Isolation and Characterization of Homogeneous Acetate Kinase from *Salmonella typhimurium* and *Escherichia coli*

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Acetate kinase from *Salmonella typhimurium* and *Escherichia coli* was purified to electrophoretic homogeneity. The amino acid compositions of both proteins were similar, and the apparent molecular weights were the same, about 40,000 for the putative monomers. The native proteins gave higher molecular weights, suggesting that the enzymes may be oligomers, perhaps with two polypeptide subunits. Steady-state kinetic studies were performed with the enzymes isolated from both organisms and the kinetic constants determined. The $K_m$ values were 0.07 and 7 mM for ATP and acetate, respectively. In contrast to earlier studies using less pure preparations, the homogenous enzymes from both strains were active only with acetate but not with propionate or butyrate. The enzyme activity was cold-labile, and the length of reaction time in the presence of Mg-ATP and acetate was dependent on protein concentration, suggesting that the monomer may not be catalytically active.

The enzyme was phosphorylated with $[\gamma-^{32}\text{P}]\text{ATP}$ and the phosphoprotein was isolated. Phosphoacetate kinase was capable of transferring the phosphate group to either ADP or acetate. The accompanying paper (Fox, D. K., Meadow, N. D., and Roseman, S. (1986) *J. Biol. Chem.* 261, 13498–13503) shows that the phosphoryl group of phosphoacetate kinase can also be reversibly transferred to Enzyme I of the phosphoeno1pyruvate:glycose phosphotransferase system.

Acetate kinase (EC 2.7.2.1, ATP:acetate phosphotransferase) catalyzes the transfer of the terminal phosphate of ATP to acetate to form the high energy mixed anhydride bond of acetyl phosphate. The reaction is reversible and requires a monovalent ($K^+$ or $NH^+$) and a divalent cation ($Mg^{2+}$ or $Mn^{2+}$) (1). Although the equilibrium lies far to the left (1–5), the reaction is generally written in the direction of acetyl phosphate formation:

$$\text{ATP} + \text{acetate} \rightarrow \text{Mg}^{2+}, K^+ \rightarrow \text{ADP} + \text{acetyl phosphate}.$$

Acetate kinase was discovered in 1944 by Lipmann in extracts of Lactobacillus delbrueckii (6). Subsequently, the activity was detected in Clostridium kluyveri, *Escherichia coli*, *Salmonella typhimurium*, yeast, and thermophilic bacteria (6–10). The enzyme is widely distributed among facultative and obligate anaerobes.

Acetate can be converted to the key metabolic intermediate, acetyl-S-CoA, by coupling acetate kinase with the enzyme phosphotransacytase (EC 2.3.1.8, acetyl-CoA:orthophosphate acetyltransferase). The overall equilibrium constant for the combined reactions is about 1 (11). Although the major pathway for acetate utilization in bacteria appears to be via these coupled reactions, it cannot be the sole pathway. Strains deleted in the structural genes for the two enzymes, ack and pta, respectively, can grow on acetate, albeit slowly (12).

Several additional physiological functions have been proposed for acetate kinase. It may be involved in the secretion of acetate (13, 14), in the synthesis of most of the ATP formed catabolically during anaerobic growth (15, 16), and acetyl phosphate may be a source of energy required for the uptake of some nutrients by whole cells (17–19).

Acetate kinase can be phosphorylated by ATP or acetyl phosphate to give the phosphoenzyme, with the phosphoryl group linked to the carboxyl group of a glutamyl residue (20). Whether the phosphoprotein is an obligatory intermediate in the reaction catalyzed by the enzyme is a matter of controversy. The phosphoprotein of the phosphoenzyme is transferred to ADP or acetate (1, 2, 22). However, steady-state kinetic data (23–25) suggest that the mechanism for the reaction is random sequential, thus leading to the conclusion that the phosphoenzyme is not in the main reaction pathway, a contention supported by stereochemical studies (26) of the reaction products.

Our interest in acetate kinase was stimulated by preliminary observations suggesting that it interacts in some manner with III$^{13}$, one of the phosphocarrier proteins of the phosphoeno1pyruvate:glycose phosphotransferase system. The latter system, designated PTS, has a variety of functions in the bacterial cell (27–30), one of which is the translocation and concomitant phosphorylation of its sugar substrates. Since the rate-determining step in cell growth is frequently the rate of sugar uptake, it is not surprising to find that the PTS is stringently regulated (30), although the molecular mecha-
Purification of Acetate Kinase

Acetate kinase is a potential regulator of the PTS. The preliminary data mentioned above suggested an association between the kinase and a PTS protein. The phosphorylated groups in both the phosphorylated PTS proteins and phosphoacetate kinase are linked via "high energy" bonds (1, 20, 30). Thus it appeared possible that a reversible phosphotransfer reaction could occur between phosphoacetate kinase and one or more of the PTS proteins. If such transfer reactions did occur, they would link sugar transport via the PTS to the tricarboxylic acid cycle via acetate kinase (31).

The hypothesis that PTS proteins and acetate kinase interact can only be rigorously tested with homogeneous preparations of the respective proteins. Although pure PTS proteins were available (32-34), homogeneous acetate kinase has not been previously isolated from Salmonella typhimurium and E. coli. Indeed, the conflicting interpretations of the data reported in the literature on the mechanism of the enzyme could be the result of working with partially purified preparations of the enzyme.

