The Carboxyl Transferase Component of Acetyl CoA Carboxylase: Structural Evidence for Intersubunit Translocation of the Biotin Prosthetic Group

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ABSTRACT An essential protein component of acetyl CoA carboxylase, isolated and extensively purified from cell-free extracts of *Escherichia coli*, has been identified as malonyl CoA:*d*-biotin carboxyl transferase. This enzyme, which does not contain covalently-bound biotin, catalyzes carboxyl transfer from malonyl CoA to free *d*-biotin, a model reaction for the second step in the carboxylation of acetyl CoA. The transcarboxylation product, after stabilization by methylation, was identified as 1'-*N*-carboxy-*d*-biotin dimethyl ester. These results indicate the presence of a biotin site on the carboxyl transferase, distinct from that on the biotin carboxylase, which carries out the first step in the overall process. In addition, the carboxyl transferase catalyzes a slower abortive decarboxylation of malonyl CoA, thus indicating that carboxyl abstraction and protonation do not require the participation of biotin.

It is now evident that the half-reactions of acetyl CoA carboxylation are catalyzed by biotin carboxylase and carboxyl transferase. Both components are devoid of biotin and have specific binding sites for free *d*-biotin, as well as for their respective substrates; hence, the acetyl CoA carboxylation mechanism must involve intersubunit translocation of the carboxylated biotinyl group, which is bound covalently to carboxyl-carrier-protein, a non-catalytic polypeptide.

It is now well established that the reactions catalyzed by acetyl CoA carboxylase and other biotin-dependent carboxylases (1, 2) proceed via the minimal 2-step reaction sequence shown below:

Unlike the carboxylases from higher organisms, which retain their structural integrity during purification (2), *Escherichia coli* acetyl CoA carboxylase is readily resolved into three essential protein components (3, 4): (a) biotin carboxylase (BC), which catalyzes the ATP- and divalent cation-dependent carboxylation of biotin (4-6) and presumably participates in the first half-reaction [Reaction (1)], (b) carboxyl-carrier-protein (CCP), a polypeptide of about 9000 daltons, which contains a covalently-bound biotin prosthetic group (7), and (c) a third protein component, referred to as *E_b* by Al-

berts, *et al.* (3), which is free of biotin and appears to function in the second half-reaction. The precise role of *E_b*, whether catalytic, structural, or otherwise, has remained obscure.

The present investigation reveals that a protein isolated from *E. coli*, having characteristics similar to those reported for *E_b*, catalyzes BC- and CCP-independent carboxyl transfer from malonyl CoA to free *d*-biotin to form free carboxybiotin. This malonyl CoA:*d*-biotin carboxyl transferase, which has been extensively purified, is devoid of biotin and is required in combination with BC and CCP for acetyl CoA carboxylation.

EXPERIMENTAL PROCEDURE

E. coli B cells (grown to $3/4$ log phase) grown on enriched medium were purchased from Grain Processing Corp., Muscatine, Iowa. *E. coli* SA 283, a biotin auxotroph, was grown in the presence of [2'- ^{14}C] *d*-biotin as described (5). Biotin carboxylase assays, materials, and other procedures not described herein were as reported (5, 6). Acetyl CoA carboxylation was determined at 30°C by measuring [^{14}C]bicarbonate incorporation into malonyl CoA under assay conditions similar to those of Alberts and Vagelos (3). [2'- ^{14}C]malonyl CoA was chemically synthesized by the method of Trams and Brady (8) and [3- ^{14}C]malonyl CoA was enzymatically synthesized according to Gregolin, *et al.* (9); both labeled thioesters were purified as described (9). Acetyl CoA was prepared by the method of Simon and Shemin (10). Protein concentration was determined spectrophotometrically (11).

Steps in the preparation of carboxyl transferase

Cell-free extracts of *E. coli* are prepared in 0.1 M potassium phosphate buffer, pH 7, using a Manton-Gaulin submicron dispersor. The enzyme is purified by fractionation with ammonium sulfate (between 25 and 42% saturation), adsorption on and elution from calcium phosphate gel, and ion-exchange chromatography on DEAE-cellulose and phosphocellulose. This procedure, which will be reported in detail elsewhere, results in preparations that are at least 200-fold purified and have a specific activity in the carboxyl transferase assay of approximately 100 milliunits per mg of protein.

