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Mechanism of Enzymic Acetylation of des-Acetyl Citrate Lyase

(enzymic activation/acetyl adenylate/acetate:enzyme ligase)

H. SCHMELLENKAMP AND H. EGGERER

Fachbereich Biologie der Universität, Biochemie I, BRD-8400 Regensburg, Universitätsstrasse 31, Germany

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ABSTRACT **A new enzyme, acetate:SH-[acyl-carrier-protein] enzyme ligase (AMP), has been purified about 30-fold from cell-free extracts of *Klebsiella aerogenes*. The enzyme, in the presence of acetate and ATP, catalyzes the acetylation of enzymically inactive des-acetyl citrate lyase to enzymically active citrate lyase (EC 4.1.3.6). Acetate and ATP could be replaced by acetyl adenylate. Acetyl-CoA can act as acetyl donor in this activation only if trace amounts of adenine nucleotides and auxiliary enzymes are present. These allow formation of acetyl adenylate in acetate- and ATP-generating reactions.**

The enzyme citrate lyase [EC 4.1.3.6; citrate(*pro-3S*)-lyase] from *Klebsiella aerogenes* was characterized recently as an acyl carrier protein-containing multienzyme complex (1, 2). Enzymic activity of the complex depends on the presence of an acetyl thioester which is bound to the prosthetic group of acyl carrier protein (2, 3). The cleavage of citrate to acetate and oxaloacetate or its reversal (Eq. **1**) takes place at the thioester level in two steps, the acyl exchange (Eq. **2**) (1, 3) and acyl lyase (1, 3) reactions (Eq. **3**), the sum of which yields Eq. **1**.

$$\text{citrate} \rightleftharpoons \text{acetate} + \text{oxaloacetate} \quad [1]$$

$$\text{acetyl-}S\text{-citrate lyase} + \text{citrate} \rightleftharpoons \text{citryl-}S\text{-citrate lyase} + \text{acetate} \quad [2]$$

$$\text{citryl-}S\text{-citrate lyase} \rightleftharpoons \text{acetyl-}S\text{-citrate lyase} + \text{oxaloacetate} \quad [3]$$

Removal of the acetyl group from the enzyme by, e.g., treatment with hydroxylamine produces the enzymically inactive species des-acetyl citrate lyase (1). This can easily be reconverted to the enzymically active species by acetylation with acetic anhydride (1, 4). These chemical interconversions prompted us to search for the corresponding enzyme-catalyzed processes.

MATERIALS AND METHODS

Biochemicals were from C. F. Boehringer & Soehne, Mannheim, with the following exceptions. Acetate:CoA ligase (AMP) (EC 6.2.1.1), specific activity 2.6 U/mg of protein, was purified (5) from baker's yeast. Growth of *K. aerogenes* (ATCC 13 882) and purification of citrate lyase, specific activity about 70 U/mg, were performed according to Dagley (6) but slightly modified (2). des-Acetyl citrate lyase was prepared as described (2). Acetyl derivatives were prepared as described: acetyl adenylate (9), acetyl-CoA (10), and acetyl phosphate (11). Acetyl pantetheine and acetyl phosphopantetheine were a gift from Dr. W. Buckel. Acetyl-CoA (2,5 μmol each sample) was purified by chromatography (TCL plastic roll Cellulose, Merck, Darmstadt) by successive runs in two solvents (I: isobutyric acid–5 N ammonia–water 66:1:33; II: *n*-butanol–glacial acetic acid–water 50:30:20; v:v) in a total yield of 65%. Acetyl-CoA was detected by UV-absorption, eluted with water after each run, and freeze-dried.

