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The Role of Four Active Site Residues in the Catalytic Mechanism of Biotin Carboxylase

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Acetyl CoA carboxylase catalyzes the biotin dependent carboxylation of acetyl CoA to form malonyl CoA in the first committed step in the formation of fatty acids (Moss and Lane, 1971) and follows the two-step mechanism shown below.

(1) enzyme-biotin + MgATP + HCO_3^- → enzyme-biotin-CO_2^- + MgADP + Pi

(2) enzyme-biotin-CO_2^- + acetyl-CoA → malonyl-CoA + enzyme-biotin

The enzyme is composed of three components: a biotin carboxylase component, a biotin carrier component, and a carboxyltransferase component. In *E.coli*, these three components can be isolated and still maintain activity in the absence of the other two. This report describes the production of several different mutants (E211A, E288A, N290A, and R292A) of biotin carboxylase and the effects of each mutation on the catalytic properties of the enzyme.

Rationale for mutations

The three dimensional structure of the biotin carboxylase component from *E.coli* has been determined using X-ray crystallography (Waldrop *et al.*, 1994). The four residues chosen for mutation (E211, E288, N290, and R292) were all in the active site of the enzyme. The four residues are also strictly conserved throughout evolution in all biotin-dependent enzymes (Galperin and Koonin, 1977). Also, these four residues are the only active site residues strictly conserved in carbamoyl phosphate synthetase (Thoden *et al.*, 1997), which is similar to biotin carboxylase in mechanism and structure. Mutations at these residues have greatly affected the activities of carbamoyl phosphate synthetase

(Stapleton *et al.*, 1996). Biotin carboxylase belongs to a family of proteins known as ATP-grasp proteins. This family shares a structural homology and each enzyme catalyzes an ATP-dependent ligation of a carboxyl group to N or S. E288 and N290 are strictly conserved in the entire family of ATP-grasp proteins (Galperin and Koonin, 1977), to which both biotin carboxylase and carbamoyl phosphate synthetase belong. All of the residues were mutated to alanine because it is the most common amino acid in proteins, located in α-helicies, β-strands, and turns, the second smallest amino acid, and is found in buried and exposed residues.

Construction of a functional mutant enzyme

The first step in making a mutant enzyme begins with the DNA that encodes for that enzyme. The gene sequence for wild-type biotin carboxylase was cloned into pGEM-7f with sites for EcoR1 and Nde1 on the 5' end and a BamH1 site on the 3' end. A technique known as polymerase chain reaction (PCR) was used to selectively mutate a single base in the wild-type DNA and amplify the DNA. The mutants were constructed using a special PCR method known as overlap-extension.

Overlap-extension was the most efficient way to make mutations in the wild-type DNA. Two overlapping fragments were made in the first round of reactions. Then these two fragments were combined in a full-length PCR reaction. Oligonucleotides were developed for the sense and nonsense strand for each mutant. These oligonucleotides contained either a single mismatch (E211A

and E288A) or a double mismatch (N290A and R292A) that changed the sequence to encode for a new amino acid and ~15 bases on either side which were complementary to the wild-type copy. Each mutant followed the same steps except for the different oligonucleotides. The fragment PCR reactions contained:

36 ul ddH_20
10 ul Template (purified from Wizard Miniprep)
10 ul 10X Thermopol buffer
40 ul dNTP's (3 mM each)
0.6 ul oligonuleotides (100 pm/ul)
1.0 ul primers (100 pm/ul)
1.0 ul $MgSO_4$ (100 mM)
1.0 Deep Vent Polymerase (2,000 units/ml)

