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INACTIVATION OF *ESCHERICHIA COLI* ACETATE KINASE BY *N*-ETHYLMALEIMIDE

PROTECTION BY SUBSTRATES AND PRODUCTS

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Summary

Acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) from *Escherichia coli* exhibited a time-dependent loss of activity when incubated with *N*-ethylmaleimide at micromolar concentrations. However, prolonged incubation did not eliminate all catalytic activity and generally about 15% of its initial activity remained. When incubated with 7.2 μM *N*-ethylmaleimide, acetate kinase was inactivated with a rate constant of 0.063 min^{-1}. Adenine nucleotides, ATP, ADP and AMP, protected the enzyme against such inactivation, but acetate up to 3.0 M and in the presence of 0.2 M $MgCl_2$ and acetyl phosphate at 24 mM did not interfere with the rate of inactivation. While both acetate and acetyl phosphate did not affect the protection rendered by AMP, the presence of acetyl phosphate altered ADP protection. However, both substrates prevented ATP from protecting the enzyme. These data suggest that the binding sites for acetate and acetyl phosphate are different from that of the adenosine binding domain, but are in close vicinity to the phosphoryl binding regions of the nucleotides.

Introduction

Acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) from *E. coli* catalyzes the phosphorylation of acetate according to the following equation:

$$ATP + acetate \overset{Mg^{2+}}{\rightleftharpoons} ADP + acetyl\ phosphate$$

The mechanistic pathway followed by this enzyme has been a matter of con-

troversy over the years. Anthony and Spector [1,2] reported that the enzyme catalyzes both ATP-ADP exchange in the absence of acetate, and acetyl phosphate-acetate exchange in the absence of added nucleoside triphosphate. They [3] later isolated a phosphorylated enzyme on incubation with ATP or acetyl phosphate and showed its chemical competence, in the sense that the phosphoryl group could be transferred either to ADP or acetate. However, the phosphoryl-enzyme intermediate was not kinetically competent, in that, although ATP-ADP exchange was seven times faster than the overall reaction, the acetyl phosphate-acetate exchange was 64 times slower [2]. Subsequently, Todhunter and Purich [4] showed that the phosphoryl group was attached to glutamic acid residue in the protein. Early kinetic investigations of Purich and Fromm [5] indicated that the enzyme followed a simple ping-pong pathway, consistent with the formation of a phosphoryl enzyme in the reaction. However, Janson and Cleland [6], through the use of chromium triphosphate, suggested a random sequential mechanism for the majority of the reaction flux and a ping-pong type mechanism to account for the ATP-ADP exchange. Subsequent kinetic studies by Webb et al. [7] using propionyl phosphate, further substantiated the random sequential mechanism. Nevertheless, Todhunter et al. [8] showed that the nucleoside diphosphate kinase activity of acetate kinase followed ping-pong kinetics. These data were consistent with the presence of a phosphoryl-enzyme intermediate. On the contrary, Skarstedt and Silverstein [9] reported that at thermodynamic equilibrium the ATP-ADP exchange rate was independent of the presence or concentration of co-substrate, whereas acetyl phosphate-acetate exchange could not be observed in the absence of adenine nucleotide and concluded that phosphoryl enzyme was not formed in the absence of nucleotides. Finally, Blatter and Knowles [10] showed that the phosphorylation of acetate by ATP proceeded with inversion of configuration at the phosphorus, suggesting that a direct phosphoryl-group transfer reaction occurred between enzyme-bound substrates.

Shortly after acetate kinase was detected in *E. coli* extract, Rose et al. [11] showed that the enzyme was strongly inhibited by sulfhydryl reagents, particularly mercuric chloride, *p*-chloromercuribenzoate and iodosobenzoate. The present report examines the functional role of the sulfhydryl group in an effort to determine its location at the active site and to elucidate its function in the catalytic mechanism.

Experimental procedure

Materials. *N*-Ethylmaleimide was obtained from Aldrich Chemical Company and recrystallized three times from heptane. All nucleotides and acetyl phosphate were products of Sigma Chemical Co., various enzymes used were also supplied from 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 per mg protein.