This paper describes the isolation and properties of homogeneous acetate kinase isolated from both S. typhimurium and E. coli. The accompanying report describes studies on the interactions between acetate kinase and the PTS proteins (31). With the pure enzyme it was possible to determine for the first time whether it could be phosphorylated autocatalytically, or whether it required another factor such as a protein kinase. Finally, the enzyme preparation described here should be useful in determining whether the phosphoprotein is an obligatory intermediate in the reaction catalyzed by acetate kinase. A preliminary report has been presented (35).

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Homogeneity**—Since many reports have appeared on the purification of acetate kinase (1-3, 21, 24, 64-67), but it had not previously been obtained in homogeneous form from enteric bacteria, several methods were used to assess the purity of the preparations described here.

The purified enzyme (from Step 8, Fig. 1) was subjected to SDS-polyacrylamide gel electrophoresis with different concentrations of both protein and polyacrylamide. Typical results are shown in Fig. 2B. Under all conditions tested, only a single polypeptide band was observed and this band corresponded to $M_r = 40,000$.

In a second set of experiments, the native protein was subjected to electrophoresis at pH 7.5 or at 8.9, and again only one protein band was detected (data not shown). Furthermore, duplicate samples of this gel were eluted (as described under "Experimental Procedures").

Finally, electrophoresis in polyacrylamide gels of different concentrations was employed (68). In this procedure, the native protein was subjected to electrophoresis at pH 8.9 in three different concentrations of acrylamide. Only a single protein band was observed (Fig. 2A).

The only impurities that would migrate identically to the

 kinetic under all of these conditions were those which had the same size, shape, and net charge at two different pH values (68).

**Amino Acid Composition**—The amino acid compositions of the final preparations were determined as described under "Experimental Procedures". Slight differences in the amino acid compositions of the proteins from S. typhimurium and E. coli were found (Table III). The largest difference was in the glycine content, with the E. coli protein containing 11 more glycine residues than that from S. typhimurium. These values were the average of at least four determinations on different hydrolysates and the difference is probably authentic. However, when contamination of a sample occurs during handling (e.g. dialysis), glycine is the most common contaminating amino acid.

The amino acid compositions of the acetate kinases isolated from Veillonella alcalescens (66) and Bacillus stearothermophilus (10) are also given in Table III for comparison. The amino acid compositions of acetate kinases from these strains are similar to those from E. coli and S. typhimurium except that the thermophilic bacterium B. stearothermophilus had

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*"Experimental Procedures," Figs. 3-6, and Tables I-III are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-4221, cite the authors, and include a check or money order for $10.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.*
measurable tryptophan and no cysteine. This lack of cysteine is characteristic of many thermophilic proteins and may be directly related to their thermostability (68, 69).

Determination of the amino acid sequence of acetate kinase from S. typhimurium by Edman degradation was not possible since the NH$_2$-terminal amino acid of acetate kinase was blocked. The nature of the blocking group was not determined.

The enzyme eluted from the Sephadex G-75 column is $7\%$ in this region. These molecular weights of different acrylamide concentrations by use of the methods of Ornstein and Davis (47, 48). Tube 1, 7.5% acrylamide; tube 2, 12% acrylamide; tube 3, 15% acrylamide. In B, under denaturing conditions, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (45, 46) using 12% polyacrylamide gels. The amount of acetate kinase (in micrograms) used was: lane 4, 3.5; lane 5, 7; lane 6, 15. Molecular weight standards were: E. coli RNA polymerase $\beta$-subunit, $M_w = 155,000$; bovine serum albumin, $M_w = 68,000$; RNA polymerase $\alpha$-subunit, $M_w = 39,000$; soybean trypsin inhibitor, $M_w = 21,500$.

Absorption Spectra of Acetate Kinase—Acetate kinase from E. coli and S. typhimurium were extensively dialyzed against 0.1% NaHCO$_3$, and the protein concentration was determined by microbiuret assay (42) with the dialysate as a reference buffer. The absorption spectra were obtained with a Perkin-Elmer (Model 557) dual beam spectrophotometer.

At an absorption maximum of 277 nm, the extinction coefficient of acetate kinase was $E_{277}^\text{Bohr} = 3.91$ (E. coli) and $E_{277}^\text{Bohr} = 3.85$ (S. typhimurium). These values reflect the content of tyrosine and phenylalanine of the proteins.

Molecular Weight of the Denatured Acetate Kinase—$^{14}$C-Labeled acetate kinase from E. coli or S. typhimurium was subjected to gel elution chromatography on Sepharose 6B in 6 M guanidine as described under "Experimental Procedures". Acetate kinase was eluted from the column between blue dextran and soybean trypsin inhibitor ($M_w = 27,000$). The molecular weight was determined by using the calculated distribution coefficient (0.146 for S. typhimurium and 0.161 for E. coli) and corresponded to a $M_w = 41,200 \pm 1,000$ for the protein isolated from S. typhimurium and $M_w = 40,000 \pm 1,000$ for the protein isolated from E. coli. The denatured, reduced, and alkylated acetate kinase was eluted in the linear portion of the calibration curve (Fig. 3A) and the accuracy of the column is $\pm 7\%$ in this region. These molecular weights of 40,000 agree well with those derived from the SDS-polyacrylamide gel electrophoresis.

