Kinetic Characterization of Mutations Found in Propionic Acidemia and Methylcrotonylglycinuria

EVIDENCE FOR COOPERATIVITY IN BIOTIN CARBOXYLASE*

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Acetyl-CoA carboxylase catalyzes the committed step in fatty acid synthesis in all plants, animals, and bacteria. The Escherichia coli form is a multifunctional enzyme consisting of three separate proteins: biotin carboxylase, carboxyltransferase, and the biotin carboxyl carrier protein. The biotin carboxylase component, which catalyzes the ATP-dependent carboxylation of biotin using bicarbonate as the carboxylate source, has a homologous functionally identical subunit in the mammalian biotin-dependent enzymes propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase. In humans, mutations in either of these enzymes result in the metabolic deficiency propionic acidemia or methylcrotonylglycinuria. The lack of a system for structure-function studies of these two biotin-dependent carboxylases has prevented a detailed analysis of the disease-causing mutations. However, structural data are available for E. coli biotin carboxylase as is a system for its overexpression and purification. Thus, we have constructed three site-directed mutants of biotin carboxylase that are homologous to three missense mutations found in propionic acidemia or methylcrotonylglycinuria patients. The mutants M169K, R338Q, and R338S of E. coli biotin carboxylase were selected for study to mimic the disease-causing mutations M204K and R374Q of propionyl-CoA carboxylase and R385S of 3-methylcrotonyl-CoA carboxylase. These three mutants were subjected to a rigorous kinetic analysis to determine the function of the residues in the catalytic mechanism of biotin carboxylase as well as to establish a molecular basis for the two diseases. The results of the kinetic studies have revealed the first evidence for negative cooperativity with respect to bicarbonate and suggest that Arg-338 serves to orient the carboxyphosphate intermediate for optimal carboxylation of biotin.

For over 20 years, site-directed mutagenesis has been used for determining the roles of active site residues in enzyme-catalyzed reactions. In designing such experiments, one is invariably faced with the question of which residue to mutate and to what to mutate this residue. Residues targeted for mutagenesis are usually identified using three-dimensional structural information or sequence alignments with homologous proteins, indicating which residues are highly conserved. Once the residues for mutagenesis are identified, they are commonly replaced with an isosteric residue or alanine (i.e. alanine-scanning mutagenesis) to minimize disruption of the protein structure. However, prior to the advent of site-directed mutagenesis, mutant human hemoglobins provided the only opportunity to study structure-function relationships in proteins. Many of these hemoglobinopathies involved amino acid replacements that would not normally be made using site-directed mutagenesis out of concern for disruption of the protein structure. Yet these mutations yielded important insight into how hemoglobin functioned (1). In this report, we take this classical approach to mutagenesis to examine the effect of three naturally occurring mutations found in biotin-dependent carboxylases on the catalytic mechanism of E. coli biotin carboxylase.

Biotin carboxylase is one component of the multifunctional enzyme acetyl-CoA carboxylase, which catalyzes the first committed step in long chain fatty acid biosynthesis (2). Acetyl-CoA carboxylase is a biotin-dependent enzyme that catalyzes the following two-step reaction as shown in Scheme 1.

1. Enzyme-biotin + MgATP + HCO₃⁻ → enzyme-biotin-CO₂⁻ + MgADP + P₅

2. Enzyme-biotin-CO₂⁻ + acetyl-CoA → malonyl-CoA + enzyme-biotin

SCHEME 1

Biotin carboxylase catalyzes the first half-reaction, which is an ATP-dependent carboxylation of biotin to form carboxybiotin. Biotin is covalently attached to the biotin carboxyl carrier protein designated enzyme-biotin in Scheme 1. The second half-reaction is catalyzed by carboxyltransferase, which involves the transfer of the carboxyl group from carboxybiotin to acetyl-CoA to make malonyl-CoA. Animal acetyl-CoA carboxylase incorporates all three of these functions on a single polypeptide chain (3), whereas in the bacterial form, each function is a separate protein (4).

