EVIDENCE FOR THE FORMATION OF A Y-PHOSPHORYLATED GLUTAMYL RESIDUE IN THE ESCHERICHIA COLI ACETATE KINASE REACTION*

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Summary

Studies of phosphorylated Escherichia coli acetate kinase, which has been reduced with [^{3}H]sodium borohydride and subjected to acid hydrolysis, suggest that [^{3}H]α-amino-δ-hydroxyvaleric acid is formed with a 20-25% overall yield. This finding is compatible with the formation of a Y-phosphorylated glutamyl residue upon incubation of enzyme with acetyl-P.

In 1970, Anthony and Spector (1) identified reaction conditions for the phosphorylation of the Escherichia coli acetate kinase (ATP: acetate phosphotransferase, EC 2.7.2.1) by either of two of its substrates, ATP or acetyl-P. The phosphorylated enzyme was isolated by gel filtration and was relatively stable to hydrolysis. Later studies by these investigators (2) demonstrated that the degree of enzyme phosphorylation increases with the relative concentration of the phosphorylating agent. Moreover, the isolated phosphoryl-enzyme reacted quantitatively with ADP to form ATP, and with acetate ions to form acetyl-P in high yield. The U-shaped pH stability profile of the phosphorylated acetate kinase at 37° was fully consistent with the formation of an enzyme bound acyl phosphate intermediary, presumably at an aspartyl or glutamyl residue on this phosphotransferase. Consistent with this view was the observation that neutral hydroxylamine

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(0.1-1.0 M) at 26° reacts rapidly with the quantitative release of inorganic orthophosphate. Although the rates of phosphorylation of ADP and acetate by the phospho-enzyme intermediate were not established, these data strongly intimated the participation of a covalent enzyme-bound phosphoryl intermediary in the catalysis of the acetate kinase reaction. Purich and Fromm (3) found that initial rate studies in the direction of ATP synthesis, as well as the use of established product and competitive inhibitor protocols, are also consistent with the following double-displacement mechanism originally proposed by Koshland (4):

$$E + \text{acetyl-P} \rightleftharpoons EX \rightleftharpoons E\text{-P} + \text{acetate}$$

$$E\text{-P} + ADP \rightleftharpoons EY \rightleftharpoons E + ATP$$

where E·X and E·Y are Michaelis complexes and E-P is the covalent intermediary.

Recently, Degani and Boyer (5) introduced the use of a borohydride reduction method to detect an acyl phosphate intermediate in the sarcoplasmic reticulum adenosine triphosphatase reaction. Their studies demonstrated that the reduction of acyl phosphates to the corresponding ω-hydroxy α-amino acids is fully consistent with the known reactivity of acyl phosphates toward negatively charged nucleophiles, such as hydroxyl ion (6,7). In this respect, their approach was completely analogous to that of Solomon and Jencks (8) who identified an enzyme-γ-glutamyl coenzyme A intermediate in the succinyl-CoA : acetoacetate coenzyme A transferase reaction. We report here similar experiments providing strong evidence for the phosphorylation of a glutamyl residue of acetate kinase at the γ-carboxyl.

RESULTS

To demonstrate that an acyl-P intermediate is indeed formed during the phosphorylation of E.coli acetate kinase by acetyl-P, the sodium borohydride technique of Degani and Boyer (5) was employed. The basic

idea is that [^{3}H]$NaBH_4$ reduction of a γ-glutamyl-P, β-aspartyl-P, or some other amino acyl-P residue (which in the latter case might form by the participation of the C-terminus an active center) would lead to the formation of an [^{3}H]ω-hydroxy amino acid or an [^{3}H]amino alcohol. Phosphorylacetate kinase was prepared by the procedure of Anthony and Spector (1,2); however, mercaptoethanol was deliberately omitted lest the fraction of the corresponding symmetrical disulfide formed by air oxidation of this thiol compete with the acyl-P intermediate in the reduction reaction. Prior to reduction the remaining unbound acetyl-P and acetate were cleanly removed from the enzyme by Sephadex G-25 gel filtration as outlined by Anthony and Spector (1,2). This minimizes the amount of acetyl-P which would also compete in the reduction by forming tritiated ethanol (5). A suitable control lacking acetyl-P in the phosphorylation reaction mix was also subjected to [^{3}H]$NaBH_4$ reduction and further processing in an identical manner. In Fig. 1, we present the elution profile of the [^{3}H]sodium borohydride reduced enzyme over Sephadex G-25. To cleanly resolve any weakly bound tritiated material and boric acid, this preparation was passed over a similar column for a second time. As found by Degani and Boyer (5), not all of the tritium radioactivity associated with the reduced enzyme results from reduction of the acyl-P residue; rather, a sizeable fraction apparently is derived from the reduction of peptide bonds. For this reason, it is not possible to precisely estimate the estent of acyl-P reduction at this point in the study, and further analysis was necessary.

