Acetyl-CoA carboxylase catalyzes the first committed step in the biosynthesis of fatty acids. The Escherichia coli form of the enzyme consists of a biotin carboxylase protein, a biotin carboxyl carrier protein, and a carboxyltransferase component. In this report the overexpression of the genes for the carboxyltransferase component is described. The steady-state kinetics of the recombinant carboxyltransferase are characterized in the reverse direction, in which malonyl-CoA reacts with biocytin to form acetyl-CoA and carboxybiocytin. The initial velocity patterns indicated that the kinetic mechanism is equilibrium-ordered with malonyl-CoA binding before biocytin and the binding of malonyl-CoA to carboxyltransferase at equilibrium. The biotin analogs, desthiobiotin and 2-imidazolidone, inhibited carboxyltransferase. Both analogs exhibited parabolic noncompetitive inhibition, which means that two molecules of inhibitor bind to the enzyme. The pH dependence for both the maximum velocity (V) and the (V/K)biocytin parameters decreased at low pH. A single ionizing group on the enzyme with a pK of 6.2 or lower in the (V/K)biocytin profile and 7.5 in the V profile must be unprotonated for catalysis. Carboxyltransferase was inactivated by N-ethylmaleimide, whereas malonyl-CoA protected against inactivation. This suggests that a thiol in or near the active site is needed for catalysis. The rate of inactivation of carboxyltransferase by N-ethylmaleimide decreased with decreasing pH and indicated that the pK of the sulfhydryl group had a pK value of 7.3. It is proposed that the thiolate ion of a cysteine acts as a catalytic base to remove the N1 proton of biocytin.

The first committed step in fatty acid biosynthesis is catalyzed by acetyl-CoA carboxylase (1). The enzyme is found in all animals, plants, and bacteria and catalyzes the biotin-dependent carboxylation of acetyl-CoA to form malonyl-CoA in the two steps of Reaction 1.

\[
\text{Enzyme-biotin + Mg}^{2+} + \text{ATP + HCO}_3^{-} \xrightarrow{\text{Mg}^{2+}} \text{enzyme-biotin-CO}_2^2^- + \text{Mg}^{2+} + \text{ATP + Pi} \quad \text{(Step 1)}
\]

\[
\text{Enzyme-biotin-CO}_2^2^- + \text{acetyl-CoA} \xrightarrow{\text{enzyme-biotin}} \text{malonyl-CoA} + \text{enzyme-biotin} \quad \text{(Step 2)}
\]

**REACTION 1**

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MATERIALS AND METHODS

E. coli strain JM109 and plasmid pGem-11zf were purchased from Amersham Promega Biotech. The expression vector, pET14b, and the host strain, E. coli BL21(DE3)pLysS, were from Novagen. The Wizard kit for plasmid isolation and purification was from Promega. All restriction enzymes, dNTPs, deep vent polymerase, and T4 DNA ligase were purchased from New England Biolabs. Primers were synthesized by Life Technologies Inc. A Sequenase Quick-Denature plasmid sequencing kit with sequenase version 2.0 was from Amersham Promega Biotech. His-binding resin, columns, and protocol were from Novagen. All other reagents were from Sigma.

Construction of Expression Vectors—The steps used to construct the expression vectors pCZB4 and pCZB5 are summarized in Fig. 1. The gene for the α subunit of carboxyltransferase, designated accA, was contained on pLS181, which was obtained from Dr. John Cronan, University of Illinois. The gene for the β subunit of carboxyltransferase, designated accD, was contained on pCS, which was obtained from Dr. Dean Tolan, Boston University.

This paper is available on line at http://www.jbc.org
The accA gene was amplified by the polymerase chain reaction using the primers (5'-CCG CCT GAG TTC TCC TTA CGC GTA ACC GTA GCT CAT CAG GCC TCA GGT TCC TGA TCC GGT AC-3') and (5'-GGA ATT CCA TAT GAG TCT GAA TTT) which incorporated the restriction sites EcoRI, NdeI and XhoI and a ribosome binding site into the 959-base pair amplified fragment. The accD gene was amplified using the primers (5'-CCG GAT CCT CAG GCC TCA GGT TCC TGA TCC GGT AC-3') and (5'-CGG CTC GAG TTC TCC TTA CGC GTA ACC GTA). The amplification of each of these two components resulted in approximately 1.5-kilobase fragments containing the restriction sites EcoRI and BamHI into the 999-base pair amplified fragment. Polymerase chain reaction cycling conditions were as follows: melting, 1 min at 94 °C; annealing, 1 min at 55 °C; and extension, 1.5 min at 72 °C for 40 cycles.

