Acetyl-CoA carboxylase catalyzes the first committed step in fatty acid synthesis. In Escherichia coli, the enzyme is composed of three distinct protein components: biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase. The biotin carboxylase component has served for many years as a paradigm for mechanistic studies devoted toward understanding more complicated biotin-dependent carboxylases. The three-dimensional x-ray structure of an unliganded form of E. coli biotin carboxylase was originally solved in 1994 to 2.4Å resolution. This study revealed the architecture of the enzyme and demonstrated that the protein belongs to the ATP-grasp superfamily. Here we describe the three-dimensional structure of the E. coli biotin carboxylase complexed with ATP and determined to 2.5Å resolution. The major conformational change that occurs upon nucleotide binding is a rotation of approximately 45° of one domain relative to the other domains thereby closing off the active site pocket. Key residues involved in binding the nucleotide to the protein include Lys-116, His-236, and Glu-201. The backbone amide groups of Gly-165 and Gly-166 participate in hydrogen bonding interactions with the phosphoryl oxygens of the nucleotide. A comparison of this closed form of biotin carboxylase with carbamoyl-phosphate synthetase is presented.

Acetyl-CoA carboxylase, found in all animals, plants, and bacteria, catalyzes the first committed step in fatty acid synthesis whereby malonyl-CoA is formed from acetyl-CoA, Mg\(^{2+}\) ATP, and bicarbonate (1). As indicted in Scheme 1, the enzyme is biotin-dependent and the reaction mechanism occurs in two steps. In Escherichia coli, acetyl-CoA carboxylase is not a single entity but rather consists of three polypeptide chains that can be isolated separately and that display distinct functional properties (2). These polypeptide chains are referred to as biotin carboxylase (which is Mg\(^{2+}\)-ATP-dependent), biotin carboxyl carrier protein, and carboxyltransferase. The E. coli biotin carboxylase is known to function as a dimer with each subunit containing 449 amino acid residues (3, 4).

The first half of the reaction mechanism, as depicted in Scheme 1, is catalyzed by biotin carboxylase and involves the carboxylation of the ureido ring of the cofactor at the N-1 position. Note that the biotin is not free but rather is covalently attached to the biotin carboxyl carrier protein through an amide linkage between its valeric acid side and an e-nitrogen of a lysine residue in the carrier protein. The current hypothesis is that the biotin carboxyl carrier protein serves as a “swinging arm” to move this activated form of biotin from biotin carboxylase to the carboxyltransferase, where it reacts with acetyl-CoA to yield the final product, malonyl-CoA. In contrast to the acetyl-CoA carboxylase from E. coli, the mammalian enzymes contain all three functions on a single polypeptide chain (5, 6).

In an effort to more fully explore the various structural/functional relationships in the biotin-dependent carboxylases, the three-dimensional x-ray structure of the E. coli enzyme was initially solved in 1994 to a nominal resolution of 2.4 Å (7). While there were no ligands or substrate analogs bound in the active site region, this initial investigation still revealed a number of interesting molecular features. Shown in Fig. 1 is a ribbon representation of one subunit of biotin carboxylase. The molecular architecture of the enzyme can be described in terms of three structural motifs referred to as the A-, B-, and C-domains. The A-domain, defined by Met-1 to Ile-103, contains five strands of parallel β-pleated sheet flanked on either side by a total of four α-helices in a similar topological arrangement to that observed in the Rossmann fold. Connecting the A- and B-domains is a helix-turn-helix motif delineated by Ala-107 to Ala-126. The two helices in this motif are bridged by Asp-115 and lie with their helical axes oriented at approximately 90° with respect to one another. As can be seen, the B-domain, formed by Val-131 to Tyr-203, extends away from the main body of the protein and contains a number of disordered surface loops. The C-domain, beginning at Arg-208, is the largest of the three motifs and is dominated by an eight-stranded antiparallel β-sheet. From this initial investigation, the tentative location of the active site was identified and shown to contain Tyr-82, His-209 to Glu-211, His-236 to Glu-241, Glu-276, Ile-287 to Glu-296, and Arg-338.