There have been no documented naturally occurring mutations in acetyl-CoA carboxylase leading to an inborn error of metabolism. However, there are two related biotin-dependent carboxylases in which naturally occurring mutations have been detected. Propionyl-CoA carboxylase is involved in the catabolism of odd chain length fatty acids and branched chain amino acids, and mutations in the human form of this enzyme result in the condition propionic acidemia (5). The enzyme 3-methylcrotonyl-CoA carboxylase is involved in the catabolism of...
leucine, and mutations in the human form of this enzyme result in methylcrotonylglycinuria (6). Both propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase contain two different types of subunits and catalyze their respective reactions via a two-step reaction sequence similar to the reaction catalyzed by acetyl-CoA carboxylase (Scheme 1). In fact, the first half-reaction in Scheme 1, the carboxylation of biotin, is identical for all three enzymes. The biotin carboxylase subunits of propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase are known as PCCα and MCCα, respectively, whereas the carboxyltransferase functions are contained in the PCCβ and MCCβ subunits. Several disease-causing missense mutations have been identified in human propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase (5–7), revealing that disease phenotypes can arise from mutations in either the α or β subunit. To date, characterization of the mutant proteins has included specific activity measurements of fibroblast extracts (8, 9) or of purified recombinant PCCβ subunit (10). A useful model system for detailed analysis of mutations in the β subunit is provided by trans-carboxylase found in Propionibacterium shermanii. In particular, the PCCβ chain shares 50% sequence homology with the 12S subunit of trans-carboxylase. Given the recent solution of the crystal structure of the 12S subunit, structure-function predictions may be made for the β subunit of propionyl-CoA carboxylase (11). Unfortunately, the PCCα subunit bears no sequence homology to the other subunits of trans-carboxylase, precluding this enzyme as a structural model for PCCα. However, the biotin carboxylase subunit of E. coli acetyl-CoA carboxylase provides an ideal model system to conduct a rigorous kinetic analysis of mutations found in the α subunits of propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase. In fact, 10 active site mutants of E. coli biotin carboxylase have been characterized to date (12–14). Kinetic characterization of these naturally occurring mutants using E. coli biotin carboxylase as a model may lend insight into the structure and function of the enzyme.

The advantage of using E. coli biotin carboxylase for kinetic analysis is that the homodimer can be isolated and retains catalytic activity in the absence of the other two components (15). Moreover, biotin carboxylase is able to utilize free biotin as a substrate instead of biotin linked to the biotin carboxyl carrier protein, thereby simplifying kinetic analysis (15). The availability of structural information is perhaps the most significant feature of E. coli biotin carboxylase that makes it the paradigm for mechanistic studies of biotin-dependent enzymes. The x-ray crystal structure of biotin carboxylase has been solved and to date is the only structural model of a biotin-dependent carboxylase (16, 17). Based on the three-dimensional structure, two mutations in the biotin carboxylase domain of human propionyl-CoA carboxylase, M204K and R374Q, were predicted to be in the active site (5, 7). According to sequence alignments of the human propionyl-CoA carboxylase and E. coli biotin carboxylase, the homologous residues in the E. coli enzyme are Met-169 and Arg-338 (Fig. 1). In patients with methylcrotonylglycinuria, only one mutation of 3-methylcrotonyl-CoA carboxylase, R385S, was predicted to lie in the active site of the biotin carboxylase domain (6). Interestingly, this residue also corresponds to Arg-338 of E. coli biotin carboxylase (Fig. 1). In this report, these three mutations, M169K, R338Q, and R385S, were constructed in E. coli biotin carboxylase and the mutant enzymes were subjected to kinetic characterization. The results not only suggest a molecular basis for understanding how these mutations cause disease but also provide the first kinetic evidence for cooperativity between the subunits of E. coli biotin carboxylase.

MATERIALS AND METHODS

Chemicals and Enzymes—[14C]-labeled sodium bicarbonate was purchased from Amersham Biosciences and had a specific activity of 0.1 mCi/mmol. His bind resin was purchased from Novagen. DEAE cellulose resin was manufactured by Whatman. Pyruvate kinase was obtained from Roche Applied Sciences. All of the other reagents were from Sigma or Aldrich.

