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EVIDENCE FOR AN ESSENTIAL ARGININE RESIDUE AT THE ACTIVE SITE OF *ESCHERICHIA COLI* ACETATE KINASE

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Escherichia coli **acetate kinase (ATP: acetate phosphotransferase, EC 2.7.2.1.) was inactivated in the presence of either 2,3-butanedione in borate buffer or phenylglyoxal in triethanolamine buffer. When incubated with 9.4 mM phenylglyoxal or 5.1 mM butanedione, the enzyme lost its activity with an apparent rate constant of inactivation of 0.079 min^{-1} and 0.051 min^{-1}, respectively. The loss of enzymatic activity was concomitant with the loss of an arginine residue per active site. Phosphorylated substrates of acetate kinase, ATP, ADP and acetylphosphate as well as AMP markedly decreased the rate of inactivation by both phenylglyoxal and butanedione. Acetate neither provided any protection nor affected the protection rendered by the adenine nucleotides. However, it interfered with the protection afforded by acetylphosphate. These data suggest that an arginine residue is located at the active site of acetate kinase and is essential for its catalytic activity, probably as a binding site for the negatively charged phosphate group of the substrates.**

Introduction

In a previous communication [1] concerning the catalytic mechanism of acetate kinase (ATP: acetate phosphotransferase, EC 2.7.2.1) isolated from *Escherichia coli*, we have reported that the enzyme was inactivated by *N*-ethylmaleimide. Through detailed kinetic analysis of the process of inactivation and the protection afforded by substrates and products, we have shown that the sulfhydryl group is located in a region different from the binding site for acetate and acetylphosphate. The proposed substrate binding topography at the active site of the enzyme is consistent with a direct in-line phosphoryl group transfer reaction proposed by Blatter and Knowles [2]. In this report, we present further support for the binding arrangement of the substrates at the active site. We show here that the active site of acetate kinase contains an essential arginine residue which can be modified by either 2,3-butanedione or phenylglyoxal.

Experimental procedure

Materials. Phenylglyoxal was obtained from Aldrich Chemical Co. and 2,3-butanedione from Eastman Kodak. All nucleotides as well as pyridoxal 5′-phosphate and acetylphosphate were supplied by Sigma Chemical Co. Various enzymes used were also products of Sigma. *E. coli* acetate kinase was obtained in the form of a crystalline suspension in 3.2 M $(NH_4)_2SO_4$ and had a specific activity of 230 units/mg protein.

Methods. Acetate kinase was assayed as described earlier [1]. Modification reactions were carried out under the conditions given in the figure legends. To minimize the possible influence of cold inactivation at 0°C and reactivation at ambient temperature [3], the enzyme solution was incubated at 27°C for at least 30 min before experimentation. Whenever substrates or products were included, they were first incubated with the enzyme. The kinetic data were fitted to the equations discussed in the text with least-squares analysis.

The modification of arginine residue was determined by amino acid analysis after acid hydrolysis. The enzyme was dialyzed against 50 mM triethanolamine buffer, pH 7.6, before modification. Protein samples were hydrolyzed at 110°C for 20–24 h in 6 N HCl in evacuated, sealed glass tubes and analysis was performed on a Beckman Model 121 Automatic Amino Acid Analyzer.

Results

Inactivation by 2,3-butanedione

Acetate kinase from *E. coli* was completely inactivated in the presence of α-dicarbonyl reagents, 2,3-butanedione and phenylglyoxal. The time course of inactivation of acetate kinase by butanedione is depicted in Fig. 1. Since the concentration of the inhibitor is much higher than that of the enzyme, the process of inactivation can be treated as pseudo-first-order kinetics. When acetate kinase was incubated with 5.1 mM butanedione in 50 mM borate buffer, pH 8.6, an apparent rate constant of inactivation was determined to be 0.051 min^{-1}. The borate ion seems to accelerate the rate of inactivation. In 50 mM triethanolamine (pH 8.5), 5.1 mM butanedione inactivated the enzyme with a half-life of 76 min compared to that of 13 min in 50 mM borate (pH 8.6). This increase in reactivity probably reflects the formation of a stable borate complex similar to that suggested by Patthy and Smith [4]. At a lower pH (pH 7.7, 50 mM borate), the rate constant of inactivation is also lowered to 0.011 min^{-1} ($t_{1/2}$ = 63 min).

