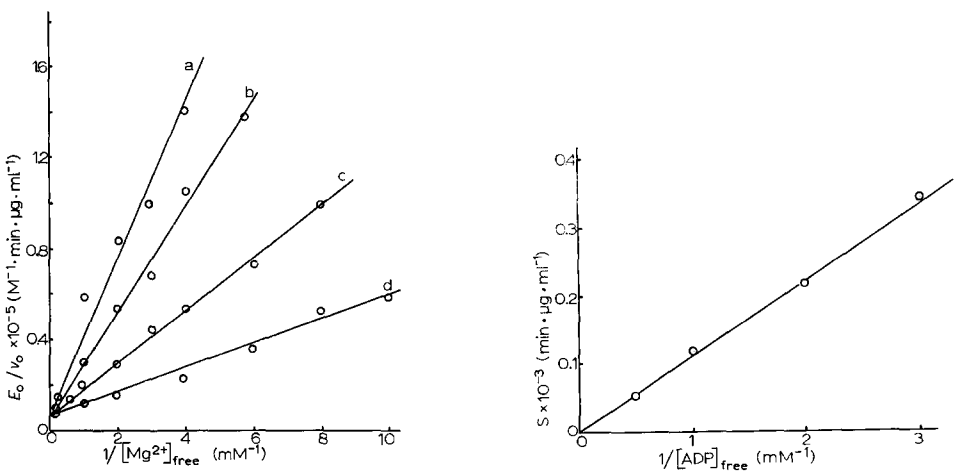


Fig. 2. Plots of Eqn. 5. Conditions: pH 8.15; [acetyl-*P*] = 3.0 mM; I = 1.0; E_0 = 0.5–1.5 μg/ml; for (a) [ADP] = 0.33 mM; for (b) [ADP] = 0.5 mM; for (c) [ADP] = 1.0 mM; for (d) [ADP] = 2.0 mM. Each line has a different slope (S), but the same intercept (P).

Fig. 3. Plot of slopes (S) obtained from Fig. 2 against 1/[ADP]. The slope and intercept of this plot lead to values of K_1K_2/k and K_6/k.

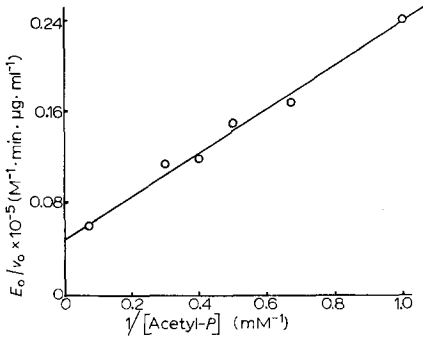


Fig. 4. Lineweaver–Burk plot for acetyl-*P*. Conditions: pH 8.15; I = 1.0; $[ADP]_{total}$ = 6.5 mM; $[Mg^{2+}]_{total}$ = 2.5 mM; E_0 = 0.5–1.0 µg/ml.

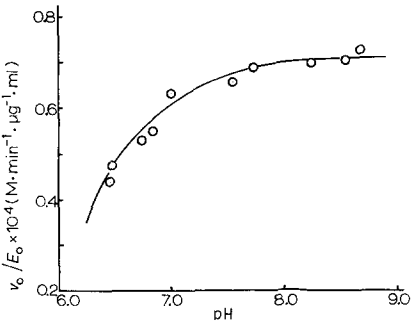


Fig. 5. pH dependance at 4°. Conditions: I = 1.0; $[Mg^{2+}]_{total}$ = 67.5 mM; $[ADP]_{total}$ = 67.5 mM; $[acetyl\text{-}P]_{total}$ = 0.04 M. Continuous line represents the calculated dependance of v_0/E_0 on pH, it being assumed that v_0 is controlled by the state of ionisation of a group of $pK_a \simeq 6.25$.