Site-directed Mutagenesis—Construction of biotin carboxylase mutants was performed by the PCR method of overlap extension as described by Blanchard et al. (12). All of the primers were synthesized by Invitrogen. Mutagenic internal primers for the M169K, R338Q, and R385S mutants were used to incorporate the desired mutation. The mutagenic primer sequences were as follows: M169K, 5’-GGGCTATGACGCTTTACCCGACCCCC-3’ and 5’-GGGCTATGACGCTTTACCCGACCCCC-3’; R338Q, 5’-CTGGCCGCTGTTGCATTTGACCCCGCCGACCCC-3’ and 5’-CTGGCCGCTGTTGCATTTGACCCCGCCGACCCC-3’; and R385S, 5’-CTGGCCGCTGTTGCATTTGACCCCGCCGACCCC-3’ and 5’-CTGGCCGCTGTTGCATTTGACCCCGCCGACCCC-3’. The entire gene of each mutant was sequenced to confirm that only the desired mutation was incorporated.

Overexpression and Purification of Biotin Carboxylase—The growth and overexpression of wild type and mutant biotin carboxylase was performed as described by Blanchard et al. (12). Biotin carboxylase was purified by nickel affinity chromatography using His tag columns as described elsewhere (12), and the eluate from the His bind resin was dialyzed overnight against buffer A (10 mM NaCl, 50 mM KH2PO4, pH 7.1) and then applied to a DEAE cellulose column. The column was washed with 14 volumes of buffer A. The flow-through and wash volumes were pooled, and the protein was precipitated by the addition of ammonium sulfate to 60% saturation. The precipitate was dissolved and dialyzed against 500 mM KCl, 10 mM HEPES, pH 7.0. The resulting solution was then concentrated by vacuum dialysis using a collodion bag apparatus. Protein concentrations were determined by the Bio-Rad protein assay with bovine serum albumin as a standard.

Kinetic Assays—The rate of ATP hydrolysis by biotin carboxylase was measured spectrophotometrically by coupling the formation of ADP to pyruvate kinase and lactate dehydrogenase and monitoring the oxidation of NADH at 340 nm. Each assay was conducted in the presence of 0.5 mM phosphoenolpyruvate, 0.4 mM NADH, 23 units of pyruvate kinase and lactate dehydrogenase, and 100 mM HEPES at pH 8. Because the K_m for biotin is high (100 mM), the ionic strength was kept constant with KCl in assays where biotin was varied.

The rate of ATP synthesis from Mg-ADP and carbamyl phosphate was measured spectrophotometrically. The formation of ATP was coupled to hexokinase and glucose-6-phosphate dehydrogenase while the reduction of NADP was followed at 340 nm. Each assay contained 0.5 mM glucose, 0.4 mM NADP, 5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, 100 mM KCl, and 100 mM HEPES at pH 8.

Initial velocities were measured using a Uvikon 810 spectrophotometer (Kontron Instruments). All of the reactions were initiated by the addition of enzyme. Kinetic parameters were calculated per active site using a molecular mass of 50 kDa for the biotin carboxylase monomer (biotin carboxylase exists as a homodimer). The temperature of the reactions was maintained at 25°C or 37°C by a circulating water bath. For assays conducted at 37°C, all of the reaction components were preincubated for 6 min at 37°C prior to mixing in quartz cuvettes. For each

1 The abbreviations used are: PCC, propionyl-CoA carboxylase; MCC, 3-methylcrotonyl-CoA carboxylase; CbmP, carbamyl phosphate.
The kinetic parameters were determined by varying [ATP] at a constant level of bicarbonate. The concentration of bicarbonate was saturating for wild type, 30 mM for R338Q, 15 mM for R338S, and 10 mM for M169K. For the three mutants, these levels of bicarbonate were saturating concentrations based on the $K_m$ values determined from Equation 1. The errors on $K_m$ and $V_{max}$ were determined by nonlinear regression analysis.

|           | $K_m$ (mM) | $V_{max}$ (min$^{-1}$) |
|-----------|------------|------------------------|
| WT        | 0.081 ± 0.003 | 0.073 ± 0.001          |
| R338Q     | 4.4 ± 0.3   | 0.16 ± 0.01            |
| R338S     | 5.1 ± 0.5   | 0.045 ± 0.002          |
| M169K     | 0.26 ± 0.02 | 0.033 ± 0.001          |

$^a$ Data taken from Ref. 8.

initial velocity measurement, the cuvette was preincubated in the spectrophotometer at 37 °C for 10 min before the addition of the reaction components.