Within recent years it has become apparent that biotin carboxylase belongs to a larger family of proteins characterized by an ATP-grasp fold (8). Other enzymes in this family include D-alanine:D-alanine ligase, glutathione synthetase, succinyl-CoA synthetase, and carbamoyl-phosphate synthetase, among others (9–12). The overall chemical transformation catalyzed by the ATP-grasp proteins is the formation of a carbon-nitrogen bond and as such they have been referred to as carboxylate-amine ligases. Members of this superfamily have a common reaction mechanism which requires Mg\(^{2+}\)-ATP for the formation of an acylphosphate intermediate. Additionally, the ATP-grasp enzymes have a common three domain molecular architecture as described for biotin carboxylase, including the helix-turn-helix motif connecting the first two domains. The

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** The atomic coordinates and structure factors (codes IDV1 and IDV2) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ The NMR structure of the ATP-grasp domain of E. coli biotin carboxylase has been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ This paper is available on line at http://www.jbc.org
The nucleotide triphosphate binding pocket is positioned at the interface between the B- and C-domains.

Biotin carboxylase from *E. coli* has served as a paradigm for mechanistic studies of biotin-dependent carboxylation reactions in part because it retains enzymatic activity when isolated from the other components and because it is one of only two presently known biotin-dependent carboxylases that will utilize free biotin as a substrate, the other being β-methylcrotonyl-CoA carboxylase (13). It is widely accepted that the reaction mechanism of biotin carboxylase proceeds through formation of a carboxyphosphate intermediate (14–16). What is not known at the present time, however, is whether the carboxyphosphate intermediate reacts directly with the enolate tautomer of biotin to form carboxybiotin or rather collapses into CO₂ and phosphate with subsequent attack of biotin on the CO₂. However, for most of the enzymes in the ATP-grasp family (e.g. D-alanine:D-alanine ligase and glutathione synthetase), there is direct attack on the carbonyl group in the acylphosphate intermediate. Since biotin carboxylase is a member of this superfamily, most likely CO₂ is not an intermediate in the reaction pathway. Moreover, recent studies on carbamoyl-phosphate synthetase have shown that carboxyphosphate does not decompose within the active site, which is consistent with direct transfer of the carboxyl group (17).

In an effort to more fully define the molecular architecture of the biotin carboxylase active site, we initiated an x-ray crystallographic analysis of the enzyme complexed with a nucleotide triphosphate. Here we describe the three-dimensional structure of biotin carboxylase with bound ATP solved and refined to a nominal resolution of 2.5 Å. For this study, a site-directed mutant protein, in which Glu-288 was replaced with a lysine residue, was constructed. Details of the active site geometry are presented in context of both this E288K protein/ATP model and a previously determined structure in which crystals of native biotin carboxylase had been soaked in a biotin-containing solution (7).

**MATERIALS AND METHODS**

**Crystallization and X-ray Data Collection for Native Biotin Carboxylase**—The native form of biotin carboxylase employed in this investigation was purified according to previously published procedures (7). For crystallization trials, the protein was concentrated to 10 mg/ml. Large single crystals were grown by microdialysis against 10 mM potassium phosphate (pH 7.0), 1 mM EDTA, 2 mM dithiothreitol, and 1 mM NaN₃ at 4 °C as described in Ref. 7. The crystals belonged to the space group P2₁2₁2₁ with unit cell dimensions of a = 561.7 Å, b = 595.8 Å, c = 180.7 Å, and one dimer per asymmetric unit.

For x-ray data collection, the crystals were mounted in quartz capillary tubes. All x-ray data were collected at 4 °C with a Bruker AXS HiStar area detector system. The x-ray source was nickel-filtered CuKα radiation from a Rigaku RU200 x-ray generator operated at 50 mA and 90 mA and equipped with Gobel focusing optics. A complete native x-ray data set to 1.9-Å resolution was collected from a total of nine crystals. These data were processed with the software package SAINT (Bruker AXS Inc.) and internally scaled with XCALIBRE. The x-ray data set was 92.1% complete to 1.9-Å resolution. Relevant x-ray data collection statistics can be found in Table I.
Least-squares Refinement of the Native Structure—The structure of biotin carboxylase, solved to 2.4-Å resolution (7), served as a starting model for the least-squares refinement of the structure described here to 1.9-Å resolution. Alternate cycles of least-squares refinement with the software package TNT (18) and manual model building reduced the overall R-factor to 18.2% for all measured x-ray data between 30- and 1.9-Å resolution. During the early stages of least-squares refinement, 10% of the x-ray data were excluded for the required calculation of R_{free}. The final R_{free} was 23.8%. In that all x-ray data are important for the Fourier synthesis, however, these data were ultimately included in the final stages of the refinement and model building. Relevant refinement statistics are listed in Table II.

