S-ACETYL PHOSPHOPANTETHEINE: DEACETYL CITRATE LYASE S-ACETYL TRANSFERASE FROM *Klebsiella aerogenes*

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SUMMARY

An enzyme has been partially purified from *Klebsiella aerogenes* which transfers an acetyl group from S-acetyl phosphopantetheine to deacetyl citrate lyase. This converts the deacetyl citrate lyase which has no enzyme activity, to citrate lyase, the active enzyme. A variety of other acetyl thioesters including acetyl CoA did not serve as acetyl donors.

Citrate (*pro*-3*S*) lyase (E.C.4.1.3.6) catalyzes the following reaction:

$$\text{Citrate} \overset{Mg^{2}}{\rightleftharpoons} \text{acetate} + \text{oxalacetate}$$

The citrate lyase isolated either from *E. coli* or *K. aerogenes* exhibits two unusual behaviours; first, it is inactivated during the course of the reaction which it catalyzes (1,2), and second, the enzyme is inactivated by high concentrations of oxalacetate (3,4). Recently it has been shown by Buckel *et al.* (5) and by ourselves (6) that the citrate lyase from *K. aerogenes* could be inactivated by treatment with hydroxylamine. This hydroxylamine inactivated enzyme could be reactivated by treatment with acetic anhydride. In addition, Buckel *et al.* (5) reported that the enzyme which had been inactivated by oxalacetate could also be reactivated with acetic anhydride. We have shown that the reaction-inactivated enzyme could also be reactivated with acetic anhydride (6). Buckel *et al.* explained these results with the following mechanism:

$$\text{E-S} \sim \text{acetyl} + \text{citrate} = \text{E-S} \sim \text{citryl} + \text{acetate}$$

$$\underline{\text{E-S} \sim \text{citryl} = \text{E-S} \sim \text{acetyl} + \text{oxalacetate}}$$

$$\text{Sum: citrate} = \text{acetate} + \text{oxalacetate}$$

They established that the acetyl group on the enzyme turned over during the course of the reaction which the enzyme catalyzed. If one assumes that the E-S-citryl is hydrolyzed slowly to E-SH (inactive, deacetyl citrate lyase), then this mechanism can explain both the reaction-inactivation and the oxalacetate inactivation.

Based on similar rates of hydroxylaminolysis of the active enzyme and an acetyl thioester and the inactivation by mercaptans, Buckel *et al.* (5) postulated that the acetyl group existed as a thioester on the enzyme. Recently Srere *et al.* (7) demonstrated that if the acetyl group is removed from the enzyme in the presence of an SH blocking compound, reacetylation could not take place until the blocking group was removed. They reported also that the citrate lyase from *K. aerogenes* contained stoichiometric amounts of phosphopantothenic acid. The inference was made that the active site contained S-acetyl-phosphopantetheine. This previous work therefore establishes that 1) active citrate lyase contains an acetyl thioester group which is necessary for enzyme activity and that 2) deacetyl citrate lyase has no enzyme activity.

It seemed likely that there existed a separate *in vivo* mechanism for the acetylation of the SH group on deacetyl citrate lyase. We looked in extracts of *K. aerogenes* for such an acetylating enzyme. This report concerns an enzyme (an acetyl transferase) that can be purified from extracts of citrate-grown *K. aerogenes* which catalyzes the activation of deacetyl citrate lyase in the presence of S-acetyl phosphopantetheine to produce active citrate lyase.

EXPERIMENTAL AND RESULTS

K. aerogenes citrate lyase was purchased from Boehringer Mannheim and purified by chromatography on Bio-gel A 1.5 m and DEAE-cellulose (2) to a specific activity of about 70. It was then deacetylated (inactivated) either by reaction-inactivation in the presence of citrate and Mg^{+2} or by treatment with neutral hydroxylamine. The deacetyl enzyme (inactive) was desalted by passing through a Sephadex G-25 column and then concentrated by dialysis against solid Sephadex G-200.

Pantetheine was prepared by reduction of pantethine (obtained from Sigma Chemical Co.) with $NaBH_4$. It was phosphorylated by ATP in a reaction catalyzed by pantetheine kinase isolated from pigeon liver according to the procedure of Novelli (8). After phosphorylation, the protein was precipitated with $HClO_4$ and the nucleotides removed by activated charcoal. Phosphopantetheine thus obtained was acetylated by acetic anhydride and the S-acetyl phosphopantetheine used without further purification.

