Regulatory Properties of Acetokinase from *Veillonella alcalescens*

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Acetokinase from *Veillonella alcalescens* catalyzes the virtually irreversible synthesis of adenosine triphosphate from acetyl phosphate and adenosine diphosphate. Kinetic analysis revealed that the enzyme was activated by acetyl phosphate and inhibited by adenosine triphosphate. Velocity curves obtained with increasing amounts of adenosine diphosphate were of the Michaelis-Menten type (rectangular hyperbolas) under all conditions employed. However, velocity curves generated by varying the level of acetyl phosphate were sigmoidal, suggesting homotropic interactions for this substrate. Alteration of the pH of the reaction mixture from 7.4 to 10 or addition of a substrate analogue, propionyl phosphate, resulted in the loss of cooperativity and changed the values of the Hill coefficient from 3 to 1. Data obtained in experiments with propionyl phosphate suggested the existence of separate effector sites on the enzyme. The inhibition curves for adenosine triphosphate were also sigmoidal with Hill coefficients ranging between 1 and 3, depending on the concentration of acetyl phosphate. Sedimentation studies with the partially purified enzyme indicated a polymeric structure with a maximum molecular weight of about 90,000 daltons; a subunit of approximately 29,000 daltons was also detected.

Much of the adenosine triphosphate (ATP) required for the growth of the obligate anaerobe *Veillonella alcalescens* in lactate-containing media comes from the degradation of pyruvate. Lactate is oxidized to pyruvate by lactate-malate transhydrogenase (2, 7) and ATP is generated from pyruvate via the following sequence of reactions:

\[
\text{Pyrurate} \rightarrow \text{coenzyme A} \rightarrow \text{acetyl coenzyme A} + \text{CO}_2 + \text{H}_2 \quad (1)
\]

\[
\text{Acetyl coenzyme A} + \text{PO}_4 = \text{acetyl phosphate} + \text{coenzyme A} \quad (2)
\]

\[
\text{Acetyl phosphate} + \text{adenosine diphosphate} \rightarrow \text{acetate} + \text{ATP} \quad (3)
\]

The oxidative decarboxylation of pyruvate (reaction 1) requires coenzyme A (CoA) and ferredoxin or another electron acceptor which can be linked to hydrogenase (10, 18). The above three-step pathway for the production of ATP from pyruvate is characteristic of a number of anaerobic organisms including clostridia (4).

Acetokinase (ATP: acetylphosphate transferase, EC 2.7.4.3), the enzyme catalyzing reaction 3, has been found in a variety of microorganisms including enteric bacteria, clostridia, lactic acid bacteria (16), propionibacteria, yeast (11), and blue-green algae (15). In most organisms, reaction 3 is freely reversible and could, therefore, function not only to generate ATP but, when coupled with phosphotransacetylase (reaction 2), could provide acetyl CoA for biosynthetic reactions. The properties of this enzyme might then be expected to reflect the role that it plays in metabolism, i.e., catabolic or amphibolic. The present investigation has disclosed that, in *V. alcalescens*, acetokinase is active only in the direction of ATP synthesis. Furthermore, the enzyme is activated by acetyl phosphate and inhibited by ATP. These observations suggest that regulation of the synthesis of ATP from acetyl phosphate represents an important control point in the energy metabolism of *V. alcalescens*.

MATERIALS AND METHODS

Growth of cultures and preparation of cell extracts. Strain 221 of *V. alcalescens* (formerly called *Micrococcus lactilyticus*) was grown in a medium containing 2% lactate, 2% peptone, 1% yeast extract, 0.5% NaCl, and 0.25% dibasic potassium phosphate at pH 7.0 to 7.2. Large quantities of cells were obtained from 14-liter cultures grown in 16-liter carboys or from 100-liter cultures grown in a 130-liter New Brunswick fermentor inoculated with 1 and 5 liters of exponential-phase cultures, respectively. After 12 to 16 hr of incubation, when the cultures had reached stationary phase, the cells were harvested by centrifugation at 4°C, washed with buffer A [0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 8.3, containing 0.01 M MgCl₂], packed by centrifuga-
tion at 25,000 \times g for 30 min in a Sorvall RC-2 refrigerated centrifuge, and stored at −20 C. All subsequent steps were performed at 0 to 4 C. Cell-free extracts were prepared by suspending the packed cell mass in buffer A to a density of approximately 0.5 g (wet weight) per ml, subjecting it to sonic treatment in a Raytheon 10-khertz sonic oscillator at maximum power for 30 min, and centrifuging to remove cell debris. Extracts prepared in this manner were stable indefinitely when stored at −20 C.