The amplified DNA fragments were digested with EcoRI and XhoI in the case of accA and XhoI and BamHI in the case of accD using conditions recommended by the enzyme supplier (New England Biolabs). The accA and accD fragments were then ligated to similarly digested pGem-11zf to generate plasmids pCZB2 and pCZB1, respectively. pCZB1 was digested with the restriction enzymes XhoI and BamHI, and the 0.9-kilobase fragment containing accD was isolated by DNA gel electrophoresis. The 0.9-kilobase fragment containing accD was then ligated to similarly digested pCZB2 resulting in a new plasmid, pCZB3, containing both accA and accD. accA and accD were subcloned out of pCZB3 and into pET22b and pET14b with the restriction enzymes NdeI and BamHI to produce the vectors pCZB4 and pCZB5, respectively. pCZB5 contains a His-tag sequence that allows for expression of carboxyltransferase with a 20-amino acid His-tag (Novagen) fused to the amino terminus of the α subunit. pCZB4 and pCZB5 were transformed into BL21(DE3)pLysS, which contains a chromosomal copy of the T7 RNA polymerase gene under the control of the lacUV5 promoter.

**Growth Conditions for Overexpression**—Bacteria were grown in LB medium supplemented with 100 μg/ml ampicillin. A fresh overnight culture from a single colony was used to inoculate 0.5 liter of medium (50 ml in 10 125-ml Erlenmeyer flasks). Attempts to do growths in 2-liter flasks resulted in an increase in insoluble protein. The cultures were grown at 37 °C until A650 reached 0.60–0.70, then lactose was added to a final concentration of 28 mM. The temperature was decreased to 30 °C upon induction, and the cultures were incubated an additional 2.5 h. The cells were harvested by centrifugation at 8,000 × g for 10 min at 4 °C.

**Purification**—Cell paste from 0.5 liter of culture was suspended in 20 ml of binding buffer (50 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl, pH 7.9) and lysed by freezing the cells and then thawing. His-binding resin was used for rapid one-step affinity purification of carboxyltransferase containing the His-tag sequence. The protocol recommended by Novagen for His-tag protein purification was followed except that carboxyltransferase was eluted from the 2.5-ml His-tag columns with a solution of 170 mM imidazole, 500 mM NaCl, 120 mM Tris-Cl, pH 7.9. The protein solution was dialyzed overnight against 0.67 mM EDTA, 10 mM KPiO4, pH 7.0, and then dialyzed overnight against 500 mM KCl, 10 mM Hepes, pH 7.0. Carboxyltransferase was concentrated by placing dialysis tubing containing the enzyme in a container and covering the tubing with polyvinylpyrrolidone. Freezing of carboxyltransferase was found to cause irreversible precipitation/denaturation, therefore all protein solutions were stored at 4 °C.

**Carboxyltransferase Activity**—Carboxyltransferase activity was measured in the reverse direction with a spectrophotometric assay in which the production of acetyl-CoA was coupled to the combined citrate synthase-malate dehydrogenase reaction requiring NAD+ reduction (10). The reaction mixture (0.5 ml) contained 100 mM Tris-Cl, pH 8.0, 10 mM l-malate, 0.6 mg/ml bovine serum albumin, 3.6 units/ml malic dehydrogenase, 6.8 units/ml citrate synthase. For inhibition studies with dithiobiotin, the ionic strength was held constant by the addition of KCl. pH studies were done in a three-component buffer system of 0.1 M Mes, 0.051 M N-ethylmorpholine, and 0.051 M diethanolamine. Over the pH range of 6.2–9.6, for which the initial velocities were measured, the ionic strength of the buffer mixture remained constant at a value of 0.1 M (11). Data were collected using a Uvikon 810 (Kontron Instruments) spectrophotometer interfaced to a PC equipped with a data acquisition program. The temperature was maintained at 25 °C by a circulating water bath with the capacity to heat and cool the thermal space of the cell compartment. NADH formation was followed spectrophotometrically at 340 nm. Specific activity is expressed as μmol/min/mg of carboxyltransferase.