The acetyl transferase reaction was initiated with S-acetyl phosphopantetheine after deacetyl citrate lyase and acetyl transferase had been preincubated with 0.4 mM dithiothreitol (DTT) for 10 min. At timed intervals, aliquots of the mixture were assayed for citrate lyase activity by the malate dehydrogenase coupled assay procedure with Mg^{+2} as the metal ion as described previously (2). Control incubations in which S-acetyl phosphopantetheine or the enzyme was omitted were run simultaneously. Acetyl transferase activity is expressed in terms of citrate lyase units formed per minute under the assay conditions. One citrate lyase unit is defined as the amount of enzyme necessary for cleavage of one μmole of citrate per minute at 25°.

Table I shows that all the three components, S-acetyl phosphopantetheine, deacetyl citrate lyase and acetyl transferase, are necessary to produce citrate lyase activity.

In our early studies we used a preparation of acetyl CoA prepared with acetic anhydride and CoA as an acetyl donor. This preparation was active as an acetyl donor as shown in Table I. When this "crude" acetyl CoA was purified on a DEAE-cellulose column by the procedure of Moffatt and Khorana (9) it lost its ability to serve as an acetyl donor. Search for an acetyl donor led us to S-acetyl phosphopantetheine. Since Lenz *et al.* (10) have indicated that commercial CoA contains small amounts of phosphopantetheine, it is likely that the activity in the unpurified acetyl CoA is due to S-acetyl phosphopantetheine. Treatment of either the "crude" acetyl CoA or

TABLE I

Components Necessary for the Acetyl Transferase Activity

Composition of Reaction Mixture	Activity units/10 min
S-Acetyl Phosphopantetheine as acetyl donor	
1. Complete system	3.2
2. Minus acetyl transferase	0.17
3. Minus acetyl transferase plus boiled enzyme	0.20
4. Minus S-acetyl phosphopantetheine	0.06
5. Minus deacetyl citrate lyase	Not detectable
"Crude" Acetyl CoA as acetyl donor	
6. Complete system	0.55
7. Minus acetyl transferase	0.14
8. Minus acetyl transferase plus boiled enzyme	0.13

Assay mixtures for the acetyl transferase contained 20 μmoles of potassium phosphate buffer, pH 7.0; 60 μg of deacetyl citrate lyase; 80 nmoles of S-acetyl phosphopantetheine and 10 μl of acetyl transferase (0.54 mg protein/ml) in a total volume of 0.1 ml. After preincubation with 0.4 mM DTT for 10 min, the reaction was initiated by an addition of S-acetyl phosphopantetheine or acetyl CoA and incubated for 10 min at 25°C. Suitable aliquots were assayed for active citrate lyase as described in the text. The enzyme employed was an aliquot from one of the fractions of the Bio-gel column, representing the peak of enzyme activity. The concentration of acetyl CoA was 2.4 mM.

S-acetyl phosphopantetheine with alkaline phosphatase caused a loss of acetyl donor ability. The following acetyl compounds did not exhibit donor activity: acetyl phosphate, acetyl imidazole, acetyl DTT, acetyl glutathione, S-acetyl pantetheine, acetyl dephosphoCoA and acetyl CoA.

Since the acetyl transferase could not be assayed in the presence of citrate lyase, it was necessary to effect a separation of the two enzymes early in the purification. A summary of a typical purification procedure starting with 10 gm of citrate-induced *K. aerogenes* is presented in Table II. Acetyl transferase could not be detected in the extracts of non-induced bacteria.