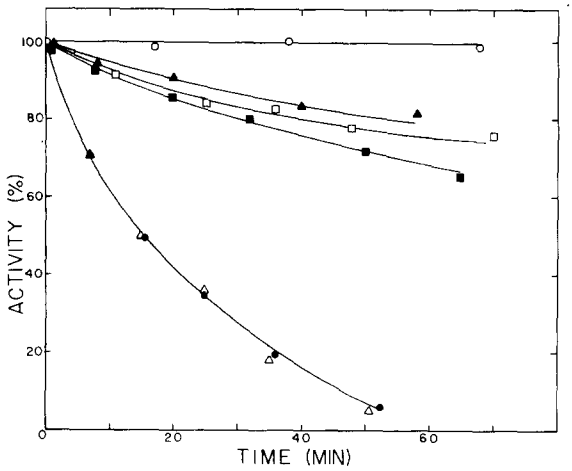

Fig. 1. Inactivation of acetate kinase by 2,3-butanedione. Acetate kinase (1.2 units) was incubated at 27°C in 50 mM borate buffer (pH 8.6) with 5.1 mM 2,3-butanedione (●———●). The enzyme activity was followed after the addition of the reagent at the time intervals indicated in the figure. In some experiments the incubation mixture also contained 1.0 M acetate (△———△), 5.2 mM ATP (□———□), 23 mM acetylphosphate (▲———▲) or 5.2 mM ADP (■———■). A parallel control experiment without the inhibitor is also shown (○———○).

Fig. 1 also shows the effect of various substrates on the rate of inactivation. Phosphorylated compounds, such as ATP, ADP, AMP and acetylphosphate decreased the rate of inactivation by butanedione. In the presence of 5.2 mM ATP the apparent rate constant of inactivation was reduced from 0.051 min^{-1} to 0.0047 min^{-1}. A similar degree of protection was observed for 5.2 mM ADP and 23 mM acetylphosphate. However, acetate up to 1.0 M did not affect the rate of inactivation.

Inactivation by phenylglyoxal

The time course of inactivation of acetate kinase in the presence of phenylglyoxal is shown in Fig. 2. In 50 mM triethanolamine buffer, pH 7.6, 9.4 mM phenylglyoxal completely inactivated the enzyme within an hour with a half-life of inactivation of 8.8 min. The presence or absence of Mg^{2+} (up to 22 mM) did not affect the rate of inactivation. To identify the

TABLE I

RELATION OF LOSS OF ACTIVITY AND ARGININE RESIDUE OF ACETATE KINASE ON INCUBATION WITH PHENYLGLYOXAL

Acetate kinase was incubated with 7.2 mM phenylglyoxal in 50 mM triethanolamine buffer, pH 7.6. At various time intervals the solution was assayed for enzyme activity and aliquots were removed for analysis of arginine content by amino acid analysis.