Enzyme fractionation. A saturated solution of ammonium sulfate, adjusted to pH 8.3, was slowly added to a prepared by sonication with a 50-ml (Calbiochem), 10 to 50 \( \mu \) moles of ATP, 20 \( \mu \) moles of CoA (Pabst Co.), 10 to 50 \( \mu \) moles of ATP, 20 \( \mu \) moles of sodium fluoride, 20 \( \mu \) moles of MgCl\(_2\), and 100 \( \mu \) moles of Tris buffer, pH 7.5. Reactions were incubated for 10 to 60 min at 37 C. A 500-\( \mu \)mole amount of neutralized hydroxylamine was added either initially or after the reaction was terminated by addition of trichloroacetic acid (10% final concentration). Acyl derivatives were detected as the hydroxamates (17).

Synthesis of phosphate donors. Acetyl phosphate and propionyl phosphate were synthesized from the corresponding anhydrides by the method of Stadtmann (18).

RESULTS

Purification of acetylkinase. The enzyme was purified approximately 100-fold by the steps shown in Table 1. The recovery was low (5 to 10%) and could not be increased by the addition of substrates or products of the reaction to various fractions obtained in the course of the fractionation. Hydroxylapatite or carboxymethyl cellulose could be used for column chromatography but were less effective than diethylaminoethyl cellulose. The enzyme was eluted as a single symmetrical peak in the latter step with a recovery of approximately 32% of the activity adsorbed to the column. These fractions were free of phosphotransacetylase, adenylyl kinase, and acyl phosphate phosphatase activity. Gel electrophoresis of material from the tubes having the highest specific activity and estimation of the intensity of staining of various protein bands indicated that fractions from step 6 were approximately 30% pure with respect to ace-}


cose given above. The rate of NADP reduction was followed spectrophotometrically at 340 nm with a Beckman DU spectrophotometer equipped with a Gilford cuvette positioner and recorder. The rate of this reduction was linear for at least 5 min under most of the conditions used in these studies.

Specific activity was expressed as micromoles per minute per milligram of protein of acetyl phosphate degraded or ATP formed. Protein was measured by the method of Lowry et al. (12).

The formation of ATP from ADP via adenylate kinase was assayed by a coupled reaction with hexokinase and glucose-6-phosphate dehydrogenase as described above for method ii, except that acetyl phosphate was omitted from the reaction mixture. Hydrolysis of acetyl phosphate due to phosphatase action or nonspecific degradation of acetyl phosphate was measured colorimetrically by the disappearance of acetyl phosphate (determined as the hydroxamate) in reaction mixtures lacking ADP. Phosphotransacetylase was prepared from \( V.\) alcaligenes \( (16)\) and assayed either by a coupled enzyme system using malic dehydrogenase, citrate synthetase, nicotinamide adenine dinucleotide, and malate (16), or by arsenolysis of acetyl phosphate (17). Acethio kinase was determined by incubating 0.2 to 0.5 ml of crude extract (20 to 25 mg of protein per ml) or ammonium sulfate fractions from the fractionation procedure for acetyl kinase with 10 \( \mu \) moles of acetate, 2 \( \mu \) moles of CoA (Pabst Co.), 10 to 50 \( \mu \) moles of ATP, 20 \( \mu \) moles of sodium fluoride, 20 \( \mu \) moles of MgCl\(_2\), and 100 \( \mu \) moles of Tris buffer, pH 7.5. Reactions were incubated for 10 to 60 min at 37 C. A 500-\( \mu \)mole amount of neutralized hydroxylamine was added either initially or after the reaction was terminated by addition of tri-chloroacetic acid (10% final concentration). Acyl derivatives were detected as the hydroxamates (17).