Assay. Acetate kinase was assayed spectrophotometrically for the production of ADP. A standard assay mixture (1 ml) contained 0.6 M sodium acetate, 40 mM $MgCl_2$, 5.4 mM ATP, 1.1 mM phospho*enol*pyruvate, 0.23 mM NADH, 5 units pyruvate kinase, 7 units lactate dehydrogenase and 50 mM triethanol-

amine with a final pH of 7.5. About 0.01 units acetate kinase were used in each assay. The decrease in absorbance at 340 nm was measured with a Unicam SP18000 spectrophotometer at 27°C using a cuvette of 1 cm light path.

Enzyme inactivation. Unless otherwise specified, acetate kinase (1.2 units) was incubated at 27°C with *N*-ethylmaleimide in 50 mM triethanolamine (pH 7.5) in a final volume of 0.1 ml. *N*-Ethylmaleimide was the last compound added and during the subsequent 30 min aliquots were removed periodically and assayed. To minimize the possible influence of cold inactivation at 0°C and reactivation at ambient temperature [7] the enzyme solution was incubated at 27°C for at least 30 min prior to the addition of reagent. Whenever substrates or products were included, they were first incubated with the enzyme. In order to avoid phosphorylation of the enzyme, all incubations were carried out in the absence of $MgCl_2$. The concentration of *N*-ethylmaleimide was determined using an extinction coefficient of 620 $M^{-1} \cdot cm^{-1}$ at 302 nm [12].

Equations. The equations used herein were derived in a manner similar to that reported earlier [13]. As shown in Scheme I, the native enzyme form E of acetate kinase is inactivated to E^* by *N*-ethylmaleimide (NEM) at a rate k_1. The inactivation will be pseudo-first order with respect to

$$E + A \overset{K_a}{\rightleftharpoons} E \cdot A$$

$$E + NEM \overset{k_1}{\rightarrow} E^*$$

$$E \cdot A + NEM \overset{k_2}{\rightarrow} E^* \cdot A \rightleftharpoons E^* + A$$

Scheme I.

NEM concentration. E can bind a substrate A with dissociation constant K_a to form an enzyme-substrate binary complex $E \cdot A$ which is inactivated by the same reagent at a rate k_2 to form $E^* \cdot A$. The latter can dissociate to form E^* and A. At a fixed concentration of *N*-ethylmaleimide, the rate of loss of the fully active (native) enzyme is

$$-\frac{dE_t}{dt} = k_1(E) + k_2(E \cdot A) \tag{1}$$

where E_t is the total amount (concentration) of fully active native enzyme at any time t, ($E_t = E + E \cdot A$). On substitution and integration, Eqn. 1 becomes

$$\ln \frac{E_t}{E_0} = -\left(\frac{k_1 K_a + k_2 A}{K_a + A}\right) t \tag{2}$$

where E_0 is the initial concentration of native enzyme. Since the catalytic rate, v, is proportional to the concentration of native enzyme, it follows that Eqn. 2 may be rewritten as

$$\ln \frac{v - v_\infty}{v_0 - v_\infty} = -K_{ob} t \tag{3}$$

where v, v_0 and v_∞, are respectively, the catalytic rate of the enzyme at time t, zero time (native enzyme) and after infinite time of incubation with inhibitor

(residual activity), and the observed apparent rate constant.

$$K_{ob} = \frac{k_1 K_a + k_2 A}{K_a + A} \tag{4}$$

Note that if the substrate concentration is zero, K_{ob} is equal to k_1. Also if the binding of A to the enzyme does not affect the rate of inactivation, then $k_2 = k_1$ and K_{ob} will be independent of the concentration of A, i.e., $K_{ob} = k_1$. On the other hand, if A totally protects the enzyme from inactivation, then $k_2 = 0$ and Eqn. 4 reduces to

$$K_{ob} = \frac{k_1 K_a}{K_a + A}$$

or

$$\frac{1}{K_{ob}} = \left(\frac{1}{k_1 K_a}\right) A + \frac{1}{k_1} \tag{5}$$

In the case where two substrates, A and B, are present,there will be four enzyme species which could be formed according to a random sequential mechanism and each could undergo inactivation by *N*-ethylmaleimide (NEM) as shown in Scheme II.