To determine whether there was a stoichiometric production of ADP and carboxybiotin, the amount of carboxybiotin produced by biotin carboxylase was determined using a $^{14}$C fixation assay and compared with the production of ADP as described elsewhere (12). The reaction mixtures contained 20 mM ATP, 70 mM bicarbonate, 100 mM biotin, 50 mM MgCl$_2$, and 100 mM HEPES at pH 8 in a total volume of 0.5 ml.

Data Analysis—the $K_m$ and $V_{max}$ were determined by nonlinear regression analysis of the velocity, as a function of [substrate] data by the Michaelis-Menten equation. For assays in which the double reciprocal plots appeared hyperbolic, the data were fitted to Equation 1 where $V_1$ and $V_2$ are maximal velocities, $K_1$ and $K_2$ are Michaelis constants, and $V$ is the substrate concentration.

$$v = \frac{V_1A}{(K_1 + A)} + \frac{V_2A}{(K_2 + A)}$$ (Eq. 1)

Hill coefficients ($n_H$) were determined by nonlinear regression analysis of the velocity versus [bicarbonate] data to Equation 2,

$$v = \frac{V_{max}[A]^{n_H}}{(K + [A])^n}$$ (Eq. 2)

where $A$ is the substrate concentration and $K$ is a constant that depends upon the interaction between substrate binding sites and the intrinsic dissociation constant of the enzyme-substrate complex.

RESULTS

Bicarbonate-dependent ATPase Activity—The binding of substrate to biotin carboxylase is ordered with ATP binding first followed by bicarbonate and then biotin (18). However, in the absence of biotin, the enzyme also catalyzes a slow bicarbonate-dependent ATP hydrolysis as shown in Scheme 2.

$$\text{HCO}_3^- + \text{MgATP} + \text{H}_2\text{O} \rightleftharpoons \text{MgADP} + \text{P}_i$$

This reaction involves the transfer of the phosphate group to the bicarbonate, forming carboxyphosphate, which quickly breaks down into carbon dioxide and inorganic phosphate (19). When ATP was varied at constant levels of bicarbonate, the kinetic parameters were determined for the three mutants and wild type. The $V_{max}$ values for the three mutants were only modestly different from the wild type enzyme. R338Q showed a 2-fold increase in $V_{max}$, and R338S and M169K showed a 2-fold decrease or less (Table I). By contrast, the $K_m$ values varied, the double reciprocal plots were hyperbolic for all three mutants (Fig. 2). All three mutants exhibited significant increases in velocity at higher concentrations of bicarbonate, suggesting substrate activation, also known as negative cooperativity. Negative cooperativity with respect to bicarbonate means that the binding of bicarbonate to one subunit of the biotin carboxylase homodimer decreases the affinity of the second subunit for bicarbonate. When the data were fitted to the Michaelis-Menten equation, the difference in the observed and the calculated values revealed a distinct pattern on a residual plot. A nonrandom residual plot indicates that the data are not accurately described by the Michaelis-Menten equation. By contrast, fitting the data to the equation for negative cooperativity (Equation 1) revealed the residuals to be random. A representative example of this type of analysis is shown in Fig. 3 for the M169K mutant. The fitting to Equation 1 also revealed that the $K_m$ for each of the mutants was greater than 30 mM. For this reason, the $K_m$ values for ATP shown in Table I are apparent $K_m$ values because saturating levels of bicarbonate could not be achieved.

Particularly intriguing is the fact that the degree of negative cooperativity by bicarbonate for the three mutants is dependent upon the concentration of biotin. To illustrate this trend, the Hill coefficients ($n_H$) under different concentrations of biotin are listed in Table II. In the presence of 60 mM biotin, M169K showed a slight 23% increase in $n_H$ relative to the reaction without biotin. The R338S and R338Q mutants displayed a more dramatic increase in $n_H$ (3- and 5-fold, respectively) with 60 mM biotin as opposed to 16 mM biotin. These trends indicate that biotin decreases the negative cooperativity of the three mutants, particularly for the two Arg-338 mutants. Assaying the two Arg-338 mutants in the absence of biotin proved difficult because the enzymes displayed almost no change in rate when bicarbonate was varied.