Purification of Acetate:SH-[acyl-carrier-protein] Enzyme Ligase (AMP) (Isolation of Fractions I and II). Buffer solutions used contained 5 mM 2-mercaptoethanol unless indicated otherwise. Enzyme units refer to the assay with acetate and ATP as substrates. The following procedure was used: (*1*) A cell-free extract (160 ml; 24 mg of protein and 120 U/ml) was prepared from 50 g of wet cells and treated with streptomycin sulfate for removal of nucleic acids as in citrate lyase purification (6, 2) but in the presence of 1 mM dithioerythritol. (*2*) The resulting solution (150 ml; 22 mg of protein and 126 U/ml) was titrated with protamine sulfate (75 ml of 0.55% solution) for removal of citrate lyase (7), and the precipitate was discarded. The supernatant (236 ml; 5.5 mg of protein and 63 U/ml) was diluted with 0.03 M phosphate buffer, pH 7.0, to 4.3 mg of protein per ml.

Fraction I (acetate:SH-[acyl-carrier-protein] enzyme ligase). (*3*) Ammonium sulfate was added to 45% saturation and the precipitate was dialyzed against 0.1 M (12 hr) and 0.03 M (3 hr) phosphate buffer, pH 7.0, to yield a 9.0-ml solution (37.5 mg of protein and 1440 U/ml). (*4*) The protein was diluted to 22.5 mg/ml, and 25.5 ml of calcium phosphate gel (8) (26.9 mg of dry weight per ml) was added. The gel was eluted twice with 37.5 ml of 0.1 M phosphate buffer, pH 7.0, containing 1 mM dithioerythritol, with a mechanically driven Potter-Elvehjem homogenizer, to yield 75.3 ml of extract. This, on treatment with ammonium sulfate (27.3 g; 60% saturation) followed by dialysis, gave 7.1 ml (15.2 mg of protein and 790 U/ml). (*5*) The solution was applied to a Sephadex G-100 column (2 × 95 cm) and eluted into 5-ml fractions with 0.03 mM phosphate buffer, pH 7.0, at a flux rate of 26 ml/hr. Enzymic activity appeared in fractions 31 to 35, which were combined (25 ml; 1.6 mg of protein and 218 U/ml) and treated with ammonium sulfate (90% saturation) to yield 2.6 ml (13.5 mg of protein and 2000 U/ml) after dialysis of the precipitate. Saccharose, 2 mg/mg of protein, was added and the solution was freeze-dried. The dry powder was initially fully active but lost some activity on storage at +4°.

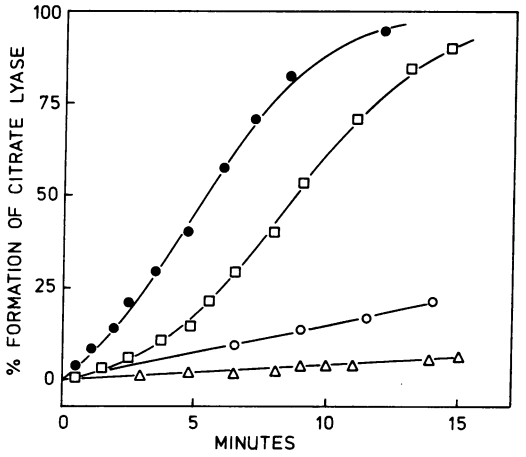


FIG. 1. Acetyl-CoA-dependent activation of des-acetyl citrate lyase (3.5 U) in the presence of Fraction I (50 μg of protein) (○); of Fraction II (250 μg of protein) (△); of Fractions I + II (50 and 250 μg of protein, respectively) when the reaction was started with acetyl-CoA (□) or with des-acetyl citrate lyase (●). The activation is shown in the presence of Fractions I and II (50 and 250 μg of protein, respectively) plus 10 mM arsenate (△); without arsenate but after preincubation of acetyl-CoA with alkaline phosphatase (8 U; Tris buffer, pH 7.2; 5 min) (△); without Fractions I and II (chemical activation by mercaptan exchange) (△). Protein used was that of the last purification step of each fraction.

Fraction II. (*6*) The supernatant of the first ammonium sulfate precipitation (45% saturation) was brought to 65% saturation, and the precipitate was dialyzed to yield 21.5 ml (39.5 mg of protein per ml). The solution was diluted to 22.5 mg of protein per ml and completely freed of acetate:SH-[acyl-carrier-protein] enzyme ligase by keeping 10 ml at 50° for 10 min. The cooled and centrifuged solution (9 ml; 16 mg of protein per ml) contained 9 U of adenylate kinase (EC 2.7.4.3) and 23 U of acetate kinase (EC 2.7.2.1) per ml.