If acetyl transferase is assayed without preincubation with DTT, a long lag phase is observed and there is a decreased formation of active citrate lyase (Table III). This lag is due to SH group requirements for both the acetyl transferase and the deacetyl citrate lyase. Preincubation of either

TABLE II

Partial Purification of the Acetyl Transferase

Step	Total Activity (Cit. lyase formed) (units/min)	Total Protein (mg)	Specific Activity (Cit. lyase formed) (units/min/mg)
Protamine Sulfate	1760	528	3.3
50% $(NH_4)_2SO_4$	799	105	7.6
Bio-gel A 0.5 m	474	27	17.4

10 gm of citrate-induced *K. aerogenes* was sonicated in 0.05 M potassium phosphate buffer and titrated with 2% protamine sulfate until all citrate lyase was precipitated. The ammonium sulfate precipitate was dissolved in a small volume of the same buffer containing 1 mM EDTA and subjected to gel filtration on a (2.5 x 50 cm) column of Bio-gel A 0.5 m equilibrated and eluted with the same buffer. Protein was assayed by the method of Warburg and Christian (11). The enzyme was assayed as described in Table I. Activity is expressed as citrate lyase units per minute.

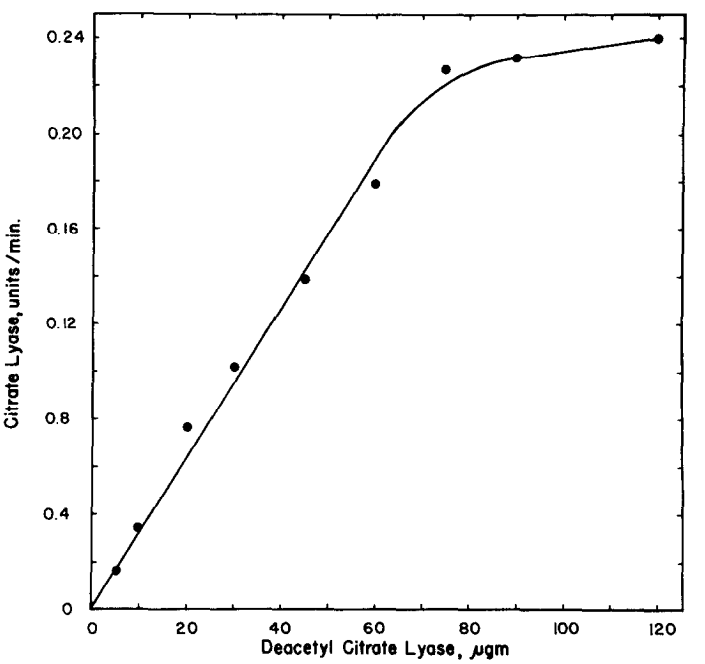

Fig. 1. Deacetyl citrate lyase concentration curve for acetyl transferase. The rate of the reaction was measured at various concentrations of deacetyl citrate lyase with 0.8 mM S-acetyl phosphopantetheine and 10.2 µg enzyme protein.

acetyl transferase or deacetyl citrate lyase with 4 x 10^{-4} M N-ethylmaleimide prior to assay results in a large decrease in the amount of active citrate lyase formed. This is consistent with our earlier findings that if the acetyl group is removed from citrate lyase in the presence of an SH blocking

TABLE III

Some Properties of the Acetyl Transferase

Time (min)	S-Acetyl Phosphopantetheine (mM)	Deacetyl Citrate Lyase (µg)	Acetyl Transferase (µl)	Total Activity (Cit. lyase units) (+DTT)	(-DTT)*	Rate of Reaction (Cit. lyase units/ min)
5	0.8	60	20 +	0.63	0.19	
10	0.8	60	20	1.28	0.38	
20	0.8	60	20	3.05	0.64	
40	0.8	60	20	6.52	2.40	
60	0.8	60	20	6.88	3.43	
10	0.8	60	1 ++			0.03
10	0.8	60	2			0.05
10	0.8	60	5			0.11
10	0.8	60	10			0.29
10	0.05	60	10 ++			0.05
10	0.10	60	10			0.09
10	0.20	60	10			0.15
10	0.30	60	10			0.19
10	0.60	60	10			0.26
10	0.80	60	10			0.27
10	1.60	60	10			0.28

+ The protein concentration of this enzyme preparation was 0.51 mg/ml.

++ The protein concentration of this enzyme preparation was 0.54 mg/ml.

* These results were obtained by omitting the DTT preincubation.

The enzyme was assayed as described in Table I.