Malonyl CoA decarboxylase assay

The rate of malonyl CoA decarboxylation is determined in a reaction mixture (0.5 ml, pH 6.7) containing 100 mM imidazole-HCl buffer, 85 μM [2'- ^{14}C]- or [3- ^{14}C]malonyl CoA ($4-6 \times 10^3$ cpm per nmol), 0.3 mg of bovine serum albumin, and up to 10 milliunits of carboxyl transferase. At 5, 10, 15,

Abbreviations: BC, biotin carboxylase; CCP, carboxyl-carrier-protein; MCD, malonyl CoA decarboxylase.

FIG. 1. Chromatography of E_b and malonyl CoA decarboxylase (MCD). (A) Calcium phosphate gel-purified enzyme (1.46 g of protein, see preparation of carboxyl transferase in *Experimental Procedure*) in 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 5 mM β -mercaptoethanol was applied to a 4.5×50 cm DEAE-cellulose column and eluted with a 2-liter linear phosphate gradient (50–400 mM, pH 7) also containing EDTA and β -mercaptoethanol. The eluted fractions were assayed for MCD activity and for the ability to restore acetyl CoA carboxylase [in the presence of 0.96 mg of a combined biotin carboxylase-carboxyl-carrier-protein preparation, calcium phosphate gel-purified enzyme from Step 3 of the biotin carboxylase purification procedure (5)]. The enzymatically-active fractions were pooled, and the protein was precipitated with 60%-saturated ammonium sulfate. (B) After dialysis against 25 mM potassium phosphate buffer, pH 7, containing 1 mM EDTA and β -mercaptoethanol, half of the protein (25 mg) recovered from A was applied to a 1.5×30 cm column of phosphocellulose. Elution was with a 500-ml phosphate gradient (25–300 mM, pH 7) containing EDTA and β -mercaptoethanol. The eluted fractions were assayed and the active fractions precipitated as in A.

and 20 min of incubation at 30°C, 0.1-ml aliquots are transferred to scintillation vials containing 0.1 ml of 6 N HCl. The acidified solutions are taken to dryness at 95°C; water and scintillator are added, and the residual acid-stable ^{14}C is determined. The $[^{14}\text{C}]\text{CO}_2$ or $[2\text{-}^{14}\text{C}]\text{acetic acid}$ generated are volatile under these conditions, whereas $[2\text{-}^{14}\text{C}]\text{malonyl CoA}$ is not.

Carboxyl transferase assay

Malonyl CoA: *d*-biotin carboxyl transferase (CT) catalyzes transcarboxylation from malonyl CoA to free *d*-biotin (Reaction 3), a model reaction for the reverse of the second step (Reaction 2).

The (free) biotin-dependent formation of $[2\text{-}^{14}\text{C}]\text{acetyl CoA}$ from $[2\text{-}^{14}\text{C}]\text{malonyl CoA}$ or of $[^{14}\text{C}]\text{carboxybiotin}$ from $[3\text{-}^{14}\text{C}]\text{malonyl CoA}$ is determined by the malonyl CoA decarboxylase assay procedure modified to include 10 mM free *d*-biotin as carboxyl acceptor. The ^{14}C -labeled fragments ($[2\text{-}^{14}\text{C}]\text{acetic acid}$ or $[^{14}\text{C}]\text{CO}_2$) derived from either radioactive

product during the work-up subsequent to the enzymatic reaction are volatilized; this procedure leaves behind the residual acid-stable ^{14}C from unused substrate. The rate of carboxyl transfer is equal to the difference between the rate of disappearance of acid-stable ^{14}C in the presence and absence of free *d*-biotin. Linear transfer rates are obtained for 10 min with up to 2 milliunits of carboxyl transferase.

One unit of carboxyl transferase catalyzes the formation of 1 μmol of free carboxybiotin per min from malonyl CoA and free *d*-biotin under these conditions.