$$E + A \overset{K_a}{\rightleftharpoons} E \cdot A$$

$$E + B \overset{K_b}{\rightleftharpoons} E \cdot B$$

$$E \cdot A + B \overset{K_{ab}}{\rightleftharpoons} E \cdot A \cdot B$$

$$E \cdot B + A \overset{K_{ba}}{\rightleftharpoons} E \cdot A \cdot B$$

$$E + NEM \overset{k_1}{\rightarrow} E^*$$

$$E \cdot A + NEM \overset{k_2}{\rightarrow} E^* \cdot A \rightleftharpoons E^* + A$$

$$E \cdot B + NEM \overset{k_3}{\rightarrow} E^* \cdot B \rightleftharpoons E^* + B$$

$$E \cdot A \cdot B + NEM \overset{k_4}{\rightarrow} E^* \cdot A \cdot B \rightleftharpoons E^* + A + B$$

Scheme II.

By the similar approach outlined above, it can be derived that

$$\frac{1}{K_{ob}} = \frac{K_a K_b K_{ba} + K_a K_{ab} A + K_a K_{ba} B + K_a AB}{k_1 K_a K_b K_{ba} + k_2 K_a K_{ab} A + k_3 K_a K_{ba} B + k_4 K_a AB} \tag{6}$$

If the presence of substrate A totally protects the enzyme from inactivation, then $k_2 = k_4 = 0$. In addition, if substrate B does not interfere with the rate of inactivation, then $k_3 = k_1$ and Eqn. 6 can be simplified to

$$\frac{1}{K_{ob}} = \left(\frac{K_{ab} + B}{k_1 K_{ba}(K_b + B)}\right) A + \frac{1}{k_1} \tag{7}$$

Note that Eqn. 7 reduces to Eqn. 5 when the second substrate B is absent. Eqn. 7 also reduces to Eqn. 5 if the presence of B does not interfere with the binding of A and vice versa, i.e., $K_{ab} = K_b$ and $K_{ba} = K_a$. If A and B are, respectively, acetyl phosphate and ATP, they will not be compatible on the enzyme surface and the ternary enzyme complex E · A · B will not be formed, in other words, K_{ab} and K_{ba} are infinite. Then Eqn. 7 can be simplified to

$$\frac{1}{K_{ob}} = \left(\frac{K_b}{k_1 K_a (K_b + B)}\right) A + \frac{1}{k_1} \tag{8}$$

Note that this equation also reduces to Eqn. 5 when B is absent. A plot of Eqn. 5 using the reciprocal of the experimentally determined apparent rate constants of inactivation, $1/K_{ob}$, against concentrations of a protecting substrate A will provide the dissociation constant of the enzyme-substrate complex. And if a second non-protecting substrate B binds competitively against A, a secondary plot of Eqn. 8 as a function of concentrations of B will yield its dissociation constant K_b. If the second substrate B can bind simultaneously with A on the enzyme surface to form E · A · B, a plot of $1/K_{ob}$ vs. the concentration of protecting substrate A will give a series of lines with different slopes as a function of the concentrations of B according to Eqn. 7. All data fitted to the above equations are treated with least-squares analysis.

Results

Inhibition by N-ethylmaleimide

Acetate kinase from *E. coli* exhibited a time-dependent inactivation in the presence of *N*-ethylmaleimide (Fig. 1). But it was observed that extending the incubation period to over 10 times the half-life of inactivation and a further addition of the inhibitor did not eliminate all its activity. A residual activity of