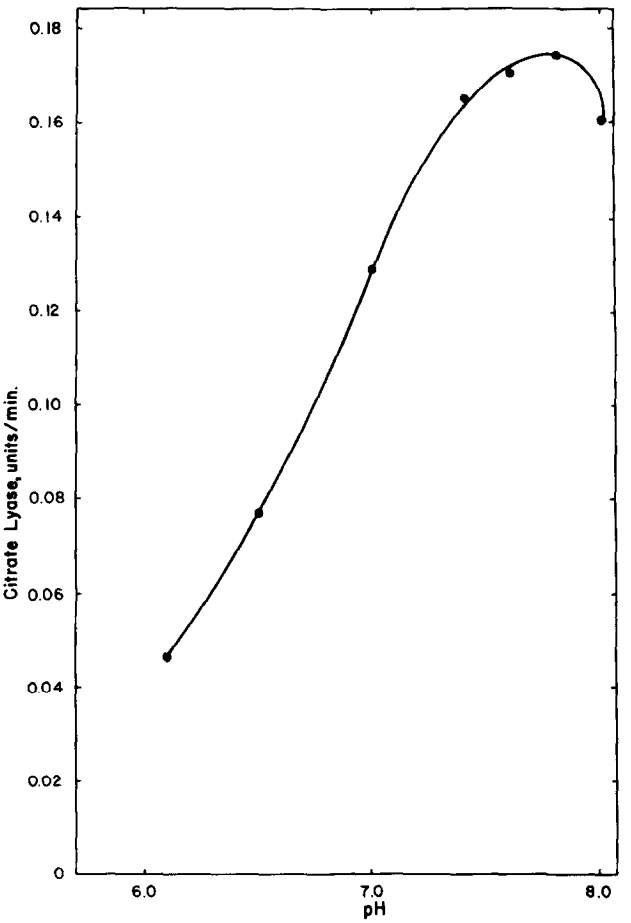

Fig. 2. Activity of acetyl transferase at different pHs. The rate of the reaction was determined with 0.2 M potassium phosphate buffer of various pH values. 60 µg deacetyl citrate lyase, acetyl transferase (10.2 µg protein) and 0.8 mM S-acetyl phosphopantetheine were used.

compound, reactivation by acetic anhydride could not be achieved before removal of the blocking group (7).

Table III shows some of the properties of this enzyme. Linear production of citrate lyase is observed up to about 40 min. The reaction rate is proportional to the amount of acetyl transferase added. The rate of acetylation follows Michaelis-Menten kinetics with respect to the concentrations of the two substrates. The maximum rate occurs at 1.6 mM S-acetyl phosphopantetheine (Table III) and 90 µg/0.1 ml (~1.8×10^{-6} M) of deacetyl citrate lyase (Fig. 1). The enzyme has an optimum activity around pH 7.7 (Fig. 2).

Most of the studies were carried out at pH 7.0 since considerable non-enzymatic acetylation occurs at higher pH values.

These data indicate that the acetyl transferase catalyzes the following reaction:

$$\underset{\text{(deacetyl citrate lyase)}}{\text{E-SH}} + \text{S-acetyl phosphopantetheine} \longrightarrow \underset{\text{(citrate lyase)}}{\text{E-S}\sim\text{acetyl}} + \text{phosphopantetheine}$$

A number of proteins have been shown to contain acetyl groups. Most of these exist as N-acetyl derivatives of N-terminal amino acid residues (12-16). In the case of hemoglobin acetylation, Marchis-Mouren and Lipmann (17) have shown that an enzyme from chicken reticulocytes catalyzes a transfer from acetyl CoA to certain hemoglobins. In addition, a histone acetylating enzyme with acetyl CoA as the acetyl donor has been described by Allfrey *et al.* (18). We are not aware of any other enzyme which contains an acetyl group that participates in the enzymic mechanism. Moreover, of the acetyl transferases reported, this enzyme is rather unique in that only S-acetyl phosphopantetheine serves as an acetyl donor.

Recently, Giffhorn *et al.* postulated deacetylation of citrate lyase to be the basis of an important control mechanism in *Rhodopseudomonas gelatinosa* (19). They found that citrate lyase is deacetylated upon exhaustion of citrate in the growth medium and reacetylation occurs after the addition of citrate to the medium. In the light of this observation, deacetylation and acetylation seems to be an interesting mechanism for the control of citrate metabolism in bacteria, with acetyl transferase playing a key role. Our present inability to detect this enzyme in non-induced cells would lead us to believe that it is a physiologically important mechanism.

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