increase in temperature for the enzyme and both to store it, and to conduct the main kinetic experiments, at 4°. The results of these experiments are given in Figs. 2–6. Previous studies have indicated that for the reverse reaction (with which we have been mostly concerned) all the non-enzymatic reactants (ADP, acetyl-*P*, Mg^{2+}) lead to saturation (Michaelis–Menten) curves. This suggests that each may be absorbed in some way by the enzyme. Similar results have been obtained for related enzymatic phosphorylations[9]. If, at constant [acetyl-*P*], we assume that the rate of reaction is proportional to the concentration of the ternary species E–ADP–Mg^{2+} (where E = enzyme), the overall scheme can be written as

$$\begin{array}{ccccccccc} & & & & E + \mathrm{ADP} & & & & \\ & & & & \Updownarrow K_5 & & & & \\ & & & & E\text{–ADP} & & & & \\ & & & & \Updownarrow K_6 & & & & \\ \mathrm{ADP} + \mathrm{Mg^{2+}} & \overset{K_1}{\rightleftharpoons} & \mathrm{ADP\text{–}Mg^{2+}} & \overset{K_2}{\rightleftharpoons} & E\text{–ADP–}\mathrm{Mg^{2+}} & \overset{K_4}{\rightleftharpoons} & E\text{–}\mathrm{Mg^{2+}} & \overset{K_3}{\rightleftharpoons} & E + \mathrm{Mg^{2+}} \\ & & & & \downarrow k & & & & \\ & & & & \text{products} & & & & \end{array} \qquad (4)$$

If it is further assumed that (i) the equilibria governed by the dissociation constants K_1 to K_6 are rapidly set up; (ii) the same ternary species arises from each path, (iii) only the ternary species reacts with acetyl-*P* to give products, (iv) there is no competitive inhibition between species, and (v) the concentrations of enzyme-containing species are small compared with $[Mg^{2+}]$, [ADP], and $[ADP\text{–}Mg^{2+}]$, then it can be shown[9] that Eqn. 5 applies. In Eqn. 5, E_0 = total enzyme concentration and the other constants are defined by Scheme 4. For such a system, when [ADP] = constant,

$$\frac{E_0}{v_0} = \left\{\left(1 + \frac{K_4}{[ADP]}\right) + \frac{1}{[Mg^{2+}]}\left(K_6 + \frac{K_1K_2}{[ADP]}\right)\right\}/k \qquad (5)$$

a plot of E_0/v_0 against $1/[Mg^{2+}]$ should be rectilinear with a slope $S = (K_6 + K_1K_2/[ADP])/k$ and an intercept $P = (1 + K_4/[ADP])/k$. Determination of S and P at various fixed values of [ADP] enables plots to be made of S, and of P, against 1/[ADP].

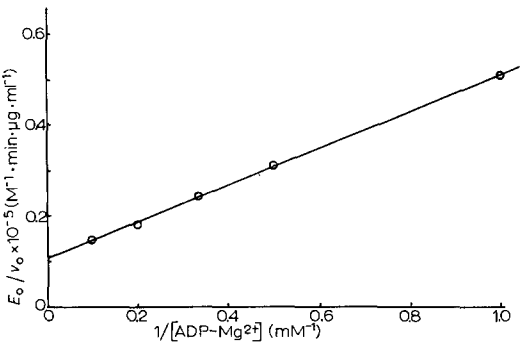


Fig. 6. Plot of Eqn. 8. Conditions: pH 8.15, I = 1.0; [Mg^{2+}] = 0.10 M; [acetyl-P]$_{total}$ = 3 mM; [ADP]$_{total}$ = 1–10 mM. Intercept = $1/k$ and slope = K_2/k.

These two plots lead to values of K_1K_2/k, K_6/k, K_4/k, and $1/k$. Since $K_1K_2 = K_3K_4 = K_5K_6$, and since $K_1 = K_{ADP}$, which is independently known (Table I), all the remaining constants can be evaluated.

On constructing the various plots (Figs. 2,3) for the present system it is found that $K_6/k \simeq 0$, $K_4/k \simeq 0$, $K_1K_2/k = 0.12$, and $1/k = 8 \cdot 10^3$. Since k is finite and $K_6/k \simeq 0$, therefore $K_6 \simeq 0$. Since K_5K_6 is also finite, $K_5 \simeq \infty$. It follows that a negligible amount of E–ADP is formed, either from free E and ADP, or by dissociation of E–ADP–Mg^{2+}. The path controlled by K_5 and K_6 can therefore be eliminated. A similar argument concerning K_4 and K_3 shows this route must also be relatively unimportant. It follows therefore that the simple Scheme 6 is probably the essential contributor to this enzymatic phosphorylation, the observed kinetics being well represented by Eqn. 7. Our values of K_2, and k are in Table I.

