Vol. 114, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1983 Pages 310-317

S-ACYLATED RESIDUES OF THE ACYL-CARRIER PROTEIN SUBUNIT OF *Klebsiella aerogenes* CITRATE LYASE

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Received May 17, 1983

SUMMARY Oxidation of the isolated deacetyl acyl-carrier protein subunit of citrate lyase from *Klebsiella aerogenes* with Cu^{2+}-o-phenanthroline complex leads exclusively to intrapeptide disulfide bridge formation indicating that the cysteamine and the cysteine residues are located in close proximity. The S-acetylation of the cysteine residue in deacetyl acyl-carrier protein subunit is catalysed by a citrate lyase ligase preparation in presence of acetate and ATP. Reaction-inactivation of citrate lyase results in deacetylation of the S-acetyl cysteamine residue of the prosthetic group but not of the S-acylated cysteine residue in the acyl-carrier protein.

Citrate lyase (EC 4.1.3.6) which catalyses the cleavage of citrate in a two step sequence of reactions (1) is a multienzyme complex containing two enzymes and an acyl-carrier protein (ACP)† (2). The most extensively studied of these complexes is the one from *Klebsiella aerogenes*, the ACP subunit of which has been shown to contain an S-acylated cysteine residue in addition to the essential S-acetyl moeity on the cysteamine residue of the CoA-like prosthetic group (3). Citrate lyase from *K. aerogenes* undergoes rapid inactivation in the course of the reaction it catalyses with loss of the essential acetyl groups to give inactive deacetyl (HS-) citrate lyase (4). The HS-citrate lyase is re-

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† *Abbreviations*: ACP, acyl-carrier protein; SDS, sodium dodecyl sulfate; DTNB, 5,5'-(2-nitrobenzoic acid); DTE, dithioerythreitol.

0006-291X/83 $1.50

activated either chemically by acetic anhydride (4) or enzymatically by citrate lyase ligase in presence of acetate and ATP(5).

Some of the properties of the acylated thiol residues of the ACP subunit of K. aerogenes citrate lyase and the probable role of citrate lyase ligase in modification of the cysteine residue of the subunit are reported here.

MATERIALS AND METHODS

Iodo [2-^{14}C] acetic acid (57 Ci/mol) was obtained from Radiochemical Centre, Amersham and diluted with cold iodoacetate to a sp. act. of 20 Ci/mol. Sodium [1-^{14}C] acetate (47.6 Ci/mol) from the Bhabha Atomic Research Centre, Bombay, was diluted with cold acetate to about 3 Ci/mol before use.

S-Carboxymethyl derivatives of cysteine and cysteamine were prepared by standard procedures (6,7).

Citrate lyase The pure enzyme (sp. act.$\sim$70 μmol.min^{-1}mg^{-1}) was obtained from K. aerogenes and assayed as described earlier (8).

Deacetylated ACP subunit Citrate lyase was dissociated with 6 M urea in the presence of 10 mM dithiothreitol and the deacetylated ACP subunit isolated in electrophoretically pure state by the procedure described by Carpenter et al.(2), a Sepharose CL-6B column (1 x 100 cm) being used for the separation. Protein in the isolated subunit preparation was determined by the method of Lowry et al.(9) using bovine serum albumin as standard.

Oxidation with o-phenanthroline-Cu^{2+} complex The separated deacetyl ACP subunit (0.8 mg) in 0.5 ml of 0.05 M potassium phosphate buffer, pH 7.4 was treated with o-phenanthroline and copper at concentrations of 100 μM and 20 μM respectively (10). The mixture was incubated for 30 min at 25°C and the reaction quenched by adding 0.5 ml of 20% SDS† solution. Aliquots of the reaction system were separately taken for SDS-polyacrylamide gel electrophoresis and thiol group estimation. Sulfhydryl groups were estimated with DTNB† (11) before and after the oxidation of the subunit.

SDS-polyacrylamide gel electrophoresis SDS-gel electrophoresis was carried out by the method of Weber and Osborn (12) in the absence of 2-mercaptoethanol.

