Mutations at Four Active Site Residues of Biotin Carboxylase Abolish Substrate-Induced Synergism by Biotin†

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Received November 9, 1998; Revised Manuscript Received January 12, 1999

ABSTRACT: Acetyl-CoA carboxylase catalyzes the first committed step in the biosynthesis of long-chain fatty acids. The *Escherichia coli* form of the enzyme consists of a biotin carboxylase protein, a biotin carboxyl carrier protein, and a carboxyltransferase protein. In this report a system for site-directed mutagenesis of the biotin carboxylase component is described. The wild-type copy of the enzyme, derived from the chromosomal gene, is separated from the mutant form of the enzyme which is coded on a plasmid. Separation of the two forms is accomplished using a histidine-tag attached to the amino terminus of the mutant form of the enzyme and nickel affinity chromatography. This system was used to mutate four active site residues, E211, E288, N290, and R292, to alanine followed by their characterization with respect to several different reactions catalyzed by biotin carboxylase. In comparison to wild-type biotin carboxylase, all four mutant enzymes gave very similar results in all the different assays, suggesting that the mutated residues have a common function. The mutations did not affect the bicarbonate-dependent ATPase reaction. In contrast, the mutations decreased the maximal velocity of the biotin-dependent ATPase reaction 1000-fold but did not affect the K_m for biotin. The activity of the ATP synthesis reaction catalyzed by biotin carboxylase where carbamoyl phosphate reacts with ADP was decreased 100-fold by the mutations. The ATP synthesis reaction required biotin to stimulate the activity in the wild-type; however, biotin did not stimulate the activity of the mutant enzymes. The results showed that the mutations have abolished the ability of biotin to increase the activity of the enzyme. Thus, E211, E288, N290, and R292 were responsible, at least in part, for the substrate-induced synergism by biotin in biotin carboxylase.

Biotin carboxylase from *Escherichia coli* catalyzes the ATP-dependent carboxylation of biotin as shown in reaction 1 below and is one component of the multienzyme complex acetyl-CoA carboxylase. Acetyl-CoA carboxylase catalyzes the committed step in long-chain fatty acid synthesis via the two-step reaction mechanism shown below (*1*):

$$\text{reaction 1:}\quad \text{enzyme}-\text{biotin} + \text{MgATP} + \text{HCO}_3^- \xrightleftharpoons{Mg^{2+}} \text{enzyme}-\text{biotin}-\text{CO}_2^- + \text{MgADP} + \text{P}_i$$

reaction 2:

$$\text{enzyme}-\text{biotin}-\text{CO}_2^- + \text{acetyl-CoA} \rightleftarrows \text{malonyl-CoA} + \text{enzyme}-\text{biotin}$$

Reaction 1 involves the phosphorylation of bicarbonate by ATP to form a carboxyphosphate intermediate, followed by transfer of the carboxyl group to biotin to form carboxybiotin. In vivo, biotin is attached to the carboxyl carrier protein (designated as enzyme−biotin in reactions 1 and 2) through an amide bond to a specific lysine residue. In the second reaction, catalyzed by carboxyltransferase, the carboxyl group is transferred from biotin to acetyl-CoA to form malonyl-CoA. Upon isolation of the three components, biotin carboxylase and carboxyltransferase retain activity, and both enzymes will utilize free biotin as a substrate. Moreover, the genes for biotin carboxylase (*2*) and carboxyltransferase (*3*) have been overexpressed, ensuring that ample amounts of protein are available. Thus, biotin carboxylase and carboxyltransferase serve as model systems for mechanistic studies of biotin-dependent carboxylases.

The three-dimensional structure of biotin carboxylase has been determined by X-ray crystallography and is the first and only structural model of a biotin-dependent carboxylase (4). With the crystal structure of biotin carboxylase in hand, it is a propitious time to begin investigating the function of active site residues in catalysis by site-directed mutagenesis. Initially, four residues were chosen for mutagenesis: E211, E288, N290, and R292 (Figure 1). These four particular active site residues were selected for analysis because they are in close proximity in the active site, and they are strictly conserved in all biotin-dependent carboxylases sequenced to date (*5*). Furthermore, of all the active site residues in biotin carboxylase, only these four residues are strictly conserved in carbamoyl phosphate synthetase, which has considerable mechanistic and structural similarities to biotin carboxylase (*6*).

† This research was supported by grants from the NIH (GM51261) and the Petroleum Research Fund of the American Chemical Society (32234-AC4).

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10.1021/bi982660a CCC: $18.00
Published on Web 02/20/1999

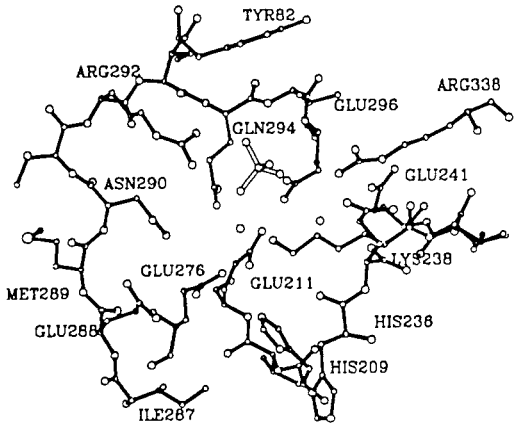

FIGURE 1: Active residues of biotin carboxylase. The drawing was taken from ref 4.