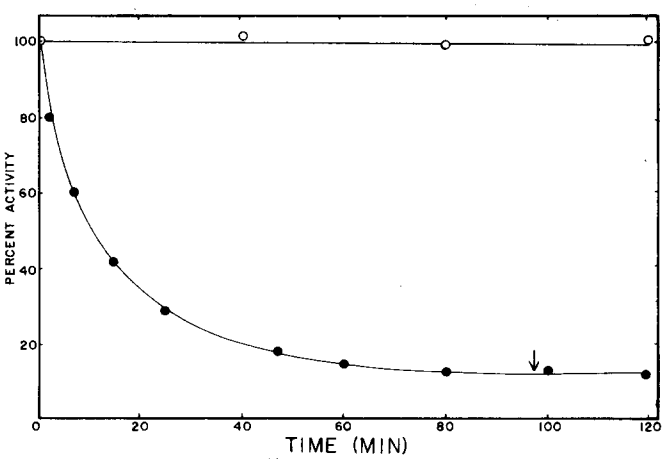

Fig. 1. Inactivation of acetate kinase by *N*-ethylmaleimide. Acetate kinase (1.2 units) was incubated at 27°C in 50 mM triethanolamine (pH 7.5) with 7.2 μM *N*-ethylmaleimide (●———●). The enzyme activity was followed after the addition of the reagent at time interval as indicated in the figure. Arrow (↓) indicates further addition of 20 μM *N*-ethylmaleimide. A parallel control experiment without the inhibitor is also shown (○———○).

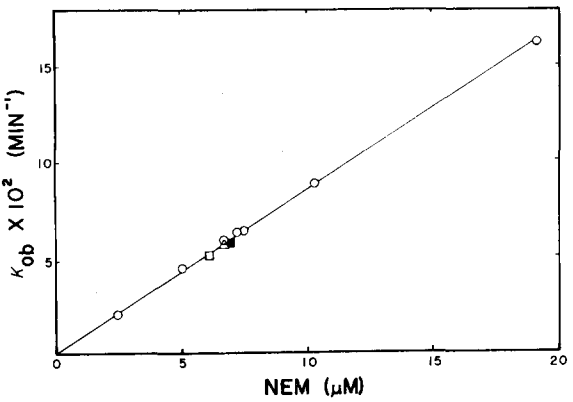

Fig. 2. Effect of acetate and acetyl phosphate on inactivation of acetate kinase by *N*-ethylmaleimide. The apparent rate constants of inactivation, K_{ob}, obtained by Eqn. 3 for the reactions in the absence of acetate or acetyl phosphate (○), in the presence of 3.0 M acetate and 0.2 M $MgCl_2$ (△), 13 mM acetyl phosphate (□) and 24 mM acetyl phosphate (■) are plotted against the concentrations of *N*-ethylmaleimide (NEM) at which the inactivations were carried out.

about 15% of the native enzyme always remained. A plot of the log of relative enzyme activity against time is non-linear unless the residual activity v_∞ is subtracted out. The apparent rate constant of inactivation, K_{ob}, was determined from the slope of a plot of $\ln(v - v_\infty/v_0 - v_\infty)$ vs. t according to Eqn. 3. The apparent rate constants of inactivation in the absence of any protecting agent are directly proportional to the concentrations of *N*-ethylmaleimide over a range of 0—19 μM (Fig. 2) suggesting that the inactivation does not involve prior association of the reagent with enzyme to form an enzyme-inhibitor complex, which would give saturation kinetics. The slope of a plot of $-\ln K_{ob}$ against ln *N*-ethylmaleimide concentration is 0.99 indicating that essentially 1 mol *N*-ethylmaleimide was bound per mole of catalytic site to produce inactivation [13,14]. Fig. 2 also shows that 3.0 M acetate in the presence of 0.2 M $MgCl_2$ and acetyl phosphate up to 24 mM did not affect the process of inactivation. It may be concluded that acetyl phosphate and acetate did not protect the enzyme against inactivation. An explanation for this observation is that neither acetate nor acetyl phosphate binds to the free enzyme. However, from the conclusion that reaction occurs via a random sequential mechanism [6,7], acetate and acetyl phosphate must interact with the free enzyme. If A in Scheme I is acetate or acetyl phosphate, k_2 may be assumed to be equal to k_1 and the apparent rate constant, K_{ob}, would be independent of the concentrations of acetate or acetyl phosphate. The apparent rate constant of inactivation of acetate kinase, K_{ob}, when incubated with 7.2 μM *N*-ethylmaleimide is 0.063 min^{-1}.