Preparation of the E288K Site-directed Mutant Protein—The required primers were synthesized by Life Technologies, Inc. Restriction grade thrombin and His-binding resin were obtained from Novagen. Pyruvate kinase was purchased from Roche Molecular Biochemicals. All other reagents were from Sigma or Aldrich.

The glutamic acid at position 288 in biotin carboxylase was replaced with a lysine residue via site-directed mutagenesis according to Ref. 19. The pair of internal primers employed to prepare the E288K mutant was as follows (with the base change underlined): 5'-TCTATTTGATC-AAAAATGACACCGG3' and 3'-AGATAAAGTAGTTTTACTTGTGGG-C5'. The entire gene of the mutant form of biotin carboxylase was sequenced to verify the mutation and to confirm that there were no other changes in the nucleotide sequence. The gene for the E288K mutant was overexpressed in E. coli with a histidine tag attached to the amino terminus. This allowed purification of the protein via nickel affinity chromatography as described elsewhere (19). For the crystallization studies, the histidine tag was removed from the mutant enzyme by cleavage with thrombin as previously reported (19). This procedure leaves behind three residues at the N terminus with the sequence Gly-Ser-His.

The rate of ATP hydrolysis by the E288K mutant form of biotin carboxylase was determined spectrophotometrically by coupling the production of ADP to pyruvate kinase and lactate dehydrogenase. No ATP hydrolysis was observed with this mutant protein, in either the presence or absence of biotin after 45 min. The rate of ATP hydrolysis by the E288K mutant protein was grown by the hanging drop method of vapor diffusion. For these experiments, 5 μl of protein were mixed with 5 μl of the precipitant solution. The protein sample, at 10 mg/ml, contained 500 mM KCl and 10 mM HEPES (pH 7.0), while the precipitant solution contained 10% polyethylene glycol) 8000, 5 mM ATP, 10 mM MgCl2, and 100 mM HEPPS1 (pH 7.5). The crystals belonged to the space group P212121 with unit cell dimensions of a = 81.3 Å, b = 115.5 Å, c = 122.4 Å and one dimer in the asymmetric unit.

An x-ray data set to 2.5-Å resolution from a single crystal was collected in a similar manner as described for the native form of biotin carboxylase. The x-ray data set was limited to 2.5-Å resolution due to the inherent diffraction qualities of this crystal form. Relevant intensity statistics are given in Table I. The three-dimensional structure of this E288K protein/ATP complex was solved by molecular replacement with the native enzyme serving as a search model. The B-domain, expected to move upon nucleotide binding, was removed from the search model. To expedite the model-building and refinement processes, the electron densities for both subunits in the asymmetric unit were averaged with the program AVE found in the software package RAVE (20, 21). An averaged model was built into the electron density, including those residues constituting the B-domain. Subsequently, the averaged model was placed back into the unit cell and refined with the software package, TNT (18). The model was adjusted within the unit cell and one final round of least-squares refinement applied. The final R-factor was 17.2% for all measured x-ray data from 30 to 2.5 Å. R_{free} was 20.3% for 10% of the x-ray data removed from the reflection file. Relevant least-squares refinement statistics can be found in Table II. In subunit I, there were three additional residues (Gly, Ser, and His) included at the N terminus due to the His tag construction. In subunit II, only one additional histidine residue at the N terminus was included in the model.

### RESULTS AND DISCUSSION

Refinement of the Apo Form of Biotin Carboxylase—The original structure of biotin carboxylase was solved to a nominal resolution of 2.4 Å (7). As reported here, the model has now been refined to 1.9 Å resolution, which has allowed for a better three-dimensional description of both the active site and the solvent structure. Two inorganic phosphate molecules, one per subunit, and 544 water molecules were included in the final refined model. The average B-value for the waters was 51 Å² while that for the phosphates was 45 Å².