Biotin carboxylase and carbamoyl phosphate synthetase are members of a superfamily of enzymes known as the ATP-grasp proteins. The enzymes of this family share structural homology which is consistent with each enzyme catalyzing an ATP-dependent ligation of a carboxyl group to N or S atom via an acyl phosphate intermediate (*5*). Other members of this family include glutathione synthetase, D-Ala:D-Ala ligase, and succinyl-CoA synthetase. A recent sequence analysis and structural comparison of the ATP-grasp family of enzymes found that E288 and N290 of biotin carboxylase were strictly conserved throughout this family of proteins which suggested these two residues played an important role in the function of the enzyme (*5*). Finally, mutagenesis of these four residues in carbamoyl phosphate synthetase (*7*) and the residue equivalent to E288 in D-Ala:D-Ala ligase (*8*) all resulted in significant decreases in activity. Thus, it is likely that mutations at these residues will result in significant effects on biotin carboxylase activity.

In this report, we describe a system for studying mutants of biotin carboxylase that are free of contamination from wild-type enzyme derived from the chromosomal copy of the gene. This is followed by examining the effects of alanine-scanning mutagenesis of residues E211, E288, N290, and R292 on a variety of reactions catalyzed by biotin carboxylase. The results indicate that mutations at each of these residues abolish substrate-induced synergism by biotin and by inference these four residues are responsible, at least in part, for the dramatic increase in rate induced by biotin.

MATERIALS AND METHODS

Chemicals and Enzymes. Escherichia coli strain JM109 and plasmid pGem-7Zf were purchased from Promega. The expression vectors pET14b and pET28b 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 GibcoBRL. Sequenase Quick-Denature Plasmid Sequencing kit with sequenase version 2.0 was from Amersham. Collodion bags were from Schleicher & Schuell. Sodium bicarbonate labeled with ^{14}C was from Amersham and had a specific activity of 0.1 mCi/mmol. His·binding resin and restriction grade thrombin were from Novagen. Pyruvate kinase was from Boehringer Mannheim. All other reagents were from Sigma or Aldrich.

Table 1: Primers Used for Site-Directed Mutagenesis of Biotin Carboxylase

plasmid	DNA sequence[a]	enzyme
pGLW29	5′-CTCGCCACGTCGCGATTCAGGTACT-3′	E211A
	3′-GAGCGGTGCAGCGCTAAGTCCATGA-5′	
pGLW8	5′-TCTATTTCATCGCAATGAACACCCG-3′	E288A
	3′-AGATAAAGTAGCGTTACTTGTGGGC-5′	
pYML6	5′-ATTTCATCGAAATGGCCACCCGTATTCAGGT-3′	N290A
	3′-TAAAGTAGCTTTACCGGTGGGCATAAGTCCA-5′	
pGLW10	5′-TCGAAATGAACACCGCTATTCAGGTAGAACA-3′	R292A
	3′-AGCTTTACTTGTGGCGATAAGTCCATCTTGT-5′	

[a] The underlined bases indicate the nucleotide positions that were changed.

Construction of Histidine-Tagged Biotin Carboxylase. To separate the plasmid copy of biotin carboxylase from the chromosomal copy of the enzyme, a poly-histidine-tag was engineered on the 5′ end of the plasmid copy of the gene. This was accomplished by using the polymerase chain reaction (PCR) to introduce restriction sites *Eco*R1 and *Nde*1 on the 5′ end of the biotin carboxylase gene and a *Bam*H1 site on the 3′ end. The sequence of the primer containing the *Eco*R1 and *Nde*1 sites was 5′-GGA ATT CCA TAT GAT GCT GGA TAA AAT TGT TAT T-3′ while the primer sequence containing the *Bam*H1 site was 5′-CGG GAT CCC GTT ATT TTT CCT GAA GAC CGA G-3′. The template biotin carboxylase gene used for the PCR reactions was contained on pGBH which was a gift from Dr. John Cronan of the University of Illinois. The PCR product was cut with *Eco*R1 and *Bam*H1 and ligated into pGEM-7f cut with the same two enzymes to form pGLW1. The entire gene for biotin carboxylase was sequenced to confirm that the PCR did not introduce any changes in the sequence. The expression plasmid for the biotin carboxylase gene was constructed by cutting pGLW1 with *Nde*1 and *Bam*H1, isolating the fragment containing the biotin carboxylase gene, and ligating into pET14b cut with the same two enzymes to create pGLW2. In pGLW2, the biotin carboxylase gene was extended on the 5′ end to code for 20 amino acids which contain a contiguous sequence of 6 histidine residues.

Site-Directed Mutagenesis. The PCR method of overlap extension (*9*) was used to make site-directed mutants of biotin carboxylase. The template for the PCR reactions was pGLW1. The pairs of internal primers used to make each site-directed mutant are shown in Table 1. The flanking primers were those used above to construct the histidine-tagged biotin carboxylase. The procedure for cloning the PCR products and insertion into the expression plasmid to make mutant forms of biotin carboxylase with a histidine-tagged sequence was the same as for the wild-type described above except that all the mutants were overexpressed using pET28b. The entire gene for each mutant form of biotin carboxylase was sequenced to verify the mutation and confirm there were no other changes in the sequence.

Growth and Purification of Wild-Type and Mutant Enzymes. Plasmids coding for wild-type or mutant forms of biotin carboxylase were transformed into *E. coli* strain BL21-(DE3)pLysS. Bacteria were grown at 37 °C in 1 L of LB medium in 2 L flasks that were inoculated with an overnight culture grown in the presence of 100 μg/mL of ampicillin for the wild-type enzyme or 30 ug/mL of kanamycin for the mutant enzymes. Cultures were grown to midlog phase and then induced with 1 g of lactose and incubated for another

2 h. The cells were harvested by centrifugation at 10400*g* for 10 min at 4 °C.