Protection by adenine nucleotides

In contrast to acetate and acetyl phosphate, adenine nucleotides, AMP, ADP and ATP, protected the enzyme against *N*-ethylmaleimide inactivation. If a plot of $1/K_{ob}$ against the concentrations of protecting nucleotide is linear, then k_2 of Eqn. 4 may be assumed to be equal to zero. Thus Eqn. 5 holds and the dis-

sociation constant, K_a, of the enzyme-nucleotide complex can be obtained from the intercept on the abscissa. This is illustrated in Figs. 3 and 4 with AMP, ADP and ATP as the protecting substances, respectively. It should be pointed out that no metal ions were included in these experiments. The protection of the enzyme against inactivation is therefore due to the binding of free nucleotide to the enzyme. Table I lists the dissociation constants of the enzyme-nucleotide complexes as calculated according to Eqn. 5. The dissociation constants decrease steadily from AMP through ADP to ATP suggesting that additional phosphoryl group contributes to binding. Although these values are lower than the corresponding K_m values determined earlier [11], they are not directly comparable since the K_a values are for the free nucleotides and the K_m values refer to their magnesium complexes.

Inclusion of 0.38 M acetate or 9.6 mM acetyl phosphate in the incubation mixture with AMP did not affect the protection rendered by AMP (Fig. 3A). As shown in Table I, the dissociation constants of enzyme—AMP complex obtained in the presence of acetate or acetyl phosphate are both 1.2 mM; these are identical within experimental error to the value of 1.4 mM obtained when AMP was incubated alone. Similarly, acetate did not affect ADP protection (Fig. 3B). In the presence of 94 mM acetate, the dissociation constant for the enzyme-ADP complex is similar to that obtained in the absence of acetate (Table I). However, inclusion of 5.3 mM acetyl phosphate increased the value of the dissocation constant of enzyme-ADP complex to 2.8 mM, which is about 2.5-times higher than that obtained in its absence (Table I).

Both acetate and acetyl phosphate affected the protection rendered by ATP as shown in Fig. 4. The data are analyzed as indicated in Scheme II. According to a random sequential mechanism, both acetate and MgATP would bind to the enzyme to form an enzyme-acetate-MgATP complex. In the absence of Mg, it would be expected that an enzyme-acetate-ATP complex would form so that both reactants are at the active site of the enzyme at the same time. A plot of $1/K_{ob}$ against ATP at various fixed concentrations of acetate (Fig. 4A) gives a

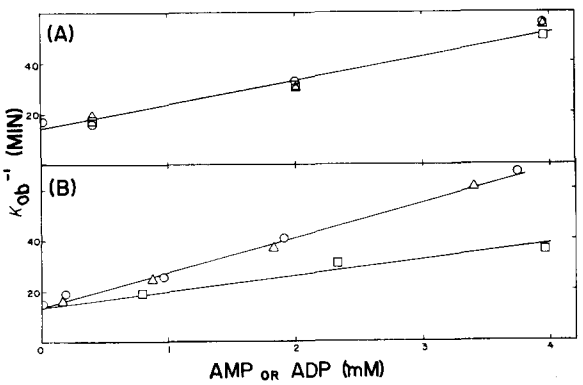

Fig. 3. Protection of acetate kinase by AMP and ADP from *N*-ethylmaleimide inactivation. The data are plotted according to Eqn. 5. (A) The inactivation was effected by 6.6 μM *N*-ethylmaleimide in the presence of various concentrations of AMP, either alone (○———○), with 0.38 M acetate (△———△), or with 9.6 mM acetyl phosphate (□———□). (B) The inactivation was carried out with 7.3 μM *N*-ethylmaleimide in the presence of various concentrations of ADP, either alone (○———○), with 94 mM acetate (△———△), or with 5.3 mM acetyl phosphate (□———□).