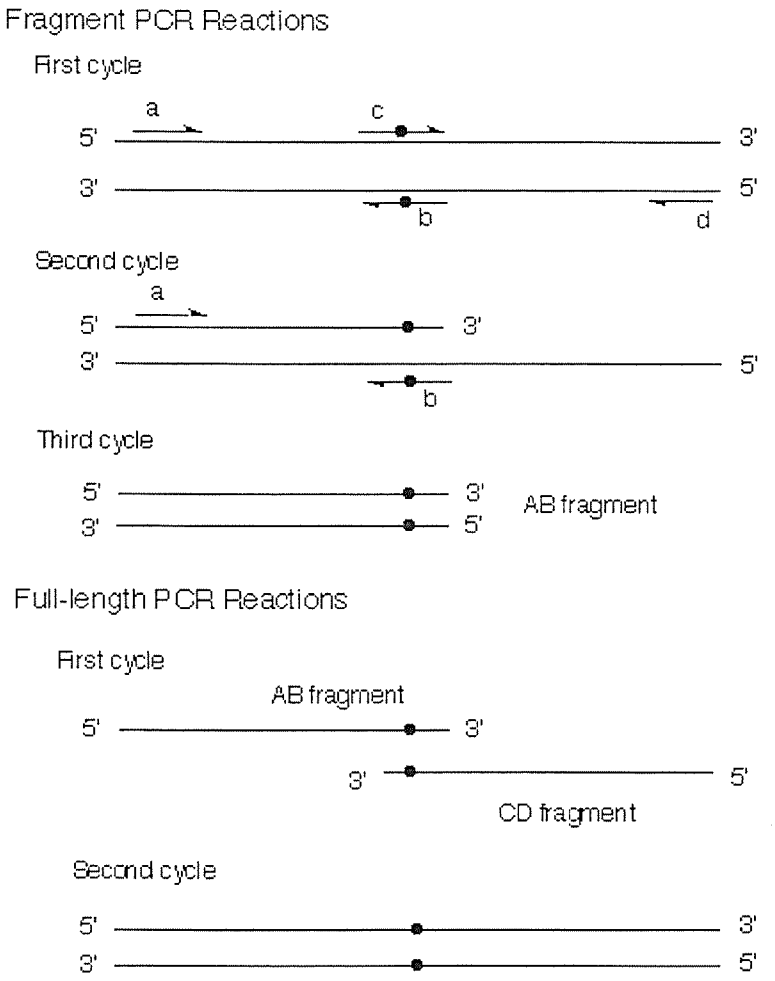
The conditions were:
94 °C 1 min
50 °C 3 min
72 °C 2 min

The PCR fragments were checked to make sure they were the right lengths by agarose gel electrophoresis. Then they were purified from the gel. The two fragments were then used in a full-length PCR reaction. The full-length PCR reaction contained:

6 ul ddH_20
10 ul 10X Thermopol buffer
40 ul dNTP's (3 mM each)
20 ul of each PCR fragment (purified from Wizard Minipreps)
0.5 ul of each primer (100 pm/ul)
1.0 ul $MgSO_4$ (100 mM)
1.0 ul Deep Vent Polymerase (2,000 units/ml)

The conditions were:
94 °C 1 min
50 °C 3 min
72 °C 2 min

A diagram of the reactions in the tubes is shown below.



The full-length PCR products were checked for the correct molecular weight on an agarose gel, and purified. The full-length mutant DNA sequence was then cloned into the expression vector pET28b.

The specific procedures for the cloning of the full-length PCR products was as follows:

20 ul of DNA
1 ul BSA (10 mg/ml)
2 ul 10X EcoR1 Buffer
1ul EcoR1 (20,000 units/ml)
1ul BamH1 (20,000 units/ml)
Place in 37 °C water bath for 1 h

The cut plasmid was purified via an agarose gel. The cut PCR products were purified directly by Wizard Minipreps for PCR products (Promega, Madison, WI.). The purified cut PCR products were then ligated into the purified, cut plasmid using the following procedure:

20 ul of PCR product
20 ul of plasmid
5 ul 10X ligase buffer
3 ul ddH_2O
2 ul T4 DNA ligase (400,000 units/ml)
Place in 16 °C water bath overnight

The ligated plasmids were transformed into competent JM109 cells and grown on agar plates containing ampicillin to select for the pGEM-7f plasmid. The plates were checked the next day for the presence of colonies. Colonies were picked and grown overnight in 10mls of LB media with ampicillin.

The next day, the cells were harvested and the plasmids were purified. The plasmids were checked for the presence of the biotin carboxylase gene by digestion with EcoR1 and BamH1. If the plasmid contained the biotin carboxylase insert, there would be a band at 3000 kb for the pGEM-7f plasmid and another band at 1347 kb for the insert. If an insert was present the plasmid was cut with Nde1 and BamH1 and ligated into the expression vector pET28b. The insert was sequenced to confirm that the mutation was at the desired location and the rest of the gene was unchanged. The expression plasmid was transformed into *E.coli* strain BL21-(DE3)pLysS.

