Citryl 1-Phosphate and the Citrate Cleavage Reaction*

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SUMMARY

Citryl 1-phosphate has been synthesized chemically. It is an active substrate of ATP: citrate lyase (EC 4.1.3.8). With coenzyme A present, the enzyme catalyzes the cleavage of citryl-1-P into oxaloacetate and acetyl-CoA. Starting with radioactive citryl-1-P, the products of cleavage have been isolated from incubation mixtures in the form of convenient derivatives which have been purified to constant specific activity. The identity of oxaloacetate as a cleavage product has been further confirmed through its specific oxidation of NADH in the presence of malate dehydrogenase (EC 1.1.1.37).

In 1953, Srere and Lipmann (1) discovered the enzyme ATP: citrate lyase which, with coenzyme A and ATP, catalyzes the irreversible cleavage of citrate

\[
\text{Citrate} + \text{ATP} + \text{CoA} \rightarrow \text{oxaloacetate} + \text{acetyl-CoA} + \text{ADP} + P_i
\]

Recently this enzyme was purified (2), and experiments on its mode of action have been described (3). More recently still, the enzyme has been specified as a site of metabolic regulation (4).

A reasonable chemical view of this complex reaction predicts that, at some stage, citryl-CoA must participate in it as a bona fide intermediate. This prediction was fulfilled in 1963, when Eggerer and Remberger (5) chemically synthesized citryl-CoA and proved that it is indeed a substrate of the enzyme. With this much settled, one is led then to ponder the role of ATP in this reaction. Does it react with citrate, directly or indirectly, to form citryl-1-P, which thereafter acts upon CoA to yield citryl-CoA? To test this idea we have synthesized citryl-1-P.

We find that, like citryl-CoA, it is an active substrate of ATP: citrate lyase. With CoA present, the enzyme easily transforms citryl-1-P into oxaloacetate and acetyl-CoA

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Citryl-1-P + CoA → oxaloacetate + acetyl-CoA + P_i (2)

In what follows we give evidence to validate Reaction 2.

Synthesis of Citryl-1-P, Trilithium Salt—To a suspension of 480 mg (2500 μmoles) of anhydrous citric acid in 15 ml of redistilled acetonalone is added (dropwise from a funnel) and over 15 min at room temperature a solution of 0.194 ml (2500 μmoles) of freshly distilled ethoxyacetylene in 5 ml of the same solvent. After 30 min the solid is completely dissolved. Occasionally the clear solution is yellow; it is easily decolorized with acid-washed charcoal. The infrared spectrum of the acetonalone solution displays strong bands at 1880 cm⁻¹ and 1800 cm⁻¹, signifying the presence in solution of an acid anhydride.

Triethylammonium phosphate (500 mg, 2500 μmoles) and 0.76 ml of redistilled triethylamine (5000 μmoles) are added directly and in rapid succession to the anhydride solution. The mixture is stirred magnetically at room temperature, and within 5 min the solid is dissolved. During this time the solution acquires a yellow color which is resistant to charcoal. The solution of triethylammonium citryl-1-P is placed in a dropping funnel and run slowly at room temperature into a stirring solution of 840 mg (7900 μmoles) of anhydrous lithium perchlorate in acetonalone. After 15 min the suspension is filtered and the solid washed successively with 30 ml of acetonalone and 50 ml of ether. Care is taken at this point to not let the solid dry to more than a thick slurry before placing it in a vacuum over phosphorus pentoxide. The yield of trilithium citryl-1-P, thus prepared, is usually about 95% of theory. The salt is deliquescent and faintly off-white in color. Each lot is freshly prepared and used within 48 hours since, even in a vacuum over phosphorus pentoxide, the compound is unstable. It gives a positive hydroxamate test with acidic hydroxylamine (6). For analysis, citric acid is assayed by the acetic anhydride-pyridine method (7, 8), and phosphorus by the method of Fiske and SubbaRow (9). The lithium content is determined with a Perkin-Elmer, model 290, atomic absorption spectrophotometer. A typical analysis follows

Trilithium citryl-1-P (C_{12}H_{10}Li_3O_{10}P)

Calculated: Citric acid 66.8, P 10.68, Li 7.2
Found: Citric acid 67.0, P 10.20, Li 7.0

Oxidation of NADH—Oxaloacetate, formed in the cleavage of citryl-1-P according to Reaction 2, becomes manifest through its power to oxidize NADH specifically in the presence of malate dehydrogenase

Oxaloacetate + NADH + H⁺ → l-malate + NAD⁺ (3)

The oxidation is followed optically, as in Fig. 1. Citryl-1-P effects the oxidation of NADH as rapidly as does an equivalent amount of ATP plus citrate. We note here that the molecular structure of citryl-1-P includes 1 asymmetric carbon atom, and that the synthetic compound must therefore be a mixture of δ and l isomers. Expectation is that only one of the isomers is enzymatically active. This is borne out in stoichiometric
experiments showing that an equivalent of synthetic citryl-1-P mediates the oxidation of only 0.5 eq of NADH. The same finding was made with synthetic citryl-coenzyme A (2). For this reason we have taken 2 μmoles of synthetic citryl-1-P to be enzymatically equivalent to the sum of 1 μmole each of ATP and citrate. Experimentally, we find that the use of 1 μmole of citryl-1-P results in a rate of oxidation that is about one-half that observed with 2 μmoles. The substrate dependency of activity displays Michaelis-Menten kinetics with an apparent \( K_m \) of \( 1.2 \times 10^{-3} \) M. Activity in the assay system of Fig. 1, with saturating concentrations of citryl-1-P, is proportional to enzyme concentration up to at least 0.06 unit of enzyme (66% pure). Citryl-1-P does not react with coenzyme A in the absence of enzyme.

