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Photoaffinity Labeling of *Klebsiella aerogenes* Citrate Lyase by *p*-Azidobenzoyl Coenzyme A†

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ABSTRACT: *p*-Azidobenzoyl coenzyme A functions as a linear competitive inhibitor for (3*S*)-citryl-CoA in the citryl-CoA oxaloacetate-lyase reaction catalyzed by the *Klebsiella aerogenes* deacetylcitrate lyase complex (K_i = 80 μM; (3*S*)-citryl-CoA K_m = 67 μM). Inactivation is irreversible on photolysis of *p*-azidobenzoyl-CoA in the presence of the deacetylcitrate lyase complex. Mg^{2+} is not required for the inactivation. Inactivation is blocked by (3*S*)-citryl-CoA in the presence of ethylenediaminetetraacetic acid. *p*-Azidobenzoyl-CoA has no effect on the acetyl-CoA:citrate CoA transferase activity of both the deacetylcitrate lyase complex and its isolated transferase subunit. The stoichiometry of the CoA ester binding has been investigated by the use of *p*-azido[^{14}C]benzoyl-CoA as a photoaffinity reagent. The labeling is exclusively on the lyase β subunit of the citrate lyase complex.

The citrate lyase multienzyme complex (EC 4.1.3.6) from *Klebsiella aerogenes* is assembled from three dissimilar subunits α, β, and γ of molecular weights 54 000, 32 000, and 10 000, respectively (Carpenter et al., 1975; Dimroth & Eggerer, 1975a). The γ subunit functions as an acyl-carrier protein (ACP)[1] with an essential acetyl function in thioester linkage (Buckel et al., 1971) on a covalently bound CoA-like prosthetic group (Oppenheimer et al., 1979). The α subunit acts as an acyl transferase involved in the formation of (3*S*)-citryl-ACP[1] in the presence of citrate with the release of acetate, while the β subunit functions as an acyl lyase catalyzing the cleavage of the citryl-ACP intermediate to oxaloacetate and acetyl-ACP[1] (Dimroth & Eggerer, 1975b). Acyl coenzyme A (acyl-CoA) derivatives such as acetyl-CoA and propionyl-CoA have been shown to serve also as substrates for the transferase reaction, while (3*S*)-citryl-CoA acts as a substrate for the lyase reaction (Buckel et al., 1973; Dimroth et al., 1977). The mechanism of action of the citrate lyase complex requires the oscillation of the prosthetic group (or of acyl-CoA in the acetyl-CoA-mediated reaction) between the active sites on the α and β subunits (Srere & Singh, 1974).

The paper reports the use of *p*-azidobenzoyl-CoA as a photoaffinity reagent for probing the acyl-CoA binding sites of the complex.

Experimental Procedures

Products. NADH, *N*-hydroxysuccinimide, sodium dodecyl sulfate ($NaDodSO_4$),[1] citrate synthase, and malate dehydrogenase were from Sigma. DTNB[1] was from Calbiochem. Sepharose CL-6B, Sephadex G-10, and Sephadex G-25 were from Pharmacia Fine Chemicals. DEAE-cellulose (DE-52) was from Whatman. *p*-Amino[*carboxyl*-^{14}C]benzoic acid (21 Ci/mol) was obtained from Radiochemicals Centre. All other products were of the highest purity obtainable commercially.

Preparation of CoA Esters. Acetyl-CoA was prepared by acetylation of CoA with acetic anhydride (Simon & Shemin, 1953). (3*S*)-Citryl-CoA was prepared enzymatically from acetyl-CoA and citrate in the presence of *K. aerogenes* deacetylcitrate lyase and EDTA[1] (Dimroth et al., 1977) and assayed with citrate synthase in the presence of DTNB.

p-Azidobenzoyl-CoA was synthesized as described by Lau et al. (1977). *p*-Azido[^{14}C]benzoyl-CoA was synthesized from *p*-amino[*carboxyl*-^{14}C]benzoic acid without excessive dilution of the radioactivity. The concentration of the reagent was determined by using ϵ_{265nm} = 22.2 mM^{-1} (Lau et al., 1977).

All acyl-CoA preparations were purified as described by Moffat & Khorana (1961) by chromatography on a DEAE-cellulose (Cl^- form) column using a linear gradient from 0 to 0.2 M LiCl containing 0.003 N HCl. Samples of the CoA esters were concentrated at 25 °C in a rotary evaporator and desalted by filtration through a Sephadex G-10 column (1.0 × 50 cm).