Molecular Weight of the Native Protein—Gel elution chromatography of acetate kinase isolated from S. typhimurium was performed as described under "Experimental Procedures." The enzyme eluted from the Sephadex G-75 column either slightly ahead of or at the position of bovine serum albumin ($M_w = 68,000$). A plot of the logarithm of the molecular weight versus $K_v$ gave a molecular weight for acetate kinase of about 70,000 (Fig. 3B). The acetate kinase proteins isolated from E. coli or S. typhimurium or anaerobically grown S. typhimurium appear to have the same molecular weight (data not shown). Because 70,000 is very near the limit of the linear fractionation range for Sephadex G-75, the molecular weight determination was repeated with Ultrogel AcA 44, which has a broader fractionation range (10,000–130,000). The results were the same: acetate kinase in its native form behaves like a molecule that is as large or larger than bovine serum albumin.

Thus, the denatured protein exhibited an apparent molecular weight of 40,000 $\pm 1,000$ by both SDS-polyacrylamide gel electrophoresis and by chromatography in 6 M guanidine, whereas the native protein showed an apparent molecular weight of about 70,000. Acetate kinases isolated from other sources also show a difference between the molecular weight of the protein under denaturing and non-denaturing conditions (1, 10, 65, 70–73), which suggests an associating system of apparently identical monomers.

Cold Lability of Acetate Kinase—Homogeneous acetate kinase that had been stored at or below 0 °C exhibited a lag of 1–3 min before the maximal initial velocity was reached. This property of cold lability was found both by Anthony and Spector (1, 22) and Webb et al. (58) using partially purified acetate kinase from E. coli. Prior incubation of the enzyme at room temperature for 1–2 h eliminated this lag (data not shown, and see Ref. 1). Another aspect of this behavior, not mentioned by earlier workers, is that the length of this lag decreases as the concentration of acetate kinase is increased. However, even when higher levels of protein were used, a lag period was still observed (about 1 min) unless the protein was first activated by incubation at room temperature (data not shown). All of these results are consistent with the hypothesis that acetate kinase is an associating system and that the monomer is catalytically inactive. It is of particular interest to note that Enzyme I of the PTS exhibits the same type of behavior (82).

Metal Requirements of the Acetate Kinase Reaction—All known kinases require divalent cations, and acetate kinase is no exception. As reported by Van Campen and Matrone (74), Rose (2), Anthony and Spector (1, 22), and others, acetate kinase requires Mg$^{2+}$ and is inhibited by Na$^+$ and Li$^+$. For example, when NaCl or LiCl were present in the incubation mixture at final concentrations of 8.8 mM, the formation of acetyl phosphate was inhibited 50 and 62%, respectively. The homogeneous proteins from E. coli and S. typhimurium showed the same requirements and specificities: Mn$^{2+}$ was as effective as Mg$^{2+}$ when used at a concentration equal to that of ATP, and CaCl$_2$ could not substitute for MgCl$_2$.

One difference between the homogeneous enzyme and the crude preparations appears to be the ratio of Mg$^{2+}$:ATP required for optimal activity. Anthony and Spector (22) found the highest activity at a Mg$^{2+}$:ATP ratio of 2:1. They concluded that the actual substrate of acetate kinase was the Mg-ATP chelate, as with most kinases. The authors suggested that perhaps Mg$^{2+}$ itself was needed for enzyme activity. When we did the same experiment with partially purified acetate kinase from E. coli K235, we obtained similar results: optimal activity occurred at a Mg:ATP ratio of 1.5:1. However, when we determined this ratio using the homogeneous enzymes from both E. coli and S. typhimurium, we found the optimum ratio was 1:1. This discrepancy may be because the last step of the enzyme purification in this work used a buffer
containing 8 mM MgCl₂, and the enzyme was also stored in this buffer. In other words, some Mg²⁺ needed to activate the enzyme may have been bound to the protein.

**Optimum pH**—The pH optimum of the acetate kinase reaction was measured in the direction of acetate phosphate synthesis. The shape of the pH curve and its optimum, pH 7.5 (data not shown), are in good agreement with that obtained by Rose et al. (2, 3), who used a partially purified preparation from E. coli. It differs, however, from the results obtained by Anthony and Spector (1, 22). They reported a pH optimum of about pH 6.4, measured in the direction of ATP synthesis and a slightly more acidic optimum for acetyl phosphate synthesis, although the shape of the curve was similar. There is no obvious explanation for these differences. They may result from differences in the conditions of the assays, in the pH optima of the reaction in each direction, in the enzymes due to genetic variation or modification during purification or growth, or impurities in the earlier preparation.

**Substrate Specificity**—Substrate specificities of the homogeneous enzyme were tested with propionate and butyrate, analogues of acetate previously reported to be active as phosphate acceptors (1–3, 75). In contrast to the earlier results (1), neither propionic acid nor butyric acid were active at concentrations similar to or greater than those required for saturation by acetate (both the spectrophotometric and the colorimetric assays). Our results agree with those obtained by other workers using acetate kinase isolated from other bacterial strains (71) but are in contrast to the results reported with E. coli (1, 3, 75). However, propionic acid is a competitive inhibitor of homogeneous acetate kinase with respect to acetate (data not shown). These differences can be explained by an impurity in the older preparations that changed the substrate specificity of the enzyme, or by a contaminating kinase(s) active with propionate and butyrate.

The nucleotide specificity of homogeneous acetate kinase from S. typhimurium was studied and found to agree well with that obtained for acetate kinases from E. coli and other bacteria (10, 64, 70, 71) and mycoplasma (72). All three of the nucleotides tested were active, although the relative apparent $V_{\text{max}}$ with ITP (77%) was less than those with ATP (100%) and GTP (125%). Since ATP and GTP are excellent substrates of the enzyme, GTP was used in studies on the interaction between acetate kinase with the PTS (31).