Assay for Acetyl-CoA-Dependent Acetylation of des-Acetyl Citrate Lyase. The incubation mixture, which was kept at 25°, contained in a total volume of 0.50 ml: 0.1 M Tris buffer, pH 7.2; 10 mM $MgCl_2$; 0.4 mM dithioerythritol; 3.5 U of des-acetyl citrate lyase; Fractions I and II; and 0.36 mM acetyl-CoA. The latter (10 μl) was added to start the reaction after a 5-min preincubation. Samples, 50 μl each, were withdrawn periodically, and citrate lyase activity was determined immediately.

Assay for Acetate:SH-[acyl-carrier-protein] Enzyme Ligase (AMP). The assay was as described above but in presence of 5 mM acetate and in the absence of Fraction II and acetyl-CoA. The reaction was started with 0.05 μmol (10 μl) of ATP. Acetate and ATP were omitted if acetyl adenylate was used to start the reaction.

Other Enzymes and Substrates were determined as described: acetate: CoA-ligase (AMP) (12), acetate kinase (13), adenylate kinase (14), citrate lyase (13), phosphotransacetylase (EC 2.3.1.8) (16), acetyl adenylate (17), acetyl-CoA (18), acetyl phosphate (19), AMP (20), ADP (20), and ATP (21). All enzyme units indicated are related to initial rates expressed in μmol/min with the exception of the acetate: enzyme ligase, where one unit catalyzes the formation of one unit of citrate lyase from the des-acetyl enzyme per min, in the assay with acetate and ATP as substrates. Indicated specific activities of des-acetyl citrate lyase are those determined before removal of the acetyl group. Protein was estimated by the method of Warburg and Christian (22), with the exception of crude extracts where the biuret method was used.

RESULTS

An enzymic activity was discovered in cell-free extracts of *K. aerogenes* which, in the presence of acetyl-CoA and magnesium, catalyzed the acetylation of des-acetyl citrate lyase (23). This activity was purified about 5-fold and separated from citrate lyase by chromatography on Sephadex G-75; it has been used for characterization of citrate lyase subunits (2). If the "activating enzyme" was incubated with [$^3H_1$]-acetyl-CoA and des-acetyl citrate lyase, then the lyase became labeled and enzymic activity was restored in parallel (23). A characteristic feature of the activation kinetics was an initial lag phase depending in duration on whether the reaction was started with either acetyl-CoA or des-acetyl citrate lyase (Fig. 1). The kinetics could indicate the formation of an intermediate or reflect a peculiarity of the enzyme. The concentration of acetyl-CoA, about 0.2–0.4 mM, necessary for enzymic acetylation of des-acetyl citrate lyase seemed low enough to imply biological significance. The results pointed to acetyl-CoA as being the direct acetyl donor in the conversion of des-acetyl citrate lyase (inactive) to citrate lyase (active). The activating enzyme would consequently consist of an acetyl transferase. However, the "activating enzyme" was separated by ammonium sulfate precipitation into two fractions, both of which were enzymically inactive, or nearly so, by themselves. The enzymic activity for formation of citrate lyase from the des-acetyl enzyme was restored if both fractions were recombined (Fig. 1). Fraction I was heat labile (complete inactivation within 5 min at 50°) and sensitive towards 0.1 mM iodoacetamide (50% inhibition within 15 min), whereas Fraction II was stable in these conditions.