Purification of Citrate Lyase. Citrate lyase complex from *Klebsiella aerogenes* (NCTC 418) was purified by a modification of the procedure described earlier (Mahadik & SivaRaman, 1968), sonic extracts being treated with ATP and acetate at final concentrations of 0.3 and 1 mM, respectively, for reactivating any deacetylcitrate lyase (Leena, 1979).

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[1] Abbreviations: ACP, acyl-carrier protein; (3*S*)-citryl-ACP, (3*S*)-citrylacyl-carrier protein; acetyl-ACP, acetylacyl-carrier protein; $NaDodSO_4$, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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Table I: Effect of Photoactivated *p*-Azidobenzoyl-CoA on Transferase and Lyase Activities of *Klebsiella aerogenes* Citrate Lyase[a]

enzyme	buffer	*p*-azidobenzoyl-CoA (μM)	% inhibition: transferase act.	% inhibition: lyase act.
deacetylcitrate lyase	50 mM Tris-HCl (pH 8.0) + 3 mM Mg^{2+}	0	0	0
		80	0	25
		112	0	42
		160	0	50
		168	0	58
	50 mM Tris-HCl (pH 8.0) + EDTA	0	0	0
		80	0	25
		160	0	48
acyltransferase subunit	50 mM Tris-HCl (pH 8.0)	0	0	
		80	0	
		160	0	

[a] Samples contained, in a total volume of 0.3 mL, 150 μg of deacetyl enzyme or about 25 μg of isolated α subunit. Aliquots were withdrawn after a 5-min irradiation and activities assayed as described under Experimental Procedures. Controls in which *p*-azidobenzoyl-CoA was absent showed no loss in the enzymatic activities.

Preparations were homogeneous in the ultracentrifuge and in polyacrylamide gel electrophoresis and had a specific activity varying between 70 μmol/mg of protein and 90 μmol/mg of protein.

Enzyme protein in the purified complex was determined from absorbance measurements at 278 nm by using the absorption index $E^{1\%}_{1cm}$ of 6.2 (Singh et al., 1976).

Preparation of Deacetylcitrate Lyase. Deacetylcitrate lyase was obtained by treatment of the purified native enzyme with neutral hydroxylamine according to the procedure of Buckel et al. (1973).

Isolation of Citrate Lyase Subunits. Component subunits were isolated in the electrophoretically pure and catalytically active state after dissociation with urea by the procedure described by Dimroth & Eggerer (1975b). Protein measurements of the isolated subunit preparations were made by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Assay of Enzymatic Activities. Citrate cleavage activity of the native enzyme complex was determined by coupled assay with malate dehydrogenase (Singh & Srere, 1971).

Acyl transferase activity of the deacetylcitrate lyase complex and of the isolated α subunit was determined from the initial velocity of the CoA transfer from acetyl-CoA to citrate in the presence of EDTA by estimation of the citryl-CoA produced with citrate synthase in the presence of DTNB (Dimroth et al., 1977; Srere et al., 1963).

Acyl lyase activity of the deacetyl enzyme complex and of the isolated β subunit was determined from the initial rate of oxaloacetate formation on cleavage of (3*S*)-citryl-CoA in the presence of Mg^{2+}, oxaloacetate being assayed with malate dehydrogenase (Buckel et al., 1973).

Kinetics. The K_m for citryl-CoA and K_i for *p*-azidobenzoyl-CoA were calculated from the double-reciprocal plots by least-squares analysis. *p*-Azidobenzoyl-CoA was tested at two different concentrations of 30 and 60 μM with varied initial concentrations of (3*S*)-citryl-CoA.

Photoactivation of p-Azidobenzoyl-CoA. Photoactivation was carried out under a Philips black light lamp (125 W) with the sample placed in a glass well kept at a distance of 2 cm. Solutions were precooled and maintained at 0–4 °C in an ice bath. Mixing was achieved by using a magnetic stirrer.

Incorporation of radioactivity into protein on photolysis in the presence of the ^{14}C-labeled reagent was measured by the filter paper disk method (Mans & Novelli, 1961). Dried disks were counted in 10 mL of dioxane-based scintillant on a Beckman Model LS-100 scintillation system.