% Activity remaining	% Arginine residue modified
100	0
75	25
45	47
25	77
7	94

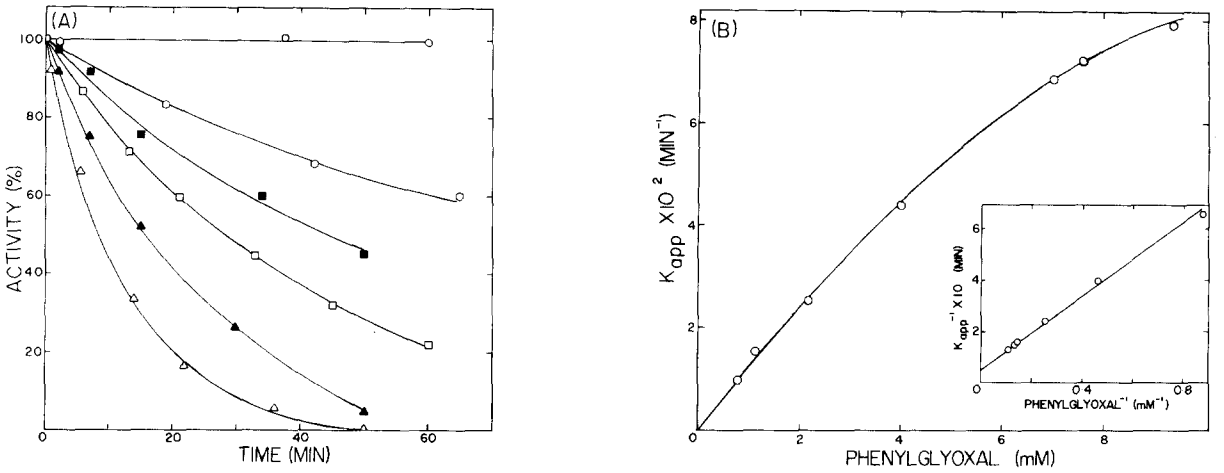

Fig. 2. Inactivation of acetate kinase by phenylglyoxal. (A) Acetate kinase was incubated at 27°C in 50 mM triethanolamine buffer (pH 7.6) with various concentrations of phenylglyoxal: 0.83 mM (○———○), 1.1 mM (■———■), 2.2 mM (□———□), 4.0 mM (▲———▲) and 9.4 mM (△———△). The enzyme activity was followed after addition of the reagent at the time intervals indicated in the figure. A parallel control experiment without the inhibitor is also shown (○———○). (B) The apparent rate constants of inactivation calculated from the pseudo-first-order kinetics as shown in (A) are plotted against the concentrations of phenylglyoxal. Insert. A double-reciprocal plot of the apparent rate constants of inactivation vs. the concentration of phenylglyoxal.

amino acid residue modified with phenylglyoxal, samples of inactivated enzyme were analyzed for their amino acid content. As shown in Table I, the loss of enzymatic activity correlated well with a progressive decrease in arginine content.

As shown in Fig. 2A and more vividly in Fig. 2B, the rate of inactivation depends upon the concentration of phenylglyoxal. At low concentrations of phenylglyoxal, where there is linear relationship between the apparent rate constant of inactivation and the concentration of the inactivator, the apparent rate constant may be expressed by the following equation as employed by Scrutton and Utter [5],

$$K_{app} = K[I]^n$$

where K_{app} is the apparent rate constant of inactivation, K, a proportionality constant and n, the order of reaction with respect to phenylglyoxal, I. Thus,

$$\ln K_{app} = \ln K + n \ln[I] \qquad (1)$$

From a plot of $\ln K_{app}$ vs. $\ln[I]$, n can be obtained from the slope of the line. Scrutton and Utter [5], and Brans and Keech [6] have used this technique to determine the order of the reaction between pyruvate carboxylase and avidin, and phospho*enol*pyruvate carboxykinase and *N*-ethylmaleimide, respectively. When the linear portion of the curve shown in Fig. 2B was replotted according to Eqn. 1, n was calculated to be 1.1 indicating that interaction of 1 mol phenylglyoxal/mol acetate kinase catalytic site was sufficient to produce inactivation. Earlier observations [7] have shown that 2 molecules phenylglyoxal react with 1 molecule arginine with the reaction of the first molecule of phenylglyoxal being rate limiting. However, the condensation of a molecule of phenylglyoxal with the guanido group of arginine to form a glyoxaline ring is sufficient to inactivate the enzyme. It is, therefore, reasonable to assume that one arginine residue is loacted at the active site of acetate kinase.

Since phenylglyoxal is more specific for arginine than butanedione [7] and it can be obtained commercially in pure crystalline form, the process of inactivation by phenylglyoxal was studied further in order to characterize more precisely the role of the arginyl residue in acetate kinase.

Substrate protection and inactivation kinetics

The exhibition of saturation kinetics of the rate of inactivation of acetate kinase by phenylglyoxal as shown in Fig. 2B suggests that the process of inactivation involves prior association of the reagent with the enzyme to form an enzyme-inhibitor complex before it reacts with the arginine residue. As it was shown earlier that 1 molecule phenylglyoxal is sufficient to inactivate the enzyme, the process of inactivation can be described by the following equation,

$$E + I \overset{K_I}{\rightleftharpoons} E \cdot I \overset{k}{\rightarrow} E^*$$

where K_I is the dissociation constant of enzyme-phenylglyoxal complex, $E \cdot I$, and k, the rate constant of inactivation of the enzyme. It can be derived from this scheme that

$$K_{app} = \frac{k[I]}{K_I + [I]} \quad \text{or}$$

$$\frac{1}{K_{app}} = \frac{1}{k} + \frac{K_I}{k[I]} \tag{2}$$

Note that when $[I] \ll K_I$, this equation can be converted to Eqn. 1 with n equal to 1.

Eqn. 2 has the form of the Michaelis-Menten equation and a double-reciprocal plot of the rate constants of inactivation, K_{app}, against phenylglyoxal concentration, [I], gives a straight line (Fig. 2B, insert) from which k and K_I are calculated from the intercepts at the ordinate and abscissa to be 0.17 min^{-1} and 12 mM, respectively.