**Enzyme modifications by DEPC and N-ethylmaleimide (NEM)** were performed in 10 mM Hepes, 500 mM KCl, pH 7.0. The reactions were initiated by the addition of one of the inactivators to the carboxyltransferase solution. Aliquots were removed at regular time intervals and assayed for remaining activity. Aliquots were of sufficiently small volume compared with the assay volume such that the inactivating reagent was diluted 100-fold. NEM and DEPC were used at 4 and 1 mM, respectively. The protection by either malonyl-CoA or biocytin or both was assayed by incubating the protective agent with the enzyme in 10 mM Hepes, 500 mM KCl, pH 7.0, for 2 min before adding the inactivating agent. The pH dependence of the rate of inactivation of carboxyltransferase by NEM was not determined in the tripart buffer system used for the rate profiles because of inactivation of NEM by diethanolamine. The buffer system used was Mes, pH 6.2–6.5, Pipes, pH 6.5–7.0, Hepes, pH 7.0–8.0.

**Data Analysis**—Data were fitted to the appropriate equation using either the computer program Enzfitter or the computer programs of Cleland (13). Initial velocities obtained by varying one of the substrates (A) were substituted in Equation 1 to yield values for the maximum velocity (V) and the Michaelis constant for the substrate (K) (11).

\[ v = \frac{V A}{K+A} \]  

**Eq. 1**

When one substrate was varied at fixed levels of the other, velocity data were fitted to Equation 2, which describes the equilibrium-ordered initial velocity pattern, where \( v \) is the experimentally determined velocity, \( V \) is the maximum velocity, \( A \) and \( B \) are the substrate concentrations, \( K_A \) and \( K_B \) are the respective Michaelis constants, and \( K_{ab} \) is the dissociation constant of \( A \).

\[ v = \frac{V_{AB}}{K_{AB}+A+B} \]  

\[ \text{Eq. 2} \]

The abbreviations used are: Mes, 2-(N-morpholino)ethanesulfonic acid; DEPC, diethyl pyrocarbonate; NEM, N-ethylmaleimide; Pipes, 1,4-piperazinediethanesulfonic acid.
Characterization of Carboxyltransferase

\[ v = \frac{VA}{K_aK_b + KA + AB} \]  
\[ (Eq. 2) \]

Data conforming to parabolic noncompetitive inhibition were fitted to Equation 3,

\[ v = \frac{VA}{K(1 + I/K_{i1} + I/K_{i2}) + A(1 + I/K_{i1} + I/K_{i2})} \]
\[ (Eq. 3) \]
in which I is the inhibitor concentration; \( K_{i1} \) and \( K_{i2} \) are the slope inhibition constants for site 1 and 2, respectively, and \( K_{i1} \) and \( K_{i2} \) are the intercept inhibition constants for site 1 and 2, respectively.

The variation of the values for \( V/V/K \), and \( k \), the rate of inactivation of carboxyltransferase by NEM as a function of pH were fitted to the log form of Equation 4. In this equation \( y \) represents the value of \( V \) or \( V/K \) at a particular pH value, \( C \) represents the pH-independent value of the parameter, \( K \), is an acid dissociation constant, and \( H \) is the hydrogen ion concentration.

\[ y = C/(1 + H/K_a) \]
\[ (Eq. 4) \]