$NaDodSO_4$–polyacrylamide gel electrophoresis of the enzyme photolyzed in the presence of the radioactive reagent was carried out according to the method of Weber & Osborn (1969). Samples were prepared by heating aliquots (50–100 μL) containing about 200 μg of protein with 1% β-mercaptoethanol in a boiling water bath for 3 min. Gels were stained with Coomassie brilliant blue R-250 and destained by diffusion. Detection of radioactivity in the protein bands was carried out by the method described by Saitoh et al. (1980).

Results

Effect of p-Azidobenzoyl-CoA. (*1*) *Acyl Transferase Activity.* *p*-Azidobenzoyl-CoA had no effect on the acetyl-CoA-mediated acyl transferase activity, both of deacetylcitrate lyase from *K. aerogenes* and of the isolated α subunit. The reagent was tried up to 160 μM final concentration.

(*2*) *Lyase Activity.* Kinetic studies were performed in the dark to see the effect of *p*-azidobenzoyl-CoA on the lyase activity of *K. aerogenes* deacetylcitrate lyase toward (3*S*)-citryl-CoA. Assay mixtures at 25 °C contained 12.5 μg of deacetylcitrate lyase, varying concentrations of (3*S*)-citryl-CoA (up to 50 μM), 0–60 μM *p*-azidobenzoyl-CoA, 50 mM Tris-HCl buffer, pH 8.0, and 3 mM $MgSO_4$, in a final volume of 1.0 mL. Double-reciprocal plots indicated that *p*-azidobenzoyl-CoA was a competitive inhibitor with a K_i of 80 μM. The K_m for (3*S*)-citryl-CoA determined in this experiment was 67 μM.

Photoaffinity Labeling of Citrate Lyase with p-Azidobenzoyl-CoA. The data on the effect of photoactivated *p*-azidobenzoyl-CoA on *K. aerogenes* deacetylcitrate lyase are summarized in Table I. Deacetylcitrate lyase treated with *p*-azidobenzoyl-CoA (final concentrations 80–168 μM) and photolyzed for 5 min showed no loss in acyl transferase activity. The photoactivated reagent was also without effect on the activity of the isolated transferase α subunit of *K. aerogenes* citrate lyase. Under these conditions the lyase activity of the deacetyl enzyme was inhibited 25%, 50%, and 58% in the presence of 80, 160, and 168 μM concentrations of *p*-azidobenzoyl-CoA, respectively. The inactivation of lyase activity was similar both in the presence of EDTA (10 mM final concentration) and in the presence of Mg^{2+} (3 mM final concentration).

Another piece of evidence that *p*-azidobenzoyl-CoA is an active-site directed photoaffinity label is the protection from irreversible inactivation of the lyase activity by (3*S*)-citryl-CoA (Table II). Inactivation in the presence of 112 μM reagent is completely blocked by 200 μM (3*S*)-citryl-CoA.

Table II: Protection of Deacetylcitrate Lyase by (3*S*)-Citryl-CoA from Inhibition by Photoactivated *p*-Azidobenzoyl-CoA[a]

p-azidobenzoyl-CoA (μM)	citryl-CoA (μM)	% inhibition
0	0	0
112	0	42
112	44	31.5
112	74	25
112	200	0

[a] The reaction mixture contained, in a total volume of 0.3 mL, 150 μg of deacetyl enzyme; 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and the indicated amounts of the inhibitor and (3*S*)-citryl-CoA. The mixture was irradiated for 5 min, and 0.05-mL aliquots were assayed for residual lyase activity.

The isolated β subunit of *K. aerogenes* citrate lyase showed very low activity; thus it was probably being extensively and irreversibly denatured during the isolation procedure as reported by Dimroth & Eggerer (1975b). The effect of *p*-azidobenzoyl-CoA on the isolated subunit was therefore not studied.

Quantitative binding studies were carried out with *p*-azido[^{14}C]benzoyl-CoA and deacetylcitrate lyase from *K. aerogenes*. A plot of the inactivation of the lyase activity toward (3*S*)-citryl-CoA as a function of the covalent incorporation of *p*-azido[^{14}C]benzoyl-CoA after photolysis is shown in Figure 1. The activity of the enzyme which had been photolyzed in the presence of various concentrations of *p*-azidobenzoyl-CoA (0–100 μM) was determined by assaying aliquots from the irradiated mixture. Controls photolyzed without reagent showed no loss of activity and were taken as 100% activity. The stoichiometry of binding was calculated by using a value of 575 000 for the molecular weight of *K. aerogenes* citrate lyase complex (Mahadik & SivaRaman, 1968).