Cell paste was suspended in binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and lysed by the freeze−thaw method. DNase was added to degrade the nucleic acids. The lysate was centrifuged at 20200*g* for 1 h at 4 °C. The soluble fraction of the crude lysate was loaded on a column of His-binding resin and then washed with binding buffer. The column was then washed with the following buffer: 60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9, 0.1% Triton X-100. This buffer allowed the removal of any proteins nonspecifically bound to the column. The column was then washed with the same buffer without Triton X-100. It was determined that biotin carboxylase without a histidine-tag bound to the His-binding resin but could be removed with the above washes. Biotin carboxylase with a histidine-tag was eluted from the column with a solution of 170 mM imidazole, 500 mM NaCl, 120 mM Tris-HCl, pH 7.9. The protein solution was dialyzed overnight against 0.67 mM EDTA, 10 mM $KHPO_4$, pH 7.0. The protein was precipitated by the addition of ammonium sulfate to 60% saturation. The precipitate was dissolved and dialyzed overnight against 500 mM KCl, 10 mM HEPES, pH 7.0. Biotin carboxylase was concentrated by vacuum dialysis using a collodion bag apparatus.

Removal of the His-Tag. For experiments in which the concentration of magnesium ion was varied, the His-tag was removed from biotin carboxylase by the following method. The protein was dialyzed overnight against 150 mM NaCl, 2.5 mM $CaCl_2$, and 120 mM Tris-HCl, pH 8.4. Restriction grade thrombin was added to the protein solution at a ratio of 1 unit of thrombin to 1 mg of biotin carboxylase and incubated 16 h at 25 °C. The protein solution was then loaded onto a His-binding column and washed with binding buffer to remove any thrombin. Biotin carboxylase with the His-tag removed was eluted from the column with 60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9, and processed as described above for the His-tag protein. Any enzyme that was not cut by the thrombin remained bound to the column and could be recovered by eluting with the same buffer used above to elute the His-tag version of the enzyme.

Steady-State Kinetic Assays. The rate of ATP hydrolysis by biotin carboxylase in the absence or presence of biotin was measured spectrophotometrically. The production of ADP was coupled to pyruvate kinase and lactate dehydrogenase, and the oxidation of NADH was followed at 340 nm. Each measurement was carried out in a volume of 0.5 mL in 1 cm path length quartz cuvettes. The reaction mixture contained 10 units of pyruvate kinase, 18 units of lactate dehydrogenase, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 8 mM $MgCl_2$, and 100 mM HEPES at pH 8.0. It was found that these amounts of coupling enzymes were necessary to ensure that the initial velocity varied linearly with enzyme concentration. Since the K_m for biotin is high (100 mM), the ionic strength of the reaction mixture was held constant with KCl when the initial velocity was measured as a function of the biotin concentration.

The rate of ATP synthesis from MgADP and carbamyl phosphate was determined spectrophotometrically with the coupled enzyme system of hexokinase and glucose-6-phosphate dehydrogenase where the production of NADPH at 340 nm was measured. Each assay mixture contained 5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, 0.5 mM glucose, 0.4 mM NADP, 8 mM $MgCl_2$, 50 mM biotin, and 100 mM HEPES, pH 8.0.

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 thermospace of the cell compartment. Reactions were initiated by the addition of enzyme. Values of V and V/K were calculated per active site using a value of 50 000 for the molecular mass of the monomer of biotin carboxylase which exists as a homodimer.

When the concentration of bicarbonate was varied, special precautions had to be taken since the endogenous level of bicarbonate at pH 8.0 is 0.5 mM (*10*), which is close to the K_m for bicarbonate in biotin carboxylase. Thus, the endogenous bicarbonate had to be removed so the bicarbonate could be varied below its K_m value. The following describes in detail the procedure used to remove the endogenous bicarbonate. Deionized water was boiled for 10 min and cooled in a glovebag under a nitrogen atmosphere. A stock solution of HEPES buffer, pH 8.0, was also prepared under nitrogen in the glovebag. Stock solutions of ATP, phosphoenolpyruvate, NADH, pyruvate kinase, and lactate dehydrogenase were made in vials containing septum. After the powder or liquid was added to the vials, they were capped and flushed with nitrogen. The nitrogen was passed through an Ascarite column to remove any contaminating CO_2. The appropriate amount of HEPES, pH 8.0, was added to each vial using a Hamilton syringe after which a cocktail containing all the assay components except bicarbonate and enzyme was made. Assays were carried out in a volume of 1 mL and contained 3 mM ATP, 60 mM biotin, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 8 mM $MgCl_2$, 20 units of pyruvate kinase, 36 units of lactate dehydrogenase, and 100 mM HEPES. A stock bicarbonate solution was prepared and stored in a gastight flask. The reactions were carried out in 1 mL cuvettes with a 1 cm path length fitted with rubber septa. Cuvettes were flushed with nitrogen, assay components were added with a Hamilton syringe, and the assays were started with the addition of enzyme.

Carboxybiotin Assay. The amount of carboxybiotin produced by biotin carboxylase was determined using a modification of the [^{14}C]bicarbonate fixation assay of Guchhait et al. (*11*). Reactions were carried out in 1.5 mL microfuge tubes in a reaction volume of 0.5 mL at 25 °C. The reaction mixture contained 9 mM ATP, 8 mM $MgCl_2$, 100 mM biotin, 70 mM $KHCO_3$, and 100 mM HEPES at pH 8.0. Each reaction mixture was supplemented with 3.5 μL (17.5 nCi) of a $NaH^{14}CO_3$ solution. The reaction was initiated by the addition of enzyme and allowed to react for 1 h. Control experiments showed that carboxybiotin decomposed after 1 h under the conditions of the reaction. The reaction was stopped by the addition of 3.6 μL of a saturated KOH solution to remove the proton of any unreacted bicarbonate, and then 200 μL of a 2.0 M $BaCl_2$/0.1 M $Ba(OH)_2$ solution was added to precipitate the carbonate. The tubes were mixed and allowed to sit for 2 min and then centrifuged at 10000*g* for 5 min. A 100 μL aliquot from the supernatant was transferred to a scintillation vial and counted to determine the amount of ^{14}C activity. An aliquot of the reaction mixture

without enzyme and without precipitation by $BaCl/Ba(OH)_2$ was counted to determine the specific activity of the bicarbonate. This activity was used to calculate the amount of carboxybiotin.