When pooled and concentrated column fractions...
TABLE 1. Purification of acetokinase

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg/ml)</th>
<th>Specific activity*</th>
<th>Purification*</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>35.6</td>
<td>1.40</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>2. 40-45% Ammonium sulfate</td>
<td>21.6</td>
<td>0.83</td>
<td>0.380</td>
<td>7</td>
</tr>
<tr>
<td>3. 45-50% Ammonium sulfate</td>
<td>17.4</td>
<td>6.00</td>
<td>4.30</td>
<td>33</td>
</tr>
<tr>
<td>4. 50-55% Ammonium sulfate</td>
<td>15.6</td>
<td>5.10</td>
<td>3.60</td>
<td>52</td>
</tr>
<tr>
<td>5. 55-65% Ammonium sulfate</td>
<td>19.0</td>
<td>1.96</td>
<td>1.40</td>
<td>19</td>
</tr>
<tr>
<td>6. Heat at 60°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step 4</td>
<td>5.3</td>
<td>18.6</td>
<td>13.2</td>
<td>32</td>
</tr>
<tr>
<td>Step 5</td>
<td>4.3</td>
<td>19.0</td>
<td>13.5</td>
<td>52</td>
</tr>
<tr>
<td>7. DEAE* gradient elution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(combined step 6)</td>
<td>0.450</td>
<td>79.0</td>
<td>56</td>
<td>32</td>
</tr>
<tr>
<td>8. Sucrose gradient 1</td>
<td>0.430</td>
<td>180</td>
<td>125</td>
<td>9</td>
</tr>
</tbody>
</table>

* Specific activity is expressed in micromoles of ATP formed per minute per milligram of protein.
* Purification is defined as the ratio of the specific activity for a given fraction relative to the specific activity of the crude extract.

(step 7, Table 1) were sedimented in a sucrose gradient, two regions showed acetokinase activity. The region having maximal activity had a relative sedimentation constant of 5.8, corresponding to an estimated molecular weight of 87,000 daltons (14); the region of lower activity had a relative value of 2.5, corresponding to an estimated molecular weight of 29,000 daltons (Fig. 1). When the material from the tubes having maximal activity (5.8S region) was pooled, concentrated, and re-sedimented, the same sedimentation pattern was again obtained. These results suggest that acetokinase from *V. alcalescens* may dissociate during sedimentation. Addition of acetylphosphate, ADP, ATP, MgCl₂, or acetate, or combinations of these compounds, to the gradients did not prevent the appearance of activity in the lower-molecular-weight regions of the gradient. The specific activities of the two regions were significantly different, with substantially lower values for the smaller molecular forms of the enzyme.

Gel electrophoresis of fractions from the sucrose gradient showed two major protein bands, both having enzymatic activity. The faster moving band (R₀ 0.3 to 0.4) contained more than 60% of the protein and had slight enzymatic activity.

Stability. The enzyme was sensitive to exposure to acid or neutral pH. For this reason, all steps beyond step 1 (Table 1) were performed at pH 8.3. The enzyme also lost a significant amount of activity when filtered through columns of Sephadex G-25 or G-75. Filtration of the crude extract resulted in the loss of over 80% of the total activity; total loss of activity was observed when more highly purified fractions (steps 6 or 7 of Table 1) were filtered. No enzymatic activity could be recovered after preparative acrylamide-gel electrophoresis of either crude extracts or partially purified fractions. However, approximately 10% of the initial starting activity could be detected in eluates from slices of gels after analytical acrylamide-gel electrophoresis. The enzyme was stable when stored in buffer A for 1 month at 4°C or for 3 months at -80°C.

Reaction requirements. Propionyl phosphate served as a phosphate donor for acetokinase, although the rate of reaction with this compound was about one-fourth the rate with acetyl phosphate (Fig. 2). This is in marked contrast to the acetokinase of *Propionibacterium shermanii* (1), which was as active with propionyl phosphate as with acetyl phosphate. Carbamyl phosphate, which can serve as a substrate for the acetokinase of *Escherichia coli* (10), is not utilized by the acetokinase of *V. alcalescens*. Other acyl phosphates were not tested with the *V. alcalescens* enzyme. The effect of propionyl phosphate on the reaction velocity with respect to acetyl phosphate will be discussed in a later section.

All dinucleotides tested were capable of serving as phosphate acceptors, with purine nucleoside diphosphates serving more effectively than pyrimidine nucleoside diphosphates (Table 2). The reaction required a divalent cation; this requirement could be satisfied by Mg²⁺ or Co²⁺ and less effectively by Ca²⁺. The reaction has a broad pH optimum between 7.5 and 9.0.