Biotin-dependent ATPase Activity—The rate of ATP hydrolysis is increased 1100-fold in the presence of biotin for the wild type enzyme (12). However, when the hydrolysis of ATP was assayed in the presence of biotin for the three mutants, the most notable result was that the $V_{max}$ of R338S and R338Q was 700- and 100-fold lower than wild type, respectively (Table III). The $V_{max}$ was also reduced in the M169K mutant but only by ~10-fold. The $K_m$ for biotin for R338S was virtually unchanged from that of wild type. The R338S and M169K mutants, however, showed a 5- and 2.5-fold reduction in apparent $K_m$, respectively. Although the latter two mutants seem to bind biotin with slightly greater affinity than wild type, it is important to note that the catalytic efficiency (V/K) is 5-fold lower for M169K and 140-fold lower for R338S. Again, the $K_m$ for the three mutants reported here are apparent $K_m$ values because it was not possible to saturate the enzymes with bicarbonate.

Although the assay for the ATPase activity measures the rate of production of ADP using a continuous assay, the production of carboxybiotin cannot be followed. Thus, the assay cannot detect whether ATP hydrolysis is uncoupled from the production of carboxybiotin in the mutants. However, carboxybiotin can be quantified to demonstrate whether it is produced at a 1:1 stoichiometry with ADP. Wild type biotin carboxylase and M169K both exhibited a normal 1:1 ratio of ADP to carboxybiotin production (Table IV). Unexpectedly, however, R338S and R338Q revealed ratios of 3.0 and 3.8, respectively. This conspicuous perturbation in the stoichiometry suggests that for the two Arg-338 mutants, the carboxylation of biotin is uncoupled from ATP hydrolysis. Of the 10 site-directed mutants of biotin carboxylase characterized to date, only one other, R238Q, has exhibited such an uncoupling (13).

ATP Synthesis Activity—E. coli biotin carboxylase is known...
to catalyze the formation of ATP from ADP and carbamyl phosphate as shown in Scheme 3.

MgADP + carbamyl phosphate → MgATP + carbamate

Scheme 3

The carbamate molecule rapidly decomposes to carbon dioxide and ammonia. This reaction mimics the reverse of the physiological reaction with carbamyl phosphate acting as a carboxyphosphate analog. Note that the ATP synthesis reaction does not include biotin, although biotin does stimulate the rate of ADP phosphorylation (12). The three mutations do not appear to have markedly altered the kinetics of the ATP syn-
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The Hill coefficients \( n_H \) were determined by fitting data from the bicarbonate-dependent ATPase assays to Equation 2. The error on \( n_H \) was derived from nonlinear regression analysis.

| Biotin   | \( n_H \) |
|----------|-----------|
| R338Q    | 0.17 ± 0.03 |
| 16       | 0.79 ± 0.07 |
| 60       | 0.20 ± 0.03 |
| 60       | 0.58 ± 0.05 |
| M169K    | 0.29 ± 0.02 |
| 0        | 0.38 ± 0.04 |

The kinetic parameters for the Biotin-dependent ATPase reaction were obtained by varying [biotin] at constant levels of ATP and bicarbonate. For all of the mutants, bicarbonate was held at the same level as in Table I. ATP concentration was saturating for M169K and wild type. For R338Q and R338S, ATP was held at the highest concentration that did not produce substrate inhibition. The errors on \( K_m \) and \( V_{max} \), and \( V/K \) were determined by nonlinear regression analysis.

| \( K_m \) [biotin] | \( V_{max} \) | \( V/K \) |
|-----------------|--------------|---------|
| WT**            | 134 ± 14     | 79 ± 3  |
| R338Q           | 143 ± 21     | 0.74 ± 0.05  | 0.0052 ± 0.0004 |
| R338S           | 25 ± 2       | 0.10 ± 0.01  | 0.0041 ± 0.0003 |
| M169K           | 56 ± 6       | 6.7 ± 0.3    | 0.12 ± 0.01    |