When the reduced enzyme was subjected to acid hydrolysis and electrophoresis at pH 1.9, essentially no net increase in the radioactivity in the regions corresponding to genuine homoserine or α-amino-δ-hydroxyvalerate was observed. Yet the sample corresponding to phosphoryl-acetate kinase did have a higher specific radioactivity than the control. Electrophoresis for a shorter period revealed a tritiated compound whose electrophoretic mobility was slightly less than synthetic homoserine lactone and corresponded to the position of genuine α-amino-δ-hydroxy valerolactone.

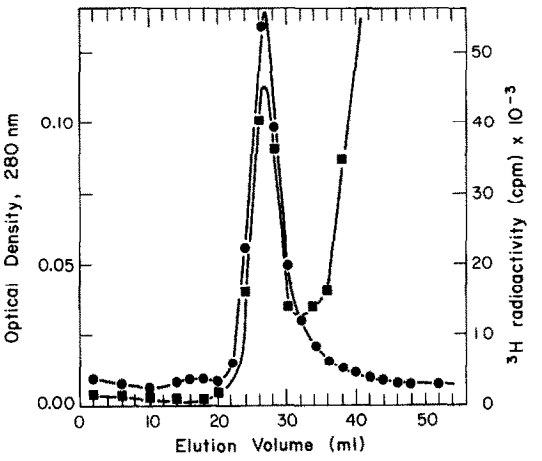


Fig. 1 : Elution profile of sodium borohydride-reduced phosphorylation acetate kinase from Sephadex G-25.
The final volume of 0.5 ml contained the following components: triethanolamine-HCl, 10 mM (pH 6.8); KCl, 20 mM; $MgCl_2$, 5 mM; glycerol, 4% (v/v); acetate kinase (Sigma), 1.5 mg; and acetyl-P (containing approximately 155,000 cpm [^{32}P]acetyl-P), 0.5 mM. A control was prepared containing no acetyl-P. Both were incubated for 1 hr at 23° after which an additional 87 mmoles of acetyl-P was added to the test sample. The enzyme samples were chromatographed over a 1 x 30 cm Sephadex G-25 column equilibrated with triethanolamine-HCl (pH 7.8) containing 20 mM KCl. Phosphoenzyme fractions were pooled on the basis of optical density at 280 nm and radiophosphorous activity. To a final volume of 2.5 ml enzyme was added 0.5 mg [^{3}H]sodium borohydride (specific radioactivity 5.7 mCi/mg) in 0.2 ml of 50% DMSO-water. Reduction mixtures were incubated 24 hours at 23° and subsequently applied to a 2 x 30 cm Sephadex G-25 column equilibrated with 10 mM K_2HPO_4 (pH 7.8) containing 100 mM KCl. Two ml samples were collected and monitored for optical density at 280 nm (●) and radioactivity of ^{3}H (■). The radioactivity values are corrected for background but not for counting efficiency.

In contrast, when the acid hydrolysates were brought to pH 12 at 60°C for approximately 1 hour and subsequently subjected to high voltage electrophoresis, the radioactivity peak exactly corresponded to the position of α-amino-δ-hydroxy valerate which would be anticipated were a γ-glutamyl-P intermediary formed in the phosphorylation reaction. These findings are presented in Fig. 2, and it is clear that the major peak corresponds to the α-amino-δ-hydroxy valerate. The horizontal bars corresponding to the α-amino-δ-hydroxy valerate and homoserine show that these amino acids are well resolved