Similar to that observed for 2,3-butanedione, the inactivation of acetate kinase by phenylglyoxal is retarted by ATP, ADP, AMP and acetylphosphate. Since Mg^{2+} is shown not to affect the rate of inactivation by phenylglyoxal, all investigations were carried out in the absence of the metal ion to prevent any complication due to the phosphorylation by ATP or acetylphosphate. However, in an experiment with ADP, inclusion of Mg^{2+} (22 mM) did not affect the degree of protection rendered by ADP.

Assuming that the protection afforded by these phosphorylated compounds is due to direct steric hindrance of the accessibility of phenylglyoxal to the arginine residue, the process of protection can be analyzed by the following scheme,

$$E + S \overset{K_S}{\rightleftharpoons} E \cdot S$$

$$E + I \overset{K_I}{\rightleftharpoons} E \cdot I \overset{k}{\rightarrow} E^*$$

where K_S is the dissociation constant of the enzyme-ligand complex, $E \cdot S$. From this scheme, it can be shown that

$$\frac{1}{K_{app}} = \frac{1}{k} + \frac{K_I}{k[I]} + \frac{K_I[S]}{kK_S[I]} \tag{3}$$

At a fixed concentration of phenylglyoxal, a plot of $1/K_{app}$ against the concentration of a protecting compound, [S], will be a straight line with a slope equal to K_I/kK_S from which K_S can be evaluated. Such plots of the protective effects of ATP, ADP, AMP and acetylphosphate are shown in Fig. 3. Inclusion of acetate up to 0.19 M in incubation mixtures of the adenine nucleotides did not affect the protection rendered by these compounds. However, addition of acetate to incubation mixtures of acetylphosphate decreased the extent of protection rendered by

TABLE II

DISSOCIATION CONSTANTS FOR VARIOUS ACETATE KINASE-LIGAND COMPLEXES

The dissociation constants were calculated according to Eqn. 3 from Fig. 3. Values in parentheses indicate the concentration of acetate present.

Ligand	K_I (mM)
ATP	2.7
ATP (86 mM)	2.7
ADP	3.6
ADP (0.18 M)	3.7
AMP	2.8
AMP (0.19 M)	2.8
Acetylphosphate	2.6
Acetylphosphate (0.32 M)	3.5