Identification of L-Malate—To verify that l-malate is in fact formed from citryl-1-P through sequential working of ATP-citrate lyase and malate dehydrogenase (Reactions 2 and 3), we prepared 1,5-14C-citryl-1-P, and, after enzymatic action, isolated l-malate as the pure d-cinchonine salt (10). 1,5-14C-Citril-1-P was prepared from 1,5-14C-acetic acid as described above. ATP-citrate lyase was isolated from rat liver by following the procedure of Inoue et al. (2) to the stage where the enzyme was purified 15-fold. The incubation mixture contained (in micromoles): dithiothreitol, 20; sodium fluoride, 1; magnesium chloride, 20; CoA, 1.9; pyridine-2,4-diketopiperazine (2 units); NADH, 2.5; Tris, pH 8.4, 400; malate dehydrogenase, 2.4 units; 1,5-14C-citryl-1-P, 20, 2 \( \times \) 10^6 cpm; ATP-citrate lyase, 6 units, in 2.7 ml. The solution was incubated at 37° for 30 min. Reaction was stopped by addition of 0.4 ml of 20% perchloric acid. After 45 min in ice, denatured protein was removed by centrifugation. To the supernatant solution was added 0.24 ml of 4 M potassium chloride and, after 2 hours at 0°, potassium perchlorate was removed by centrifugation. To remove nucleotides, the supernatant solution was stirred with acetic-washed charcoal for 15 min and filtered. The filtrate was brought to pH 7.5 with potassium hydroxide, carrier L-malic acid (6.7 mg, 50 Mmoles) was added, and the solution was applied to a column (1 by 10 cm) of Dowex 1-X8 (20/40 mesh) in the formate form. The solution was eluted stepwise with 10 ml each of 1.5 M, 2.5 M, and 4.9 M formic acid (11). Malic acid appears in the middle fraction. This was concentrated to dryness in a flash evaporator, and the residue was dissolved and concentrated twice more to dryness from acetone. For ease of crystallization, the size of the residue was enlarged with 40 mg (300 μmoles) of additional L-malic acid in acetone. The solution was brought to reflux, 110 mg (375 μmoles) of d-cinchonine were added, and refluxing continued for 30 min. After cooling overnight the crude crystals of d-cinchonine-L-malate were recrystallized three times from the minimal volume of water to constant melting point (195-196°, uncorrected) and constant specific activity.

Identification of Acetyl-CoA—This was achieved by transfer of the acetyl group of acetyl-CoA (Reaction 2) to the amino function of p-nitroaniline in the presence of arylamine acetyltransferase (EC 2.3.1.5) (12)

Acetyl-CoA + p-nitroaniline → p-nitroacetanilide + CoA (4)

Labeled p-nitroacetanilide, prepared in this way from 1,5-14C-citryl-1-P, was isolated and purified to constant specific activity. The incubation mixture contained in 3.2 ml (in micromoles): dithiothreitol, 20; CoA, 3.0; NADH, 2.5; Tris, pH 8.4, 400; malate dehydrogenase, 2.4 units; 1,5-14C-citryl-1-P, 20, 2 \( \times \) 10^6 cpm; ATP-citrate lyase (purified 29-fold), 5 units; p-nitroaniline, 1; arylamine acetyltransferase (References 13 and 14) (purified 13-fold), 19 units. After incubation at 37° for 30 min, the reaction was stopped with 0.4 ml of 20% perchloric acid. The vessel was iced for 2 hours and denatured protein removed by centrifugation. After neutralization with sodium hydroxide, recrystallized p-nitroacetanilide (80 mg) was added to the supernatant solution and the mixture heated to reflux to dissolve the solid. The solution stood overnight at 0°. The crude crystals of 14C-p-nitroacetanilide were recrystallized four times from the minimal volume of water to constant melting point (213-214°, uncorrected) and constant specific activity.

These experiments, we believe, strongly attest the validity of Reaction 2. They clearly establish citryl-1-P as an active substrate of ATP-citrate lyase, and, in consequence, it may be fair to designate citryl-1-P an intermediate in the enzymatic cleavage of citrate. The isotopic experiments of Inoue et al. (3) on the aggregate reaction encourage the view that the putative intermediate remains firmly fixed to the enzyme throughout its brief participation in the larger reaction. When enzyme is incubated with 1,5-14C-citryl-1-P in the absence of CoA, radioactivity is found fixed to protein. This activity can be dislodged with coenzyme A. An enzyme-citrate complex, prepared from ATP plus citrate in the absence of CoA, was earlier described by Inoue et al. (3).