These observations suggested the operation of a more complicated reaction sequence. Investigation of the substrate revealed that chromatographically purified acetyl-CoA was enzymically inactive as were acetyl phosphate, acetyl pantetheine, and acetyl phosphopantetheine, and further that commercial coenzyme A and hence acetyl-CoA prepared from it contained trace amounts of AMP (about 0.3%; w:w), ADP (<0.01%), and ATP (about 0.1%) as impurities. Involvement of the adenine nucleotides in the activation reaction seemed very probable since the acetyl-CoA-dependent acetylation of des-acetyl citrate lyase was completely abolished in the presence of arsenate or by preincubation of acetyl-CoA with alkaline phosphatase (EC 3.1.3.1) (Fig. 1). The inhibition with arsenate suggested the involvement of phosphotransacetylase (24), and the inhibition with alkaline phosphatase suggested the participation of ATP in the enzymic acetylation of des-acetyl citrate lyase. Therefore, assuming the operation of a sequence that would generate acetate and ATP from acetyl-CoA and the adenine nucleotides, the involvement of acetyl adenylate in transfer of the acetyl group seemed feasible. This conclusion was substantiated by the finding of an acetate:enzyme ligase (AMP) in Fraction I which additionally contained acetate kinase as an

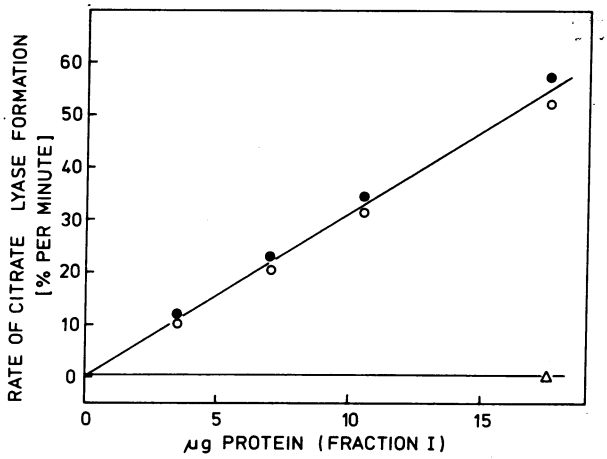
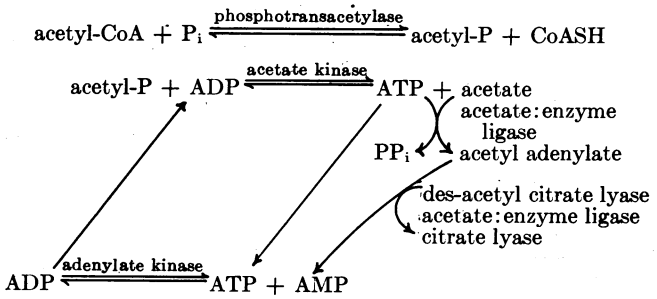


FIG. 2. Citrate lyase formation in 1 min with or without 10 mM arsenate in the presence of 3.5 U of des-acetyl citrate lyase, 5 mM acetate, 1 mM ATP, and Fraction I (○) (protein as indicated on the graph); in the absence of acetate and ATP but in the presence of 1 mM acetyl adenylate (●). The initial rate of citrate lyase formation in the presence of acetyl-CoA and Fractions I + II (17.5 μg and 250 μg, respectively) was zero (△) during this first minute. Protein used was that of the last purification step of each fraction.

impurity. Fast reactivation without a lag phase was observed if the des-acetyl lyase was incubated with acetate, ATP, and Fraction I. The rate of citrate lyase formation was dependent on enzyme concentration (Fraction I) and was not inhibited by 10 mM arsenate (Fig. 2). The formation of acetyl adenylate was indicated by the formation of acetyl hydroxamate on incubation of acetate, ATP, and Fraction I in the presence of hydroxylamine. The extent of hydroxamate formation was about 5-fold higher than the presence of acetate kinase in Fraction I could account for. In agreement with this indication, acetate and ATP could be replaced equally well by chemically prepared acetyl adenylate (Fig. 2); Mg^{2+} was not required, and arsenate was not inhibitory in this reaction. The K_M values for acetate, ATP, Mg^{2+}, and acetyl adenylate were determined as 0.66 mM, 0.8 μM, 1.6 mM, and 13 μM, respectively. These values provide a reasonable explanation for operation of the acetyl-CoA-dependent system to be described below.