Bacterial Growth and Enzyme Purification

Bacteria were grown in 1 L of LB media at 37 °C in 2 L flasks. Each flask was inoculated with an overnight growth of the specific mutant containing 30 ug/ml kanamycin to select for the pET28b mutant plasmid. The growths were induced with 1 g of lactose and allowed to grow for another 2 h. Cells were harvested by centrifugation at 10400g for 10 m at 4 °C. The cell pellet was resuspended 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, and the lysate was centrifuged at 20200g for 1 h at 4 °C. The supernatant was loaded onto a column of His-binding resin. This poly-histidine region at the N-terminus allowed the enzyme to bind the resin letting the other proteins flow through the column. To prevent any non-specific binding of proteins, the column was washed with a Triton X-100 solution (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9, 0.1% Triton X-100). Biotin carboxylase was eluted using a solution of 170 mM imidazole, 500 mM NaCl, 120 mM Tris-HCl, pH 7.9. The eluant was dialyzed overnight against 0.67 mM EDTA, 10 mM $KHPO_4$, pH 7.0. The protein was precipitated by ammonium sulfate at 60% saturation, and 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.

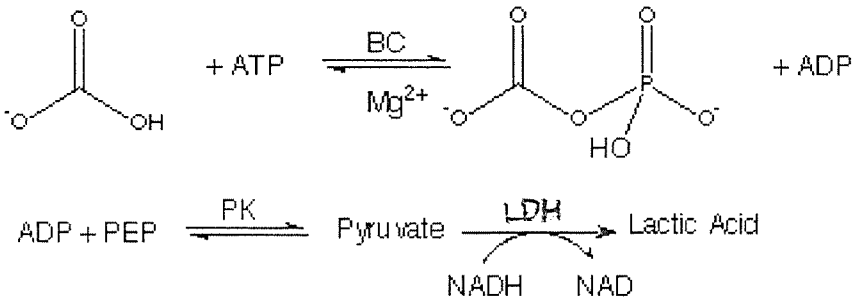
Enzymatic Assays

Biotin carboxylase catalyzes two different reactions; a bicarbonate- or biotin-dependent ATP hydrolysis reaction and an ATP synthesis reaction using

MgADP and carbamyl phosphate. Data were collected for both reactions by measuring the absorbance at 340 nm using a Uvikon 810 spectrophotometer interfaced to a PC equipped with a data acquisition program. The temperature was maintained at 25 °C by a circulating water bath.

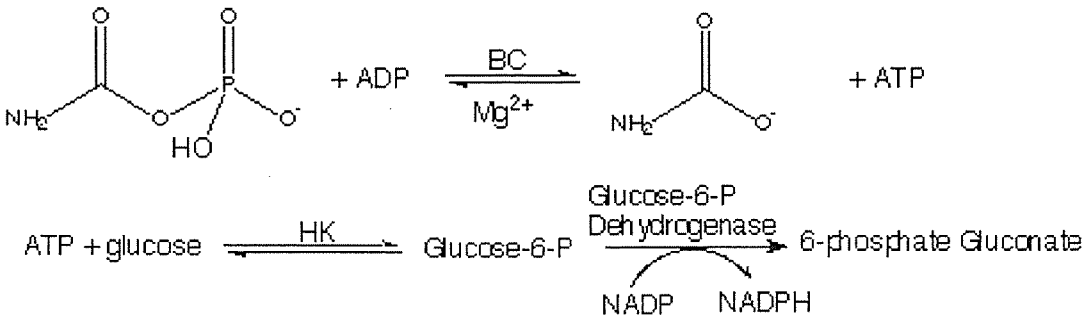
For the ATP hydrolysis reaction, the appearance of ADP was measured (with and without biotin) using a coupled reaction of pyruvate kinase, lactate dehydrogenase, and NADH (Scheme 1). Each measurement was carried out in a volume of 0.5 mL in 1cm path length quartz cuvettes. The reaction mixture contained 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, pH 8.0. Since the Km for biotin is high, the ionic strength was controlled for by the addition of KCl.

Scheme 1



In the ATP synthesis reaction, the rate of ATP appearance was measured by a coupled reaction of hexokinase, glucose-6-phosphate dehydrogenase, and NADPH (Scheme 2). 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.