The amount of ADP produced was also measured to determine if there was stoichiometric production of carboxybiotin and ADP. The reaction conditions were exactly the same as they were for the carboxybiotin assay above except no radiolabeled bicarbonate was added. Upon mixing the reaction components, aliquots were removed immediately and after a 1 h incubation. The amount of ATP in each aliquot was measured by end-point analysis. The end-point analysis was conducted in a total volume of 1.0 mL and contained 7.5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, 0.5 mM glucose, 0.4 mM NADP, 8 mM $MgCl_2$, and 100 mM HEPES, pH 8.0. The amount of ADP produced was determined from the difference between the amount of ATP at the beginning of the reaction and the amount of ATP left after a 1 h incubation.

Analysis of Kinetic Data. The parameters K_m and V_{max} were determined by nonlinear regression analysis of the velocity versus substrate concentration data using the program Enzfitter. The data were fitted to eq 1 where v is the initial velocity, V_m is the maximal velocity, K_m is the Michaelis constant, and A is the substrate concentration:

$$v = V_m A/(K_m + A) \quad (1)$$

When nonlinear double-reciprocal plots were observed, the data were fitted to eq 2 which describes substrate activation where V_1 and V_2 are the maximal velocities, K_1 and K_2 are the Michaelis constants, and A is the substrate concentration:

$$v = V_1 A/(K_1 + A) + V_2 A/(K_2 + A) \quad (2)$$

RESULTS

Use of the Histidine-Tag To Eliminate the Endogenous Wild-Type Biotin Carboxylase. 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 after 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*. Since fatty acid synthesis is essential for bacterial growth, it would be impossible to remove the chromosomal copy of biotin carboxylase and sustain bacterial growth while overexpressing an inactive mutant form of biotin carboxylase. A previous study of mutants of *E. coli* biotin carboxylase made no effort to separate the chromosomal and plasmid copies of the enzyme (*12*). It is imperative to be able to separate the two different (in the case of a mutant) copies of the enzyme so there are no ambiguities in the interpretation of the kinetics. Therefore, to separate the chromosomal copy of biotin carboxylase from the plasmid copy, a six-histidine-tag was added to the amino terminus of the plasmid copy of biotin carboxylase. Both the histidine-tagged biotin carboxylase and biotin carboxylase without a histidine-tag bound to a nickel column. However, biotin carboxylase without a histidine-tag was removed with buffer containing a low concentration of imidazole (60 mM) while biotin carboxylase with a histidine-tag required a higher concentration of imidazole to be removed. The histidine-tag on the amino terminus did not affect the activity of the enzyme since the turnover numbers for the histidine-tagged and non-histidine-tagged proteins were virtually the same: 78.6 ± 3.2 min^{-1} and 106.0 ± 3.6 min^{-1}, respectively. Thus, a system is in place so that site-directed mutagenesis studies on *E. coli* biotin carboxylase can be performed without interference from wild-type enzyme derived from the chromosomal copy of the gene.

Table 2: Kinetic Parameters for the Bicarbonate-Dependent ATPase Reaction

	V_{max} (min^{-1})	$K_{m_{ATP}}$ (mM)	V/K_{ATP} (min^{-1} mM^{-1})
WT	0.073 ± 0.001	0.081 ± 0.003	0.90 ± 0.05
E211A	0.015 ± 0.001	0.014 ± 0.003	1.07 ± 0.29
E288A	0.014 ± 0.001	0.051 ± 0.008	0.27 ± 0.06
N290A	0.027 ± 0.001	0.040 ± 0.009	0.68 ± 0.18
R292A	0.016 ± 0.001	0.041 ± 0.010	0.39 ± 0.12

[a] The kinetic parameters were determined by varying [ATP] at a constant saturating level of bicarbonate. The standard errors on V_{max} and K_m were determined from the nonlinear regression analysis. The error on V/K was calculated by standard propagation of the errors from V_{max} and K_m.

Bicarbonate-Dependent ATPase Reaction. The carboxylation of biotin by biotin carboxylase requires the hydrolysis of ATP. Therefore, it is not surprising that biotin carboxylase will catalyze the hydrolysis of ATP in the absence of biotin but at a much slower rate (1100-fold slower) (eq 3):

$$MgATP + H_2O \rightarrow MgADP + P_i \quad (3)$$

The hydrolysis of ATP in the absence of biotin is bicarbonate-dependent (*13*). Studies with ^{18}O-labeled bicarbonate show that one oxygen of bicarbonate is found in the product phosphate, suggesting that the enzyme is forming carboxyphosphate which, in the absence of biotin, rapidly decomposes (*14*). The K_m for ATP and the maximal velocity of this partial reaction were determined for the wild-type enzyme and the four mutant enzymes and are shown in Table 2. The mutations decreased the V_{max} 5-fold at the most while the Michaelis constants for ATP were also slightly decreased. If the catalytic efficiency (V/K_{ATP}) of the reaction is considered, the mutations did not appear to significantly affect the bicarbonate-dependent ATPase activity of the enzyme.