RESULTS

Isolation of E_b , an essential component of the carboxylase system that possesses malonyl CoA decarboxylase activity

Investigations in this laboratory (J. Moss, unpublished observations) have shown that several biotin-dependent carboxylases catalyze a slow, avidin-insensitive, decarboxylation of their respective carboxylated acceptor substrates, e.g., malonyl CoA decarboxylation by liver acetyl CoA carboxylase. Hence, these enzymes can labilize the α -carboxyl group of their carboxylated acceptors (e.g., malonyl CoA) and insert a proton without the participation of the biotin prosthetic group. Our suspicion that the E_b component of the *E. coli* acetyl CoA carboxylase system might catalyze this abortive reaction proved correct and provided a means to assay and follow this component during fractionation. After partial resolution from the biotin carboxylase and carboxyl-carrier-protein components by ammonium sulfate and calcium phosphate gel fractionation, the malonyl CoA decarboxylase activity was purified further by ion-exchange chromatography on DEAE-cellulose (Fig. 1A) and phosphocellulose (Fig. 1B). In order to determine whether the enzyme having malonyl CoA decarboxylase activity is an essential component of the acetyl CoA carboxylase system, the column fractions were assayed both for biotin-independent malonyl CoA decarboxylase activity and for their ability to restore acetyl CoA carboxylase activity to an enzyme preparation containing

TABLE 1. Reconstitution of acetyl CoA carboxylase activity

Enzyme	Specific enzyme activity			Acetyl-CoA carboxylated/5 min (nmol)
	Biotin carboxylase (munits/mg)	Malonyl-CoA decarboxylase (munits/mg)	Carboxyl transferase (munits/mg)	
BC-CCP*	2.9	0.05	0.2	3.5
MCD†	0.0	8	96	0.0
BC-CCP* + MCD†	---	---	---	40

* Combined biotin carboxylase (BC)- and carboxyl-carrier-protein (CCP)-containing enzyme preparation; calcium phosphate gel purified enzyme from Step 3 of the biotin carboxylase purification procedure (5). 0.96 mg was used to measure acetyl CoA carboxylase activity.

† Pooled malonyl CoA decarboxylase (MCD)-containing fractions from phosphocellulose chromatography, free of the biotin-containing carboxyl-carrier-protein (Fig. 1B). 28 μg was used to measure acetyl CoA carboxylase activity.

‡ ---, not determined.

biotin carboxylase and carboxyl-carrier-protein. It is evident (Fig. 1A and B) that the elution patterns for malonyl CoA decarboxylase and the restoration of acetyl CoA carboxylase activities are superimposable, suggesting that a single enzymatic component is responsible for both activities. It is also evident (Table 1) that acetyl CoA carboxylation catalyzed by the pooled phosphocellulose column fractions is absolutely dependent upon the addition of the biotin carboxylase-carboxyl-carrier-protein preparation. The phosphocellulose-purified enzyme obtained from an *E. coli* biotin auxotroph (Strain SA 283) grown on [2-¹⁴C]biotin of high specific activity (57 Ci/mol) was free of radioactivity, indicating that E_b was completely resolved from the biotin-containing carboxyl-carrier-protein.

Since this essential component of the acetyl CoA carboxylase system also appears to decarboxylate malonyl CoA, it is evident that it must possess a binding site for malonyl CoA. This, and the fact that the abortive reaction is chemically related to the reverse of Reaction 2, suggested that this component may function as a carboxyl transferase.

E_b-catalyzed carboxyl transfer from malonyl CoA to free biotin

In addition to malonyl CoA decarboxylation, the enzyme also catalyzes carboxyl transfer from malonyl CoA to free *d*-biotin, which serves as a model for the reverse of partial Reaction 2 of the overall process. This model reaction can be followed indirectly, by determining the rate of *d*-biotin-dependent formation of [¹⁴C]acetyl CoA from [2-¹⁴C]malonyl CoA, or directly, by measuring carboxybiotin formation. As illustrated in Fig. 2, the rate of acetyl CoA formation is enhanced about 12-fold by 10 mM *d*-biotin and is proportional to enzyme concentration. In the presence of *d*-biotin and [¹⁴C]carboxyl-labeled malonyl CoA, an acid-labile compound is formed that has stability properties characteristic of *N*-carboxybiotin, i.e., it is stable to gassing with CO₂ (see Fig. 3). In addition,

FIG. 2. Effect of free *d*- and *l*-biotin on the rate of E_b-dependent acetyl CoA formation from malonyl CoA. The rate of formation of [2-¹⁴C]acetyl CoA from [2-¹⁴C]malonyl CoA was determined by the carboxyl transferase assay procedure using phosphocellulose-purified E_b-MCD (Fig. 1B; carboxyl transferase specific activity, 100 milliunits per mg). Free *d*- and *l*-biotin were added at 10 mM and 16 mM, respectively.