The apparent $K_m$ values for the nucleotides, determined at single "saturating" concentration of acetate, were 0.94 mM for Mg-ATP, 1.10 mM for Mg-GTP, and 0.78 mM for Mg-ITP. These are quite similar to those obtained by Anthony and Spector (1, 22), although the relative $V_{\text{max(app)}}$ values differ in that they found that GTP was only 70% as effective as ATP.

**Determination of Kinetic Constants**—Initial velocities at different concentrations of the substrates were determined as described under "Experimental Procedures." Lineweaver-Burk and Hanes-Woolf (76) plots were used to analyze the initial velocity data. No evidence of nonhyperbolic behavior was seen. The kinetic constants were determined graphically from secondary plots (Lineweaver-Burk or Hanes-Woolf) of the initial velocity data. Furthermore, there was good agreement between the values for the kinetic constants calculated with all of the plotting methods used.

The $V_{\text{max}}$ values for the forward and reverse reaction are 2000 and 2600 μmol/min/mg, respectively, measured at 30 °C. The kinetic constants for the homogeneous acetate kinase from both E. coli and S. typhimurium were the same within experimental error. The $K_m$ values found were for: acetate, 7.0 mM; Mg-ATP, 0.07 mM; acetyl phosphate, 0.16 mM; and Mg-ADP, 0.50 mM. These values agree well with those of Janson and Cleland (23) and differ considerably from the apparent kinetic constants reported by some earlier workers (1–3, 10, 62, 64, 68, 74, 75) and are much closer to the concentrations of the substrates found in bacterial cells (18, 73).

Of great interest to us were the patterns observed in the primary plots of the initial velocity data. In our hands, homogeneous acetate kinase showed the same behavior observed by previous workers (23, 24, 75). Initial velocity plots for the reaction either in the direction of ATP or acetyl phosphate synthesis show patterns of lines which are characteristic of a mechanism involving a ternary complex or a mechanism that includes a ternary complex such as that suggested by Purich and co-workers (25) or Janson and Cleland (23), where a covalent intermediate can be formed but may not be in the major pathway of the reaction.

The effects of the products of the forward and reverse reactions on the initial velocities of these reactions were studied to help elucidate the mechanisms of acetate kinase and to determine whether, in some cases, product inhibition plays an important role in the kinetics of this enzyme. Acetate kinase from both E. coli and S. typhimurium was studied, and the type of inhibition and the inhibition constants were found to be the same or similar to those found by Janson and Cleland (23), and support a random sequential mechanism or a mechanism such as that suggested by these workers.

**Phosphorylation of Acetate Kinase by $\gamma$-32P]ATP**—Both Anthony and Spector (22) and Webb et al. (58) succeeded in phosphorylating partially purified acetate kinase and in isolating the phosphoprotein. In our attempts to phosphorylate acetate kinase, we tested the conditions used by both groups with slight modifications. Under these conditions, homogeneous acetate kinase from both E. coli and S. typhimurium was successfully phosphorylated. An elution profile of the phosphoprotein from the Sephadex G-25 SF column is shown in Fig. 4A. The phosphoprotein was eluted in the void volume of the column, well separated from the unreacted ATP. The column buffer was 20 mM potassium pyrophosphate, pH 8.6, containing 10 mM β-mercaptoethanol. (The use of pyrophosphate was recommended by Anthony (1), to prevent binding of ATP to the enzyme.) Two experiments were performed to verify that the 32P in the labeled protein was not simply $\gamma$-32P]ATP noncovalently bound to the enzyme. After isolation from the gel filtration column, a portion of the labeled protein fraction was applied immediately to thin layer chromatography plates (polyethyleneimine cellulose) and subjected to chromatography using System I described under "Experimental Procedures" (Fig. 4B). None of the radioactivity was found in the ATP spot; the majority of the counts were associated with the enzyme (at the origin) or with inorganic phosphate. Also, a double-labeled experiment was performed. Acetate kinase was phosphorylated in the presence of [3H]ATP and $\gamma$-32P]ATP of equal concentrations and specific activities (as count/min/mmol). The phosphoenzyme was isolated as described and a sample of the fraction was subjected to thin layer chromatography on polyethyleneimine cellulose as described above. Only background 3H counts were found associated with the phosphoenzyme (Fig. 4C). Therefore, the association of 32P with the enzyme appears to be covalent and involves neither ATP, ADP, nor AMP.

One of the difficulties in isolating phosphaacetate kinase was variability in the extent of phosphorylation of the enzyme. A summary of a number of phosphorylation experiments is shown in Table IV. Acetate kinase isolated from both E. coli and S. typhimurium exhibited this variable phosphorylation. These findings are similar to those of Webb et al. (58). To
date, only Anthony and Spector (1, 22) have reported reproducible phosphorylation of acetate kinase. An examination of the data in Table IV as well as those of Webb et al. (58) shows no definite pattern that can account for this variability. Some conditions are obviously unsatisfactory, such as performing the chromatography on the gel filtration column at room temperature (Experiments 2 and 3, Table IV) and use of pH 7.0 buffer. Increasing the molar ratio of ATP to acetate kinase or increasing the final concentration of ATP does not always result in greater incorporation of $^{32}$P (Experiments 5, 7, and 8, Table IV). However, experiments to determine the rate of phosphorylation in which a sample of the reaction mixture was treated with EDTA to stop the reaction and immediately applied to a thin layer chromatography plate and chromatographed (System II) showed that as much as 75% of the enzyme could be phosphorylated at 5 s; therefore, the variability of the extent of phosphorylation is certainly dependent in large part on the instability of the phosphoenzyme and perhaps by slight contaminants in the media (such as trace metals) which vary from one experiment to another and which can affect the rate of spontaneous hydrolysis of the phosphoprotein.