The assays in Table 2 were carried out at a fixed, saturating level of bicarbonate. To ensure that the level of bicarbonate used in the assay was saturating, the Michaelis constant for bicarbonate was determined by varying bicarbonate at a fixed level of ATP and biotin. These measurements proved to be difficult to obtain because the endogenous level of bicarbonate at pH 8.0 was about 0.5 mM (*10*) and the apparent K_m for bicarbonate in the wild-type biotin carboxylase was 0.37 ± 0.04 mM. The apparent K_m values for bicarbonate for E211A, E288A, N290A, and R292A were 0.17 ± 0.03, 1.21 ± 0.14, 0.040 ± 0.004, and 0.050 ± 0.005, respectively. The apparent Michaelis constants for E211A and E288A were close to wild-type while the values for N290A and R292A were about 10 times less than the wild-type. It is

Table 3: Kinetic Parameters for the Biotin-Dependent ATPase Reaction[a]

	V_{max} (min^{-1})	$K_{m_{biotin}}$ (mM)	V/K_{biotin} (min^{-1} mM^{-1})
WT	78.6 ± 3.2	134.0 ± 13.8	0.59 ± 0.08
E211A	0.14 ± 0.01	33.6 ± 4.9	0.0042 ± 0.0009
E288A	0.44 ± 0.02	67.6 ± 6.4	0.0065 ± 0.0009
N290A	0.31 ± 0.02	60.1 ± 8.8	0.0052 ± 0.0011
R292A	0.39 ± 0.02	123.6 ± 14.2	0.0032 ± 0.0005

[a] The kinetic parameters were determined by varying [biotin] at constant saturating levels of bicarbonate and ATP. The standard errors on V_{max} and K_m were determined from the nonlinear regression analysis. The error on V/K was calculated by standard propagation of the errors from V_{max} and K_m.

Table 4: Ratio of Carboxybiotin to ADP Formed[a]

	μmol of carboxybiotin/μmol of ADP
WT	1.00
E211A	1.03
E288A	0.92
N290A	1.29
R292A	1.38

[a] The amounts of carboxybiotin and ADP formed were determined as described under Materials and Methods section.

clear that the mutations did not significantly increase the K_m of bicarbonate, and therefore these residues did not appear to play a major role in binding bicarbonate.

Biotin-Dependent ATPase Reaction. In contrast to the bicarbonate-dependent ATPase reaction, when the hydrolysis of ATP in the presence of biotin was examined the mutant enzymes had significantly decreased activity. Table 3 shows the maximal velocity and K_m for biotin for the wild-type and four mutant enzymes. In the wild-type enzyme, the presence of biotin increased the maximal velocity of ATP hydrolysis dramatically, whereas the presence of biotin only increased the maximal velocity about 10-fold for the four mutant enzymes. Apparently, this was not due to the inability of biotin to bind to the mutant enzymes because the K_m values for biotin in the mutants were not significantly increased compared to the value for the wild-type enzyme. Moreover, comparison of the catalytic efficiencies in the presence of biotin (V/K_{biotin}, Table 3) and absence of biotin (V/K_{ATP}, Table 2) revealed that in the presence of biotin all four mutations have resulted in an over 400-fold decrease in catalytic efficiency of the enzyme.

Since the ATPase assay involved detecting the production of ADP with the coupling enzymes pyruvate kinase and lactate dehydrogenase, there was a question as to whether the mutant enzymes were producing carboxybiotin. For example, has the hydrolysis of ATP been uncoupled from the formation of carboxybiotin, suggesting that the mutations have affected the carboxyl transfer step? Or, do the mutant enzymes show a 1:1 stoichiometry for the formation of ADP and carboxybiotin? To answer these questions the formation of carboxybiotin in the wild-type and four mutant enzymes was determined with a radioactive assay using ^{14}C-labeled bicarbonate. Table 4 shows the ratio of the micromoles of carboxybiotin formed to the micromoles of ADP formed for the wild-type and four mutant enzymes. The ratio of the micromoles of carboxybiotin formed to the micromoles of ADP formed for the four mutant enzymes was very close to one, which indicated that the mutations were not preventing the carboxylation of biotin.

Table 5: Kinetic Parameters for the ATP Synthesis Reaction[a]

	$K_{m_{CbmP}}$ (mM)	V_{max} (min^{-1})	$K_{m_{ADP}}$ (mM)	V/K_{ADP} (min^{-1} mM^{-1})
WT	11.2 ± 1.3	52.3 ± 3.2	0.08± 0.01	653.8 ± 121.7
E211A	0.51 ± 0.15	0.22 ± 0.01	0.18 ± 0.01	1.2 ± 0.1
E288A	2.3 ± 0.2	0.23 ± 0.01	0.23 ± 0.03	1.0 ± 0.2
N290A	*b*	0.27 ± 0.01	0.40 ± 0.02	0.68 ± 0.06
R292A	2.8 ± 0.1	0.30 ± 0.01	0.83 ± 0.13	0.36 ± 0.07

[a] The kinetic parameters were determined by varying carbamoyl phosphate concentration (CbmP) at constant saturating levels of ADP or varying [ADP] at constant saturating levels of CbmP. The standard errors on V_{max} and K_m were determined from the nonlinear regression analysis. The error on V/K was calculated by standard propagation of the errors from the V_{max} and K_m. [b] Data shown under Results.