Electron density corresponding to the region surrounding the bound phosphate in subunit I is displayed in Fig. 2a with the hydrogen bonding pattern between this anion and the protein highlighted in Fig. 2b. Note that both O₁ and O₂ of Glu-296 lie at 3.0 and 2.6 Å, respectively, from one of the phosphoryl oxygens of the phosphate moiety. This close positioning of the negatively charged phosphate to the carboxylate of Glu-296 argues for a proton shared between the two moieties. There are five direct contacts between the protein and the phosphate, which range in length from 2.7 to 3.1 Å. Three waters also form hydrogen bonds with the phosphoryl oxygens of the phosphate with lengths ranging from 2.5 to 3.0 Å. Both the guanidinium

### Table I

| Intensity statistics | Native biotin carboxylase | E288K protein/ATP complex |
|----------------------|---------------------------|---------------------------|
| Resolution range (Å) | 30.0–1.9/1.99–1.90        | 30.0–2.5/2.61–2.50        |
| Integrated reflections | 775,589/6,147             | 823,377/4,968             |
| Independent reflections | 784,898/7,671            | 775,747/3,543            |
| Data completeness (%) | 92.1/1.9                | 92.6/3.1                 |
| Average/average σ (I) | 15.7/1.9                | 9.6/1.9                  |
| R_{sym} (%)<sup>a</sup> | 5/21.9                  | 7.2/29.7                 |

<sup>a</sup> R_{sym} = (∑|I| - 1/|∑|/|∑|) × 100.

### Table II

| Least-squares refinement statistics | Native biotin carboxylase | E288K protein/ATP complex |
|------------------------------------|---------------------------|---------------------------|
| Resolution limits (Å) | 30.0–1.9                  | 30.0–2.5                  |
| R_{overall} (%)<sup>b</sup> | 18.2                     | 17.2                      |
| R_{working} (%)                  | 18.0                     | 17.1                      |
| R_{free} (%)                      | 23.8                     | 20.3                      |
| No. of reflections used           | 78,489                   | 37,574                    |
| No. of protein atoms              | 6644                     | 6921                      |
| No. of solvent atoms<sup>c</sup> | 554                      | 192                       |
| Weighted root-mean-square deviations from ideality | | |
| Bond length (Å)                   | 0.014                    | 0.012                     |
| Bond angle (degrees)              | 2.38                     | 2.35                      |
| Planarity (trigonal) (Å)          | 0.008                    | 0.005                     |
| Planarity (other planes) (Å)      | 0.013                    | 0.010                     |
| Torsional angle (degrees)<sup>d</sup> | 17.5                     | 19.5                      |

<sup>b</sup> R_{factor} = (∑|F_o| - |F_c|)/|∑|F_o|/|∑|F_c| × 100, where F_o is the observed structure factor amplitude and F_c is the calculated structure factor amplitude.

<sup>c</sup> In addition to 544 waters, there were two inorganic phosphate molecules built into the native biotin carboxylase model. In the E288K protein/ATP complex, there were 128 water molecules and two ATP molecules built into the electron density.

<sup>d</sup> The torsional angles were not restrained during the refinement.
groups of Arg-292 and Arg-338 and the ε-amino group of Lys-238 form a positively charged pocket that is ideally suited for the binding of small, negatively charged molecules. In that one of the substrates for biotin carboxylase is bicarbonate, it is possible that this region of polypeptide chain is responsible for binding this molecular species. Strikingly, phosphate ions have also been observed in similar positions in other ATP-grasp proteins solved in this laboratory, including carbamoyl-phosphate synthetase and N⁵-carboxyaminoimidazole ribonucleotide synthetase, both from *E. coli* (22, 23).

In the model of biotin carboxylase presented here, four amino acid residues adopt multiple conformations: Glu-188 and Asp-419 in subunit I and Asp-135 and His-432 in subunit II. The overall temperature factors for subunits I and II were 37.7 and 41.3 Å², respectively. Subunit I was somewhat better ordered with respect to the electron density with breaks occurring only at the loops defined by Ser-161 to Met-169 and Ala-189 to Ala-195. In subunit II, the breaks in the electron density occurred at Lys-159 to Met-169 and Ala-187 to Tyr-199 and many residues in the B-domain had to be modeled as alanines. The last observable residues in subunits I and II are Leu-446 and Glu-448, respectively. The only significant outliers in the Ramachandran plot occur at Phe-84 (φ = 46°, ψ = −116°) and Ala-226 (φ = 53°, ψ = −158°) in both subunits. The electron density in these regions is unambiguous. Phe-84 is located within a tight turn that connects the fourth β-strand of the A-domain to an α-helix, while Ala-226 is positioned in the first β-strand of the C-domain.