** Data taken from Ref. 8.

The effect of temperature on activity—Clearly, it seems that the diseases caused by the homologous mutations of human biotin carboxylase are attributable, at least in part, to their decreased activity. The impaired \( V_{max} \) of the biotin-dependent ATPase reaction by the homologous biotin carboxylase mutants corroboration this notion. However, because the assays on the biotin carboxylase mutants were carried out at 25 °C, denaturation of the protein structure at body temperature (37 °C) could not be ruled out. Thus, the biotin-dependent ATPase reaction was carried out at 37 °C for R338Q, R338S, and M169K. Compared with the data collected at 25 °C, the \( V_{max} \) at 37 °C was 2-fold higher for both wild type and R338Q. R338S displayed a 3-fold increase in \( V_{max} \) at 37 °C, whereas M169K displayed a 7-fold increase. Because the activity of the three mutants increased at 37 °C, it can be surmised that the overall protein structure was not damaged by the physiological temperature and that the kinetics observed for the mutants are representative of those in vivo. This assertion is further confirmed by Western blot analysis of fibroblast extracts from patients carrying the mutations M204K and R338S. Biotin carboxylase subunits of propionyl-CoA carboxylase were detected in the patient fibroblasts with the M204K mutation. Biotin carboxylase subunits of 3-methylcrotonyl-CoA carboxylase were also detected in patient fibroblasts carrying the R338S mutation. The presence of these proteins also argues against any intrinsic instability and subsequent degradation of the enzyme due to the mutations.

** Data taken from Ref. 10.

** Data taken from Ref. 9.

** Data taken from Ref. 10.

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tive sites of the enzyme are not at the interface between the two subunits (16). That is, the active sites are not composed of residues from both subunits (also referred to as a shared active site). Kinetic analyses of four hybrid dimers had $V_{\text{max}}$ values that were 0.4–3.6% of the $V_{\text{max}}$ value for dimers composed of two wild type subunits. Thus, the two subunits of biotin carboxylase do not function independently. If the two subunits acted independently, the $V_{\text{max}}$ values of the hybrids would have been approximately half of the $V_{\text{max}}$ of the wild type enzyme.

All four mutations exhibited a dominant negative effect on the function of the wild type active site, suggesting that there is communication between the subunits. The observation of negative cooperativity in the N290A, R338S, R336Q, and M169K mutants is consistent with the notion of communication between the subunits and substantiates the possibility that the binding of substrate to one subunit affects the kinetics of the other subunit.

The fact that a point mutation results in cooperative behavior suggests that multisubunit proteins are teetering between cooperative and noncooperative behavior. There are numerous examples of single amino acid substitutions bestowing cooperativity or abolishing cooperativity in proteins. Examples are found in hemoglobin (1), aspartate transcarbamylase (21), pyruvate kinase (22), and the aspartate receptor (23). Given the precarious balance between cooperative and noncooperative behavior, is the negative cooperativity observed here simply an artifact of the mutations or does it provide any insight into the function of the enzyme? It is tempting to speculate that the mutant forms of biotin carboxylase that exhibit negative cooperativity represent evolutionary relics. This notion is supported by noting that the composition of the early atmosphere contained high levels of carbon dioxide (24). Negative cooperativity was detected only with respect to bicarbonate, not ATP or biotin. Negative cooperativity with respect to bicarbonate could have conferred an evolutionary advantage to the organism by decreasing the sensitivity of the enzyme to bicarbonate concentration. This would allow the enzyme to be active over a broader range of bicarbonate concentrations than would be allowed by Michaelis-Menten kinetics (25). As the level of CO2 in the environment stabilized, negative cooperativity of biotin carboxylase would have ceased to confer any advantage for the organism. Thus, the gene coding for the enzyme would mutate to create an enzyme obeying Michaelis-Menten kinetics.