![Figure 1](http://jb.asm.org) Sedimentation of acetokinase in a sucrose gradient. A 2.5-mg amount of protein from a fraction with a specific activity of 100 (step 7, Table 1) was sedimented through a 13-ml sucrose gradient.
and various fractions of \textit{V. alcalescens} showed that this enzyme was either absent or present in extremely small amounts. Thus, acetate could not be activated either by ATP or by ATP and CoA, although transfer of CoA from succinyl CoA or propionyl CoA can occur (19).

**Inhibitors.** As expected, activity was inhibited by ethylenediaminetetraacetate (EDTA), and this inhibition could be reversed by MgCl$_2$. At 5 mM MgCl$_2$, 50% inhibition of acetokinase was obtained with approximately 7 mM EDTA (Fig. 3). A concentration of 25 mM MgCl$_2$ reversed this inhibition but did not reverse the inhibitory effects of nucleoside triphosphate, which also inhibited the enzyme. Acetokinase was also inhibited by \textit{p}-hydroxymercurobenzoate and \textit{N}-ethylmaleimide, with 50% inhibition occurring at 10$^{-5}$ and 5 $\times$ 10$^{-3}$ M, respectively.

Figure 3 also demonstrates that the reaction was inhibited by ATP. A 50% inhibition was seen at 3 mM concentration of this nucleotide. ATP could be replaced by guanosine triphosphate, inosine triphosphate, and cytidine triphosphate. The inhibition by ATP and other nucleoside di- and triphosphates was dependent on the presence of Mg$^{2+}$ (added at four times the concentration of the nucleotide), indicating that the actual inhibitor was the nucleotide-Mg$^{2+}$ complex. Increasing concentrations of MgCl$_2$ did not inhibit the reaction until a relatively high concentration was reached (0.1 M). The concentration of ADP required for inhibition was approximately 20 times greater than the \textit{S}_0.5 value, and thus was not a factor in the usual assays for acetokinase. Adenyl

**Reversibility of the reaction and acetothiokinase assays.** Acetokinase from \textit{V. alcalescens} showed little or no activity in the direction of acetyl phosphate synthesis from acetate and ATP. The reaction could not be "pulled" in this direction by trapping acetyl phosphate (by reaction with hydroxylamine) or by coupling with phosphotrans-acetylase. In this respect, the enzyme resembled the acetokinase of yeast (11). Acetokinases from \textit{E. coli}, \textit{Streptococcus hemolyticus} (16), and \textit{P. shermanii} (1), on the other hand, have been reported to be freely reversible.

Assays for acetothiokinase with crude extracts

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**TABLE 2. Effect of various nucleoside phosphates as acceptors in the acetokinase reaction**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Acetyl phosphate utilized ((\mu)moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine diphosphate</td>
<td>6.50</td>
</tr>
<tr>
<td>Inosine diphosphate</td>
<td>7.60</td>
</tr>
<tr>
<td>Guanosine diphosphate</td>
<td>4.50</td>
</tr>
<tr>
<td>Thymidine diphosphate</td>
<td>3.30</td>
</tr>
<tr>
<td>Adenosine monophosphate</td>
<td>0</td>
</tr>
<tr>
<td>No nucleotide</td>
<td>0</td>
</tr>
</tbody>
</table>

* Each sample contained 20 \(\mu\)g of protein from a fraction having a specific activity of 180 (step 8, Table 1), 20 \(\mu\)moles of acetyl phosphate, and 10 \(\mu\)moles of the nucleoside diphosphate in the standard reaction mixture. Residual acetyl phosphate was determined as the hydroxamate after 60 min of incubation.

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**FIG. 2. Initial velocity of acetokinase as a function of the substrate concentration.** The rates of reaction were determined by measuring ATP formation by coupling acetokinase with glucose, hexokinase, glucose-6-phosphate dehydrogenase, and NADP. The formation of reduced NADP was followed spectrophotometrically at 340 nm by using method ii as described in Materials and Methods. Each sample contained 0.45 \(\mu\)g of protein from a fraction with a specific activity of 180 (step 8, Table 1).