ATP Synthesis Reaction. Biotin carboxylase catalyzes the transfer of the phosphoryl group of carbamoyl phosphate to ADP to form ATP and carbamic acid. The carbamic acid rapidly decomposes into carbon dioxide and ammonia (eq 4). Carbamoyl phosphate is thought to act as an analogue of the carboxyphosphate intermediate in the forward reaction (*15*):

$$\text{MgADP} + \text{carbamoyl-P} \rightarrow \text{MgATP} + CO_2 + NH_3 \quad (4)$$

The reaction rate is stimulated by the presence of biotin, which does not participate in the chemistry of the reaction but was proposed to activate the enzyme via a conformational change (*15*). The kinetic parameters of this reaction for the wild-type and four mutant enzymes are listed in Table 5. The Michaelis constants for cabamoyl phosphate were all lower than the wild-type. It is interesting to note that when carbamoyl phosphate was varied at a constant level of ADP for the N290A mutant, the double-reciprocal plots were concave downward which indicated substrate activation (Figure 2B). When the data for the N290A mutant were fitted to eq 2, the best-fit parameters were $V_1 = 0.23 \pm 0.01$ min^{-1}, $V_2 = 0.18 \pm 0.01$ min^{-1}, $K_1 = 0.03 \pm 0.01$ mM, and $K_2 = 5.2 \pm 1.0$ mM. Substrate activation was also observed in carbamoyl phosphate synthetase in which the equivalent asparagine residue (N301) was mutated to aspartate (*7*). In contrast to the Michaelis constants for carbamoyl phosphate, the Michaelis constants for ADP were all higher in the mutants. The most striking difference between wild-type enzyme and the four mutant enzymes was that the maximal velocity for ATP synthesis was decreased by 2 orders of magnitude in all four mutant enzymes. This means that all four mutations decreased the catalytic efficiency (V/K_{ADP}, Table 5) of phosphoryl transfer about 1000 times.

Since the ATP synthesis reaction is considered a model reaction for the carboxyphosphate intermediate, it was surprising that the catalytic efficiency was decreased while the catalytic efficiency of the bicarbonate-dependent ATPase reaction was not affected. However, one difference between the ATP synthesis reaction and the bicarbonate-dependent ATPase reaction was that the former required biotin to stimulate the activity. Given that the biotin-dependent ATPase reaction was affected by all four mutations, perhaps biotin did not stimulate the ATP synthesis reaction in the mutant enzymes. To test this hypothesis, the initial velocity of the ATP synthesis reaction for the wild-type and four mutant enzymes was measured at fixed carbamoyl phosphate and ADP concentrations and increasing amounts of biotin.

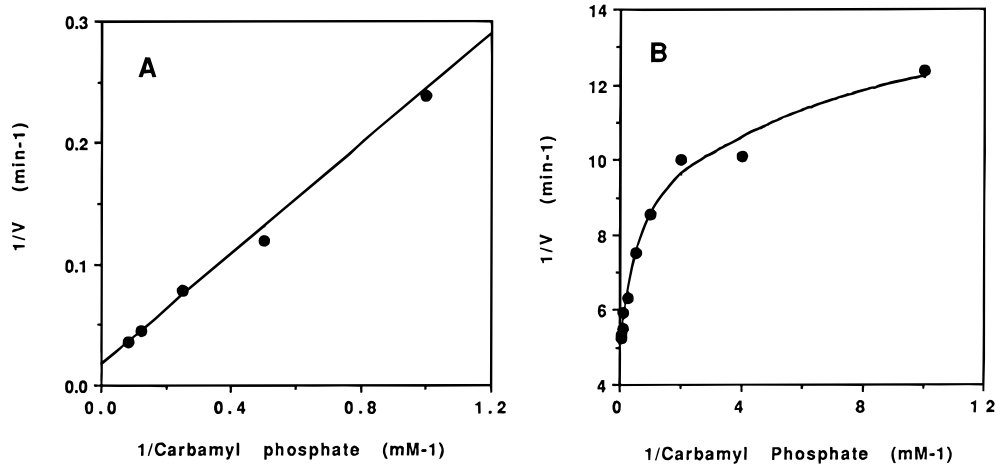

FIGURE 2: Double-reciprocal plots for the ATP synthesis reaction for wild-type biotin carboxylase (A) and the N290A mutant of biotin carboxylase (B). Carbamyl phosphate is varied at a constant saturation level of ADP. The points are the experimental velocities, and the line is derived from the best fit of the data to eq 1 for wild-type biotin carboxylase and to eq 2 for the N290A mutant.

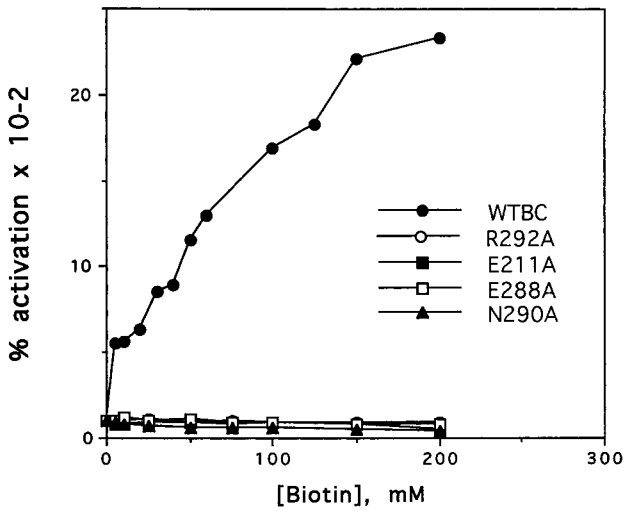

FIGURE 3: Activation of the ATP synthesis reaction by biotin for the wild-type biotin carboxylase (WTBC) and the four mutants of biotin carboxylase: E211A, E288A, N290A, and R292A. Initial velocities were measured at a constant level of carbamoyl phosphate and ADP with increasing amounts of biotin. The degree of activation of the reaction by biotin is expressed as the percent increase in the rate with biotin compared to the rate in the absence of biotin.