$$Mg^{2+} + ADP \overset{K_1}{\rightleftharpoons} ADP\text{–}Mg^{2+} \overset{K_2}{\rightleftharpoons} E\text{–}ADP\text{–}Mg^{2+} \xrightarrow[[\text{Acetyl-}P]]{k} \text{products} \tag{6}$$

$$\frac{E_0}{v_0} = \left(1 + \frac{K_1K_2}{[Mg^{2+}]\,[ADP]}\right)/k \tag{7}$$

Experiments illustrated in Fig. 4 show that, at constant [ADP] and [Mg^{2+}], variation of [acetyl-P] leads to a saturation curve, with an apparent Michaelis constant for acetyl-P, K_m = 4 mM. This suggests that acetyl-P is adsorbed, as well as ADP–Mg^{2+}. Since the value of $K_{\text{acetyl-}P}$ is relatively large, at the concentrations we have used, [acetyl-P]$_{free} \simeq$ [acetyl-P–Mg^{2+}] × constant. An alternative view therefore is that acetyl-P–Mg^{2+}, rather than acetyl-P, is adsorbed. It is not possible to distinguish between these possibilities on the basis of our results.

Experiments to determine the pH dependance of the reaction, under conditions of enzyme saturation, are shown in Fig. 5. Suitable concentrations appended to Fig. 5, are, for Mg^{2+} and ADP, deduced from Eqn. 8, which is equivalent to Eqn. 7. When [ADP–Mg^{2+}] $\simeq$ 10 K_2, then $v_0/E_0 \simeq k$.

$$\frac{v_0}{E_0} = k/\left(1 + \frac{K_2}{[ADP\text{–}Mg^{2+}]}\right) \tag{8}$$

Under these saturating conditions the observed pH dependance closely parallels an

acid–base ionisation curve for a group of $pK_a \simeq 6.25$. There is no maximum between pH 6.4 and 8.7. For these runs reaction mixtures (without enzyme) were made up in the usual way, except that for each the pH was roughly adjusted by suitable addition of a small volume of 1.0 M HCl. The resulting mixture was divided into three portions, two for use as duplicate kinetic runs, the third for determination of the exact initial pH. Normally these runs were allowed to proceed to about 30% completion and gave excellent rectilinear plots of [acetyl-*P*] against time. This extended zero-order behaviour suggests that saturating conditions were achieved. As in the main runs at pH 8.15 the final pH after a run was complete was always within $\pm$ 0.1 unit of the initial value. Since $[\text{acetyl-}P]_{\text{initial}}$ was larger in these experiments, smaller samples (0.1 ml) were taken, and these added to an appropriate hydroxylamine–buffer mixture, remembering[13] that for the estimation of acetyl-*P* according to LIPMANN AND TUTTLE[14] the pH should be about 7.1.

It was because the velocity is roughly independent of pH between pH 8.0 and 8.6 that a value pH $>$ 8 was most suitable for the main experiments.

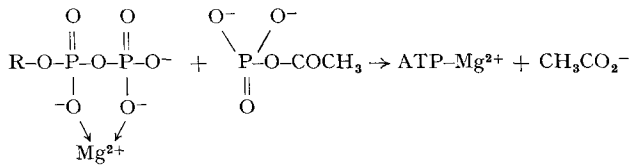
Work with other kinases has sometimes[8–10] indicated that the presence of large concentrations of one, or more, of the reactants has an inhibitory effect. Our conclusions about the dissociation constants K_3 and K_5 imply, however, that for acetate kinase neither Mg^{2+}, nor ADP, should act as a competitive inhibitor. We have been able to show that a relatively large concentration of Mg^{2+} has only a minor disturbing effect on the kinetics by examining a plot of Eqn. 8 at pH 8.15 under conditions of a large, effectively constant concentration of free Mg^{2+} and of a low, varying total concentration of ADP, the ADP existing effectively completely as the ADP–Mg^{2+} complex. The plot (Fig. 6) of E_0/v_0 against $1/[\text{ADP–Mg}^{2+}]$ leads to the values $K_2 = 3.7$ mM and $k = 1 \cdot 10^{-4}\ \text{M} \cdot \text{min}^{-1} \cdot (\mu\text{g} \cdot \text{ml}^{-1})^{-1}$. These values are quite similar to those in Table I, which were obtained using the same total acetyl-*P* concentration, but much smaller Mg^{2+} concentrations (Fig. 2). The similarity between the values suggests that comparitively very large concentrations of Mg^{2+} do not materially affect the kinetics so as to inhibit the reaction.