We are continuing this work and hope soon to report fully upon it.

REFERENCES


The Regulation of Pyruvate Kinase of Escherichia coli by Fructose Diphasphate and Adenylic Acid*

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SUMMARY

The regulatory properties of pyruvate kinase from Escherichia coli were investigated. Both fructose 1,6-diphosphate and AMP were found to activate the enzyme. These two positive effectors, however, had fundamentally different effects on the sigmoidal plots of rate-concentration data with P-enolpyruvate as the variable substrate. While fructose-1,6-di-P changed the $V_{\text{max}}$ but not the sigmoidality of the rate-concentration curves, AMP only changed the $K_m$ and converted the sigmoidal plot into a hyperbola. The enzyme lost its activity on storage at 4°C but could be reactivated by dithiothreitol by a time-dependent process. Sucrose density gradient centrifugation revealed that dithiothreitol caused a "dimerization" of the enzyme.

Phosphoenolpyruvate is metabolized in the enteric bacteria (1) on the one hand into oxalacetate by P-enolpyruvate carboxylase (anaplerotic channel) and on the other into pyruvate by pyruvate kinase (catabolic channel). We have shown earlier (2-5) that the P-enolpyruvate carboxylase of Salmonella (and Escherichia coli) is subject to a large number of controls of diverse kinds including a precursor activation (6) by fructose 1,6-diphosphate. In order to better understand the nature of partitioning of P-enolpyruvate into the anaplerotic and the catabolic channels, we have now studied the control of pyruvate kinase. It is already known that this enzyme from yeasts (7, 8) and various animal tissues (9) is activated by fructose-l, 6-di-P. We show below that the E. coli enzyme in addition is activated by AMP.

To prepare pyruvate kinase, E. coli B cells in the late log phase of growth were harvested and broken by sonic disruption in 0.05 M Tris-HCl buffer, pH 7.5. Dithiothreitol was added to a final concentration of 1 mM and this concentration was maintained in all the steps reported below.

The crude extract was treated with protamine sulfate (20 ml of 2% solution per 100 ml of extract) and the enzyme was precipitated by ammonium sulfate between 0.4 and 0.5 saturation. The precipitate was discarded and the supernatant after adjustment of the pH to 7.5 was dialyzed overnight against 0.01 M Tris-HCl, pH 7.5 and pH was adjusted to 5.0 by adding 20% acetic acid dropwise. The heavy precipitate was discarded and the supernatant after adjustment of the pH to 7.5 was dialyzed overnight against 0.01 M Tris-HCl, pH 7.5 with 0.3 M ammonium sulfate, pH 7.5. Pyruvate kinase was precipitated from the eluate by adding ammonium sulfate to 0.45 saturation. The precipitate was extracted successively by 24.5% (w/v) and 21.5% ammonium sulfate. The latter extract contained pyruvate kinase (about 12-fold purified) and was free of adenylate kinase and P-enolpyruvate carboxylase.

Pyruvate kinase as prepared by this method and assayed by a spectrophotometric method (see legend to Fig. 1) exhibits a pH optimum of 7.0 in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, and, unlike the enzyme from yeast, animals, and plants is not activated by potassium ions. In initial velocity studies, P-enolpyruvate yields sigmoidal plots (velocity against substrate) in the presence of 1.25 mM (saturating) ADP (Fig. 1). Much as expected fructose-1,6-di-P activates pyruvate kinase ($K_m = 0.2$ mM at P-enolpyruvate and ADP concentrations of 0.1 mM and 0.125 mM, respectively), but even at a concentration of 1.0 mM, the initial velocity plots for P-enolpyruvate remain sigmoidal (Fig. 1). If Hill plots (10) are any indication of

![Fig. 1. Double reciprocal plots of initial velocity data using P-enolpyruvate (PEP) as the variable substrate. A, a coupled spectrophotometric assay method was used in which the pyruvate formed is reduced with NADH in the presence of excess lactate dehydrogenase. The assay mixture contained 0.15 mM NADH, 1.25 mM ADP, 10 mM MgCl2, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 7.0), and 50 lμg of crystalline beef heart lactate dehydrogenase (Sigma, Type III) in a total volume of 3.0 ml. The reaction was started by the addition of pyruvate kinase and was followed at 25°C at 340 mλ with a Gilford model 2600 recording spectrophotometer. Specific activity is defined as a change of 0.1 absorbance unit per min per mg of protein with this assay system. A, the lower curve represents data obtained in the presence of 1.0 mM fructose-1,6-di-P and the upper curve in the absence of effectors; 15 lμg of enzyme preparation were used for each assay. B, same as A except that the lower line was obtained in the presence of 0.5 mM AMP and 28 lμg of enzyme preparation were used for each assay.](http://www.jbc.org/content/243/2/448/F1)
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