**FIG. 3. Effect of EDTA and ATP in the presence of MgCl$_2$ on the activity of acetokinase.** Each sample contained 7.5 \(\mu\)moles of ADP, 25 \(\mu\)g of protein from a fraction with a specific activity of 113 (step 7, Table 1), 14 \(\mu\)moles of acetyl phosphate, and the specified concentrations of EDTA and MgCl$_2$. The reaction was terminated after 3 min and assayed for the disappearance of acetyl phosphate.
acid had no effect on the enzyme at a concentration of $10^{-2}$ M. ATP produced by acetokinase as an end product could inhibit the reaction. To minimize this inhibition, in assays where the effect of ATP was not being measured, acetokinase was coupled to hexokinase or to hexokinase and glucose-6-phosphate dehydrogenase, and the reaction was measured during the initial 1 to 3 min. The effects of increasing concentrations of ADP and ATP on various kinetic parameters will be discussed.

**Inactivation of acetokinase by heat, dilution, or treatment with urea.** The rate of inactivation of acetokinase at 60°C was studied as a function of the concentrations of substrates and end products. The enzyme was most rapidly inactivated in the presence of ATP and MgCl₂ and less rapidly when ADP was substituted for ATP (Fig. 4A). In the presence of adenylic acid plus MgCl₂ inactivation of the enzyme proceeded at the same rate as in the control lacking nucleotides. ATP alone had less effect than the combination of ATP and MgCl₂. Figure 4B shows that addition of acetyl phosphate plus MgCl₂ did not affect the rate of inactivation, but that acetyl phosphate was able to partially reverse the effect of ATP-Mg²⁺. A concentration of 100 mM acetate (experiment not shown) had no effect.

Similar effects of acetyl phosphate and ATP-Mg²⁺ were observed with diluted preparations which had been inactivated by storage in the cold in the presence of nucleotides. However, this inactivation was partially reversed by subsequent incubation with the components of the assay mixture. Figure 5 shows that the activity of fractions exposed to ATP or ADP was low immediately after removal of ADP or ATP. After approximately 10 min of incubation with the complete reaction mixture, the enzyme slowly regained activity, although fractions previously stored with ATP or ADP did not attain an activity comparable to that of the control. On the other hand, the enzyme fraction which had been stored with a mixture of acetyl phosphate and ATP-Mg²⁺ was not inactivated (or, alternatively, rapidly regained activity when subsequently incubated with the complete reaction mixture). Comparable results were obtained upon exposure of diluted enzyme preparations to 1 M urea.

In toto, the data on inactivation show that acetyl phosphate and ATP-Mg²⁺ exert opposite effects on acetokinase. The latter complex induces an enzyme state that is more labile to inactivation than the untreated enzyme, whereas acetyl phosphate counteracts the effect of the ATP-Mg²⁺ complex.

**Kinetic properties.** The reaction velocity of acetokinase was a nonlinear function of the acetyl phosphate concentration, i.e., velocity curves for this substrate were sigmoidal, suggesting cooperative binding of acetyl phosphate (Fig. 2). The velocity curves for propionyl phosphate were also sigmoidal, and both acyl phosphates yielded Hill coefficients ($n$) near 2.5. However, acetyl phosphate was the most effective phosphate donor for ATP synthesis, with a $V_{max}$ approximately four times that obtained with propionyl phosphate. The $S_0.5$ values calculated from these curves were 3.3 mM for acetyl phosphate and 4.0 mM for propionyl phosphate. In contrast to the cooperative effects observed for acetyl phosphate, the velocity curves for increasing concentrations of ADP were hyperbolic, and $n$ was equal or nearly equal to 1 (Fig. 2). The apparent $K_M$ for ADP, calculated from data obtained at saturating concentrations of acetyl phosphate, was 0.11 mM.