Partly as a result of crystalline packing, the B-domain of the biotin carboxylase subunit is splayed significantly away from...
the main body of the enzyme. The overall $B$-values for all backbone atoms of the A-, B-, and C-domains in subunit I are 31, 56, and 28 Å$^2$, respectively. These domains in subunit II show comparable $B$-values of 34, 72, and 38 Å$^2$. While caution must be applied in interpreting temperature factors, clearly the combination of residues forming the B-domains of biotin carboxylase demonstrate conformational flexibility, which may be important for the proper functioning of the enzyme.

**Structure of the E288K Protein/ATP Complex**—The original structure of biotin carboxylase, albeit without any bound nucleotides to unambiguously define the active site, was significant in that it allowed the three-dimensional architecture of the enzyme to be defined. Interestingly, all attempts to soak crystals in nucleotide analogs cracked the crystals within minutes (7). Co-crystallization attempts of biotin carboxylase with nucleotide analogs were also unsuccessful. In an effort to more fully characterize the active site of this ATP-grasp enzyme, a site-directed mutant protein in which Glu-288 was replaced with a lysine residue was prepared. While this amino acid substitution completely abolished the ability of the enzyme to hydrolyze ATP, it allowed crystals of biotin carboxylase to be grown in the presence of ATP. As such, this structure represents the first model for any ATP-grasp protein with ATP bound in the active site rather than a nucleotide analog such as AMPPNP.

Electron density corresponding to the ATP moiety is shown in Fig. 3. Unlike that observed for carbamoyl-phosphate synthetase, for example, there are no bound magnesium ions. Rather, the ε-amino group of Lys-288 is located in a position normally occupied by such cations, i.e. wedged between β- and γ-phosphoryl oxygens. In native biotin carboxylase, Glu-288 might serve as a ligand to one of the magnesium ions known to be important for catalysis. Indeed, in carbamoyl-phosphate synthetase, Glu-299, the structural counterpart of Glu-288 in biotin carboxylase, serves as a bidentate ligand to one of the metals wedged between the β- and γ-phosphoryl oxygens (24). Likewise, Glu-841, in the second ATP binding site of carbamoyl-phosphate synthetase, the 2'- and 3'-hydroxyl groups of the nucleotide ribose are anchored to the protein via hydrogen bonding to the side chain carboxyl of Glu-215 (or Glu-761 in the second half of the large subunit). The topological equivalent residue in biotin carboxylase is His-209, which is located ~4 Å from the ribose hydroxyl groups. Since the structure reported here is that of a site-directed mutant protein, it is possible that the ATP has been shifted somewhat from its normal binding position in the active site. Consequently, it can be speculated that in the native protein, His-209 plays a role in the binding of the nucleotide to biotin carboxylase. Interestingly, substitution of His-209 with an alanine residue increases the $K_m$ for free biotin by about 10-fold and the $K_m$ for ATP by approximately 70-fold. Two peptidic NH groups, provided by Gly-165 and Gly-166 of the B-domain, participate in hydrogen bonds with the phosphoryl oxygens of the nucleotide as indicated in Fig. 4b. These residues are disordered in the model for native biotin carboxylase. The structurally equivalent residues in carbamoyl-phosphate synthetase, namely Gly-721 and Gly-722 in the B-domain, participate in hydrogen bonds with the phosphoryl oxygens of the nucleotide as indicated in Fig. 4b. These residues are disordered in the model for native biotin carboxylase. The structurally equivalent residues in carbamoyl-phosphate synthetase, namely Gly-721 and Gly-722 in the B-domain, participate in hydrogen bonds with the phosphoryl oxygens of the nucleotide as indicated in Fig. 4b. These residues are disordered in the model for native biotin carboxylase.