It will be seen from Figure 1 that the decrease in enzyme activity is linear with the irreversible incorporation of ^{14}C-labeled reagent until about 30% inactivation. To check whether the incorporation of ^{14}C-labeled reagent under limiting conditions was selectively at the active site or not, the photolysis with *p*-azido[^{14}C]benzoyl-CoA (50 μM) was carried out in the presence of (3*S*)-citryl-CoA (200 μM) and EDTA (10 mM). This reaction was carried out in the absence of Mg^{2+} to prevent action of the enzyme on the substrate, the absence of the metal having been shown to have no effect on inactivation with photoactivated *p*-azidobenzoyl-CoA (Table I). Under these conditions the enzyme retained its full lyase activity, and the incorporation of radioactivity was completely blocked. In the presence of 50 μM of the radioactive aryl-CoA derivative, the enzyme was inactivated 25% in the absence of the substrate. This would suggest that the linearity up to at least 25% inactivation represents specific labeling of the active site by *p*-azidobenzoyl-CoA.

On the basis of this assumption an estimate of the total active sites per mole of enzyme was found by extrapolation of the linear part of the plot by fitting of the least-squares line for the data up to 25% inactivation. This extension was observed to include also the value for 30% inactivation. The stoichiometry of the number of active sites per mole of enzyme obtained in this manner was 7.4. The extended extrapolation would make this only an approximate estimate.

At concentrations of the reagent required for a higher degree of inactivation, photolysis apparently results in significant nonspecific incorporation even in the presence of Tris which functions as scavenger (Bayley & Knowles, 1977). Nonspecific binding of *p*-azidobenzoyl-CoA has also been reported for acylglycine *N*-acyltransferase of beef liver at values above about 30% inhibition (Lau et al., 1977).

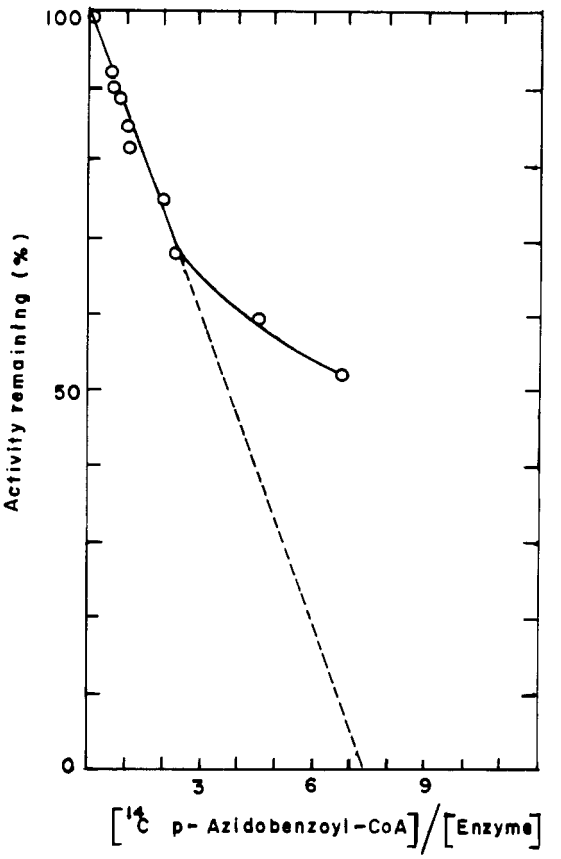

FIGURE 1: Determination of the number of active sites of lyase β subunits per mole of deacetylcitrate lyase complex accessible to *p*-azidobenzoyl-CoA. The irradiated mixture in 300 μL contained 75 μg of deacetylcitrate lyase, 50 mM Tris-HCl, pH 8.0, 3 mM $MgSO_4$, and varying amounts of *p*-azido[^{14}C]benzoyl-CoA (0–100 μM). After 5-min photolysis, a 50-μL aliquot was withdrawn for the determination of lyase activity and a 200-μL sample withdrawn for estimation of ^{14}C incorporation.

An enzyme sample photolyzed in the presence of the ^{14}C-labeled *p*-azidobenzoyl-CoA was dissociated, and the constituent subunits were separated by NaDodSO$_4$–polyacrylamide gel electrophoresis. The electrophoretic profile of the separated subunits and the radioactivity distribution in the gel are represented in Figure 2. The presence of activity exclusively in the gel region containing the lyase subunit would confirm the evidence obtained from the kinetic data, Figure 1, and Table II that the active site of this subunit is specifically labeled on photolysis in the presence of the azidoaryl-CoA.