![Fig. 4. Effect of nucleotides and acetyl phosphate on the inactivation of acetokinase by heat. A concentrated enzyme preparation (10 mg of protein/ml) with a specific activity of 80 (step 7, Table I) was diluted 1:1 with 50 mM Tris buffer containing the adenylates and acetyl phosphate or MgCl₂, or both as indicated, and incubated at 60°C. Samples were removed at the indicated times, rapidly cooled to 4°C, diluted 1:50 with 50 mM Tris buffer, pH 8.3, and assayed spectrophotometrically as described for method ii in Materials and Methods. A, Effects of adenylates; B, effects of ATP and acetyl phosphate.](http://jb.asm.org/)
effects were observed: a reduction of maximal velocity and an activation at low acetyl phosphate concentrations. Inhibition by a substrate analogue would be expected. As shown in Fig. 6A, this inhibition was competitive. However, n values for acetyl phosphate would be expected to be independent of propionyl phosphate. Figure 6B reveals that addition of propionyl phosphate altered the velocity curve for acetyl phosphate from the characteristic sigmoidal curve to a rectangular hyperbola, i.e., activation occurred at low acetyl phosphate concentrations. Hill coefficients calculated from these curves gave values of 1.0, 1.45, and 1.89 in the presence of 2.65, 1.33, and 0.75 mM propionyl phosphate, respectively. This lowering in cooperativity with respect to acetyl phosphate suggests that the competitive inhibitor can react at a separate regulatory site on the enzyme. Similar activations by competitive inhibitors have been obtained with isocitric dehydrogenase from Neurospora (3) and with aspartic transcarbamylase (8). Attempts to inactivate or destroy the postulated regulatory sites on acetokinase by heat treatment, exposure to urea, or exposure to p-hydroxymercuribenzoate without appreciably affecting the catalytic site were unsuccessful.

As shown in Fig. 7, the n values for acetyl phosphate are a function of the pH of the reaction mixture. The curves obtained at pH 8.3 and 10.0 showed a decrease in cooperativity with little or no effect on the maximum velocity. Separate experiments established that the enzyme was not inactivated during the short time of exposure to these pH values at low acetyl phosphate concentration. One interpretation of these results is that interaction of acetyl phosphate with the regulatory sites is affected at alkaline pH values, whereas the catalytic properties of the enzyme remain essentially unchanged.

The effects of various fixed concentrations of acetyl phosphate on the velocity of the reaction in the presence of different concentrations of ADP are depicted in Fig. 8. The intersection of
The effect of adding the second substrate (ADP-Mg$_{2+}$ in this instance) would be to displace the equilibrium to a new position, as the enzyme-acetyl phosphate-ATP-Mg$_{2+}$ complex was formed. Under these conditions, the reaction between acetyl phosphate and the enzyme is not rate-limiting and attainment of the maximum velocity depends on the concentration of ADP-Mg$_{2+}$.

As stated earlier, nucleoside triphosphates inhibit acetokinase. Figure 9 demonstrates that inhibition of the enzyme by ATP was cooperative and that the degree of cooperativity was determined by the concentration of acetyl phosphate. At high concentrations of acetyl phosphate, the n value for inhibition approached 3, and at lower concentrations of acetyl phosphate (near the S$_{0.5}$ for this substrate) the n value for inhibition was close to 1, i.e., the inhibition curves became nearly hyperbolic. The main effect of ATP was to increase the S$_{0.5}$ for acetyl phosphate while not significantly altering either the n value or V$_{max}$ for the acyl phosphate. Thus, both acetyl phosphate and ATP (or more correctly, the ATP-Mg$_{2+}$ complex) exert cooperative effects on acetokinase and show the same range of n values.

**DISCUSSION**

Acetokinase from *V. alcalescens* has properties typical of an allosteric enzyme, with acetyl phosphate, one of the substrates, acting as a positive effector and ATP, one of the products, serving as a negative effector. One interpretation of allosteric interactions (14) is that they are mediated by effector sites distinct from the active site of the enzyme and that the transition from a state of low

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![Graph](https://via.placeholder.com/150)

**Fig. 7.** Effect of pH on enzyme velocity at different concentrations of acetyl phosphate. Reaction mixtures and conditions of assay as in Fig. 6.

![Graph](https://via.placeholder.com/150)

**Fig. 8.** Reciprocal plot of velocity as a function of ADP concentration at four concentrations of acetyl phosphate. Initial rates were determined spectrophotometrically as described for method ii in Materials and Methods. Each sample contained 2.4 µg of protein from a fraction having a specific activity of 180 (step 8, Table 1).