**Chemical Competence of Phosphorylated Enzyme**—In order to demonstrate that the phosphorylation occurred at the active site, experiments were performed to determine whether the phosphoryl group could be transferred from the phosphoenzyme to ADP and acetate. When the phosphoenzyme was incubated with ADP, $^{32}$P counts were transferred to ADP. The extent of transfer from the phosphoenzyme was minimal about 30%. When corrected for the hydrolysis (43–46%) of the phosphoprotein, about 50% of the $^{32}$P from the $^{32}$P phosphoenzyme was transferred to ADP within 1 min.

Attempts to demonstrate the transfer of $^{32}$P to acetate were hindered by the lack of resolution between inorganic phosphate and acetyl phosphate on the thin layer chromatography (polyethyleneimine cellulose) plates. Although the conditions of chromatography used were those previously described (1), reproducibility of the results was not sufficient to determine stoichiometry. Semi-quantitative estimates of phosphate transfer were obtained, however, as indicated in Fig. 5. Incubation of the $^{[32]P}$ phosphoprotein with acetate and MgCl$_2$ caused the concomitant loss of radioactivity from the phosphoenzyme at the origin and its appearance in a spot which migrated with the mobility of acetyl phosphate.

The extent of transfer of the $^{32}$P was as high as 50% and was dependent upon the presence of both Mg$^{2+}$ and acetate (Fig. 5B). Incubation of the phosphoprotein in the buffer without acetate or Mg$^{2+}$ resulted in 50–70% hydrolysis of the phosphate bond of the phosphoenzyme.

**Nature of the Phosphoryl Linkage in the Phosphoenzyme**—Preliminary characterization of the enzyme-phosphate bond of acetate kinase was accomplished by studying its stability as a function of pH and its susceptibility to hydrolysis by hydroxylamine. The stability of the phosphoenzyme at 37 °C in buffers of various pH values is shown in Fig. 6. The linkage is acid-labile and more stable at alkaline pH values, with the region of greatest stability between pH 9 and 13. The phosphoenzyme is very unstable in 1 N KOH (data not shown, Ref. 1) where phosphoramidates (e.g. 3-phosphohistidine) are stable (77) and it is susceptible to hydrolysis by neutral hydroxylamine (data not shown), suggesting that phosphoacetate kinase is an acylphosphate. These data agree with those of Anthony and Spector (1, 21, 22) and Todhunter and Purich (20), who found evidence for an acylphosphate linkage in the phosphoprotein isolated from partially purified preparations of acetate kinase.

**Kinetic Competence of the Enzyme**—If the phosphoenzyme is an obligatory intermediate in the reaction, the rate of phosphorylation of the protein by ATP should be at least equal to or greater than the catalytic rate of the reaction (i.e. the rate of formation of acetyl phosphate by acetate kinase in the presence of acetate and ATP). Therefore, preliminary experiments were conducted with Drs. Narlin Beatty and M. Daniel Lane (The Johns Hopkins University) with a rapid quench apparatus similar to that described by Ballou and Palmier (61). Using $^{[32]P}$ATP and the homogeneous enzyme, incubations were conducted from 10 ms to 5 s at 22 °C, the reactions were quenched, and the $^{32}$P-phosphoprotein was measured. The apparent rate constant for protein phosphorylation was about one-third of the $k_{cat}$ for acetyl phosphate formation at this temperature. It must be emphasized that this is not only a preliminary value, but, because of technical problems involved in making the measurements, it is a minimal value. Nevertheless, the apparent rate constant for phosphorylation of the kinase is well within an order of magnitude of the catalytic rate constant. In view of the significance of

### Table IV

**Purification of Acetate Kinase**

| Experiment | pH of reaction mixture | MgCl$_2$ | ATP | Ratio of $[\gamma-^{32}P]$ | pH of isolation column buffer | Temperature of chromatography | Phosphorylation of acetate kinase (mol/mol) |
|------------|------------------------|----------|-----|-------------------------|-------------------------------|-----------------------------|--------------------------------------|
| 1          | 7.2                    | 5.0      | 1.3 | 131:1                   | 7.0                          | 22                          | 22.6                                 |
| 2          | 7.2                    | 5.0      | 0.69| 78:1                    | 7.0                          | 22                          | 0                                    |
| 3          | 7.2                    | 5.0      | 1.3 | 156:1                   | 7.0                          | 22                          | 6.2                                  |
| 4          | 7.2                    | 5.0      | 1.3 | 131:1                   | 8.6                          | 4                           | 28.0                                 |
| 5          | 7.2                    | 5.0      | 1.3 | 156:1                   | 8.6                          | 4                           | 42.0                                 |
| 6          | 7.4                    | 8.5      | 1.0 | 480:1                   | 8.5                          | 4                           | 48.0                                 |
| 7          | 7.4                    | 8.5      | 2.0 | 406:1                   | 8.5                          | 4                           | 27.0                                 |
| 8          | 7.4                    | 8.5      | 1.0 | 760:1                   | 8.5                          | 4                           | 25.0                                 |
these two values with respect to the mechanism of action of the enzyme, more extensive studies will be conducted to assess accurately the relative rates of the two reactions.