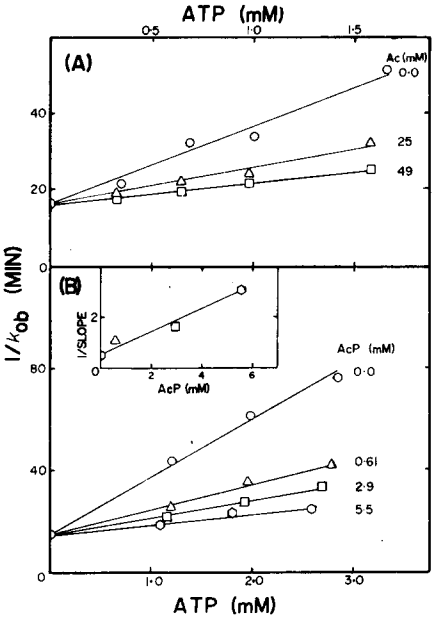

Fig. 4. Effect of acetate and acetyl phosphate on the protection of acetate kinase by ATP against *N*-ethylmaleimide inactivation. (A) The inactivation of acetate kinase was effected by 6.9 μM *N*-ethylmaleimide in the presence of varying concentrations of ATP at various levels of acetate (Ac) as indicated in the figure. The data are plotted according to Eqn. 7. (B) The inactivation of acetate kinase was effected by 7.1 μM *N*-ethylmaleimide in the presence of varying concentrations of ATP at various levels of acetyl phosphate (AcP) as indicated in the figure. The data are plotted according to Eqn. 8. Insert. The reciprocals of the slopes were plotted against acetyl phosphate concentrations.

series of lines which converge at the ordinate as predicted by Eqn. 7. Since Eqn. 7 contains dissociation constants of an enzyme-acetate-ATP complex, K_{ab} and K_{ba}, K_b cannot be calculated. In the case of acetyl phosphate and ATP, the simultaneous presence of a transferable phosphoryl group will cause these com-

TABLE I

DISSOCIATION CONSTANTS FOR VARIOUS ACETATE-LIGAND COMPLEXES

The dissociation constants for adenine nucleotides were calculated according to Eqn. 5 from Figs. 3 and 4. That for acetyl phosphate was evaluated according to Eqn. 8 from Fig. 4B.

Ligand	K_a or K_b (mM)
AMP	1.4
AMP (1.4 M acetate) *	1.2
AMP (9.6 mM acetyl phosphate)	1.2
ADP	1.1
ADP (94 mM acetate)	0.96
ADP (5.3 mM acetyl phosphate)	2.8
ATP	0.73 **
	0.81 ***
Acetyl phosphate	1.4

* Values in parentheses indicate the concentration of second substrate present.
** Calculated from Fig. 4A.
*** Calculated from Fig. 4B.

pounds to bind to the enzyme competitively against each other. The effect of acetyl phosphate on ATP protection is analyzed with Eqn. 8. As shown in Fig. 4B, acetyl phosphate competitively removes the protection rendered by ATP. A secondary plot of 1/slope vs. acetyl phosphate concentrations gives a dissociation constant of enzyme-acetyl phosphate complex of 1.4 mM. This value is comparable to that for AMP (Table I).

Discussion

E. coli acetate kinase is readily inhibited by sulfhydryl group specific reagents, *N*-ethylmaleimide, *p*-chloromercuribenzoate and mercuric chloride. All these reagents did not eliminate totally the catalytic activity of acetate kinase even with incubation time exceeding 10 times the half-time of inactivation, but rather reduced it to about 15% of the initial activity. Complete recovery of catalytic activity could be obtained by adding an excess of mercaptoethanol to *p*-chloromercuribenzoate, and mercuric ion inactivated acetate kinase while similar treatment of *N*-ethylmaleimide-modified enzyme gave no reactivation. These observations suggest inactivation results from mofidication of sulfhydryl group which apparently reacts completely with the sulfhydryl reagents. Due to the rapid rate of inactivation even with low concentrations of mercuric ion and *p*-chloromercuribenzoate (0.2 μM), accurate rate constants of inactivation were difficult to obtain and therefore the kinetic data are reported only for the slower inactivation reagent *N*-ethylmaleimide.