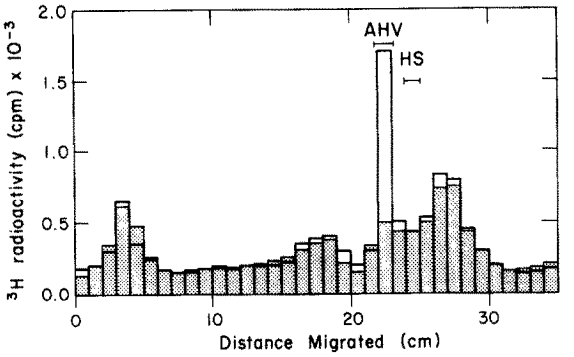


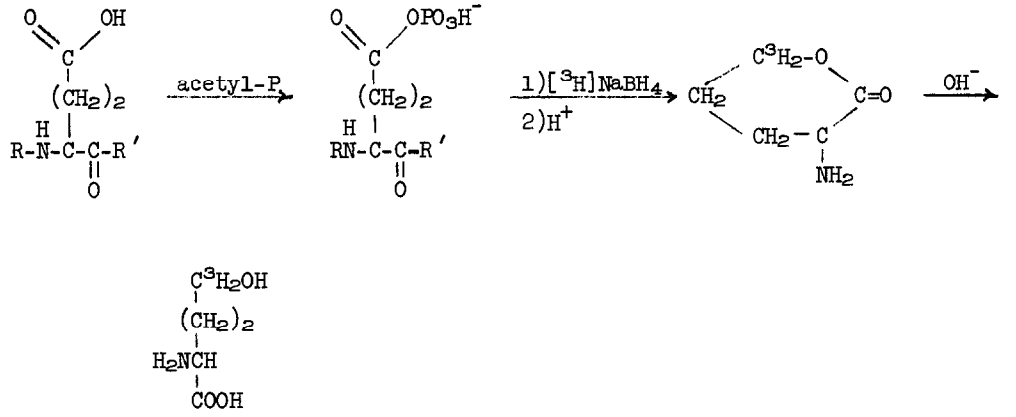
Fig. 2: High voltage paper electrophoresis of acid hydrolysates of [^{3}H]sodium borohydride-treated, phosphorylated acetate kinase after alkaline treatment to hydrolyze any cyclic lactones. After rechromatography of the reduced enzyme fractions over a second Sephadex G-25 column, the protein samples were lyophilized and subsequently hydrolyzed anaerobically in 6 N HCl at 110°C for 24 hours. The acid hydrolysates were twice lyophilized to remove any traces of HCl. To minimize the amount of ω-hydroxy amino acid present as the cyclic lactone, the samples were heated to 60° for 1 hour after the pH was adjusted to 12 with sodium hydroxide. High voltage electrophoresis in 7% formic acid and sample analysis was carried out as described elsewhere (5). The uncorrected tritium radioactivity is presented for the phosphorylated (☐) and control samples (▩). The bars marked AHV and HS refer to the positions of genuine α-amino-δ-hydroxy valerate and homoserine, respectively.

under these conditions. Thus it appears that the 6-membered cyclic lactone of glutamate was formed during acid hydrolysis; a similar finding of homoserine lactone was observed by Degani and Boyer (5).

To further verify that the radioactive compound observed in the high voltage paper electropherogram was α-amino-δ-hydroxy valerate, saturated solutions of homoserine and α-amino-δ-hydroxy valerate were separately added to aliquots of the radioactive protein hydrolysate. After two recrystallizations of these ω-hydroxy amino acid samples, it was observed that the specific radioactivity only remained constant in the sample containing the added α-amino-δ-hydroxy valerate.

DISCUSSION

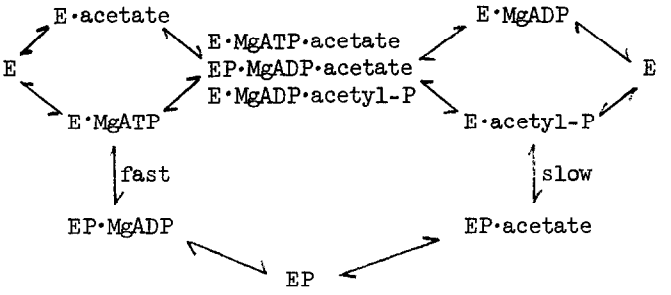
The following scheme is suggested by the results of the experiments presented in this report. Activity measurements of the acetate kinase



Scheme I

suggest that the enzyme is approximately 70% pure (1,2). On the basis of recovered [^{3}H]α-amino-δ-hydroxy-valerate and a counting efficiency of 45%, we estimate that approximately 20-25% of phosphorylated enzyme is reduced. This value compares rather favorably with the 65% efficiency of conversion of acetyl-P to ethanol under similar conditions (5). Similarly, Degani and Boyer (5) found that around 50% of phosphorylated sarcoplasmic reticulum ATPase was recovered as radioactive homoserine. No doubt, the efficiency of the reduction is affected by the availability of the acyl-P to the reductant as well as its stability to hydrolysis which is a competing reaction. Moreover, it has been recognized for some time that acid hydrolysis results in partial loss of hydroxy amino acids (9). Thus, it is clear that the recovered ω-hydroxy amino acid represents a rather substantial fraction of total phospho-enzyme.

It remains, however, to be demonstrated that this phosphoryl-enzyme intermediary is an essential facet of the catalytic mechanism. While considerable data weighs in favor of this proposal (1-3), recent kinetic studies at pH 7 suggest the reaction sequence may involve a random addition mechanism as shown in the following scheme, where the major pathway is



Scheme II

proposed to involve ternary complexes, and the formation of free phospho enzyme lies on a less active path (10). This proposal is supported in part by results of the use of chromium complexes of adenine nucleotides acting as competitive inhibitors (11) by the procedure of Fromm and Zewe (11). It is apparent that Janson and Cleland (10) were forced to consider this complex mechanism in an attempt to reconcile the exchange kinetic data (1,2) with their random addition model. On the other hand, the studies of Purich and Fromm (3) suggest that the Ping Pong mechanism best fits their data, and that the phosphoryl-enzyme is a reasonable and obligate intermediary. Thus, it will be necessary to determine if the rates of enzymic-phosphorylation and subsequent transphosphorylation to a suitable acceptor substrate are consistent with the active participation of the phosphoryl-enzyme intermediate. Further, it will be also of interest to clarify the factors accounting for the discrepancy in rate data (3,10). In any event, it is now clear that a Y-phosphorylated glutamyl residue of acetate kinase is formed during the phosphorylation of enzyme in the absence of a suitable phosphoryl acceptor.

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