Insight into the specificity of the ligase (Table 1) was obtained in attempts to synthesize acetyl-CoA on incubation of acetate, ATP, coenzyme A, and the enzyme ligase preparation. This was performed by use of the citrate synthase assay described for determination of acetate:CoA ligase (AMP) (12). If our ligase is identical with this enzyme or if it is different but acting nonspecifically, then acetyl-CoA must be formed in the sequence acetate → acetyl adenylate → acetyl-CoA. However, no acetyl-CoA was found in these conditions (Exp. 1). Acetyl-CoA was formed in this sequence only if Fraction I was substituted for acetate:CoA ligase (AMP) (Exp. 2). Since the enzyme ligase preparation (Fraction I) contained acetate kinase, the addition of phosphotransacetylase should allow the formation of acetyl-CoA in the sequence acetate → acetyl phosphate → acetyl-CoA. This was observed (Exp. 3). Addition of arsenate partially inhibited this acetyl-CoA production because arsenate effects partial hydrolysis of acetyl-CoA on phosphotransacetylase under assay conditions (Exp. 4). If, on the other hand, coenzyme A was substituted for des-acetyl citrate lyase, then fast formation of citrate lyase occurred (Exp. 5) and neither rate nor extent of formation was influenced by arsenate (Exp. 6). As expected, the acetate:CoA ligase (AMP) could not substitute for the enzyme ligase in the assay used for activation of des-acetyl citrate lyase. We conclude from these results that the ligase from *K. aerogenes* is specific for acetylation of the des-acetyl lyase and tentatively suggest the name acetate:SH-[acyl-carrier-protein] enzyme ligase (AMP). In this designation SH-[acyl-carrier-protein] indicates the acetyl acceptor, i.e., the mercaptan group of a cysteamine residue of the acyl carrier protein of citrate lyase. In providing a functionally active prosthetic group by acetylation, the effect of the ligase is similar to that of biotin:N^{ϵ}-enzyme ligase (AMP) and lipoate:N^{ϵ}-enzyme ligase (AMP). These enzymes introduce the prosthetic group by covalent attachment of biotin and lipoate, respectively, to ε-amino groups of specific lysyl residues of the corresponding apoenzymes (25).

With these results as a background, the elucidation of the acetyl-CoA-dependent system was a simple task. As determined by known methods, the heat-stable and iodoacetamide-insensitive Fraction II was found to contain adenylate kinase as well as acetate kinase, which was also present in Fraction I. Phosphotransacetylase was absent in Fractions I and II but present in the des-acetyl lyase preparation. The relative activities found in the incubation mixture of a representative activation experiment in the presence of Fractions I and II (50 and 250 μg, respectively, of the last purification step of each) were (units in parentheses) acetate:SH-[acyl-carrier-protein] enzyme ligase (AMP) (1.5), acetate kinase (0.1), adenylate kinase (0.14), des-acetyl citrate lyase (3.5), and phosphotransacetylase (3.9). Inorganic phosphate, about 1 mM, was derived from the enzyme solutions. The sequence of reactions that lies between acetyl-CoA and citrate lyase may therefore be described as outlined in *Scheme 1*.



Scheme 1. Mechanism of acetyl-CoA-dependent acetylation of des-acetyl citrate lyase.

Adenylate kinase generates ADP from AMP and ATP (the main impurities present in acetyl-CoA), and phosphotransacetylase produces acetyl phosphate from acetyl-CoA and inorganic phosphate. By subsequent action of acetate kinase the intermediates acetyl phosphate and ADP are converted to acetate and ATP. This can be used for ADP production as described above or react with acetate on the ligase to yield acetyl adenylate, the final acetyl donor for des-acetyl citrate lyase. The operation of the sequences shown in *Scheme 1* was further demonstrated as follows. If acetyl-CoA (0.18 mM), ADP (1.0 mM), and P_i (3 mM) were incubated with Fraction I (50 μg of protein of the last purification step) and des-acetyl