FIG. 3. Kinetics of formation of an acid-labile carboxyl transfer product. The carboxyl transferase assay reaction mixture contained [3-¹⁴C]malonyl CoA (6400 cpm per nmol) and 15 μg of phosphocellulose-purified E_b-MCD (Fig. 1B; carboxyl transferase specific activity, 98 milliunits per mg). Acid-stable ¹⁴C was determined according to the malonyl CoA decarboxylase assay procedure, and ¹⁴C stable to gassing with CO₂ was determined as described in the legend to Table 2.

the carboxyl transferase is highly specific in that *l*-biotin does not enhance the rate of acetyl CoA formation (Fig. 2) and is completely inactive as an acceptor at concentrations ranging from 2 to 20 mM. These results show unequivocally that this enzyme, which is an essential component of the *E. coli* acetyl CoA carboxylase system, has binding sites for both malonyl CoA and *d*-biotin, the model prosthetic group, and that it catalyzes carboxyl transfer between the two compounds.

The *K_m* values for malonyl CoA and *d*-biotin in the carboxyl-transferase reaction are 0.10 mM and 3.5 mM, respectively. Since the *K_m* for free biotin in this reaction is nearly two orders of magnitude lower than that in the biotin carboxylase-catalyzed ATP-dependent carboxylation of free biotin, the carboxyl transferase component appears to have a much higher affinity for biotin than does the biotin carboxylase component. Interestingly, 2-imidazolidone (100 mM), an analogue of the functional ureido ring of biotin, activates (*K_A* = 100 mM) [3-¹⁴C]malonyl CoA decarboxylation although it does not appear to serve as a carboxyl acceptor. The binding of imidazolidone, presumably at the biotin site may promote a conformational change at the malonyl CoA site that enhances the abortive decarboxylation.

Identification of 1'-*N*-carboxy-*d*-biotin as carboxyl transfer product

The acid-labile product formed by incubating [3-¹⁴C]malonyl CoA and *d*-biotin with the carboxyl transferase component was characterized after stabilization with diazomethane. As indicated in Table 2 (A), after a 15-min incubation of [3-¹⁴C]malonyl CoA and *d*-biotin with carboxyl transferase, about 70% of the [3-¹⁴C] carboxyl group of malonyl CoA was converted to a form stable to gassing with CO₂, but labile in acid. 30% of the [¹⁴C]CO₂ was presumably lost by enzymatic decarboxylation of malonyl CoA and by nonenzymatic decarboxylation of *N*-carboxybiotin (*t*_{1/2} about 60 min, unpub-

Fig. 5. Hypothetical scheme for intersubunit translocation of the carboxylated biotin prosthetic group of acetyl CoA carboxylase. BC refers to biotin carboxylase, CCP to carboxyl-carrier protein, and CT to carboxyl transferase.

both catalyze model reactions with free biotin that account for their respective catalytic functions in the half-reactions (Reactions 1 and 2) of the overall process. It is evident that both the biotin carboxylase and carboxyl transferase components contain distinct biotin binding sites. Furthermore, these sites are highly specific since free *l*-biotin cannot replace free *d*-biotin in either model reaction (Fig. 2 and ref. 5). On the other hand, the biotin-binding sites of the two enzymes differ markedly with respect to their apparent affinities for free *d*-biotin; biotin carboxylase and carboxyl transferase have K_m values for free biotin of 170 mM (5) and 3 mM, respectively. Interestingly, both enzyme components have far lower affinities for free carboxybiotin. Enzymatically-synthesized, free, [14 C]carboxy-*d*-biotin preparations (containing 10–15 mM free [14 C]carboxy-*d*-biotin and approximately equimolar free *d*-biotin) were not decarboxylated by biotin carboxylase in the presence of ADP, Pi, Mg^{2+} , hexokinase, and glucose, nor did they undergo carboxyl transfer to acetyl CoA in the presence of carboxyl transferase.

It is apparent that the bicyclic ring of the biotinyl prosthetic group of the carboxyl-carrier-protein must oscillate between two catalytic sites, i.e., biotin carboxylating and carboxyl transferring, which are housed on different subunits (see Fig. 5). The present investigation provides the first direct structural evidence for intersubunit translocation of the carboxylated biotinyl group and is supported by kinetic evidence for "ping-pong" mechanisms obtained with two other biotin-dependent enzymes, i.e., bacterial methylmalonyl CoA:pyruvate transcarboxylase (15, 16) and liver acetyl CoA carboxylase (17, 18).

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