RESULTS

Overexpression and Purification of Carboxyltransferase—The genes for the \( \alpha \) and \( \beta \) subunits of carboxyltransferase are not contiguous on the \( E. coli \) genome, yet they are presumably expressed stoichiometrically to give the \( \alpha_2\beta_2 \) tetramer of carboxyltransferase. Therefore, an overexpression system needed to be developed such that the genes for the \( \alpha \) and \( \beta \) subunits were contiguous and expressed stoichiometrically. The gene for the \( \alpha \) subunit was expressed using the ribosomal binding site from the pET vector. Thus, what was needed was an intercistronic region that allowed for efficient expression of the gene for the \( \beta \) subunit. To accomplish this, a 12-base pair intercistronic region that was very similar to that found between the genes for the catalytic and regulatory subunits of \( E. coli \) aspartate transcarbamylase was used (14). Like carboxyltransferase, aspartate transcarbamylase is composed of two different subunits, a catalytic subunit and a regulatory subunit. The genes for each subunit are contiguous on the \( E. coli \) genome and are expressed stoichiometrically so that intact aspartate transcarbamylase is composed of six catalytic subunits and six regulatory subunits. The 12-base pair intercistronic region that was incorporated between the genes for the \( \alpha \) and \( \beta \) subunits of carboxyltransferase contains a ribosomal binding site and an \( XhoI \) site and allowed the gene for the \( \beta \) subunit to be co-overexpressed with the \( \alpha \) subunit.

When bacteria containing pCZB5 (Fig. 1) were grown at 37 °C after induction, the genes for both the \( \alpha \) and \( \beta \) subunits were expressed very well. Unfortunately, the enzyme was found in inclusion bodies. To obtain enzyme in the soluble fraction, bacteria were grown at 30 °C after induction. His-tag carboxyltransferase was purified to apparent homogeneity using a nickel column (Fig. 2), and control experiments verified that the native carboxyltransferase did not bind to the nickel column. The average yield of carboxyltransferase was 6 mg from 3 g of \( E. coli \) cells. In contrast, it takes 2 kg of \( E. coli \) cells to obtain 6 mg of carboxyltransferase when the genes are not overexpressed (2). Attempts to remove the His-tag from carboxyltransferase by treatment with thrombin resulted in complete degradation of the \( \alpha \) subunit. Because the presence of the His-tag did not hinder the catalytic ability of the enzyme, the His-tag carboxyltransferase was left intact. The \( \alpha \) and \( \beta \) genes for carboxyltransferase were sequenced and compared with published sequences to ensure that the polymerase chain reaction did not introduce any mutations (5–7).

The kinetic parameters for carboxyltransferase-catalyzed transcarboxylation from malonyl-CoA to biotin and two biotin analogs are shown in Table I. Biotin methyl ester and biocytin had maximal velocities 3 orders of magnitude higher than biotin. Biocytin is biotin with lysine attached to the carboxyl group of the valeric acid side chain via an amide linkage with the \( \epsilon \)-amino group (Fig. 3). Because biotin reacted considerably slower than the other two analogs and biotin methyl ester had to be dissolved in dimethylformamide, all of the subsequent studies utilized biocytin as substrate.

Velocity Studies—The kinetic mechanism of carboxyltransferase was investigated by determining the initial velocity pattern. When malonyl-CoA was varied at several fixed levels of biocytin an intersecting pattern was obtained, indicating the sequential addition of malonyl-CoA and biocytin prior to product release (Fig. 4A). When biocytin was varied at several fixed levels of malonyl-CoA the pattern was intersecting; however, the intersection point was on the vertical axis (Fig. 4B). A replot of the slopes of the data versus the reciprocal of the biocytin concentration when malonyl-CoA was varied went

| Substrates       | \( K_m \)  | \( V_{max} \) |
|------------------|-----------|-------------|
|                  | \( \mu \text{mol/min} \) | \( \mu \text{mol/min} \cdot \text{mg} \) |
| Biotin           | 2.2 ± 0.3 | 0.040 ± 0.003 |
| Biotin methyl ester (DFM) | 19.3 ± 2.0 | 25.3 ± 1.2 |
| Biocytin         | 8.25 ± 0.03 | 22.40 ± 0.03 |