The sulfhydryl residue modified by the group specific reagents probably does not participate directly in the catalytic mechanism otherwise it would be expected that all catalytic activity would be lost upon modification. The properties of the modified enzyme are currently under investigation. It would be of interest to know whether the K_m or V of the enzyme is affected by the modification of the sulfhydryl group. Protection experiments as summarized in Table I indicate that adenine nucleotides prevent *N*-ethylmaleimide inactivation while acetate and acetyl phosphate do not affect the rate of inactivation. The protection rendered by ATP is not due to phosphorylation of the enzyme glutamate residue [4] for the following reasons: (a) Magnesium which is required for phosphorylation is omitted in this experiment; (b) adenylyl imidodiphosphate which cannot phosphorylate the enzyme behaved exactly the same was as ATP (Wong, S., unpublished data) and (c) acetyl phosphate which would also phosphorylate the enzyme in the presence of Mg did not render any protection under identical conditions. The inability of acetate and acetyl phosphate to protect the enzyme may be explained by the fact that the sulfhydryl group is away from the acetate and acetyl phosphate binding region. The presence of an adenine nucleotide, however, sterically prevents the sulfhydryl group from reacting with the group specific reagent. The different bonding sites for acetate or acetyl phosphate and ADP or ATP are consistent with the observation of Anthony and Spector [2] on the differential effect of mercurials on partial exchange reactions and the kinetic data which suggested that the reaction pathway is random sequential rather than ping-pong bi-bi [6,7].

The effect of acetate and acetyl phosphate on the protection rendered by adenine nucleotides may be explained in terms of a random mechanism

involving an enzyme-substrate ternary complex as shown in Scheme II. In the case of acetate and ATP, the existence of an enzyme-acetate-ATP complex is possible although no Mg is included. In our investigation on functional groups, we have found that acetate kinase was inactivated by phenylglyoxal. Such inactivation was prevented by ATP, ADP, AMP and acetyl phosphate. However, we have found that acetate has no effect on ATP protection, contrary to that observed in this work. Such data suggest the existence of an enzyme-acetate-ATP complex. The presence of the first substrate in the enzyme-substrate binary complex affects the binding of the second substrate to form the ternary complex. Thus K_{ab} is not equal to K_b, nor K_{ba} equal to K_a. Similar substrate interference to form a ternary complex probably exists between acetyl phosphate and ADP. Since acetate and acetyl phosphate do not affect the protection rendered by AMP (Fig. 3A), the presence of acetate or acetyl phosphate does not affect the binding of AMP to form an enzyme ternary complex. Thus it can be assumed that K_{ab} is equal to K_b and K_{ba} equal to K_a.

Relief of acetate kinase from ATP protection in the presence of acetyl phosphate cannot be explained by the formation of an enzyme-substrate ternary complex because such a complex probably does not form. It is conceivable that the binding site for the phosphate of acetyl phosphate overlaps with that of the γ-phosphate of ATP. Thus the two molecules will compete with each other for the enzyme. Fig. 4B shows the competitive kinetics between these two molecules in accordance with Eqn. 8.

Although the nucleotide protection against N-ethylmaleimide inactivation was studied in the absence of Mg where reaction cannot occur, some implication may be made on the mechanism of reaction in the presence of a metal ion.

The fact that acetate, acetylphosphate and AMP do not interfere with each other on binding to the enzyme indicates that the carboxyl group of acetate and the phosphoryl group of acetyl phosphate can accommodate the phosphoryl group of AMP. However, acetyl phosphate and acetate interfered with the binding of ADP and ATP, respectively. These data suggest that the phosphoryl group of acetyl phosphate and the carboxyl group of acetate are in close vicinity to the β-phosphoryl group of ADP and the γ-phosphoryl group of ATP, respectively, such that they interfere with each other. However, phosphoryl group transfer cannot occur because of the absence of a metal ion. The close approximation of the substrate binding domains suggests that direct transfer of the phosphoryl group between the participating substrates may occurs. Blatter and Knowles [10] have recently shown that the transfer of γ-phosphoryl group of ATP to acetate proceeded with inversion of configuration at the phosphorus and suggested a direct in-line phosphoryl group transfer reaction between enzyme-bound substrates. Our results presented here tend to support such a hypothesis.

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