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![Graph](https://via.placeholder.com/150)

**Fig. 9.** Reaction velocity of acetokinase as a function of ATP at different concentrations of acetyl phosphate. The reaction mixtures were as indicated for Fig. 5 except that hexokinase and glucose were omitted, and 7.5 µmoles of ADP was added. The reaction was terminated after 5 min, and acetyl phosphate was assayed according to method i described in Materials and Methods.
affinity for a substrate to one of high affinity occurs as the effector sites are filled. The multiplicity of active sites and effector sites implied by this model is most readily explained on the basis of a polymeric enzyme structure.

The present experiments with acetokinase show that, depending on the conditions of assay, both the velocity curves for acetyl phosphate and the inhibition curves obtained with ATP could vary from rectangular hyperbolas (Michaelis-Menten kinetics) to curves having a pronounced sigmoidicity. Both ligands yielded approximately the same range of Hill coefficients with \( n \) approaching 3 for curves having the greatest sigmoidicity. For concentrations of acetyl phosphate giving maximal homotropic effects (near the \( S_{0.5} \) value), inhibition by ATP was essentially noncooperative (hyperbolic). Conversely, the greatest homotropic interactions for ATP were found as saturating concentrations of acetyl phosphate. These observations suggested that the allosteric ligands exerted the strongest cooperative effects on two different states of the enzyme.

Additional support for this suggestion came from studies of inactivation of acetokinase by heat, dilution, and exposure to urea. The stability of the enzyme in any of the latter three conditions was not affected by acetyl phosphate, acetate, or ADP when tested in the presence and absence of Mg\(^{2+}\). However, addition of ATP and Mg\(^{2+}\) significantly decreased the stability of the enzyme. This ATP-Mg\(^{2+}\)-accelerated loss of enzyme activity could be partially reversed by the addition of acetyl phosphate. Thus, ligands capable of exerting cooperative effects also were able to interact with acetokinase to promote states of different stability.

Evidence that acetokinase possesses effector sites distinct from catalytic sites came from experiments with propionyl phosphate. Addition of this analogue of acetyl phosphate affected the maximum velocity in a manner indicating competitive inhibition. In addition, the shape of the velocity curve obtained with increasing concentrations of acetyl phosphate was altered from sigmoidal to rectangular hyperbola typical of Michaelis-Menten kinetics, i.e., the enzyme was activated by propionyl phosphate at low acetyl phosphate concentrations. This would be expected if an effector site were blocked by propionyl phosphate. Similar activations attributed to the binding of substrate analogues to effector sites have been reported for other enzymes (3, 8).

Lastly, acetokinase appeared to be a polymeric enzyme. The most enzymatically active species had a molecular weight of approximately 90,000, although lower molecular weight species could also be detected.

The virtual irreversibility of the reaction mediated by the \( V. \) alcalescens acetokinase indicates that the primary role of the enzyme is in the synthesis of ATP. The regulatory properties of the enzyme, however, would preclude rapid accumulation of large amounts of ATP even if large quantities of ADP were available.

On the contrary, the properties of the enzyme would promote the maintenance of a fairly large steady-state pool of acetyl phosphate. Thus, the sigmoidal response of the enzyme to acetyl phosphate, the relatively high concentration of acetyl phosphate required to saturate the enzyme (the \( S_{0.5} \) value is approximately 3 mM), and the fact that ATP inhibition is maximal at saturating concentrations of acetyl phosphate would all serve to maintain a pool of acetyl phosphate. Evidence has been obtained (unpublished data) that extracts of \( V. \) alcalescens do in fact contain fairly large quantities of acetyl phosphate and can accumulate acetyl phosphate as a consequence of pyruvate degradation. Under conditions favoring the formation of a high steady-state level of acetyl phosphate, synthesis of acetyl CoA via phosphotransacetylase could occur. As shown elsewhere (manuscript in preparation), ATP may also regulate the conversion of acetyl phosphate to acetyl CoA by phosphotransacetylase, whereas the reverse reaction (acetyl CoA + phosphate \( \rightarrow \) acetyl phosphate + CoA) is inhibited by increasing concentrations of acetyl phosphate. The net result of these effects of ATP and acetyl phosphate would be that, if sufficiently high concentrations of ATP were accumulated, the concentration of acetyl CoA would increase. Thus, the supply of this essential biosynthetic precursor would depend, in part at least, on the regulation of acetokinase.

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**LITERATURE CITED**