DISCUSSION

Our work leads to three principal conclusions concerning the mechanism of the acetate kinase reaction at 4°: (i) the true substrate for the reverse reaction is the ADP–Mg^{2+} complex; (ii) the ternary complex formed between this species and the enzyme either dissociates to the same species or, on interaction with acetyl-*P* (or acetyl-*P*–Mg^{2+}) also bound to the enzyme at a suitable adjacent site, leads to the products ATP and acetate; (iii) under conditions of enzyme saturation the pH profile closely resembles a dissociation curve for a group of $pK_a \simeq 6.25$.

A survey of the literature concerning other phosphate kinases suggests that the reaction scheme we have found for acetate kinase may eventually prove to be common to the majority of this group of enzymes. It is clear that they all possess a site especially designed to accommodate ADP–Mg^{2+}. Since acetyl-*P* (or acetyl-*P*–Mg^{2+}) is adsorbed, but does not then rapidly lead to acetate and *E*-phosphate[1] (because otherwise the enzyme would catalyse the hydrolysis of acetyl-*P* in the absence of ADP), we may assume that acetyl-*P* is adsorbed at a site suitably adjacent to ADP–Mg^{2+}, and that these two adsorbed species interact in a synchronous displace-

ment reaction, *e.g.* Reaction 9 in which R represents the organic residue of ADP. This view is in keeping with that



(9)

of ROSE[1]. We have written Reaction 9 in terms of acetyl-*P* rather than of acetyl-*P*–Mg^{2+}. There are two reasons for this: (i) since P–O bond-breaking is involved for acetyl-*P*, were Mg^{2+} coordinated in the phosphate oxygen atoms, although this would facilitate attack by the $-O^-$ group of ADP, it would hinder the P–O cleavage. (ii) More importantly, if acetyl-*P*–Mg^{2+} is involved, microscopic reversibility will require that $ATP(Mg^{2+})_2$ is involved in the forward process. There is no evidence for this[1].

Various structures have been suggested for the free ADP–Mg^{2+} complex in solution[3]. Probably more than one exists. We assume that the dominant complex is that shown in Eqn. 9. (Most of the evidence points in this direction.) The Mg^{2+} have at least two roles to play in the acetate kinase reaction. First, by reducing the triple charge on the ADP molecule to a single charge they facilitate the approach of ADP to the acetyl-*P* dianion. (The same sort of effect operates in the metal ion-catalysed hydrolysis of acyl phosphates by OH^- (ref. 11).) Secondly, since free ADP does not interact with the enzyme, the Mg^{2+} must facilitate the adsorption. Whether this again is due to the reduction of the negative charge, or owing to specific adsorption of the magnesium atom (as well as of other parts of the molecule), or to both these factors, is uncertain. Since IDP can replace ADP, the amino group of the latter cannot be essential to the adsorption. The heterocyclic residues of ADP and of IDP are, however, very similar; perhaps the thiol group of the enzyme engages a basic centre in these residues.

Our finding that, under saturating conditions, the pH profile between pH 6.4 and 8.7 exhibits no maximum, but corresponds closely to the dissociation curve for a group with $pK_a \simeq 6.25$, is contrary to the conclusions of ROSE[15] and of SAGERS *et al.*[2]. A wide variety of pH profiles have been observed in related enzymatic phosphorylations[4–9,17]. A result similar to ours was obtained by NODA *et al.*[8] (for creatine kinase) in one of the more careful previous investigations of a kinase. Moreover, earlier work with creatine kinase had suggested a pH maximum in this case also. It is clear that care is needed in determining pH profiles. (We find that acetate kinase is very susceptible to any slight contact with calomel reference electrodes.) Although complicated phenomena can underlie pH profiles which apparently reflect a simple ionisation, there is every reason to assume a simple explanation until this proves to be inadequate. Our results suggest that the enzyme becomes less efficient when a group of $pK_a \simeq 6.25$ becomes protonated. This group could well be the essential $-O^-$ group in the adsorbed $[ADP–Mg^{2+}]^-$ complex (Eqn. 9)[18].

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