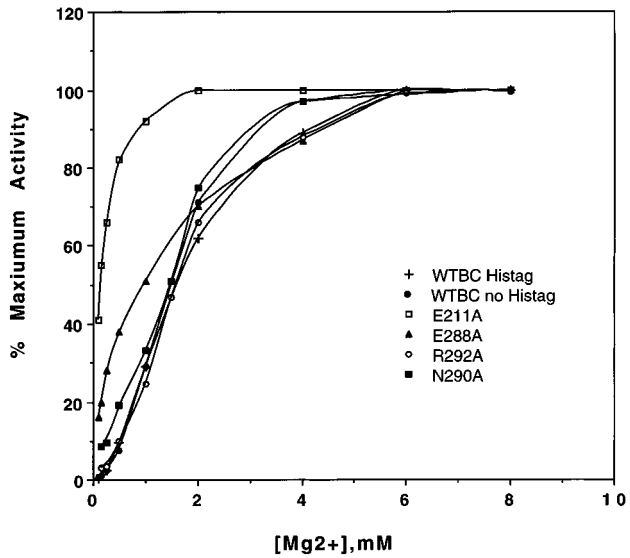

FIGURE 4: Effect of magnesium on the activity of wild-type biotin carboxylase (WTBC) and the four mutants of biotin carboxylase: E211A, E288A, N290A, and R292A. Initial velocities were measured at a constant level of bicarbonate, ATP, and biotin with increasing amounts of $MgCl_2$. The degree of activation of the reaction by $MgCl_2$ is expressed as a percentage of the maximal activation for wild-type and each individual mutant.

As shown in Figure 3 it was very apparent that while biotin stimulated the activity of the wild-type enzyme by 2000%, it had no effect on the activity of all four mutant enzymes.

Effect of Magnesium. Biotin carboxylase requires 2 equiv of magnesium for activity. One equivalent is complexed to ATP, while the role of the other equivalent is unknown. The effect of mutations at E211, E288, N290, and R292 on the ability of magnesium to stimulate the activity of wild-type and mutant biotin carboxylase was determined. To obviate any potential problems caused by the presence of the His-tag, all assays utilized enzymes in which the His-tag was removed by thrombin cleavage. The initial velocity was measured for the wild-type and four mutant enzymes by holding the concentrations of bicarbonate, ATP, and biotin constant and varying the concentration of $MgCl_2$ (Figure 4). The dependence of the initial velocity on [$MgCl_2$] was similar for wild-type, E288A, N290A, and R292A enzymes, suggesting that the mutations did not dramatically affect the binding of magnesium. The E211A mutant enzyme actually required a lower concentration of $MgCl_2$ to achieve the same level of activation as the wild-type enzyme, which suggests that magnesium may have bound tighter to that mutant. The dependence of the initial velocity versus [$MgCl_2$] for wild-type with and without the His-tag was virtually identical. This suggests that the other experiments at saturating $MgCl_2$ were not affected by the His-tag.

DISCUSSION

With the determination of the crystal structure of biotin carboxylase, the first and only three-dimensional model of a biotin-dependent carboxylase, it is time to begin studying the functions of individual amino acids in catalysis. However, before any site-directed mutagenic analysis could be performed, a system had to be developed that allowed the mutant biotin carboxylase to be separated from wild-type biotin carboxylase derived from the chromosomal copy of the gene. This was accomplished by adding a histidine-tag to the amino

terminus of the mutant biotin carboxylase which allowed the mutant enzyme to be separated from the wild-type enzyme by nickel affinity chromatography. Kinetic analysis of the mutant enzyme can now be interpreted without ambiguity.

The four residues investigated in this report (E211, E288, N290, and R292) were selected for mutagenesis because they are the only active site residues in biotin carboxylase that are strictly conserved both in biotin-dependent carboxylases and in carbamoyl phosphate synthetase, an enzyme with a mechanism similar to biotin carboxylase. In fact, mutagenesis of the equivalent residues in carbamoyl phosphate synthetase resulted in significant decreases in activity (*7*). Another common aspect of these four residues in biotin carboxylase is that they are close to one another in the active site (Figure 1). This is consistent with the fact that mutations at these four residues all result in the same effects in a variety of different assays, suggesting the mutated residues have a common function. In fact, examination of the data revealed a common pattern exhibited by all four mutant enzymes. First, the V_{max} of the bicarbonate-dependent ATPase activity was not dramatically affected by the mutations. In contrast, the V_{max} for the biotin-dependent ATPase activity was decreased by approximately 300-fold (Table 3). Second, the ATP synthesis reaction was also affected by the mutations where V_{max} was suppressed more than 100-fold. The ATP synthesis reaction, however, required biotin to stimulate activity, and biotin did not stimulate the activity in the mutant enzymes. Thus, it was clear that all reactions involving biotin are affected by the mutations. Since the K_m values for biotin in the mutant enzymes were similar to the wild-type value in the biotin-dependent ATPase reaction (Table 4), it appeared that biotin bound to the mutant enzymes but was unable to increase the activity of the ATPase and ATP synthesis reactions.

An explanation for the effect of the mutations on the activity of biotin carboxylase begins with the observation that for wild-type biotin carboxylase the binding of biotin accelerated the rate of ATP hydrolysis about 1100-fold. This dramatic increase in the reaction rate of one substrate (ATP) caused by the binding of another substrate (biotin) is an example of the phenomenon called substrate-induced synergism and is found in a number of enzymes (*16*). For example, citrate synthase will catalyze the exchange of the α-protons of acetyl-CoA only in the presence of malate, an analogue of the substrate oxaloacetate (*17*). In another example, the exchange rate between ATP and ADP in succinyl-CoA synthetase is stimulated by the presence of succinyl-CoA (*16, 18*). Since mutations at residues E211, E288, N290, and R292 have affected the ability of biotin to stimulate ATP hydrolysis and ATP synthesis, this suggests that these four residues are responsible, at least in part, for the substrate-induced synergism observed in biotin carboxylase. It is not clear whether the four residues in question, E211, E288, N290, and R292, interact directly with biotin, or if mutations at these four residues exert their effect on biotin indirectly.