A close-up view of the ATP binding pocket in biotin carboxylase is displayed in Fig. 4a, and a schematic of the hydrogen bonding pattern between the protein and the ligand is given in Fig. 4b. In that the structure of the E288K protein/ATP complex was solved to 2.5-Å resolution, the actual hydrogen bonding pattern may change somewhat when higher resolution x-ray data are eventually obtained. These crystals were quite difficult to grow, and a search for a more promising crystal form is under way. Even in light of this caveat, however, this complex has provided significant insight into nucleotide binding to biotin carboxylase. Most of the binding pocket is formed by residues located in the B- and C-domains. Lys-116, which is located in the connecting region between the A- and B-domains, provides a key electrostatic interaction with one of the α-phosphoryl oxygens of the ATP. When Lys-116 is changed to a glutamine, the $K_m$ for ATP increases by 57-fold. Likewise, N$^\text{ε2}$ of His-236 lies within hydrogen bonding distance to both the β,γ-bridging oxygen and one of the γ-phosphoryl oxygens. From Fig. 4a, it can be seen that the adenine portion of the nucleotide is wedged up into a loop defined by Glu-201 to Leu-204. The aromatic side chain of Tyr-203 forms an edge-on stacking interaction with the purine ring of ATP. In carbamoyl-phosphate synthetase, the 2'- and 3'-hydroxyl groups of the nucleotide ribose are anchored to the protein via hydrogen bonding to the side chain carboxylate of Glu-215 (or Glu-761 in the second half of the large subunit). The topologically equivalent residue in biotin carboxylase is His-209, which is located ~4 Å from the ribose hydroxyl groups. Since the structure reported here is that of a site-directed mutant protein, it is possible that the ATP has been shifted somewhat from its normal binding position in the active site. Consequently, it can be speculated that in the native protein, His-209 plays a role in the binding of the nucleotide to biotin carboxylase. Interestingly, substitution of His-209 with an alanine residue increases the $K_m$ for free biotin by about 10-fold and the $K_m$ for ATP by approximately 70-fold. Two peptidic NH groups, provided by Gly-165 and Gly-166 of the B-domain, participate in hydrogen bonds with the phosphoryl oxygens of the nucleotide as indicated in Fig. 4b. These residues are disordered in the model for native biotin carboxylase. The structurally equivalent residues in carbamoyl-phosphate synthetase, namely Gly-721 and Gly-722 in the second half of the large subunit, act as a trigger for closure of the B-domain upon nucle-
otide binding (24). Note that the γ-phosphoryl group of ATP is located at approximately 8 Å from where the inorganic phosphate was observed binding in native biotin carboxylase.

The movement of the B-domain upon ATP binding to biotin carboxylase is extensive, as can be seen in Fig. 5. There is an overall rotation of −45° of the B-domain relative to the A- and C-motifs, which results in some atoms moving by more than 8 Å. The largest shifts begin at Pro-132 and continue to Leu-204 whose aliphatic side chain abuts one face of the adenine ring of ATP. The conformational flexibility of the B-domain is not unexpected, in that 15% of the residues found in the region defined by Pro-132 to Leu-204 are glycine residues and there is stretch of five glycines in tandem beginning at Gly-162. Indeed, the B-domains (between the apo- and liganded forms of biotin carboxylase) superimpose with an r.m.s. value of 12.2 Å, while the A- and C-domains are quite similar with r.m.s. deviations of 0.33 and 0.54 Å, respectively, for all backbone atoms. Approximately 85% of the ATP surface area is buried upon binding to biotin carboxylase.

Comparison of Biotin Carboxylase with Carbamoyl-phosphate Synthetase—Of the various members of the ATP-grasp superfamily whose three-dimensional structures are now known, the architecture of carbamoyl-phosphate synthetase appears to be the most closely related to that of biotin carboxylase. The large subunit of carbamoyl-phosphate synthetase contains two MgATP binding sites formed by Met-1 to Glu-403 and Asn-554 to Asn-936, respectively (24). These two regions of carbamoyl-phosphate synthetase are remarkably similar and correspond with an r.m.s. deviation of 0.93 Å for 1076 structurally equivalent backbone atoms. Fig. 6 shows a comparison of the polypeptide chain backbones for biotin carboxylase and carbamoyl-phosphate synthetase (Met-1 to Glu-403). For this comparison, the A-, B-, and C-domains were fitted separately.