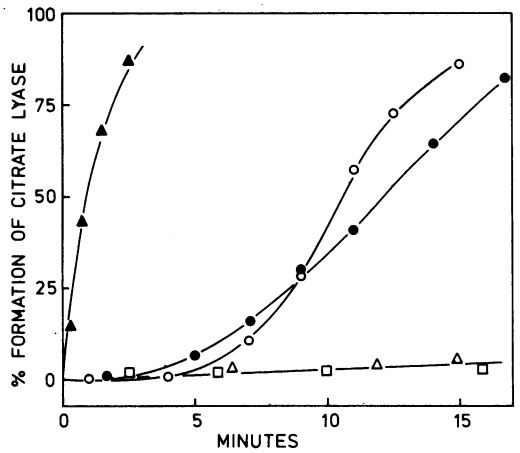


FIG. 3. Kinetics of citrate lyase formation from the des-acetyl enzyme (3.5 U) in presence of Fractions I and II (50 and 250 μg of protein, respectively) and substrates as follows. Crude acetyl-CoA (○); purified acetyl-CoA (△); 6 μM AMP + 0.04 μM ADP + 0.6 μM ATP without (□) and with (●) purified acetyl-CoA; in the absence of acetyl-CoA and Fraction II but in the presence of 5 mM acetate plus 0.2 mM ATP (▲). Protein used was that of the last purification step of each fraction.

citrate lyase (3.5 U), then ATP (0.15 mM) was formed, as was shown by the hexokinase assay (21). The presence of phosphotransacetylase in the lyase preparation permitted the formation of acetyl phosphate from acetyl-CoA, and the presence of acetate kinase in Fraction I permitted the formation of ATP from acetyl phosphate as shown in *Scheme 1*. The participation of phosphotransacetylase in this reaction was further strengthened by the lack of ATP formation in parallel runs in the presence of 10 mM arsenate. No reactivation of des-acetyl citrate lyase took place if it was incubated with Fractions I and II and purified acetyl-CoA (0.2 mM), free of adenine nucleotides. If, however, the adenine nucleotides were added in parallel experiments in the concentrations found to be present in the crude acetyl-CoA, then the rate of citrate lyase formation was nearly equal to that observed in use of acetyl-CoA, containing the adenine nucleotides (Fig. 3).

Our findings are in contrast to a recent report of Singh *et al.* (26), who described the activation of des-acetyl citrate lyase by an acetyl transferase from *K. aerogenes*. The "transferase" was shown to need specifically acetyl phosphopantetheine as a substrate. This, however, was an impure preparation (26), which certainly contained acetate and very probably acetyl pantetheine and small amounts of adenine nucleotides as impurities. We, therefore, believe that this "transferase activity" arose from reactions shown in *Scheme 1*.

TABLE 1. *Specificity of acetate:SH-[acyl-carrier-protein] enzyme ligase*

Exp.	Reagents: Added	Reagents: Omitted	Acetyl-CoA formed, μmol	Citrate lyase formed, U
(1)	None	None	0	—
(2)	U of acetate:CoA ligase (AMP)	Fraction I	0.15	—
(3)	7 U of phosphotrans-acetylase	None	0.12	—
(4)	7 U of phosphotransacetyl-ase + 10 μmol of arsenate	None	0.05	—
(5)	3.5 U of des-acetyl citrate lyase	CoA	—	3.42
(6)	3.5 U of des-acetyl citrate lyase + 10 μmol of arsen-ate	CoA	—	3.30

The complete system in a total volume of 1.0 ml contained: 0.1 M Tris buffer, pH 7.0; 0.1 mM CoA; 0.16 mM glutathione; 1.0 mM NAD; 3.3 mM *S*-malate; 3.3 mM $MgCl_2$; 2 mM ATP; 0.15 mM acetate; 4.4 U of citrate synthase (EC 4.1.3.7); 11 U of malate dehydrogenase (EC 1.1.1.40); and Fraction I (35 μg of protein containing 1.1 U of acetate:enzyme ligase and 0.1 U of acetate kinase). Additions and omissions were as indicated. The reactions were started by addition of Fraction I, and acetyl-CoA formation was followed from the increase in NADH absorption at 366 nm (d = 1 cm; Photometer Eppendorf). For test of citrate lyase formation (Expts. 5 and 6) samples, 50 μl each, were withdrawn periodically and citrate lyase activity was determined immediately.

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