The question now is what are the possible molecular mechanisms for the substrate-induced synergism by biotin and conversely what are the possible molecular defects caused by these four mutations? Jencks (*19*), in his seminal review article on how binding interactions of substrates and enzymes can be used for catalysis, proposed three possible mechanisms for substrate-induced synergism. Applying these general principles to our system, we propose three possible mechanisms for substrate-induced synergism by biotin on biotin carboxylase. First, the substrate-induced synergism by biotin may be a manifestation of the destabilization of the Michaelis complex through geometric strain, electrostatic interactions, or desolvation of the reacting groups. Second, the four residues in question may prevent nonproductive binding of biotin. Thus, when either residue is mutated, biotin binds nonproductively, precluding substrate-induced synergism by biotin. Third, the most common explanation for substrate-induced synergism is an induced fit mechanism in which the binding of the substrate, in this case biotin, causes a conformational change in the enzyme to convert it into a catalytically competent state. Distinguishing between these mechanisms will require a variety of crystallographic and spectroscopic studies which are underway.

There is precedent from site-directed mutagenesis studies of other enzymes for the involvement of substrate binding interactions in catalysis. For instance, the mutation of Arg 86 to Gln in tyrosyl-tRNA synthetase resulted in a decrease in maximal velocity by 4 orders of magnitude. However, the K_m for ATP for the mutant was observed to be similar to the K_m for ATP for wild-type. The interpretation was that the ATP was misaligned for catalysis, resulting in a decrease in maximal velocity (*20*). Furthermore, a recent review concluded that binding interactions are a major component of enzyme catalysis and that disruption of those interactions by mutation can be manifested as a decreased maximal velocity with little effect on the K_m for the substrates (*21*), which is exactly the situation seen here with these four mutations of biotin carboxylase.

In summary, we have described a system for site-directed mutagenesis studies of *E. coli* biotin carboxylase in which the mutant enzyme is free of contamination from wild-type enzyme coded by the chromosomal copy of the gene. This system was used to investigate the function of four active site residues, E211, E288, N290, and R292, in the catalytic mechanism of the enzyme. Mutations at all four residues gave similar results in which biotin was unable to stimulate ATP hydrolysis and the synthesis of ATP. In other words, mutations at these four residues abolished substrate-induced synergism by biotin. Therefore, the conclusion was that E211, E288, N290, and R292 were responsible, at least in part, for the substrate-induced synergism by biotin.

ACKNOWLEDGMENT

We thank Dr. Jack Kirsch of the University of California at Berkeley for very helpful discussions. We also thank Dr. John Cronan of the University of Illinois for pGBH. We thank the members of the laboratory and Dr. Tom Blanchard for their critical reading of the manuscript.

REFERENCES

1. Wakil, S. J., Stopps, J. K., and Joshi, V. C. (1983) *Annu. Rev. Biochem. 52*, 537−579.
2. Li, S.-J., and Cronan, J. E., Jr. (1992) *J. Biol. Chem. 267*, 855−863.
3. Blanchard, C. Z., and Waldrop, G. L. (1998) *J. Biol. Chem. 273*, 19140−19145.
4. Waldrop, G. L., Rayment, I., and Holden, H. M. (1994) *Biochemistry 33*, 10249−10256.

5. Galperin, M. Y., and Koonin, E. V. (1997) *Protein Sci. 6*, 2639−2643.
6. Thoden, J. B., Holden, H. M., Wesenberg, G., Raushel, F. M., and Rayment, I. (1997) *Biochemistry 36*, 6305−6316.
7. Stapleton, M. A., Javid-Majd, F., Harmon, M. F., Hanks, B. A., Grahmann, J. L., Mullins, L. S., and Raushel, F. M. (1996) *Biochemistry 35*, 14352−14361.
8. Shi, Y., and Walsh, C. T. (1995) *Biochemistry 34*, 2768−2776.
9. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. (1989) *Gene 77*, 51−59.
10. Asada, K. (1982) *Biological Carboxylations in Organic and Bio-organic Chemistry of Carbon Dioxide* (Inoue, S., and Yamazaki, N., Eds.) John Wiley & Sons, New York.
11. Guchhait, R. B., Polakis, S. E., Dimroth, P., Stoll, E., Moss, J., and Lane, M. D. (1974) *J. Biol. Chem. 249*, 6633−6645.13.
12. Kazuta, Y., Tokunaga, E., Aramaki, E., and Kondo, H. (1998) *FEBS Lett. 427*, 377−380.
13. Climent, I., and Rubio, V. (1986) *Arch. Biochem. Biophys. 251*, 465−470.
14. Ogita, T., and Knowles, J. R. (1988) *Biochemsitry 27*, 8028−8033.
15. Polakis, S. E., Guchhait, R. B., Zwergel, E. E., Lane, M. D., and Cooper, T. G. (1974) *J. Biol. Chem. 249*, 6657−6667.
16. Bridger, W. A., Millen, W. A., and Boyer, P. D. (1968) *Biochemistry 7*, 3608−3616.
17. Eggerer, H. (1965) *Biochem. Z. 343*, 111−138.
18. Wolodko, W. T., Brownie, E. R., O'Connor, M. D., and Bridger, W. A. (1983) *J. Biol. Chem. 258*, 14116−14119.
19. Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219−410.
20. Fersht, A. R., Knill-Jones, J. W., Bedouelle, H., and Winter, G. (1988) *Biochemistry 27*, 1581−1587.
21. Cannon, W. R., Singleton, S. F., and Benkovic, S. J. (1996) *Nat. Struct. Biol. 3*, 821−833.

BI982660A