The two A-domains for carbamoyl-phosphate synthetase and
biotin carboxylase superimpose with an r.m.s. deviation of 1.6 Å for 97 structurally equivalent α-carbons. There are three regions in the A-domains where the two polypeptide chains significantly deviate from one another. The first region occurs at Arg-10 in biotin carboxylase, where there is an 11-residue insertion in carbamoyl-phosphate synthetase (beginning at Ala-15). The second and third regions occur at Ser-33 and Gly-52, where there are 2- and 8-residue insertions in biotin carboxylase, respectively, relative to carbamoyl-phosphate synthetase at Ser-49 and Pro-68.

A comparison of the B-domains reveals that there is only one insertion in biotin carboxylase relative to carbamoyl-phosphate synthetase. This 4-residue insertion occurs at Ala-189 in biotin carboxylase and Ser-199 in carbamoyl-phosphate synthetase. Accordingly, the polypeptide chains for the B-domains of biotin carboxylase and carbamoyl-phosphate synthetase superimpose with an r.m.s. value of 2.0 Å for 63 structurally equivalent α-carbons.

The C-domains of biotin carboxylase and carbamoyl-phosphate synthetase correspond with an r.m.s. deviation of 1.6 Å for 116 structurally equivalent α-carbons. Interestingly, the last 40 residues of biotin carboxylase, which fold into one β-strand and three α-helices, have no structural counterparts in carbamoyl-phosphate synthetase. There are two small insertions in carbamoyl-phosphate synthetase at Asn-236 and Asn-289, which correspond to the regions beginning at Cys-230 and Glu-280 in biotin carboxylase. Additionally, there is an 11-residue insertion in carbamoyl-phosphate synthetase relative to biotin carboxylase (beginning at Leu-332 in carbamoyl-phosphate synthetase and Leu-321 in biotin carboxylase). By far, the most significant deviation in the polypeptide chains occurs at Ala-341 in biotin carboxylase (Asn-363 in carbamoyl-phosphate synthetase), where there is a 26-residue insertion in biotin carboxylase.

Modeling of the Biotin Carboxylase Active Site with Biotin and ATP—In the initial structural investigation of biotin carboxylase, native apo-crystals were soaked in a saturated solution of Ag+/biotin in an attempt to identify the active site region of the enzyme (7). While the crystals only diffracted to 3.0 Å resolution and the silver ion dissociated from the complex, it was possible to unambiguously orient the biotin into the difference electron density map due to the presence of the sulfur in the tetrahydrothiophene ring of the cofactor. Note that the inorganic phosphate ion observed in the original structure of biotin carboxylase was displaced upon biotin binding.

Now that the structure of biotin carboxylase in its closed form is known, it has been possible to examine the relative orientations of biotin and ATP in the active site region of the enzyme as shown in Fig. 7. This model was constructed by simply superimposing the C-domain of the native biotin car-
bovine carboxylase with bound biotin (open form) onto the C-domain of the E288K mutant protein (closed form). Approximately 77% of the biotin is buried in this model.

While the reaction mechanism of biotin carboxylase is still not completely understood, it can be envisioned to occur as outlined in Scheme 2. According to this scheme, the carboxylation of biotin requires two key structural elements: 1) a base for the abstraction of the proton from N-1 of the ureido ring; and 2) amino acid residues responsible for stabilization of the enolate anion. As shown in Fig. 7, the γ-phosphate of ATP lies adjacent to the 1’ nitrogen of biotin and both the side chains of Glu-276 and Lys-288 are positioned near the ureido ring. In the native form of biotin carboxylase, Lys-288 is a glutamate residue, and on the basis of homology with carbamoyl-phosphate synthetase, probably serves as a ligand to one of the required magnesium ions. Glu-276 probably does not function as the base as well in light of recent site-directed mutagenesis experiments, where it was replaced with a glutamine. Although the catalytic activity of this mutant protein was reduced from 10 to 21 units, and on the basis of homology with carbamoyl-phosphate synthetase, probably serves as a ligand to one of the required magnesium ions. Glu-276 and Lys-288 are positioned near the ureido ring. In the active site of biotin carboxylase, co-crystallization of ATP and biotin within the active site geometry of biotin carboxylase, co-crystallization studies of the E288K protein with various nucleotides and biotin analogs as well.

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