Characterization of the S-acylated residues The S-acetyl residue involved in the reaction mechanism was characterized by a modification of the method described for fatty acid ACP (13). Citrate lyase (1 mg) was reaction inactivated in a total volume of 0.2 ml containing 0.2 mmol, citrate (pH 8.0); 0.16 μmol, $MgSO_4$ by incubation for 15 min at 30°C. An aliquot was checked for citrate lyase activity by coupled assay with malate dehydrogenase (14) to confirm complete inactivation. The free sulfhydryl groups of the reaction-inactivated enzyme were blocked with cold iodoacetate. The thioester linkages were then cleaved with neutralized hydroxylamine and dithiothreitol and the liberated thiol groups reacted with iodo [^{14}C] acetate. The S- [^{14}C] carboxymethylated residue in the protein was characterized by paper electrophoresis of the acid hydrolysate. The experimental conditions used for the differential labeling and characterization of the

[^{14}C]carboxymethyl residue were similar to those described by Dimmoth et al.(3). A mixture of reference compounds, S-carboxymethyl cysteine and S-carboxymethyl cysteamine (2 μmol each), was also separated under identical conditions on a separate strip of paper and their positions after migration visualised by spraying with 0.1% ninhydrin in acetone. The strip with the hydrolysed S-carboxymethylated enzyme was cut into pieces at the end of the run and radioactivity located using a dioxane based cocktail and a Beckman LS-100 C counter.

Citrate lyase ligase The acetate: HS- acyl-carrier protein enzyme ligase (AMP) was partially purified from K. aerogenes as described earlier (15). The ligase preparation was free from citrate lyase activity.

[^{14}C]-acetyl citrate lyase The [^{14}C]-acetyl labeled enzyme was prepared by acetylation of HS-citrate lyase with citrate lyase ligase in presence of [1-^{14}C]-acetate and ATP. Citrate lyase from K. aerogenes was deacetylated with neutralized hydroxylamine (16) before the DEAE-cellulose step in the purification procedure (17) and excess reagent separated by filtration through Sephadex G-25 (1.5 x 40 cm) using 0.05 M potassium phosphate buffer, pH 7.6 containing 1.6 mM $MgSO_4$. An aliquot withdrawn from the protein fraction showed complete loss of activity. The deacetyl-enzyme (250 mg) in 5 to 6 ml of the phosphate buffer was treated with citrate lyase ligase in presence of ATP and acetate (8 uCi), final concentrations of 6 mM and 0.5 mM, respectively; and incubated at 25°C for 30 min. Adequate citrate lyase ligase was used to reactivate to about 50% of the initial activity. The protein was then precipitated by addition of an equal volume of cold saturated ammonium sulfate solution and collected by centrifugation for 30 min at 50 000 g and 4°C, dissolved in a minimum volume of 0.05 M potassium phosphate buffer, pH 7.6 and subjected to DEAE-cellulose chromatography and gel filtration through Sepharose CL-6B essentially as described earlier (17).

The radioactivity distribution in the subunits was determined after dissociation of [^{14}C]-acetyl citrate lyase (200 μg) with 1% SDS in the absence of 2-mercaptoethanol followed by SDS-gel electrophoresis. The gel was stained, destained and the regions corresponding to the subunits were cut and checked for radioactivity as described by Saitoh et al. (18).

The extent of deacetylation of [^{14}C]-acetyl citrate lyase on reaction-inactivation was estimated by exhaustive dialysis of the completely inactivated enzyme against 0.05 M potassium phosphate buffer, pH 7.5 and determination of the remaining protein-bound radioactivity.

RESULTS

Oxidation of deacetyl ACP The effect of o-phenanthroline-Cu^{2+} on the content of -SH groups of the isolated deacetyl ACP subunit is shown in Table 1. The content of 2-SH/mol in deacetyl ACP is in agreement with the presence of a cysteine and a cysteamine residue in the ACP molecule (19). Oxidation with the reagent

Table 1. Oxidation of sulfhydryl groups of deacetyl ACP by o-phenanthroline-Cu^{2+} complex.

System	-SH/mol of ACP[a]
Deacetyl ACP	1.9
Deacetyl ACP + o-phenanthroline-Cu^{2+} complex	0.3

The isolated deacetyl ACP was oxidized with o-phenanthroline-Cu^{2+} complex (as described under Materials and Methods) and the reduction in thiol groups estimated. The values given are an average of three separate estimations.