FIG. 2. Structure of biotin and various biotin analogs.
versus shaped curves. Because the replots of the slopes and intercepts versus inhibition (Fig. 5). Replots of both the slopes and intercepts of various concentrations of desthiobiotin gave noncompetitive inhibition of the enzyme. Reciprocal plots of the velocity of formation of a dead-end complex between the inhibitor and the enzyme. Polakis et al. (15) determined that desthiobiotin and 2-imidazolidone were not alternate substrates for carboxyltransferase so the observed inhibition is the result of formation of a dead-end complex between the inhibitor and the enzyme. Reciprocal plots of the velocity versus biocytin at various concentrations of desthiobiotin gave noncompetitive inhibition (Fig. 5). Replots of both the slopes and intercepts versus the desthiobiotin concentration resulted in parabola-shaped curves. Because the replots of the slopes and intercepts versus the desthiobiotin concentration were both parabolic the data were fitted to an equation that describes noncompetitive inhibition where only the slopes and intercepts are parabolic functions of the inhibitor concentration (Equation 3). For comparison the data were also fitted to equations that describe noncompetitive inhibition where only the slope or the intercept is a parabolic function of inhibitor concentration. The data fit best to Equation 3 based on the fact that the average least squares of the residuals was the lowest for Equation 3. The inhibition constants for the two inhibitor binding sites are given in Table II. It should be noted that all inhibition constants are given in Table II. The fact that the inhibition data conform to noncompetitive inhibition means that two molecules of desthiobiotin bind to the enzyme simultaneously.

The biotin analog 2-imidazolidone also acted as a parabolic noncompetitive inhibitor of carboxyltransferase, although it was a much worse inhibitor than desthiobiotin. The inhibition constants are given in Table II. It should be noted that although 2-imidazolidone is not an alternate substrate, it was found to stimulate the decarboxylation of malonyl-CoA (15). Therefore, the rates of decarboxylation of malonyl-CoA at each level of 2-imidazolidone were subtracted from the velocities obtained in the presence of biocytin. The rate of decarboxylation of malonyl-CoA by 2-imidazolidone was 5% or less of the rate of carboxyl group transfer.

**pH Dependence of V and V/K for Biocytin**—The effect of pH on the carboxyltransferase reaction was determined over the pH range of 6.2–9.6 by varying the biocytin concentrations at a saturating fixed level of malonyl-CoA. Under these conditions, the reaction examined was that of biocytin with the enzyme malonyl-CoA complex. As shown in Fig. 6, both log V and log (V/K) biocytin decreased with decreasing pH, although the (V/K)biocytin only decreased 2-fold. Fitting the data to Equation 4 yielded a pK of 7.52 ± 0.10 for the V profile and 6.21 ± 0.10 for the V/K profile. However, because data could not be obtained below pH 6.2 due to enzyme instability, the pK value in the V/K profile can only be estimated to be 6.2 or less.

**Inactivation Studies**—DEPC reacts preferentially with histidine residues to yield an N-carbethoxyhistidyl derivative (12). Incubation of carboxyltransferase with 1 mM DEPC resulted in a time-dependent loss in activity with a pseudo-first order rate constant of 0.09 min⁻¹. However, neither biocytin nor malonyl-CoA provided significant protection against DEPC inactivation, indicating that the histidine is probably not in the active site. Modification of the poly-His-tag by DEPC is not occurring because nickel sulfate did not protect against the modification.

Carboxyltransferase was found to be susceptible to inactivation by the sulfhydryl-modifying reagent NEM. NEM (4 mM) inactivated carboxyltransferase with a pseudo-first order rate constant of 0.06 min⁻¹. In the presence of 330 μM malonyl-CoA, almost complete protection (90%) against inactivation by NEM...
was observed. Biocytin provided no protection against inactivation by NEM. The rate of inactivation of carboxyltransferase by NEM decreased with decreasing pH (Fig. 7). Fitting the data to Equation 4 yielded a pK value of 7.32 ± 0.16.

DISCUSSION

Acetyl-CoA carboxylase plays a critical role in metabolism in that it catalyzes the committed and regulated reaction in the synthesis of long chain fatty acids. The metabolic significance of this enzyme is evident from the fact that inhibitors directed against the plant or mammalian form of the enzyme can be either herbicides or plasma lipid-lowering drugs. These agents work by inhibiting the carboxyltransferase component of acetyl-CoA carboxylase. Despite the apparent importance of acetyl-CoA carboxylase to agriculture and medicine, very little work has been done on the carboxyltransferase component, presumably because there has never been an adequate source of the enzyme. With the overexpression system described here significant amounts of protein have been obtained, allowing for a more rigorous characterization of carboxyltransferase than has been done previously (15).