**DISCUSSION**

This paper reports the isolation of homogeneous acetate kinase from the enteric bacteria, *S. typhimurium* and *E. coli*. The two preparations showed the same molecular weights (about 40,000 for the monomers) and similar amino acid compositions. These results are expected, since the two bacterial species are closely related, and other proteins from these two organisms, such as the PTS proteins, HPr and III$^{abc}$, are very similar or identical (30). The presence of little or no tryptophan in the two kinase preparations is not unique, since the same result has been obtained with acetate kinases isolated from mesophilic bacteria (Table III).

As is evident from the many papers published on this subject (1–3, 21–25, 61–72), acetate kinase has been very resistant to purification when enteric bacteria such as *E. coli* were used as a source of the enzyme. One of the most persistent problems is the extreme lability of the kinase after partial purification, as in Step 5 of this procedure. At Step 4, the enzyme is relatively stable to storage when frozen at or below \(-20^\circ\text{C}\). However, after the DEAE-Sephadex column (Step 5), we found it essential to continue as rapidly as possible, even in the presence of glycerol, which has a marked stabilizing effect on the enzyme (1, 39). The homogeneous preparations were stable to storage.

The effect of glycerol on the enzyme is important in a practical sense, but it may also have physiological implications. The mechanisms by which glycerol stabilizes enzymes, particularly those that are cold-labile, are unknown (78, 79), but it has been suggested that glycerol is effective because it lowers the dielectric constant of the medium (1) and acetate kinase may prefer a hydrophobic environment. Both acetate kinase and phosphotransacetylase can associate to some extent with bacterial membranes (80), and acetate kinase with the highly purified membrane vesicles (81) isolated in this laboratory. The membrane-associated activity did not noticeably differ from the soluble enzyme in preliminary kinetic studies (data not shown).

Acetate kinases isolated from various bacterial species have been reported to form oligomers (10, 65, 70, 72). The present studies suggest that the enzymes from the enteric bacteria behave similarly. Two independent methods give a molecular weight for the fully denatured monomer of 40,000, whereas the native protein elutes from a gel column as a protein fraction of $M, = 68,000–70,000$, which would be expected for an associating system in rapid equilibrium. More definitive evidence is obviously required concerning the putative association, and we are now attempting to collect this information. One important aspect of the current experiments will be to determine the reasons for the cold lability of the enzyme and for the lag period in the reaction when it is warmed to 36 °C. Both of these properties are shown by Enzyme I of the PTS (32, 82, 83), a self-associating monomer-dimer system whose catalytically active form may be the dimer.

Partially purified acetate kinase (1, 21, 22, 58) was phosphorylated when incubated with ATP or acetyl phosphate. However, these preparations were not more than 20% pure (based on specific activities relative to the homogeneous protein), and therefore it is conceivable that the phosphorylation reactions were catalyzed by contaminating protein kinases or that acetate kinase phosphorylated other proteins in the preparations. The present studies show, however, that the homogeneous proteins are phosphorylated autocatalytically. As in earlier work (Ref. 58; however, see Ref. 1), the extent of phosphorylation was highly variable, depending on conditions of incubation and separation of the phosphoprotein from excess substrate, and never exceeded 0.4 mol/mol of enzyme monomer. We do not know the reason for the limited extent of phosphorylation, and the explanation may be relatively trivial (e.g., instability of the phosphoenzyme during isolation). However, more important and subtle explanations (e.g., the association-dissociation referred to above) are possible.

The phosphoryl linkage to the protein has all of the properties expected for an acylphosphate rather than a phosphoramidate, phosphoserine, phosphothreonine, or phosphotyrosine (84–88). This conclusion is based on the stability of the phosphonyl linkage as a function of pH, its reactivity with hydroxylamine, and the lack of reactivity with pyridine. The phosphonyl linkage does not show precisely the same pH stability profile as acetyl phosphate, but in at least one instance, this result has been attributed to the local environment of the acylphosphate group in phosphoproteins (89).

The homogeneous phosphoenzyme can transfer the phosphoryl group to ADP and to acetate (see “Results”) and catalyzes the expected phosphate exchange reactions between substrate-product pairs (data not shown). These results are consistent with a model for the enzymatic reaction involving the phosphoenzyme as an obligatory intermediate (1, 21, 22) in the overall reaction. This interpretation is supported in part by our preliminary attempts to compare the rate of protein phosphorylation with the overall catalytic rate of the complete reaction (briefly described under “Results”). An extensive study of this type may show that the rate of kinase phosphorylation is sufficiently rapid so that the phosphoprotein can participate in the overall reaction.

There is considerable evidence against a model for the reaction which requires the phosphoenzyme to be an obligatory intermediate. Stereochemical experiments (26) indicate that an odd number of phosphate transfer reactions occur in the overall reaction because there is an inversion in the configuration of the transferred phosphate. If the phospho- transfer sequence was ATP $\rightarrow$ kinase $\rightarrow$ acetate, then two transfers would have taken place and would have given a different result. However, the stereochemical results should be reviewed in view of the possibility that the catalytic unit may be a dimer, in which case an odd number of phosphate transfers are possible with the phosphoenzyme as an intermediate. Spector (90) has offered another explanation for the stereochemical results involving a triple displacement mechanism on the surface of the phosphoenzyme.