[a] Mr = 9378 (20).

results in a decrease to about 0.3 -SH/mol. SDS-gel electrophoresis of aliquots from the reaction mixture showed that the molecular weight of the oxidized subunit was unchanged. This was established by electrophoresis along with the unoxidized ACP. Interpeptide cross-linking is apparently excluded and oxidation results exclusively in intrapeptide disulfide bridge formation between the thiol groups of the cystamine residue on the prosthetic group and of the cysteine residue in the polypeptide chain of the ACP subunit.

Acetylation of HS-citrate lyase by citrate lyase ligase The distribution of the radioactive label in the subunits of $[^{14}C]$-acetyl citrate lyase obtained on acetylation of HS-citrate lyase by citrate lyase ligase in presence of $[^{14}C]$-acetate and ATP is shown in Figure 1. Acetylation by the ligase is specifically of the ACP subunit, radioactivity being associated exclusively with the region in the gel containing the 10 000 daltons ACP (γ) subunit, the 54 000 daltons acyl transferase (α) and the 32 000 daltons acyl lyase (β) subunits carrying no radioactive label.

S-Acylated residues of reaction-inactivated citrate lyase The characterization of the S-acylated residues present in reaction-inactivated citrate lyase is shown in Figure 2. It can be seen from Figure 2 that total reaction-inactivation of citrate lyase

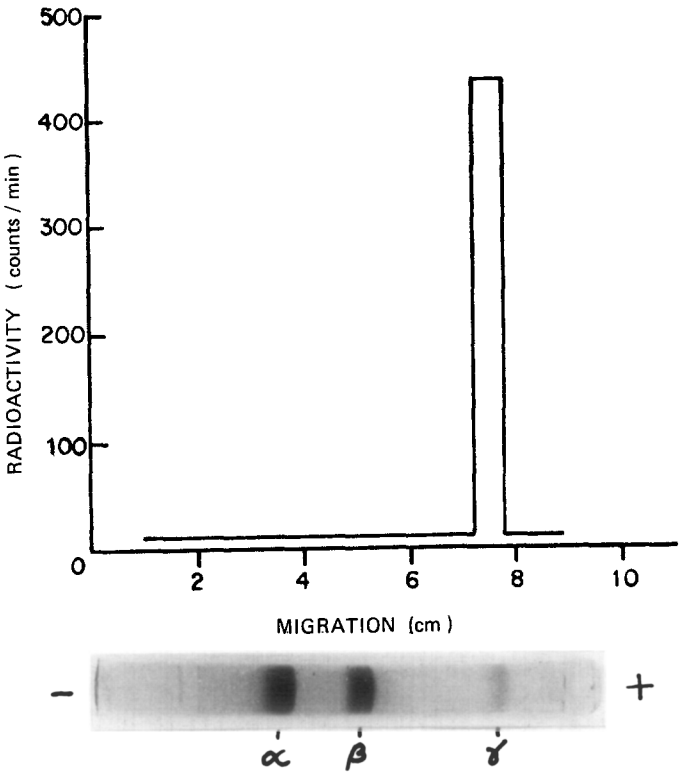

Figure 1. SDS-polyacrylamide gel electrophoresis of [^{14}C]-acetyl citrate lyase prepared as described under Materials and Methods. The gel was stained and sliced for determination of protein bound radioactivity. The histogram shows the profile of radioactivity in the separated acyl transferase (α), acyl lyase (β) and ACP (γ) subunits.

results in the complete loss of acetyl groups on the cysteamine moeity of the prosthetic group, while S-acetylated cysteine residues are still present in the inactivated enzyme. Reaction-inactivation results in the selective deacetylation of the acetyl-S-cysteamine residues of the prosthetic groups.