A major experimental problem with site-directed mutagenesis studies of E. coli enzymes is contamination of the mutant enzyme with wild-type enzyme derived from the chromosomal copy of the gene. In most cases this problem can be overcome by removing the chromosomal copy of the gene. If the enzyme is involved in an essential metabolic pathway, then for mutant enzymes with very little to no activity, a metabolic intermediate subsequent to the step in question can be added to the culture medium to sustain bacterial growth. Unfortunately, none of the metabolic intermediates after the step catalyzed by acetyl-CoA carboxylase are transported into E. coli. Because fatty acid synthesis is essential for bacterial growth, it would be impossible to remove the chromosomal copy of carboxyltransferase and sustain bacterial growth while overexpressing a mutant form of carboxyltransferase with very little to no activity. Because one of the uses of the overexpression system for carboxyltransferase will be for production of mutant enzymes, the genes for the α and β subunits of carboxyltransferase were subcloned into pET14b to produce the expression vector pCZB5 (Fig. 1). Using this expression vector carboxyltransferase is produced with a His-tag sequence fused to the NH₂ terminus of the α subunit which allows the enzyme to be purified by affinity chromatography using a nickel column. The chromosomal copy of the enzyme, which lacks the His-tag sequence, does not bind to the nickel column. Therefore, the

![FIG. 5](image-url)  
**FIG. 5.** Dead-end inhibitor of the reaction catalyzed by carboxyltransferase. Inhibition by desthiobiotin at 0 mM (●), 20 mM (○), 60 mM (■), and 100 mM (▲) with respect to biocytin at concentration of 0.3 mM malonyl-CoA is shown. Enzyme activity was measured at 25 °C in 100 mM Tris buffer at pH 8.0. Velocities are expressed as μmol of acetyl-CoA formed/mg of carboxyltransferase per minute. Data were fitted to Equation 3.

![FIG. 6](image-url)  
**FIG. 6.** Variation of pH of log V and log (V/K) biocytin for the reaction catalyzed by carboxyltransferase. Initial velocity data were obtained by varying biocytin at a fixed malonyl-CoA concentration of 4 mM. The curves for V (●) and V/K (▲) represent the best fits of the data to Equation 4.

### TABLE II

| Inhibition constants | Inhibitor | Desthiobiotin | 2-Imidazolidone |
|---------------------|----------|---------------|----------------|
|                     |          | mm  | mm           |
| K₁1₁  | 0.054 ± 0.019 | 1.1 ± 0.5 |
| K₁₂   | 2.9 ± 1.0   | 1.2 ± 0.6 |
| K₂₁₁  | 0.27 ± 0.55 | 38 ± 520 |
| K₂₂   | 3.2 ± 1.4   | 1.4 ± 0.9 |

The error for the inhibition constants is the standard error. It was derived from the nonlinear regression analysis and was calculated from the square root of the variance of the parameter (22).
His-tag version of carboxyltransferase was purified and characterized to lay the groundwork for future site-directed mutagenesis studies.

The intersecting initial velocity pattern when malonyl-CoA and biocytin are varied indicates that the kinetic mechanism of carboxyltransferase is sequential. That the lines intersected on the 1/V axis when biocytin was varied at several fixed levels of malonyl-CoA (Fig. 4B) and that a replot of the slopes of the data versus the reciprocal of the biocytin concentration went through zero suggest that the kinetic mechanism is equilibrium-ordered with malonyl-CoA binding before biocytin and the binding of malonyl-CoA at equilibrium. An equilibrium-ordered pattern means that the biocytin does not form a binary complex with the enzyme but only combines with the enzyme-malonyl-CoA complex.