Kinetic data from some other laboratories using partially purified preparations of the kinase (23, 24) also argue against the phosphoenzyme as an important kinetic intermediate. Our results agree with the earlier reports. That is, the data suggest a sequential rather than the well-behaved ping-pong mechanism required by the phosphoenzyme model. Under certain conditions, however, sequential mechanisms can appear to be ping-pong and vice versa. For instance, a ping-pong mechanism, where the product of the first reaction is only slowly released from the enzyme or where it cannot be released until the second substrate binds, would yield intersecting lines in the Lineweaver-Burk plots, suggesting a sequential mechanism (a ping-pong mechanism should give parallel lines).

Complex models have been proposed for the mechanism of the reaction which attempt to assimilate all of the apparently conflicting observations into one scheme (23–25, 90). The phosphoenzyme has been incorporated into these models, but the major flux of the reaction components does not involve the phosphoprotein, and in this way the models satisfy the
kinetic and stereochemical data.

The availability of the homogeneous enzyme will significantly aid in resolving the question of mechanism, first by permitting physical studies on the protein to determine whether it is, in fact, an associating system and the nature of the catalytic unit, and second, by permitting studies on the protein and substrate phosphorylation reactions using rapid quench kinetics.

It is, of course, entirely possible that the phosphoprotein plays no role in the catalytic reaction but that it has other physiological functions. The accompanying paper [31] shows, in fact, that there is phosphotransfer between phosphoacetate kinase and Enzyme I of the PTS, a reaction that may be important in regulating sugar uptake by the cell.

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Purification of Acetate Kinase

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Supplementary Material to "Isolation and Characterization of Homogeneous Acetate Kinase from Micrococcus lysodeikticus and Bacillus subtilis"

Protein Stability

All commercial reagents were of the highest available purity. Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymethyl)aminomethane, Tris(hydroxymeth
Introduction

- The second set of conditions for phosphorylation was adapted from the work of Webb et al. (18). In a volume of 100 µl, the phosphorylation reaction contained 40 mM PIATP, 2.4 µM [γ-32P]ATP (1.3 × 10⁶ cpm/µg; 137 MBq/µg), 0.13 µM PEP, 100 mM KCl, 20 mM Tris-Cl (pH 7.4), and 10% glycerol. The reaction was incubated at room temperature for 15 min and stopped by the addition of sodium citrate. The reactions were subjected to gel filtration as described below.

Purification of Phosphorylated Acetate Kinase

- Phosphorylated Acetate Kinase Assay - Sephadex G-15 was equilibrated with Buffer A (pH 7.4) containing 10 mM PEP and 100 mM KCl. The column was expanded with Buffer A, containing 40 mM PIATP, 10 mM PEP, and 0.13 µM [γ-32P]ATP. The active fractions were pooled and concentrated in the Pellicon apparatus as described above, except that the buffer was changed to Buffer C.

Purification of Acetate Kinase

- Phenylphosphate Chromatography on DE-52 - Solution A was equilibrated with Buffer C containing 0.13 µM [γ-32P]ATP. The resin was then placed in buffer C and extensively washed. A phosphoamidite (P) on GST was affixed to the resin (approximately 500-800 bed volumes) and washed overnight with buffer C.

- The concentrated pool from the G-75 column (Step 4) was diluted with 2 volumes of Buffer C and transferred to the column at a flow rate of 2 ml/min. After 3 days, the column was washed extensively with Buffer C to remove all radioactivity, followed by Buffer C containing 0.13 µM [γ-32P]ATP.

- The column was eluted with 3 volumes of Buffer C, followed by Buffer C containing 0.13 µM [γ-32P]ATP. The active fractions were pooled and concentrated in the Pellicon apparatus as described above, except that the buffer was changed to Buffer C.

- The product of the second gel filtration step was again subjected to affinity chromatography on DE-52 to eliminate contaminating proteins which were eluted after acetate kinase. The active fractions were pooled and concentrated in the Pellicon apparatus as described above, except that the buffer was changed to Buffer C.

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Purification of Acetate Kinase

Table I. Purification of acetate kinase from L. lactis subsp. lactis

| Purification Step | Total Protein (mg) | Total Activity (nmol/hr) | Specific Activity (nmol/hr/mg) | Purification | Recovery |
|------------------|-------------------|--------------------------|-------------------------------|-------------|----------|
| 1. Crude extract | 3,265             | 92,100                   | 28.3                          | 1           | 100      |
| 2. Phenylmethylsulfonyl fluoride | 3,265 | 193,000 | 5.98 | 1 | 100 |
| 3. DEAE-cellulose column (KCl elution) | 144 | 1,165 | 8.10 | 2 | 57 |
| 4. DEAE-cellulose column (KCl elution) | 128 | 1,000 | 7.82 | 2 | 57 |
| 5. BioGel P-2 (0.5% KCl gradient) | 1,152 | 1,152 | 10.0 | 4 | 10 |
| 6. BioGel P-2 (0.5% KCl gradient) | 1,120 | 1,120 | 10.0 | 4 | 10 |
| 7. BioGel P-2 (0.5% KCl gradient) | 1,120 | 1,120 | 10.0 | 4 | 10 |
| 8. BioGel P-2 (0.5% KCl gradient) | 1,120 | 1,120 | 10.0 | 4 | 10 |
| 9. BioGel P-2 (0.5% KCl gradient) | 1,120 | 1,120 | 10.0 | 4 | 10 |