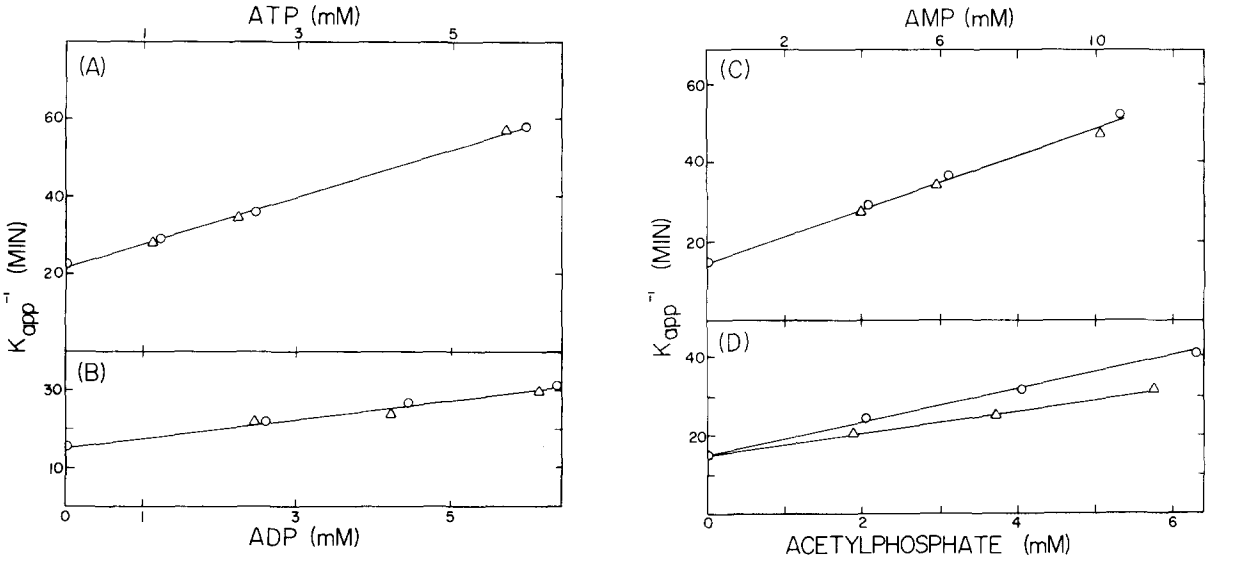

Fig. 3. Effect of various ligands on inactivation of acetate kinase by phenylglyoxal. The data were plotted according to Eqn. 3. (A) The inactivation was effected by 4.0 mM phenylglyoxal in the presence of various concentrations of ATP, either alone (o———o) or with 86 mM acetate (△———△). (B) The inactivation was carried out with 7.6 mM phenylglyoxal at various concentrations of ADP, either alone (o———o) or with 0.18 M acetate (△———△). (C) Acetate kinase was inactivated by 7.3 mM phenylglyoxal in the presence of various concentrations of AMP, either alone (o———o) or with 0.19 M acetate (△———△). (D) Acetate kinase was inactivated by 7.0 mM phenylglyoxal in the presence of various concentrations of acetylphosphate, either alone (o———o), or with 0.32 M acetate (△———△).

acetylphosphate (Fig. 3D). Table II lists the dissociation constants of the enzyme-ligand complexes as calculated from Fig. 3 according to Eqn. 3. These dissociation constants are close to the corresponding K_m values determined by Rose et al. [8], although they are not directly comparable since the K_s values are for the free nucleotides and the K_m values refer to their magnesium complexes. However, these values are 2–4-times higher than those obtained from protection kinetics against *N*-ethylmaleimide inactivation [1]. The reason for this difference is not clear. One possible explanation may be that the substances can bind at the active site in several different binding modes, some of which will protect against *N*-ethylmaleimide inactivation and some against phenylglyoxal inactivation. Table II also shows that the dissociation constants of the enzyme-adenine nucleotides complexes are not affected by the presence of acetate. However, acetate increases the dissociation constant of the enzyme-acetylphosphate complex.

Discussion

The recognition of the involvement of a particular amino acid residue in the mechanism of action of enzymes has been facilitated by the availability of specific modification agents. Various dicarbonyl reagents have been shown to be suitable for the identification of functional arginine residue [4,7,9–11] at the active site. Although the selectivity of none of these has been proven ideal, phenylglyoxal was shown to be highly specific toward the guanido group of arginine residue under mild conditions [7]. Using these group specific reagents, coupled with amino acid analysis, we have shown that an arginine is required for the enzymatic activity of *E. coli* acetate kinase. Both butanedione (Fig. 1) and phenylglyoxal (Fig. 2) can completely inactivate the catalytic activity of the enzyme. Amino acid analysis on the phenylglyoxal-inactivated enzyme showed that the loss of activity followed a concomitant decrease in

arginine content, whereas other amino acids were not affected. In addition, incubation of the enzyme with 22 mM pyridoxal 5′-phosphate for 2 h did not affect the activity of the enzyme.

That the essential arginine is located at the active site of acetate kinase comes from the experiments of substrate protection. Adenine nucleotides and acetylphosphate prevented or retarded the inactivation of the enzyme by both butanedione (Fig. 1) and phenylglyoxal (Fig. 3). On the other hand, acetate did not protect the enzyme. Since only the phosphorylated compounds protected the enzyme from inactivation, the data suggest that the arginine residue might serve as a binding site for the negatively-charged phosphate group of the substrates.

Not only does acetate not render the enzyme any protection against butanedione (Fig. 1) and phenylglyoxal inactivation (Fig. 3), it also does not interfere with the protection rendered by the adenine nucleotides. It does, however, compete with acetylphosphate for the enzyme (Fig. 3D), thus increases the dissociation constant for the enzyme-acetylphosphate complex (Table II). These observations are consistent with those reported earlier [1]. However, in the previous work [1] acetate was shown to relieve the ATP protection against *N*-ethylmaleimide inactivation. This suggests that the acetate binding domain is different from that of the ATP such that an enzyme-acetate-ATP complex exists in accordance with the random sequential mechanism pathway followed by the enzyme [12,13].

Relief of acetate kinase from acetylphosphate protection in the presence of acetate is in agreement with that observed on the effect of *N*-ethylmaleimide inactivation [1] giving further support to the notion that acetate and acetylphosphate have a common binding site which is different from that of the adenosine binding domain. The data obtained in this work are, therefore, consisitent with the suggestion [2] that the enzyme can follow a direct in-line phosphoryl group transfer reaction between enzyme-bound substrates.

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