In addition to negative cooperativity, the two Arg-338 mutants (R338Q and R338S) revealed that arginine plays a significant role in carboxyl transfer to biotin. The 3:1 ratio of ADP produced to carboxybiotin produced indicates that cleavage of ATP is uncoupled from carboxyl transfer to biotin. What function could Arg-338 serve in the chemistry of carboxyl transfer? One possibility would be to abstract the N1– proton from biotin to allow for carboxylation. However, finding an active site residue that acts as a catalytic base in biotin carboxylase has been unsuccessful to date despite the mutation of 10 active site amino acids. Furthermore, there is virtually no precedent for an arginine acting as a general base in enzymic reactions. An alternate explanation of the role of Arg-338 begins with the work of Levert et al. (13) who implicate Lys-238 as an essential residue in the production of carboxybiotin. Similar to the Arg-338 mutants, mutation of Lys-238 resulted in uncoupling of ATP hydrolysis and biotin carboxylation. It is interesting to note that all of the site-directed mutants of biotin carboxylase that have been characterized, only mutations of Lys-238 and Arg-338 have resulted in the uncoupling of ATP hydrolysis and biotin carboxylation. Lys-238 acting as a catalytic base was ruled out because the $pK_a$ value was found to be greater than 9.5. The $K_m$ for ATP for R238Q was 50-fold higher than that of wild type, whereas the $K_m$ for ADP was only 5-fold higher. Similarly, R338Q and R338S displayed a 50-fold increase in the $K_m$ for ATP along with a modest 3-fold increase in the $K_m$ for ADP. Given the similarity in kinetics between K238Q and the two Arg-338 mutants, it is likely that their catalytic roles are similar. Since Levert et al. (13) concluded that Lys-238 interacted with the γ-phosphate of ATP, it may be inferred that Arg-338 also interacts with the γ-phosphate. Levert et al. (13) went on to conclude that if Lys-238 binds to the γ-phosphate group of ATP and in turn binds the phosphate group of carboxyphosphate, Lys-238 may help orient the phosphate group to act as a base for extraction of the proton from the 1′-nitrogen of biotin. Thus, Arg-338 may act in concert with Lys-238 in the alignment of the phosphate group of carboxyphosphate to carry out substrate-assisted catalysis, as illustrated in Fig. 4. Although this model seems inconsistent with the observation that the $K_m$ for CbmP (a carboxyphosphate analog) for the two Arg-338 mutants was not significantly different from that of wild type, it should be noted that CbmP is not the physiological substrate. In fact, the amide nitrogen of CbmP can act as a hydrogen bond donor, whereas the carbonyl oxygen of carboxyphosphate can act as a hydrogen bond acceptor. The fact that CbmP can have different hydrogen bonding properties may cause it to be positioned differently from carboxyphosphate in the active site. Thus, although CbmP is an analog of a physiological intermediate, it may not exactly mimic the binding of carboxyphosphate.

Does the kinetic analysis of the mutants found in patients with propionic acidemia and methylcrotonylglycinuria correlate with the clinical phenotype? The patient with the mutation M204K in propionyl-CoA carboxylase exhibited normal growth and development when treated with a protein-restricted diet (26). This mild clinical phenotype is consistent with the observation that the overall function of the homologous mutation in E. coli biotin carboxylase (M169K) did not severely cripple the enzyme. The mutant enzyme had a maximal velocity 10% of the wild type value and exhibited negative cooperativity with respect to bicarbonate. However, all of the other aspects of the kinetics of the M169K mutant were close to wild type and, most importantly, the ratio of ADP to carboxybiotin production was 1:1. This is in contrast to the R338S mutant, which showed a decreased production of carboxybiotin relative to ADP and a maximal velocity that was 100-fold less than the wild type value. The fact that the R338S mutation resulted in a defective E. coli biotin carboxylase is consistent with the severe phenotype exhibited by the patient carrying the homologous mutation (R338S) in 3-methylcrotonyl-CoA carboxylase (6, 27).
clinical phenotype of the patient with the mutation R374Q in propionyl-CoA carboxylase was not available.

It is important to note that although the M169K and R338S mutations both reduced the $V_{\text{max}}$, neither mutation completely abolished the carboxylation of biotin. Likewise, the residual activity of the homologous mutant carboxylases in both patients was sufficient to allow compensation for the deficiency through a modified diet (26, 27). By decreasing the flux of metabolites through the amino acid catabolic pathway, the buildup of toxic intermediates can be reduced. However, the severity of the two phenotypes was quite different, reflecting the importance of the individual roles of the two mutated residues. Despite the protein-restricted diet, the patient carrying the R385S mutation sustained irreversible brain damage and delayed development. In contrast, the patient carrying the M204K mutation had normal growth and development. Thus, the classical approach of studying naturally occurring disease-causing mutations in proteins allows genotype-phenotype correlations to be made.

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