Partial deacetylation was also observed on reaction-inactivation of [^{14}C]-acetyl citrate lyase. The specific radioactivity of the active enzyme and of the reaction-inactivated complex are shown in Table 2. The presence of about 30% of the initial radioactivity in protein bound form after total reaction-inactivation and the finding that the cysteamine residues of the prosthetic

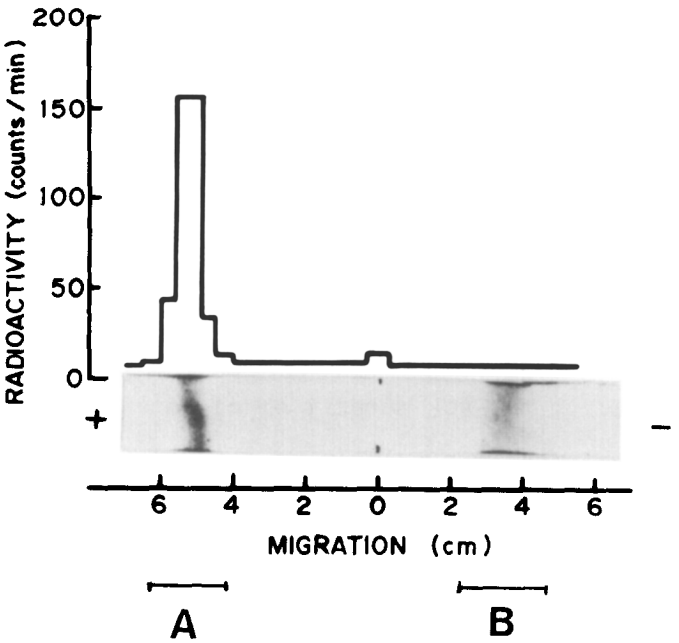

Figure 2. The histogram shows the distribution of radioactivity between the S-carboxymethyl derivatives formed after differential labeling of S-acetylated residues of reaction-inactivated citrate lyase with iodo[^{14}C] -acetate. See text for details. A, S-carboxymethyl cysteine; B, S-carboxymethyl cysteamine.

groups in the inactivated complex are fully deacylated would indicate that the [^{14}C]-acetyl moeities are retained only on the cysteine residues of the ACP subunits. The results are suggestive of the role of citrate lyase ligase in the modification of the

Table 2. Effect of reaction-inactivation on protein-bound radioactivity of[^{14}C]-acetyl citrate lyase.

Enzyme	cpm/mg
[^{14}C]-acetyl citrate lyase	13070
[^{14}C]-acetyl citrate lyase after reaction-inactivation	4120

[^{14}C]-Acetyl citrate lyase (430 µg) was incubated in a total volume of 1 ml of the reaction mixture containing 1 mmol citrate; 3 µmol, $MgSO_4$ and 50 µmol, Tris-HCl buffer, pH 8.0. Aliquots tested after 15 min incubation at 30°C showed complete loss of citrate lyase activity. Radioactivity was measured as described in the text.

cysteine residues of the ACP subunits to acetyl-S-cysteines in addition to the acetylation of the prosthetic groups in presence of acetate and ATP. The acetylation of the cysteine residue occurs even under conditions where HS-citrate lyase is only partially reactivated.

DISCUSSION

The presence of an S-acylated cysteamine and an S-acylated cysteine residue in the ACP subunit of citrate lyase from K. aerogenes has posed the problem of the nature of the biologically active acyl-carrier in the enzyme complex (3). Affinity labeling of HS-citrate lyase with iodoacetate in presence of excess DTE was shown by Dimroth and Eggerer (21) to result exclusively in the carboxymethylation of the cysteamine residue to yield a carboxymethylated complex inaccessible to reactivation either chemically with acetic anhydride or enzymatically by citrate lyase ligase. The present studies provide direct evidence that the cysteamine residue functions as the biologically active acyl-carrier, the reaction-inactivated citrate lyase being devoid of acetyl-S-cysteamine while still retaining S-acetylated cysteine residues.

Numerous instances of the presence of amino acid residues with modified side chains as constituents of proteins have been reported in the literature (22). The in vivo mechanisms of such modifications are not often understood. The data presented here suggest the involvement of citrate lyase ligase in the acetylation of cysteine residues in the ACP subunits of the K. aerogenes citrate lyase complex. The possible function of this modification might be to protect against intrapeptide disulfide bridge formation with the juxtaposed sulfhydryl residue on the prosthetic group of the enzyme in the deacetylated form.

ACKNOWLEDGEMENTS

A.B., S.S. and L.S.H. received fellowship awards of the Council of Scientific and Industrial Research, New Delhi, India. Communication No. 3271 from the National Chemical Laboratory.

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