Table II. Purification of acetate kinase from E. coli

| Purification Step | Total Protein (mg) | Total Activity (nmol/hr) | Specific Activity (nmol/hr/mg) | Purification | Recovery |
|------------------|-------------------|--------------------------|-------------------------------|-------------|----------|
| 1. Crude extract | 1,138             | 29,000                   | 25.3                          | 1           | 100      |
| 2. DEAE-cellulose column (KCl elution) | 350 | 7,780 | 22.3 | 4 | 10 |
| 3. DEAE-cellulose column (KCl elution) | 350 | 7,780 | 22.3 | 4 | 10 |
| 4. BioGel P-2 (0.5% KCl gradient) | 880 | 80 | 88 | 11 | 10 |
| 5. BioGel P-2 (0.5% KCl gradient) | 880 | 80 | 88 | 11 | 10 |
| 6. BioGel P-2 (0.5% KCl gradient) | 880 | 80 | 88 | 11 | 10 |
| 7. BioGel P-2 (0.5% KCl gradient) | 880 | 80 | 88 | 11 | 10 |

Fig. 1. Determination of the molecular weight of acetate kinase under denaturing and non-denaturing conditions. Reduced and alkylated proteins of known molecular weight and acetate kinase from either L. lactis or E. coli were chromatographed on a Sepharose 2B column in 0.1% GES/0.01 M DTT as described in "Experimental Procedures." The molecular weights are plotted as a function of their FPLC elution volumes (fraction numbers 1-20). The proteins eluting at 10,000 daltons were calculated as acetate kinase (A). The samples were chromatographed on an 10% SDS-polyacrylamide gel and stained with Coomassie blue. The gel was scanned for radioactivity. The specific activity of the acetate kinase was determined by measuring the radioactivity in the gel bands corresponding to the molecular weight of 10,000 daltons.

Fig. 2. Effect of ATP on the activity of acetate kinase. The activity of acetate kinase was measured in the presence of varying concentrations of ATP. The assay was performed in a microcentrifuge tube containing 200 μl of 50 mM MOPS (pH 7.4) buffer, 100 μM [γ-32P]ATP, 10 μM MgCl₂, and 100 μM acetate. The reaction was initiated by adding 10 μl of enzyme solution and stopped after 5 min by adding 10 μl of 10% TCA. The sample was then spotted onto a Whatman P-81 paper and dried for 1 h. The radioactivity was determined by liquid scintillation counting.

Fig. 3. Separation of acetate kinase from other proteins. The samples contained acetate kinase and other proteins derived from E. coli. The proteins were separated by SDS-PAGE and stained with Coomassie blue. The gels were scanned for radioactivity. The specific activity of acetate kinase was determined by measuring the radioactivity in the gel bands corresponding to the molecular weight of 10,000 daltons.

Fig. 4. Characterization of acetate kinase with [γ-32P]ATP. The reaction mixture contained 200 μl of 50 mM MOPS (pH 7.4), 10 μM MgCl₂, and 100 μM acetate. The reaction was initiated by adding 10 μl of enzyme solution and stopped after 15 min by adding 10 μl of 10% TCA. The samples were then spotted onto a Whatman P-81 paper and dried for 1 h. The radioactivity was determined by liquid scintillation counting.
Fig. 5. Transfer of $^{32}$P from $^{32}$P-phosphoacetate kinase to acetate.

Phosphoacetate kinase (P-M) was prepared and isolated as shown in Fig. 4B. Immediately after isolation, samples from the fractions containing phosphoacetate kinase of the Sephadex G-25 column were used in the transfer experiment. Equal volumes of the phosphoprotein in the column buffer were mixed with a solution containing the necessary compounds so that in the final incubation mixture the concentrations were: 125 mM potassium acetate, pH 7.2; 10 mM MgCl₂; 10 mM glycerol; 120 mM NH₄Cl, and 10 mM DTE. The reaction was allowed to proceed for 10 min at room temperature. At the end of 60 min, the reaction was stopped by spotting 10 to 20 μl onto a thin layer chromatography plate and subjected to chromatography with System II (*Experimental Procedures*) at 4°C. Complete reaction mixture A, without MgCl₂. The control incubation without acetate gave the results identical to those shown in B.

Fig. 6. Stability of the phosphoprotein in phosphoacetate kinase as a function of pH. Phosphoacetate kinase was isolated as described in Fig. 4B. The phosphoprotein was immediately diluted into an equal volume of different buffers and incubated at 25°C for 15 min. Hydrolysis of the phosphoprotein was followed by the release of $^{32}$P (counts per min) from the origin after chromatography on a thin-layer chromatography plate in System I (*Experimental Procedures*). The values shown are those determined by diluting the identical volume of column buffer without enzyme to the same molarity and measuring the pH of the resulting solution. The final concentrations of the buffers at the different pH values are: pH 1.3; 40 mM HC₃O₂; pH 5.6, 7.0, and 8.0, 50 mM potassium phosphate buffer; pH 4.5, 50 mM potassium pyrophosphate buffer; pH 3.7, 6.1 M KCl; pH 5.6, 7.0, and 8.0, 50 mM potassium phosphate buffer; pH 12, 40 mM EDTA; pH 13, 100 mM KCl.