Discussion

p-Azidobenzoyl-CoA has been shown to have no inhibitory effect on the acyltransferase activity of *K. aerogenes* deacetylcitrate lyase. That the enzyme complex might have hindered the photoaffinity reagent from reaching the active site on the transferase α subunit has been ruled out, since the isolated α subunit is also not inhibited by *p*-azidobenzoyl-CoA on photoactivation. The active site of the transferase subunit itself apparently does not accept *p*-azidobenzoyl-CoA, possibly on account of the bulky aryl group.

The substrate binding site of the lyase subunit, however, accepts the aryl-CoA ester. The kinetic data establish that *p*-azidobenzoyl-CoA acts as a potent competitive inhibitor of the acyl lyase activity. The reagent presumably binds specifically to the CoA ester sites of the lyase subunits. Again during photolysis a linear relationship is observed between incorporation of the ^{14}C-labeled reagent and loss in activity

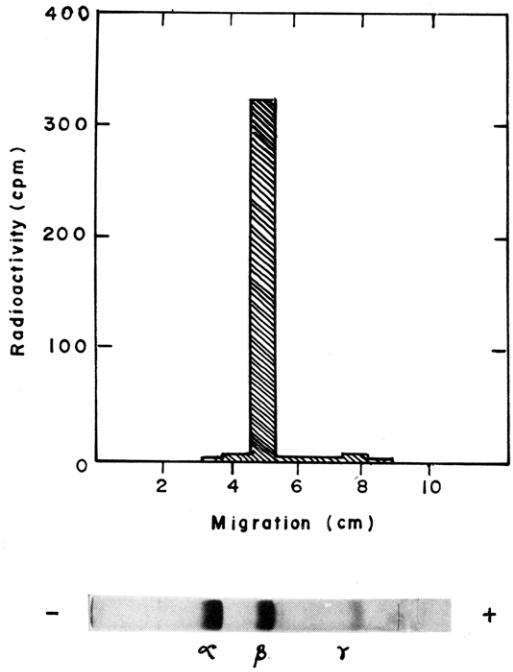

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of deacetylcitrate lyase labeled by photolysis in the presence of *p*-azido-[^{14}C]benzoyl-CoA. The irradiated mixture contained, in 0.5 mL, 400 μg of deacetylcitrate lyase, 50 mM Tris-HCl, pH 8.0, 3 mM $MgSO_4$, and 300 μM *p*-azido[^{14}C]benzoyl-CoA. An aliquot of about 0.2 mL was taken for electrophoresis. Gels were stained and sliced for determination of covalently bound radioactivity as described under Experimental Procedures. The histogram shows the profile of radioactivity in the separated subunits.

under limiting conditions of the reagent, the label being exclusively on the lyase β subunit. The reagent would thus be useful for selectively labeling the lyase active sites, having no action on the acetyl-CoA binding sites of the acyltransferase α subunits.

The lyase activity requires the presence of divalent metal ions such as Mg^{2+} (Dimroth & Eggerer, 1975b; Buckel et al., 1973). Divalent metal binding has been shown more recently to bring about a conformational change in the enzyme complex from *K. aerogenes* (SivaRaman & SivaRaman, 1979). In the present studies, the binding of *p*-azidobenzoyl-CoA to the lyase subunit and the subsequent irreversible inactivation on photolysis have been shown to occur both in the presence of Mg^{2+} and in the presence of an excess of EDTA. These results might suggest that (3*S*)-citryl-CoA binding is independent of divalent metals, while its cleavage requires the presence of Mg^{2+}.

Although *K. aerogenes* citrate lyase has been shown to have a hexameric $\alpha_6\beta_6\gamma_6$ structure (Carpenter et al., 1975; Dimroth & Eggerer, 1975a), careful estimation of the prosthetic group components have indicated that only four acyl-carrier proteins carry pantetheine moieties (Singh et al., 1976). This has posed the problem of the function of subunit combinations which are devoid of prosthetic groups. An earlier suggestion has been made that these subunits might function in citrate cleavage mediated by acetyl-CoA (Robinson et al., 1976). The approximate estimate of about seven sites on the lyase subunit complex obtained in the present investigation is suggestive of the fact that all the lyase subunits are accessible to citryl-CoA. Thus in the intact organism the cleavage of citrate can proceed both via the built-in prosthetic group and through the acyl-CoA-mediated reaction.

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