Scheme 2



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 the following equation where v is the initial velocity, V_m is the maximal velocity, K_m is the Michaelis constant, and A is the substrate concentration:

$$v = V_m A / (K_m + A)$$

When nonlinear double-reciprocal plots were encountered, the following equation was used where V_1 and V_2 are the maximal velocities, and K_1 and K_2 are the Michaelis constants, and A is the substrate concentration:

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

Results

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

Kinetic Parameters for the Biotin-Dependent ATPase Reaction

	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

ATPase Reaction

The parameters for the bicarbonate-dependent ATPase reaction were not significantly affected. However, the biotin-dependent ATPase reaction was greatly affected. The Km of biotin remained relatively the same, but the Vmax was decreased by 300-fold. This decreased the V/K value, or the catalytic efficiency by 100-fold.

Kinetic Parameters for the ATP Synthesis Reaction

	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 ± 0.1	0.23 ± 0.03	1.0 ± 0.2
N290A	*	0.27 ± 0.1	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

* gave nonlinear double-reciprocal plot and will be described in the discussion

ATP Synthesis Reaction

The kinetic parameters for the ATP synthesis reaction were affected in the same ways that the biotin-dependent ATPase reaction was affected. That is, the Km for the substrates remained relatively the same, but the Vmax was decrease 100-fold, which reduced the V/K value by 1000-fold. There was a unique occurrence while varying the levels of CbmP at constant levels of ADP for the

N290A mutant. The Lineweaver-Burke plot (which should be linear) was nonlinear. Fitting the data to the equation for nonlinear double-reciprocal plots the kinetic parameters were determined to be $V_1 = 0.23 \pm 0.01$ 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.

Discussion

The nonlinear Lineweaver-Burk plot (Fig. 1) which was obtained from the N290A CbmP assay is an interesting occurrence. This type of result suggests substrate activation, which means that the binding of a substrate to one active site in the dimer, will increase the binding of that substrate in the other active site. There is precedence for this in that it was observed in the N301D mutation in carbamyl phosphate synthetase (Stapleton, M.A., et al., 1996). The N301 residue is analogous to the N290 residue in biotin carboxylase.

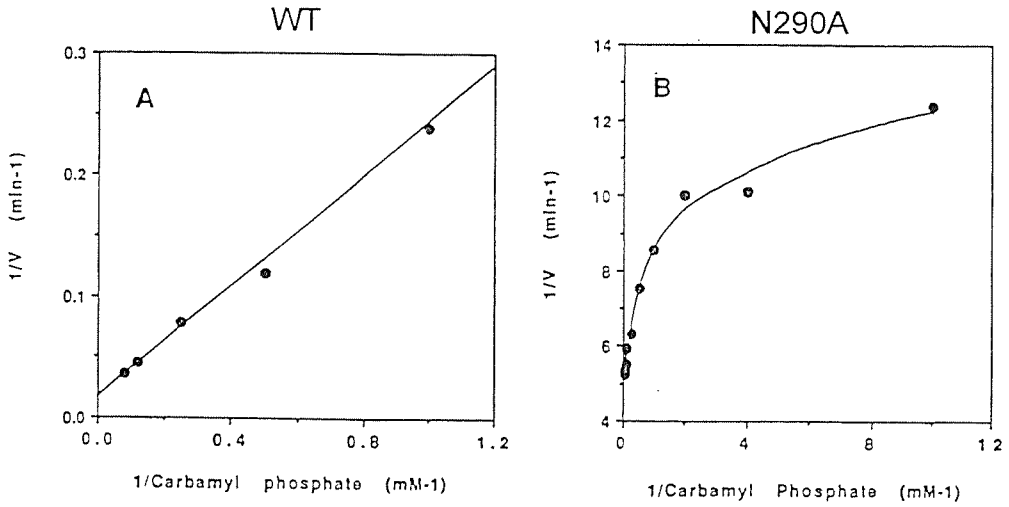


Fig 1: Double-reciprocal plots for the ATP synthesis reaction for wild-type biotin carboxylase and N290A

The kinetic parameters for the bicarbonate-dependent ATPase reaction remained unchanged. This suggests that the mutants had no affect on the reaction. However, the biotin-dependent ATPase reaction and the ATP synthesis reaction each showed dramatic changes to the kinetic parameters, the K_m was the same, but the V_{max} was significantly reduced. The common thread of these two reactions is the presence of biotin. The fact that the K_m for biotin in the mutants is relatively the same as WT suggests that the biotin is still binding. However, the reduction in V_{max} suggests that the mutations have affected the ability of biotin to productively contribute to the catalytic mechanism of the enzyme.

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