Dead-end inhibition studies would be extremely helpful to define further the kinetic mechanism. However, no suitable inhibitor was found. Because the assay for carboxyltransferase involves detecting the production of acetyl-CoA using citrate synthase, the inhibition studies precluded the use of CoA analogs and thus were limited to analogs of biotin. Although not very informative mechanistically, inhibition by two analogs of biotin proved very interesting and worth noting. Both desthiobiotin and 2-imidazolidone were parabolic noncompetitive inhibitors of the carboxyltransferase reaction when either biocytin or malonyl-CoA was varied. In the case of dead-end inhibitors like desthiobiotin and 2-imidazolidone, parabolic noncompetitive inhibition occurs only when two molecules of the inhibitor bind to the enzyme(16). One inhibitor site (site 1) may be at or near the binding site for biocytin, and the second inhibitor is probably at another location on the enzyme (site 2) because saturating amounts of substrate do not relieve inhibition totally. This interpretation would help to explain why the error for the inhibition constant for site 1 at saturating levels of substrate is greater than the parameter. Also, it is not clear why there is a second inhibitor binding site. It should be noted, however, that the carboxyltransferase component of E. coli acetyl-CoA carboxylase is inhibited by guanosine 5′-diphosphate-3′-diphosphate (ppGpp) and guanosine 5′-triphosphate-3′-diphosphate (pppGpp) (17). These nucleotides have been shown to inhibit RNA synthesis during amino acid starvation in what is referred to as the stringent response (18). Fatty acid synthesis is also reduced during amino acid starvation. The decrease in fatty acid synthesis is thought to be mediated by inhibition of carboxyltransferase by ppGpp and pppGpp. The second inhibitor site on carboxyltransferase may be the same as the pppGpp inhibition because it is found in the stringent response (18).

The intersecting initial velocity pattern when malonyl-CoA and biocytin are varied indicates that the kinetic mechanism of carboxyltransferase is sequential. That the lines intersected on the 1/V axis when biocytin was varied at several fixed levels of malonyl-CoA (Fig. 4B) and that a replot of the slopes of the data versus the reciprocal of the biocytin concentration went through zero suggest that the kinetic mechanism is equilibrium-ordered with malonyl-CoA binding before biocytin and the binding of malonyl-CoA at equilibrium. An equilibrium-ordered pattern means that the biocytin does not form a binary complex with the enzyme but only combines with the enzyme-malonyl-CoA complex.

The pH dependence of the reaction suggests that an enzymic residue acts as a base in catalysis. The activity for both the log V and the log (V/K) biocytin parameters decreased at low pH indicating that a group must be deprotonated for activity. The group has a pK value of 7.5 in the V profile. The V profile indicates the pK values of groups in the enzyme substrate complex that are required for catalysis. Thus, it is very probable that the observed base acts in a catalytic role because it is found in the V profile.

The inactivation of carboxyltransferase by NEM and the protection against inactivation by malonyl-CoA suggest that an active site cysteine residue is needed for catalysis. Like the pH dependence of the V and V/K parameters, the pH dependence of the rate of inactivation of carboxyltransferase by NEM decreased with decreasing pH. The pK value derived from the pH dependence of inactivation (7.32 ± 0.16) was, within error, the same as the pK value seen in the V profile (7.52 ± 0.10). Thus, given the similarity of the pH rate profiles and the pH dependence of the rate of inactivation of carboxyltransferase by NEM it is plausible that the residue that must be deprotonated for activity is a cysteine. The reverse reaction of carboxyltransferase requires an activated biocytin molecule that has the proton removed from the 1′-nitrogen prior to carboxylation. The thiolate ion of the cysteine suggested above may be the catalytic base that abstracts the N1′ proton of biocytin. It has been proposed that the thiolate ion of cysteine abstracts the N1′ proton of biotin in biotin carboxylase (19) and pyruvate carboxylase (20, 21).

Verification of this mechanism in terms of which cysteine residue is involved would be aided greatly by a three-dimensional structure of carboxyltransferase followed by site-directed mutagenesis studies. To this end, overexpression of the gene for carboxyltransferase now provides us with ample amounts of enzyme for structure/function studies such as crystallography and site-directed mutagenesis experiments. The His-tag carboxyltransferase allows mutant enzyme to be separated from the wild-type chromosomal copy so that there are no ambiguities in the interpretation of the kinetics of the mutant enzyme. And finally, crystals of the carboxyltransferase have been obtained and the crystal structure determination is currently underway.

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